Interdisciplinary investigations of phage predation dynamics and generalised transduction of antimicrobial resistance in *Staphylococcus aureus*

QUENTIN LECLERC

Thesis submitted in accordance with the requirements
for the degree of

Doctor of Philosophy
of the
University of London

JUNE 2022

Department of Infectious Disease Epidemiology
Faculty of Epidemiology and Population Health
LONDON SCHOOL OF HYGIENE & TROPICAL MEDICINE

Funded by the UK Medical Research Council

Research group affiliations:
Centre for Mathematical Modelling of Infectious Diseases, LSHTM, UK
Antimicrobial Resistance Centre, LSHTM, UK
Institute for Infection and Immunity, SGUL, UK

Declaration

I, Quentin Leclerc, confirm that the work presented in the thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signature

Date
06/06/2022

Abstract

Antimicrobial resistance (AMR) is a major global public health threat, typically represented by bacteria becoming resistant to antibiotics, and hence harder to treat. A particular concern is multidrug resistance, which can arise as bacteria acquire new AMR genes via horizontal gene transfer (HGT). In the important nosocomial pathogen *Staphylococcus aureus*, bacteriophage (phage, viruses of bacteria) are the major drivers of HGT of AMR by the process of transduction. In an initial systematic review, I found that dynamics of transduction and the overall contribution of this process to the global spread of AMR are unclear. In this thesis, I aimed to fill this research gap through an interdisciplinary approach, combining mathematical modelling, lab work, and analysis of routinely collected hospital data.

I first investigated the dynamics of phage and *S. aureus*, including generalised transduction of AMR, by developing a novel mathematical model representing these dynamics, and generating *in vitro* data to parameterise this model. I estimated rates of generalised transduction, and showed that this process consistently leads to generation of multidrug-resistant bacteria, even in the absence of a selection pressure. Within-host however, phage may often be present alongside antibiotics. These may either act in synergy to kill bacteria, or antibiotics may limit phage predation and instead exert a selective pressure on multidrug-resistant bacteria generated by phage via generalised transduction. I extended my model to include antibiotic pharmacodynamics, and parameterised this by generating additional *in vitro* data. By analysing this extended model, I identified timings and concentrations of phage and antibiotics which maximise bacteria killing, whilst minimising the risk of multidrug resistance evolution and selection. Finally, I translated these findings to an *in vivo* setting by analysing 20 years of routinely collected pseudonymised hospital data on more than 20,000 patients colonised or infected by *S. aureus*. Using antibiograms of more than 70,000 isolates, I identified evidence of within-host AMR phenotypic diversity, and changes in that diversity over time, potentially mediated by transduction.

Overall, the work presented in this thesis clarifies some of the complex phage-bacteria dynamics in *S. aureus*, and highlights the important role played by phage in AMR spread through generalised transduction.

Acknowledgments

Science is always a team effort, and this thesis is no exception!

Firstly, thank you Gwen, for being simply the best supervisor anyone could ever have. Thank you for your enthusiasm, your passion, your patience, everything you have taught me, and for always being there when I needed you during this incredible, unpredictable journey. I will be forever grateful for everything you have done for me. Fortunately, I'm typing this on my computer, otherwise there would be some emotional tear drops on the page…!

Thank you Jodi for all the incredibly valuable advice you have shared with me over the years. Our discussions have been essential to get me to ask the right questions, put both work and life into perspective, and spark my curiosity whenever I felt stuck. I will always try to remember all your precious guidance, particularly a comment you made at the beginning of my PhD: "you will have to learn how to supervise your supervisors". Well, I guess I can say that it was a pleasure to supervise you too then! Thank you for everything.

This thesis has benefited from the many interactions I had with different colleagues. I would like to thank Katherine Atkins for helpful discussions on the search strategy for the systematic review in Chapter 2, José Penadés and Nuria Quiles for providing the JP8488 *S. aureus* strain used as a positive control for the 80α lysogeny PCR in Chapter 3, Caroline Memmi for helpful discussions on antibiotic bioavailability and *in vivo* antibiotic concentrations in Chapter 4, and Joseph Standing and his research group for advice regarding antibiotic pharmacodynamics modelling in Chapter 4. I would also like to thank Louis Grandjean, Helen Dunn, James Hatcher, the GOSH Digital Research Environment (in particular Mohsin Shah), and other staff at Great Ormond Street Hospital for support around the analyses presented in Chapter 5.

Thanks to all the people at CMMID I have interacted with over the years. Whether we've shared an office, had a coffee, or just talked randomly in the corridor one day, you've contributed to the great time I had in this group.

Thanks to all the wonderful members of the Knight Group, old and new: Nichola, Naomi, Jake, Naomi, Tresco, Yalda, Alastair, and Max! Special thanks to Nichola for your wise advice and tequila shots, Naomi for the lunch breaks in the sun when I really needed them, and Jake for… well… just being an absolute legend – this final year was a blast thanks to your constant banter and climbing coaching.

On a more personal note, I would like to thank all the friends who have been there for me these past few years in London, Paris, and elsewhere. Special thanks to the best sciency flatmates Jules and Caroline, you've clearly contributed to this work (cakes + fancy dishes = energy = work), and I'm happy to have found two people I can play "guess the infectious disease" with at dinner! Thanks to the best fajitas flatmates Antoine and Anatole, you've made all my years in London so much fun, that it's clear I wouldn't have stuck around for so long if you hadn't been there. Thanks to Lucas, Violette and Claire, even though we've all spent the last few years in different parts of the world, I always look forward to our goûters and apéros, whether they're virtual or in the real world. Thank you Thomas for all the boardgames, and your relaxed attitude that inspires me! And thank you Sirina for all the pub nights, endless discussions, for your honesty, and all the questions you've ever asked (including the famous "can I ask you a question?").

Finally, a big big merci to my parents and my brother, merci de m'avoir fait confiance, de m'avoir offert les opportunités qui m'ont amenées jusqu'ici, et d'avoir toujours été là pour me ramener sur Terre quand je me perdais un peu trop dans mon univers scientifique !

COVID-19 impact statement

The majority of the work presented as part of this PhD was completed during the COVID-19 pandemic, which started in 2020. Following LSHTM guidelines, this section describes the impact of COVID-19 on my research plan.

1. Details on how disruption caused by COVID-19 has impacted the research

A key aspect of my thesis, presented in Chapter 3, is the simultaneous generation of data in the lab, and development of models to reproduce and analyse the dynamics seen in this data. When the first lockdown was announced in the UK in March 2020, I had to stop all the experiments I was conducting, and was only able to return in August 2020, delaying my lab work by approximately 4 months. Since the data was necessary for the development of the models, I could not make any progress on the modelling in Chapter 3 either during this period. As I had already spent time developing and validating my experiments even before my upgrading in April 2019, I knew that they would work, therefore I had built my entire analysis plan around this data that I knew I was going to obtain. For this reason, I had already planned to use the resulting models in subsequent Chapters of my PhD. Consequently, this delay in completing my experiments and generating the data to inform the models impacted my entire project. I was not able to make up for the lost time upon my return to the lab, as these experiments were on fixed time-points over 24 hours.

In addition, I chose not to conduct any lab work involving co-cultures of bacteria, phage, and antibiotics for Chapter 4. This was due to the high uncertainty of the situation, as the risk of further lockdowns meant that I could not be confident I would be able to complete this lab work within the timeline of my PhD.

2. Description of how the planned work would have fitted within the thesis' narrative

Additional lab work in Chapter 4 would have substantially strengthened the analysis, either confirming the hypotheses generated or leading to the discovery of new interactions in this system, and hence refining the model. This could also have opened

new possibilities for further exploration of the joint effect of antibiotics, phage predation, and transduction of AMR on bacteria, perhaps to identify clearer thresholds that affect these dynamics. Finally, an original objective of this thesis was to simultaneously explore the dynamics of *S. aureus* within-host evolution and between-host transmission, by integrating my model in an individual-based model of *S. aureus* transmission in hospital. This could have generated interesting insights on how AMR spread within *S. aureus* bacterial populations affects the prevalence of AMR in humans, but this analysis was not feasible due to the delays in the project timeline.

3. Summary of any decisions / actions taken to mitigate for any work or data collection/analyses that were prevented by COVID-19

I was granted a 3 months extension by my funding body to make up for some of the delays in the project. Since I could not conduct further experiments in Chapter 4 to grow bacteria, phage, and antibiotics together, I instead focused on extending the model developed in Chapter 3 and using it to predict the dynamics we might see in such *in vitro* experiments. The resulting hypotheses I have generated are now being investigated by other members of the research group who are conducting experiments with bacteria, phage, and antibiotics.

As I could not work on my thesis for several months in 2020, I instead developed research skills by taking part in the COVID-19 response within the Centre for Mathematical Modelling of Infectious Diseases (CMMID) at LSHTM. I completed the following analyses, including one on the impact of COVID-19 on AMR:

1) **Leclerc QJ**, Fuller NM, Knight LE, Funk S, Knight GM. What settings have been linked to SARS-CoV-2 transmission clusters? *Wellcome Open Res*. 2020;5: 83. doi:10.12688/wellcomeopenres.15889.2 (**Contributions**: developed and maintained the database of SARS-CoV-2 clusters, extracted and analysed information from these clusters, wrote the manuscript. This work received substantial international scientific, media, and public attention, as it highlighted early during the pandemic the importance of indoor transmission. Altmetric attention score: 933, Scopus citations: 159)

2) **Leclerc QJ**, Nightingale ES, Abbott S, Jombart T. Analysis of temporal trends in potential COVID-19 cases reported through NHS Pathways England. *Sci Rep*. 2021;11: 7106. doi:10.1038/s41598-021-86266-3 (**Contributions**: developed and maintained the code to extract COVID-19 cases from NHS Pathways and number of COVID-19 deaths in the UK, analysed and compared these numbers, wrote the manuscript. This work was used to inform real-time analysis of COVID-19 transmission in the UK.)
3) Endo A, **Leclerc QJ**, Knight GM, Medley GF, Atkins KE, Funk S, Kucharski AJ. Implication of backward contact tracing in the presence of overdispersed transmission in COVID-19 outbreaks. *Wellcome Open Res*. 2021;5: 239. doi:10.12688/wellcomeopenres.16344.3 (**Contributions**: discussed the analysis, reviewed the code, edited the manuscript. This work was used to support the implementation of backward contact tracing to limit COVID-19 transmission in various countries.)
4) Knight GM, Glover RE, McQuaid CF, Olaru ID, Gallandat K, **Leclerc QJ**, Fuller NM, Willcocks SJ, Hasan R, van Kleef E, Chandler CIR. Antimicrobial resistance and COVID-19: Intersections and implications. *eLife.* 2021;10: e64139. doi:10.7554/eLife.64139 (**Contributions**: discussed the analysis, edited the manuscript. This work was the first to extensively discuss the complex changes on AMR emergence, transmission, and burden that may result from the COVID-19 pandemic.)
5) Jombart T, Ghozzi S, Schumacher D, Taylor TJ, **Leclerc QJ**, Jit M, Flasche S, Greaves F, Ward T, Eggo RM, Nightingale E, Meakin S, Brady OJ. Real-time monitoring of COVID-19 dynamics using automated trend fitting and anomaly detection. *Philosophical Transactions of the Royal Society B: Biological Sciences*. 2021;376: 20200266. doi:10.1098/rstb.2020.0266 (**Contributions**: developed and maintained the code to extract COVID-19 cases from NHS Pathways and number of COVID-19 deaths in the UK, reviewed other analysis code, edited the manuscript. This work built upon the analyses conducted in reference 2 in this list.)
6) **Leclerc QJ**, Fuller NM, Keogh RH, Diaz-Ordaz K, Sekula R, Semple MG, Atkins KE, Procter SR, Knight GM. Importance of patient bed pathways and length of stay differences in predicting COVID-19 hospital bed occupancy in England. *BMC Health Serv Res*. 2021;21: 566. doi:10.1186/s12913-021-

06509-x (**Contributions**: developed the mathematical model, conducted the analyses, wrote the manuscript. This work supported hospital bed management at University College London Hospital during the COVID-19 pandemic.)

In addition, I volunteered my time to help maintain the data pipeline which members of CMMID relied on for multiple COVID-19 projects, and translated the CoMIX contact survey from English to French, to be used in Belgium. However, as mentioned above since I already had developed a clear research plan around *S. aureus*, antimicrobial resistance, and phage, I chose not to include any of this work, as this would have reduced the coherence of my thesis.

List of research outputs produced as part of this thesis

Research papers

1) **Leclerc QJ**, Lindsay JA, Knight GM. Mathematical modelling to study the horizontal transfer of antimicrobial resistance genes in bacteria: current state of the field and recommendations. *Journal of the Royal Society Interface*. 2019 Aug 30;16(157):20190260.
2) **Leclerc QJ**, Wildfire J, Gupta A, Lindsay JA, Knight GM. Growth-Dependent Predation and Generalized Transduction of Antimicrobial Resistance by Bacteriophage. *mSystems*. 2022 Mar 21:e00135-22.
3) **Leclerc QJ**, Lindsay JA, Knight GM. Modelling the synergistic effect of bacteriophage and antibiotics on bacteria: killers and drivers of resistance evolution. *bioRxiv*. 2022 Jan 1. (undergoing revisions for resubmission to PLoS Computational Biology)

Conference and workshop presentations

Oral presentations

1) **Leclerc QJ**, Wildfire J, Gupta A, Lindsay JA, Knight GM. New insights into phage predation dynamics and the importance of horizontal transfer of AMR by generalised transduction in *S. aureus*. *StaphGBI 2021*, online. 2021 Jul.
2) **Leclerc QJ**, Wildfire J, Gupta A, Lindsay JA, Knight GM. New insights into phage predation dynamics and the importance of horizontal transfer of AMR by transduction. *National PhD Training Program in AMR Annual Conference*, online. 2021 Aug.
3) **Leclerc QJ**, Wildfire J, Gupta A, Lindsay JA, Knight GM. Modelling the dual nature of bacteriophage in the context of antimicrobial resistance: bacterial predation and horizontal gene transfer by transduction. *EPIDEMICS8*, online. 2021 Dec.
4) **Leclerc QJ**, Wildfire J, Gupta A, Lindsay JA, Knight GM. Modelling to reveal the joint effect of bacteriophages and antibiotics on AMR evolution. *RESIST2 workshop*, online. 2022 Mar.

5) **Leclerc QJ**. Interdisciplinary AMR research: the future of AMR science?. *Microbiology Society Annual Conference*, Belfast, UK. 2022 Apr.

Poster presentations

1) **Leclerc QJ**, Lindsay JA, Knight GM. Mathematical modelling to study the horizontal transfer of antimicrobial resistance genes in bacteria: current state of the field and recommendations. *Modelling the World's Systems*, Washington, USA. 2019 May.
2) **Leclerc QJ**, Lindsay JA, Knight GM. Mathematical modelling to study the horizontal transfer of antimicrobial resistance genes in bacteria: current state of the field and recommendations. *National PhD Training Program in AMR Annual Conference*, Bristol, UK. 2019 Aug.
3) **Leclerc QJ**, Lindsay JA, Knight GM. Mathematical modelling to study the horizontal transfer of antimicrobial resistance genes in bacteria: current state of the field and recommendations. *EPIDEMICS7*, Charleston, USA. 2019 Dec.
4) **Leclerc QJ**, Gupta A, Lindsay JA, Knight GM. An interdisciplinary approach to reveal the dynamics of generalized transduction of antimicrobial resistance genes. *Microbiology Society Annual Conference*, Edinburgh, UK. 2020 Mar. *(note: cancelled due to COVID-19)*
5) **Leclerc QJ**, Wildfire J, Gupta A, Lindsay JA, Knight GM. An interdisciplinary approach to reveal the dynamics of generalized transduction of antimicrobial resistance genes. *Microbiology Society Annual Conference*, online. 2021 Apr.
6) **Leclerc QJ**, Wildfire J, Gupta A, Lindsay JA, Knight GM. An interdisciplinary approach to reveal the dynamics of generalized transduction of antimicrobial resistance genes. *31st ECCMID*, online. 2021 Jul.
7) **Leclerc QJ**, Knight GM. How does bacterial diversity quantitatively affect antimicrobial resistance evolution and hence the impact of interventions for control? *EPIDEMICS8*, online. 2021 Dec.
8) **Leclerc QJ**, Lindsay JA, Knight GM. Modelling the synergistic effect of antibiotics and bacteriophage to drive antimicrobial resistance evolution in Staphylococcus aureus by transduction. *Microbiology Society Annual Conference*, Belfast, UK. 2022 Apr.

Table of contents

List of tables and figures

List of abbreviations

AMR: antimicrobial resistance

BHIA: brain heart infusion agar

BHIB: brain heart infusion broth

CA-MRSA: community-associated methicillin-resistant *Staphylococcus aureus*

CC: clonal complex

ECDC: European Centre for Disease Prevention and Control

DIC: deviance information criteria

DNA: deoxyribonucleic acid

DRIVE: Digital Research, Informatics and Virtual Environments unit

DRP: double-resistant progeny

GOSH: Great Ormond Street Hospital

HA-MRSA: hospital-associated methicillin-resistant *Staphylococcus aureus*

HGT: horizontal gene transfer

LA-MRSA: livestock-associated methicillin-resistant *Staphylococcus aureus*

MGE: mobile genetic element

MHB: Mueller-Hilton broth

MIC: minimum inhibitory concentration

MLST: multilocus sequence typing

MOI: multiplicity of infection

MRSA: methicillin-resistant *Staphylococcus aureus*

MSSA: methicillin-susceptible *Staphylococcus aureus*

PBP: penicillin-binding protein

PBP2a: penicillin-binding protein 2

PBS: protein-binding saline

PCR: polymerase chain reaction

PKPD: pharmacokinetics and pharmacodynamics

RNA: ribonucleic acid

SCC*mec*: staphylococcal cassette chromosome *mec*

ST: sequence type

WHO: World Health Organization

WTA: wall teichoic acid

1 Introduction

1.1 Outline of the thesis

In this Introduction, I describe the global public health threat of antimicrobial resistance (AMR), focusing on antibiotic resistance in bacteria, before presenting *Staphylococcus aureus* as a clinically important pathogen, made more dangerous by the acquisition of multiple resistance genes. I then introduce bacteriophage (phage) as organisms that substantially interact with *S. aureus*. I explain how phage can lead to the transfer of AMR genes in *S. aureus* by transduction, and present how mathematical modelling can help to reveal the dynamics of phage predation and transduction of AMR.

This thesis is structured following the London School of Hygiene & Tropical Medicine "research paper thesis" style, with the work divided between a series of papers written during the project, and each paper corresponding to a chapter. For each paper included, I first give an overview of the work conducted and how it fits in the thesis, then I directly include the corresponding manuscript. Note that the references in Chapters 2, 3, 4 and 5 are independent from the references in the Introduction (Chapter 1) and General Discussion (Chapter 6). The Supplementary Material for each Chapter is included in the Appendix of this thesis.

In Chapter 2, I include a published systematic review I conducted on mathematical models used to study horizontal gene transfer of AMR in bacteria (Leclerc, Lindsay and Knight, 2019). In Chapter 3, I present my published research article combining laboratory work and mathematical modelling to reveal the dynamics of phage predation and generalised transduction in *S. aureus* (Leclerc *et al.*, 2022). In Chapter 4, I include my follow-up work extending the model from Chapter 3 to study the joint effect of antibiotics and phage capable of transduction on bacteria, available as a preprint at the time of writing (Leclerc, Lindsay and Knight, 2022). In Chapter 5, I present an analysis of routinely collected hospital data to identify evidence of within-host AMR diversity in *S. aureus*, not yet publicly available. The final General Discussion Chapter then brings together and discusses the conclusions from this research.

1.2 The global public health threat of antimicrobial resistance

1.2.1 The biology of antimicrobial resistance

1.2.1.1 Bacteria and antibiotics

The human body is host to 10^{13} bacteria on average, which is the same order of magnitude as the number of human cells (Sender, Fuchs and Milo, 2016). The majority of the time, these microscopic organisms in our bodies are harmless, existing as commensal organisms in different organs such as our gut, skin, or nose (Sender, Fuchs and Milo, 2016). However, some bacteria are able to cause infections, when they rapidly proliferate and interfere with the normal function of our body. If our immune system is unable to eradicate the causative bacterial population, these infections can be life-threatening or lead to severe sequelae in individuals. Bacterial infections are therefore responsible for a significant burden of disease worldwide (Murray *et al.*, 2022). To treat them, the main weapon at our disposal are antibiotics. These are molecules that specifically target and kill bacteria (bactericidal) or prevent their growth (bacteriostatic), allowing the immune system to bring the infection under control (Silverman and Holladay, 2014).

One of the most important early antibiotics was penicillin, discovered by Alexander Fleming in 1928 (Fleming, 1929). This bactericidal molecule binds to penicillin-binding proteins (PBPs), inactivating them irreversibly (Waxman and Strominger, 1983). PBPs are required in the construction of the bacterial cell wall, therefore their inhibition leads to instability in the cell wall, and eventually bacterial lysis (Sauvage *et al.*, 2008). When it was first made widely available in the 1940s, penicillin was used to treat various blood, respiratory, and sexually transmitted bacterial infections (Dawson and Hobby, 1944; Herrell, 1944).

Since then, other antibiotics, often derived from compounds naturally produced by fungi or bacteria, have been discovered or engineered, targeting different bacterial mechanisms (Hutchings, Truman and Wilkinson, 2019). The main antibiotic classes, alongside example antibiotic, mode of action, and resistance, are presented in Table 1.1. Antibiotics have substantially reduced the number of deaths due to bacterial infections, partly due to their accessibility. Although some antibiotics will only be given to patients in a healthcare setting (e.g. vancomycin, generally delivered intravenously), they are often taken orally and directly obtained by individuals in pharmacies after being prescribed by a doctor (e.g. nitrofurantoin, frequently prescribed for urinary tract infections). However, antibiotic consumption is less regulated in many settings with low healthcare infrastructure, and individuals may gain access to antibiotics without a prescription (Do *et al.*, 2021). As discussed in the next section, this unregulated use of antibiotics is important in the context of AMR. In any case, antibiotics are most often taken empirically, before the exact nature of the pathogen responsible for an infection is known (Versporten *et al.*, 2018).

Table 1.1: Main antibiotic classes, with examples of antibiotics and resistances. Highlighted in bold are antibiotics and resistance mechanisms particularly relevant in this thesis.

Class	**Example antibiotic**	**Mechanism of action**	**Example resistance mutation/gene (example bacteria where the resistance has been identified)**	**Mechanism of resistance**	**Reference**
Aminoglycosides	Gentamicin	Binds to the ribosome 30S subunit, leads to erroneous protein synthesis	*aacA/aphD* (*S. aureus*)	Aminoglycoside-modifying enzyme, inactivates the antibiotic	(Rouch *et al.*, 1987)
Carbapenems	Meropenem	Binds to penicillin-binding proteins, inhibits cell wall synthesis	*blaKPC* (*K. pneumoniae*)	Beta-lactamase, degrades antibiotic molecules	(Ghasemnejad, Doudi and Amirmozafari, 2019)
Cephalosporins	Ceftriaxone	Binds to penicillin-binding proteins, inhibits cell wall synthesis	*ampC* (*E. coli*)	Beta-lactamase, degrades antibiotic molecules	(Jacoby, 2009)
Glycopeptides	**Vancomycin**	**Binds to cell wall peptides, prevents cross-linking and cell wall synthesis**	***vanA* (*S. aureus*)**	**Alters the peptide terminal, prevents antibiotic binding**	(Périchon and Courvalin, 2009)
Lipopeptides	Daptomycin	Inserts into the cell membrane, causes leakage of ions and cell death	*cls* (*S. aureus*)	Alters the cell membrane composition, prevents antibiotic insertion	(Tran, Munita and Arias, 2015)
Lincosamides	Clindamycin	Binds to the ribosome 50S subunit, inhibts protein synthesis	***ermB* (*S. aureus*)**	**rRNA methylase causing a conformational change in the ribosome, prevents antibiotic binding**	(Schmitz *et al.*, 2000)
Macrolides	**Erythromycin**	**Binds to the ribosome 50S subunit, inhibts protein synthesis**			

Nitrofurans	Nitrofurantoin	Damages DNA and proteins non-specifically	*nfsA* (*E. coli*)	Encodes oxygen-insensitive nitroreductase, prevents antibiotic activation	(Osei Sekyere, 2018)
Oxazolidinones	Linezolid	Binds to the ribosome 50S subunit, inhibits protein synthesis	23S rRNA gene (*S. aureus*)	Point mutation in the 23S rRNA gene, prevents antibiotic binding	(Foster, 2017)
Penicillins	**Flucloxacillin**	**Binds to penicillin-binding proteins, inhibits cell wall synthesis**	***mecA* (*S. aureus*)**	**Encodes an alternative penicillin-binding protein, prevents antibiotic binding**	(Pinho, de Lencastre and Tomasz, 2001)
Polypeptides	Colistin	Changes the permeability of the outer membrane, causes cell leakage	*mcr-1* (*E. coli*)	Alters cell membrane lipids, prevents antibiotic binding	(Li *et al.*, 2020)
Quinolones	Ciprofloxacin	Binds to topoisomerases, prevents changes in DNA topology and cell division	*norA* (*S. aureus*)	Efflux pump, removes the antibiotic from within the cell	(Yoshida *et al.*, 1990)
Streptogramins	Quinupristin/dalfopristin	Binds to the ribosome 50S subunit, inhibits protein synthesis	*vgaA* (*S. aureus*)	Encodes a binding protein, removes the antibiotic from within the cell	(Gentry *et al.*, 2008)
Sulfonamides	Sulfamethoxazole	Competes with bacterial enzymes to prevent synthesis of folic acid, prevents bacterial DNA synthesis and growth	*dfrA* (*S. aureus*)	Encodes an alternative dihydrofolate reductase, prevents antibiotic binding	(Dale *et al.*, 1995)

Tetracyclines	**Tetracycline**	**Binds to the ribosome 30S subunit, inhibits protein synthesis**	***tetK* (*S. aureus*)**	**Efflux pump, removes the antibiotic from within the cell**	(Khan and Novick, 1983)
Others	Chloramphenicol	Binds to the ribosome 50S subunit, inhibits protein synthesis	*cfr* (*S. aureus*)	rRNA methylase causing a conformational change in the ribosome, prevents antibiotic binding	(LaMarre *et al.*, 2013)

1.2.1.2 Definition of AMR

“I would like to sound one note of warning. [...] The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant.” - Alexander Fleming, Nobel Lecture 1945

Unfortunately, antibiotics are not infallible, as highlighted by Alexander Fleming during his Nobel Prize Lecture (citation above). Antimicrobial resistance (AMR) describes resistance in microorganisms to treatments against them, and therefore includes antibiotic resistance in bacteria. Resistance in bacteria is generally defined by measuring the minimum concentration of an antibiotic required to prevent growth of these bacteria (“minimum inhibitory concentration”; MIC) (Wheat, 2001). For each antibiotic, there are cut-off MIC values used to determine whether bacteria are classified as susceptible or resistant (Wheat, 2001). The most commonly used guidelines for this purpose are from the European Committee on Antimicrobial Susceptibility Testing (*EUCAST: Clinical breakpoints and dosing of antibiotics*, 2022) and the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2022). Although this binary susceptible/resistant classification is useful clinically to identify which antibiotics should be used to treat infections, it does not fully capture the true diversity of AMR. In reality, two bacteria labelled as resistant to the same antibiotic can show variations in their actual resistance levels, with one being completely insensitive to the antibiotic, and the other still affected by a slight reduction in growth rate (Mueller, de la Peña and Derendorf, 2004). Understanding this diversity is essential to identify how resistant bacteria may persist, further adapt, and be transmitted to other individuals.

1.2.1.3 Mechanisms of AMR

Antibiotic resistance in bacteria can be spontaneously acquired by mutations in the bacterial DNA, or following acquisition of a gene encoding a resistance mechanism. An example of resistance acquired by mutation is linezolid resistance in *S. aureus*, due to a point mutation in the 23S ribosomal RNA gene which prevents the antibiotic molecules from binding to their target on the ribosome (Meka *et al.*, 2004). An example

of resistance following gene acquisition is a new efflux pump encoded by the *tetK* gene in *S. aureus* which actively removes tetracycline molecules from within the cell before they can bind to the bacterial ribosomes (Trzcinski *et al.*, 2000).

Some resistances can be intrinsic as opposed to acquired, where an antibiotic is inherently unable to affect a bacterium because its target is not available, such as vancomycin resistance in Gram-negative bacteria (Miller, 2016). These are bacteria with an outer membrane of lipopolysaccharides, as opposed to Gram-positive bacteria which only have a peptidoglycan cell wall (Salton and Kim, 1996). Vancomycin molecules are too large to pass through the porins naturally present in the Gram-negative outer membrane, and therefore cannot reach their target peptides and prevent cell wall synthesis (Miller, 2016).

Further examples of mechanisms of resistance to each of the antibiotic classes are presented in Table 1.1. As there are many resistance mechanisms, the overall resistance profiles (defined as the unique combination of resistances and susceptibilities of a bacteria to multiple antibiotics) can vary even between bacteria belonging to the same species, and the presence of multiple resistances in a single bacterium is increasingly common (Tanwar *et al.*, 2014). In addition, there are instances where a single gene can provide resistance to multiple classes of antibiotics, such as the *ermB* gene providing resistance to lincosamides and macrolides (Table 1.1). It is important to note that, by itself, the appearance and acquisition of new antibiotic resistance mechanisms by bacteria is a random process, and that antibiotics are the evolutionary pressure selecting for these resistances (Lipsitch and Samore, 2002). Accessibility, presented as a strength of antibiotics in the previous section, is therefore also a weakness, as misuse of antibiotics is a key factor responsible for increases in antibiotic resistance, for example via inappropriate antibiotic prescribing (e.g. for respiratory viral infections) or incorrect antibiotic usage by individuals (e.g. not completing a course of antibiotics).

Fortunately, the resistance mechanisms described above often impose a fitness cost on bacteria, reducing their growth rate and their ability to compete for resources with other organisms which may be present in their environment (Melnyk, Wong and Kassen, 2015). For example, a conformational change preventing antibiotic binding

might reduce the ribosome efficiency to translate messenger RNA to proteins (Powers and Noller, 1991), and an efflux pump will require additional bacterial resources to be operational (Alonso *et al.*, 2004). In settings where antibiotics are absent, this reduces the competitiveness of bacteria expressing AMR genes versus bacteria not expressing these genes (Melnyk, Wong and Kassen, 2015). These fitness costs can limit the increase in the prevalence of resistant bacteria under natural circumstances, and prevent bacteria from accumulating multiple resistances (Levin *et al.*, 1997). However, there are often multiple different genes granting resistance to the same antibiotic, hence two bacteria resistant to the same antibiotic may not be resistant via the same mechanism, and may not be affected by the same fitness cost. Importantly, bacteria carrying one or more antibiotic resistance genes can continue to evolve over time, acquiring compensatory mutations in the resistance genes or elsewhere in the genome to overcome these fitness costs (Andersson and Hughes, 2010; Knight, Budd and Lindsay, 2013). This includes mutations on mobile genetic elements (MGEs) which may carry these resistance genes (further discussed in the next section), such as plasmids (Zwanzig *et al.*, 2019). Persistence of resistance in bacteria despite the absence of antibiotic use is therefore increasingly seen (Dorado-Morales *et al.*, 2021).

1.2.2 AMR and public health

1.2.2.1 Consequences of AMR

AMR is one of the greatest global public health challenges we are facing (World Health Organization, 2015). For almost all of the antibiotics currently available, bacteria displaying resistance have been identified (Ventola, 2015). Since the majority of antibiotics are prescribed empirically, before the causative pathogen and its potential resistance are known, then all other things equal, a higher rate of AMR will directly lead to more antibiotic treatment failures (Versporten *et al.*, 2018). This translates to worse health outcomes for individuals, including prolonged illness such as recurrent urinary tract infections by antibiotic-resistant bacteria, which require further usage of antibiotics and hence further worsen the problem of AMR (Wagenlehner *et al.*, 2022).

Due to AMR, many common infections that could previously be cured with a single antibiotic dose now require complex treatment strategies (World Health Organization, 2021). The significance of this problem is illustrated by the global number of deaths due to infections by antimicrobial-resistant organisms (not bacteria only), which was initially estimated at around 700,000 per year (O'Neill, 2016). This same report suggested that if policies remained unchanged, this number could increase up to 10 million by 2050 (O'Neill, 2016). Unfortunately, we are fast progressing towards the worst case scenario, as the most recent estimate of AMR burden calculated that 1.2 million (95% uncertainty interval: 0.911–1.71) deaths in 2019 alone may be attributable to infections by antibiotic-resistant bacteria (Murray *et al.*, 2022). This same study estimated that 275,000 (161,000–439,000) years lived with disability may be attributable to infections by antibiotic-resistant bacteria in 2019, illustrating the impact of this problem on the long-term health of individuals. In addition, AMR leads to financial costs for society, by reducing the productivity of individuals infected by resistant bacteria, and requiring supplementary medical interventions to treat these individuals. Attempts to estimate the economic cost of AMR concluded that this could reach several billions of dollars per country per year (Shrestha *et al.*, 2018).

1.2.2.2 Interventions against AMR

To reduce the threat posed by AMR, many interventions have been suggested and are still being developed. For example, antibiotic stewardship regroups several interventions which aim to encourage individuals to use antibiotics more responsibly, reducing the selection pressure for resistant bacteria (King *et al.*, 2016; Price *et al.*, 2018). Stewardship can include incentives for doctors to reduce their antibiotic prescription rates, only prescribing them when necessary, or informing patients about the function of antibiotics and why these are not always the default answer to every health problem. Similarly, rapid diagnostic tests to identify resistant bacteria faster and prevent unnecessary antibiotic use are in active development (Vasala, Hytönen and Laitinen, 2020). Separately from antibiotic stewardship which aims to preserve the value of existing antimicrobials, novel antimicrobial agents are being investigated, both new antibiotics and alternative compounds to target bacteria via a wide range of mechanisms, overcoming existing resistances (Vila, Moreno-Morales and Ballesté-Delpierre, 2020). Improved economic incentives are required to encourage the development of these new antibiotics, such as the new subscription model recently

launched in the UK (Mahase, 2020). Finally, interventions against infections in general, not specifically targeting AMR (e.g. vaccines, improved sanitation), can still be effective to directly reduce the incidence of infections by resistant bacteria. Such non-specific interventions can also provide an indirect effect against AMR, as a general decrease in bacterial infections will reduce antibiotic usage, and hence selection for resistance.

1.3 The problem of methicillin-resistant *Staphylococcus aureus*

1.3.1 Biology of *S. aureus*

Staphylococcus aureus (*S. aureus*) is a Gram-positive bacterium first identified in 1884 (Rosenbach, 1884). It belongs to the *Staphylococcus* genus, and possesses the spherical shape (coccal) that characterises this group of bacteria (Foster, 1996; Taylor and Unakal, 2022). *S. aureus* bacteria typically form grape-like clusters, and the "aureus" (Latin for "golden") suffix reflects their golden-yellow colour when grown on blood agar plates. *S. aureus* replicate fairly rapidly, with a doubling-time of approximately 30 minutes in rich medium at 37 degrees Celsius (Missiakas and Schneewind, 2013). These bacteria are facultative anaerobes, able to survive in a wide range of environments with or without oxygen (Foster, 1996; Taylor and Unakal, 2022).

The complete genome size of *S. aureus* bacteria is approximately 2.8 million base-pairs (Holden *et al.*, 2004). The current nomenclature used to structure *S. aureus* populations into lineages originates from multilocus sequence typing (MLST) (Enright *et al.*, 2000). This technique focuses on the identification of seven housekeeping genes in the *S. aureus* chromosome (i.e. genes that should always be present in all isolates). MLST defines the sequence type (ST) of an isolate depending on the versions (alleles) of each housekeeping gene carried by that isolate. Isolates with at least 5 alleles in common between their STs are then grouped into clonal complexes (CCs), named according to the predicted ancestor ST (e.g. isolates belonging to CC22

all are predicted to have ST22 as ancestor) (Feil *et al.*, 2003). Almost all *S. aureus* isolates contain a type I restriction-modification system, which degrades foreign DNA introduced in the bacteria. Importantly, the *hsdS* genes which control the specificity of this system vary between lineages (Waldron and Lindsay, 2006). Consequently, isolates belonging to one CC consider DNA fragments from other CCs as foreign and target them for degradation. This represents a barrier to the exchange of genetic material between CCs, and explains the independent evolution of CCs (Lindsay, 2014). MLST has now been replaced for lineage identification by more powerful molecular and easy to use techniques, such as *spa* typing which identifies variations in the gene encoding for surface protein A (Harmsen *et al.*, 2003), and microarrays which classify isolates according to hundreds of core variable genes (Lindsay *et al.*, 2006),

In addition to their core genome, *S. aureus* possess a large number of mobile-genetic elements (MGEs) (Alibayov *et al.*, 2014). These are genetic elements that can move within a bacterium, such as transposons (DNA sequences which can relocate in a genome via excision and reinsertion), or between bacteria, such as plasmids (extrachromosomal DNA capable of independent replication). As discussed later in this Introduction, MGEs frequently encode AMR genes or virulence factors, increasing the pathogenicity of the bacteria carrying them (Alibayov *et al.*, 2014). Due to the restriction-modification systems mentioned above which restrict the movement of genetic material between *S. aureus* lineages, the distribution of MGEs varies between lineages (McCarthy and Lindsay, 2012, 2013; McCarthy, Witney and Lindsay, 2012).

1.3.2 Epidemiology of *S. aureus*

1.3.2.1 *S. aureus* colonisation in humans

S. aureus is generally a commensal bacteria in humans, with approximately 20% of individuals colonised by *S. aureus* at any given time, typically in the nose and on the skin (den Heijer *et al.*, 2013). Colonisation is most likely to be transient, with individuals being repeatedly colonised for periods of 2-6 months at a time (Miller *et al.*, 2014). This colonisation is asymptomatic (i.e. without symptoms), and therefore generally

undetected. Individuals colonised by *S. aureus* are a reservoir for these bacteria, where they can replicate and diversify by exchanging genes (discussed later in this Introduction). Colonisations can directly impact infections, since individuals are more likely to be infected by the strains they carry (von Eiff *et al.*, 2001; Huang and Platt, 2003).

1.3.2.2 *S. aureus* infections in humans

S. aureus is an opportunistic pathogen, with infections normally controlled by the immune system in healthy individuals (Fournier and Philpott, 2005). The most common *S. aureus* infections are minor skin infections, which are resolved without any treatment (Ryu *et al.*, 2014; O'Gara, 2017). *S. aureus* bacteria are not normally able to able to cross skin and mucosal barriers to reach organs where they may cause severe infections. Individuals suffering from severe *S. aureus* infections, such as bloodstream infections, are therefore often immunocompromised, and thus found in hospitals and other care facilities (O'Gara, 2017). Hence, *S. aureus* infections in humans are often healthcare-associated, also called "nosocomial infections". In Europe in 2020, more than 70,000 *S. aureus* isolates from bloodstream infections were recorded in the European Centre for Disease Prevention and Control (ECDC) Surveillance ATLAS (European Centre for Disease Prevention and Control, 2022).

S. aureus is identified as the causative pathogen of an infection by analysing a sample taken from the infected patient in a microbiology diagnostic laboratory. Common diagnostic methods include evidence of bacterial growth by plating the sample on selective culture media (Baird and Lee, 1995), coagulase agglutination test (van Griethuysen *et al.*, 2001), and Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (Dubois *et al.*, 2010). Uncomplicated *S. aureus* skin infections can be treated using topical antibiotics (applied directly to the skin) such as mupirocin (Tong *et al.*, 2015). Treatment of *S. aureus* bloodstream infections involves antibiotics, typically anti-staphylococcal penicillins such as flucloxacillin, delivered to patients intravenously for at least two weeks (Kimmig *et al.*, 2021). Prophylactic treatment is also essential in cases where patients are known to be at high risk of infection, such as after a surgery.

1.3.2.3 *S. aureus* in other ecosystems

Aside from humans, *S. aureus* can be found in many different environments, including surfaces within households (Uhlemann *et al.*, 2011; Fritz *et al.*, 2014), and environment-mediated transmission likely plays a key role in the spread of these bacteria. *S. aureus* can also be found in animals, including pets which may be an important source of infection for humans (Davis *et al.*, 2012; Bierowiec, Płoneczka-Janeczko and Rypuła, 2016). *S. aureus* infections in livestock can cause diseases such as chicken pododermatitis and cow mastitis (Smith, 2015). Although livestock-associated *S. aureus* lineages are different from lineages found in humans, suggesting that cross-species infections are uncommon, animals represent an important reservoir for *S. aureus* antibiotic resistance genes (McCarthy *et al.*, 2011; McCarthy, Lindsay and Loeffler, 2012; Moodley *et al.*, 2012; Larsen *et al.*, 2022).

1.3.3 The problem of AMR in *S. aureus*

1.3.3.1 Methicillin-resistant *S. aureus*

Penicillin was originally the antibiotic of choice to treat *S. aureus.* However, resistance rapidly appeared amongst bacterial populations, with more than 90% of *S. aureus* now carrying penicillinases, enzymes which actively degrade penicillin molecules (Lowy, 2003). Other antibiotics from the penicillin class were therefore introduced to control *S. aureus* infections, such as methicillin, which is not affected by penicillinases. Unfortunately, antibiotic resistance was again selected for as a consequence of this introduction (Chambers and DeLeo, 2009). Methicillin-resistant *S. aureus* (MRSA) refers to strains of *S. aureus* resistant to all beta-lactam antibiotics (as opposed to methicillin-susceptible *S. aureus*, MSSA). Beta-lactam antibiotics include penicillins, but also cephalosporins and carbapenems. Methicillin resistance in staphylococcal bacteria was first identified in the 1960s (Barber, 1964), with all MRSA strains found to carry the *mecA* genetic element (Kuhl, Pattee and Baldwin, 1978; Beck, Berger-Bächi and Kayser, 1986). This *mecA* gene was then shown to encode PBP2a, which has a reduced affinity for beta-lactam antibiotics compared to the regular bacterial PBP (Pinho, de Lencastre and Tomasz, 2001). This alternative protein therefore grants

beta-lactam resistance to *S. aureus* by preventing the antibiotics from inhibiting cell wall synthesis.

Later, it was discovered that the *mecA* gene was part of a DNA cassette in the *S. aureus* chromosome, named “staphylococcal cassette chromosome *mec*” (SCC*mec*) (Katayama, Ito and Hiramatsu, 2000). Different types of SCC*mec* have been identified, carrying other resistance genes alongside *mecA* (International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements, 2009). Alongside the clonal complexes mentioned previously, the SCC*mec* type can be used to structure *S. aureus* isolates into groups, as current evidence suggests that SCC*mec* elements are rarely gained or lost by *S. aureus* (Hanssen and Ericson Sollid, 2006; Scharn, Tenover and Goering, 2013; Maree *et al.*, 2022).

1.3.3.2 Epidemiology of MRSA

As for all *S. aureus* strains, MRSA can be responsible for infections, notably of the blood or the skin (Tong *et al.*, 2015). As for MSSA, MRSA infections are often healthcare-associated (HA-MRSA). MRSA can also colonise individuals asymptomatically (Kluytmans, van Belkum and Verbrugh, 1997). This again highlights the importance of understanding this pathogen in general, not only in an infection context, as individuals colonised by an MRSA strain will then be more likely to be infected by this same strain. In 2020, 13.2% of *S. aureus* isolates recorded in the ECDC Surveillance ATLAS were MRSA (European Centre for Disease Prevention and Control, 2022). MRSA is a significant public health threat, responsible for the second highest burden of disease amongst the antibiotic-resistant pathogens most commonly isolated from blood or cerebrospinal fluid in Europe in 2015 (Cassini *et al.*, 2019). MRSA was estimated to be the only pathogen-drug combination responsible for more than 100,000 deaths globally in 2019 (Murray *et al.*, 2022).

In addition, the incidence of community-associated MRSA (CA-MRSA) is increasing, indicating that this problem goes beyond healthcare environments (Kong, Johnson and Jabra-Rizk, 2016). The type of SCC*mec* elements varies between CA- and HA-MRSA, with type V more commonly found in CA-MRSA, as opposed to types I, II and III for HA-MRSA (Diep *et al.*, 2006). CA-MRSA often carry genes encoding Panton-Valentine leucocidin, a toxin associated with increased severity for skin and soft-tissue

infections (Shallcross *et al.*, 2013). Although CA-MRSA isolates are epidemiologically distinct from HA-MRSA, the increased incidence in severe infections caused by CA-MRSA translates to more patients infected by this *S. aureus* group being admitted to hospitals, and therefore an increased prevalence of CA-MRSA in healthcare settings (Lindsay, 2010). The relative importance of CA- and HA-MRSA varies geographically, with HA-MRSA being more common in Europe, while in the United States the burdens of CA- and HA-MRSA are similar (Otter and French, 2010). Livestock-associated MRSA (LA-MRSA) represents another distinct group of MRSA which typically colonise pigs, and carry tetracycline resistance genes (Wulf and Voss, 2008). Although LA-MRSA may not transmit efficiently between humans, individuals in close contacts with colonised animals (e.g. farmers, veterinarian etc.) may become colonised, and therefore be themselves at higher risk of MRSA infection (Lindsay, 2010). For these reasons, and due to the potential for strains to rapidly gain further resistances, MRSA is listed on the antibiotic-resistance priority pathogens list from the World Health Organization, and the ESKAPE list (Rice, 2008; Tacconelli *et al.*, 2018).

Once *S. aureus* has been identified as the causative pathogen in an infection, further tests are routinely conducted to distinguish between MSSA and MRSA. These include a latex agglutination test to detect the presence of PBP2a, a polymerase chain reaction to detect the *mecA* gene, or an antibiotic susceptibility test to detect beta-lactam resistance (van Griethuysen *et al.*, 1999). MRSA bloodstream infections are generally treated using intravenous vancomycin, or daptomycin (Kimmig *et al.*, 2021). These antibiotics are less convenient than those used for MSSA treatment, as they have a higher risk of causing nephrotoxicity (kidney damage) (The National Institute for Health and Care, 2022b, 2022a). In addition, these are antibiotics with poor bioavailability (capacity to reach their target site in the human body whilst still active), which further complicates their usage as they must be administered over long durations with careful monitoring of concentrations (Patel, Preuss and Bernice, 2022).

1.3.3.3 Other antibiotic resistances in *S. aureus*

Alongside broad beta-lactam resistance in MRSA, *S. aureus* strains can carry many different genes or mutations giving resistance to essentially all classes of antibiotics currently available for treatment (Foster, 2017). Multiple resistances are most common in MRSA, whilst MSSA isolates tend to be resistant to only one or two antibiotics

(Chambers and DeLeo, 2009). Importantly, many of these resistance genes are on MGEs, allowing them to spread rapidly in a bacterial population (discussed later in this Introduction) (Haaber, Penadés and Ingmer, 2017).

Of particular interest is fluoroquinolone resistance, as antibiotics from this class are not used to treat *S. aureus* infections. Instead, fluoroquinolone resistance in MRSA may be evidence of bystander selection, where usage of this antibiotic to treat an unrelated infection in an individual selects for resistance in *S. aureus* present in the same individual (Knight *et al.*, 2012; Tedijanto *et al.*, 2018). Alternatively, this may be due to co-selection, whereby selection for one antibiotic resistance in a bacterial population jointly selects for resistance to a different antibiotic, even in the absence of this second antibiotic. This can occur because of linkage, when the two resistance genes are co-located on a single MGE, and are therefore frequently present together in bacteria. Interestingly, fluoroquinolone exposure is a risk factor for MRSA colonisation (Couderc *et al.*, 2014), hence understanding the mechanism behind selection of this resistance could reveal insights into potential strategies to control the incidence of MRSA. Vancomycin resistance is a major preoccupation since, as mentioned above, this is currently the antibiotic of choice to treat severe MRSA infections (McGuinness, Malachowa and DeLeo, 2017). Widespread vancomycin resistance would substantially complicate the treatment of MRSA infections, and require alternative treatment strategies. Resistance can be acquired via the *vanA* gene, which prevents vancomycin from disrupting cell wall synthesis (Périchon and Courvalin, 2009). This gene is currently rare in *S. aureus*, but commonly found in enterococci, which are commensals of the human gut (French, 1998). Events where *S. aureus* acquired *vanA* from enterococci have previously been described (McGuinness, Malachowa and DeLeo, 2017).

1.3.3.4 Diversity in *S. aureus* populations

As highlighted above, there are many characteristics which can define *S. aureus* isolates. These characteristics are not uniformly distributed temporally and geographically. As mentioned above, there are distinct MRSA groups, each with their own reservoir (HA-MRSA, CA-MRSA, LA-MRSA), although these can cross-over between their respective settings (e.g. LA-MRSA colonisation in humans, or CA-MRSA infections in healthcare environments). The relative prevalence of the 10 main

S. aureus lineages in humans varies over time (Lindsay *et al.*, 2006); in the UK for example, the dominant MRSA lineage changed from CC30 to CC22 between 1999 and 2009 (Wyllie, Paul and Crook, 2011; Knight *et al.*, 2012). The distribution of *S. aureus* lineages also varies between countries and regions (Grundmann *et al.*, 2010). Similar geographical and temporal variations have been seen between the 13 different types of MRSA SCC*mec* elements (Hanssen and Ericson Sollid, 2006; Singh-Moodley *et al.*, 2020). It is currently unclear why variations in the dominant lineages or SCC*mec* elements occur, highlighting the importance of understanding how *S. aureus* strains evolve, and the selection pressures affecting them.

There can also be substantial diversity even between *S. aureus* isolates present within the same population (e.g. within the same host). Firstly, joint colonisation by both MRSA and MSSA is possible, with previous studies finding this to occur in 21% of hospital patients (Mongkolrattanothai *et al.*, 2011). This same study also detected multiple genotypes in 30% of patients, identified using multiple-locus variable-number tandem-repeat fingerprinting. Isolates in a single host can vary according to their antibiotic resistances, which is strongly linked to the MGEs they carry (Haaber, Penadés and Ingmer, 2017). A study from 2015 looking at within-host MRSA populations found that 24% of patients sampled carried multiple strains, each resistant to different antibiotics (Stanczak-Mrozek *et al.*, 2015). Finally, we see variation in the types of biological entities that coexist alongside *S. aureus* populations, bacteriophage, presented in the next section of this Introduction.

Information on this within-host diversity can be valuable to track transmission of *S. aureus* between individuals (Hall *et al.*, 2019). In addition, monitoring changes in AMR diversity over time may provide valuable insights into the frequency of AMR evolution within-host and the drivers of this evolution, which we must understand to design efficient interventions against this public health threat (Winstanley, O'Brien and Brockhurst, 2016). Although the substantial genetic diversity identified in the studies above suggests that gain and loss of genetic material occurs frequently in *S. aureus*, these studies only assessed individual within-host diversity at single timepoints. There is therefore a lack of longitudinal data to improve our currently limited understanding of how this diversity may change over time.

1.4 Bacteriophage: abundant predators of *S aureus*

1.4.1 Phage form a key part of the *S. aureus* ecosystem

1.4.1.1 Phage life cycles

Bacteriophage (phage) are viruses of bacteria, and likely the most abundant biological entities on Earth (Clokie *et al.*, 2011). There are more than 10 families of phage, defined by their morphology (shape, presence or absence of a protein tail etc.) and genome (size, single or double-stranded etc.) (Hyman and Abedon, 2012). Their main interaction with bacteria is a predator-prey relationship, as phage can infect and lyse bacteria through the "lytic cycle" (Figure 1.1). This process begins with a phage binding to a bacterium, and injecting its genetic material. The phage will then take over bacterial machinery to replicate, degrading bacterial DNA in the process. Importantly, as this process relies on bacterial machinery, the bacteria must be themselves capable of growth in order for the phage to replicate (Kokjohn and Sayler, 1991; Hadas *et al.*, 1997; Santos *et al.*, 2014). This replication process leads to multiple copies of the phage genome being created, as well as new phage capsids to package these genomes. Recognition of the phage DNA for packaging is mediated by the terminase subunit of the capsid. In *cos*-type phage, the terminase recognises specific *cos* sites at the beginning and end of a complete phage genome, allowing for precise packaging (Casjens and Gilcrease, 2009). Meanwhile, the terminase in *pac*-type phage only recognises a *pac* site to initiate packaging, and halts this process by making a non-specific cut in the DNA once the capsid is full, leading to the packaging of between 102-110% of the full phage genome in each capsid (Casjens and Gilcrease, 2009). Ultimately, the lytic cycle causes the bacterial cell to lyse, busting open to release the new phage in the surrounding environment.

In addition to this, some phage can undergo a "lysogenic cycle" upon initially entering the bacterium (Figure 1.1). In that case, the phage genome integrates in the bacterial chromosome, forming what is known as a "prophage", and remains in a dormant state. During that time, the lysogenic bacterium may undergo several rounds of replication, each time copying the prophage as part of its genome. Lysogenic bacteria typically acquire lysogenic immunity, protecting them from lytic infection by the same phage as

their prophage (Lwoff, 1953). Eventually, the prophage may excise from the chromosome, often upon activation of bacterial signalling pathways indicating stress, such as the SOS response. This can occur following bacterial DNA degradation by ultraviolet light or chemicals (e.g. mitomycin C) (Howard-Varona *et al.*, 2017). Upon excision of the prophage, the lytic cycle may then begin as detailed above.

The predator-prey relationship between phage and bacteria has led to a constant arms race between these organisms (Hampton, Watson and Fineran, 2020). Recent efforts have uncovered a substantial diversity in phage resistance mechanisms present in bacteria, with tools such as the PADLOC database to reveal and record new defence systems (Payne *et al.*, 2021). In addition to mechanisms such as restriction-modifications systems (mentioned earlier in this Introduction), CRISPR-Cas systems are a famous example of a bacterial defence mechanism (Barrangou *et al.*, 2007). These systems are composed of proteins (Cas) which target foreign DNA by matching it to fragments stored in a genetic library (CRISPR), allowing the bacteria to recognise and cleave phage DNA as soon as it enters the cell. Resistance can also be mediated by bacterial surface modification mutations, which prevent phage from binding to bacteria and initiating the infection process (Hampton, Watson and Fineran, 2020). Note that while such surface modifications are possible in *S. aureus*, current evidence suggests that these are exceedingly rare due to the fitness cost incurred (Dalen, Peschel and Sorge, 2020). Inversely, phage have developed mechanisms to neutralise these defences, such as anti-CRISPR systems (Bondy-Denomy *et al.*, 2013). There is therefore constant co-evolution between bacteria and phage, shaping the structure of these populations in various environments (Koskella and Brockhurst, 2014).

The ability for phage to undergo either the lytic or lysogenic cycle varies depending on the infected bacteria, environmental conditions, and between phage families. Phage only capable of undergoing the lytic cycle are referred to as “lytic phage”, while phage capable of entering the lysogenic cycle are called “temperate phage”. The lysogenic cycle requires the phage to carry specific integrase genes which allow them to integrate and persist in the bacterial chromosome (Deghorain and Van Melderen, 2012).

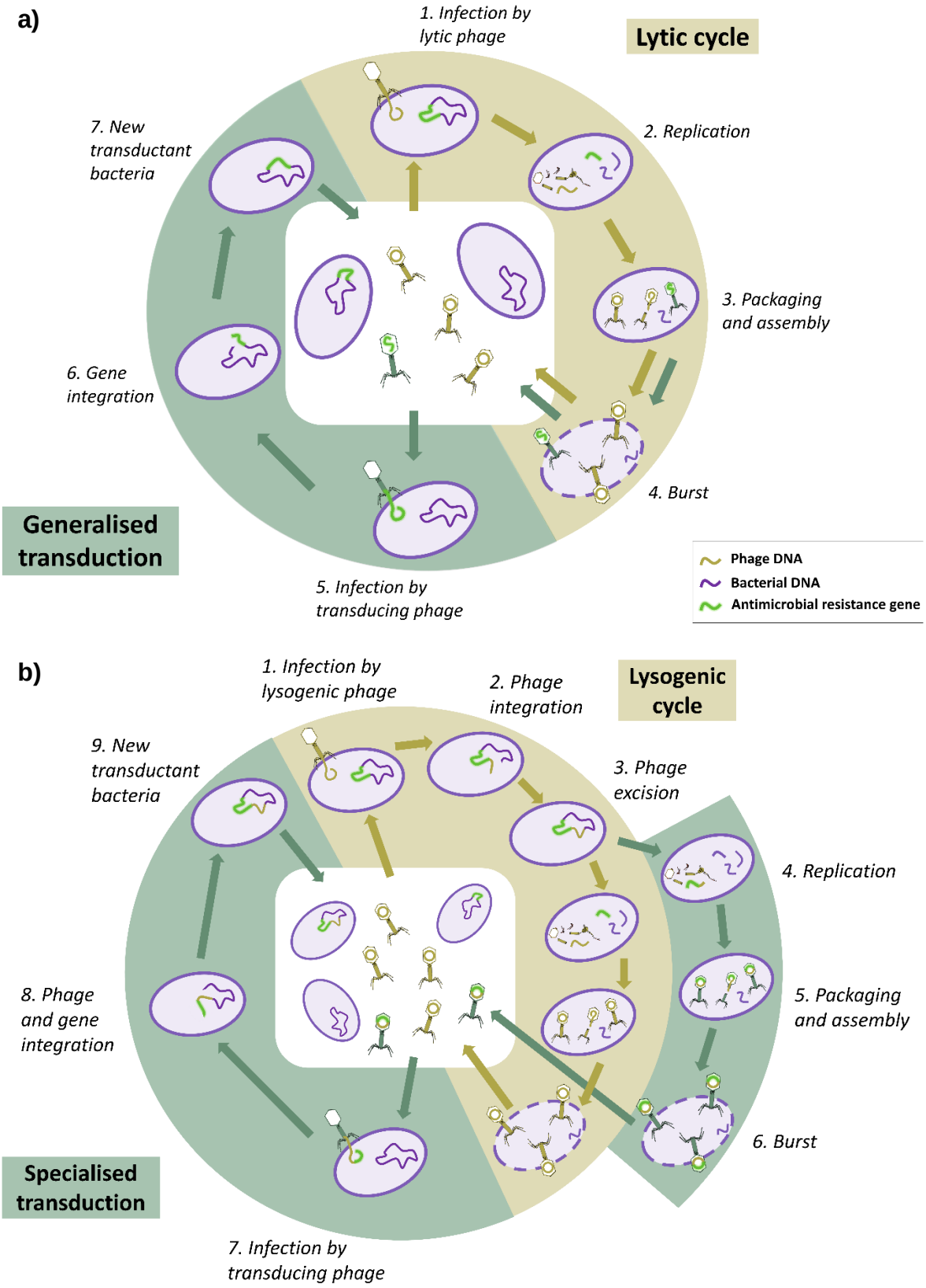


Figure 1.1: Phage life cycles and transduction. a) Lytic cycle and generalised transduction. b) Lysogenic cycle and specialised transduction. Figure adapted from (Leclerc *et al.*, 2022)**.**

1.4.1.2 Phage of *S. aureus*

Phage and *S. aureus* frequently coexist, with all known strains of *S. aureus* carrying at least one prophage, although the distribution of prophage varies between *S. aureus* lineages (McCarthy, Witney and Lindsay, 2012). Phage-bacteria coexistence has been observed in multiple species, and suggests the existence of mutualistic interactions between phage and bacteria, in addition to their typical prey-predator relationship (Shkoporov, Turkington and Hill, 2022). In *S. aureus*, this coexistence is partly explained by the fact that phage frequently carry genes that are beneficial to the bacteria, encoding immune evasion and virulence factors for example (Deghorain and Van Melderen, 2012). However, it is interesting to note that phage of *S. aureus* rarely encode AMR genes in their own genome (Kondo, Kawano and Sugai, 2021; Nepal *et al.*, 2021). A previous study of patients colonised with MRSA also detected free phage particles capable of lysing *S. aureus* in at least 50% of these patients (Stanczak-Mrozek *et al.*, 2015). Phage of *S. aureus* are mostly part of the *Siphoviridae* family, with long non-contractile tails and icosahedral capsids (Deghorain and Van Melderen, 2012). *S. aureus* phage genomes range from less than 20 to more than 125 kilo-base pairs (Kwan *et al.*, 2005), with the most common size being approximately 45 kilo-base pairs, equivalent to 1.6% of the complete *S. aureus* genome size (Deghorain and Van Melderen, 2012). Commonly studied bacteriophage of *S. aureus* include 80α and ϕ11, both labelled as temperate phage (i.e. able to enter the lysogenic cycle and integrate in the genome) since they carry integrase genes (Xia and Wolz, 2014).

1.4.2 Phage as a solution to MRSA

1.4.2.1 Phage therapy

The predator-prey relationship of phage and bacteria makes phage particularly interesting as novel antimicrobial agents, providing a potential solution to treat infections by antibiotic-resistant bacteria (Brives and Pourraz, 2020). Phage therapy was originally introduced in the early 20th century, and relies on using the lytic abilities of phage to decrease a bacterial population during an infection. Phage therapy design first requires the identification of phage that are highly lytic against the pathogen of interest, with multiple pipelines currently being developed for this purpose (Gelman *et*

al., 2021). The attractiveness of phage to treat bacterial infections is in part due to their narrow spectrum. Phage can only bind to specific receptor proteins present on their target bacteria, as opposed to antibiotics, which are less specific and can affect other bacteria in the same environment as the target, potentially causing undesirable side effects (Lin, Koskella and Lin, 2017). In *S. aureus* for example, the only known receptor for phage binding is wall teichoic acid (Park *et al.*, 1974).

1.4.2.2 Current status of phage therapy against MRSA

The ever-increasing risk of multidrug resistance in MRSA makes phage therapy an attractive alternative treatment strategy (Walsh *et al.*, 2021). Phage therapy against *S. aureus* is not currently commercially available, and treatment of patients is mostly restricted to compassionate use, to attempt to treat infections by bacteria resistant to all available antibiotics. Clinical trials of phage therapy against *S. aureus* are currently ongoing in patients with chronic or wound infections (Adaptive Phage Therapeutics, Inc., 2022; Centre Hospitalier Universitaire de Nīmes, 2022). Preliminary results suggest that phage therapy is safe and well tolerated in patients, but information on efficacy is limited (Fish *et al.*, 2016; Ooi *et al.*, 2019; Petrovic Fabijan *et al.*, 2020). Aside from these trials, there are ongoing *in vitro* efforts to identify other phage that may be used therapeutically (Lehman *et al.*, 2019; Berryhill *et al.*, 2021).

Several practical and regulatory challenges currently prevent widespread use of phage therapy to treat *S. aureus* infections (Brives and Pourraz, 2020). Firstly, the unique nature of phage as “living antimicrobials” leads to complex pharmacokinetics and pharmacodynamics (PKPD), which describe the fate of an antimicrobial (here, phage) during treatment, and how it affects its target (bacteria) (Payne and Jansen, 2003). This complexity arises due to the ability of phage to replicate whilst killing bacteria during treatment, as opposed to antibiotics PKPD which are mostly dependent on the initial concentration of antibiotics provided (Payne and Jansen, 2000; Nielsen and Friberg, 2013). Interestingly, this complexity could be leveraged in phage therapy design, by administering an initial dose of phage which by itself is too low to clear all the bacteria bacteria, yet will be able to ultimately achieve this as the phage will replicate whilst killing the bacteria (Payne and Jansen, 2001). This corresponds to active therapy, as opposed to passive therapy which requires a higher dose of phage to clear the bacteria without the need for replication. Secondly, bacterial resistance to

phage is a major preoccupation, as resistance evolution is frequently observed *in vitro* (Berryhill *et al.*, 2021). To reduce the risk of bacteria developing resistance to the phage during treatment, phage cocktails are being considered instead of single phage strains (Molina *et al.*, 2021). Phage cocktails consist of multiple different phage species, each with different infection mechanisms. This reliance on multiple infection mechanisms guarantees that the bacteria cannot easily become resistant to all the phage in the cocktail by acquiring a single resistance mechanism (Abedon, Danis-Wlodarczyk and Wozniak, 2021). Instead, they would have to acquire multiple resistance mechanisms, which is unlikely to occur in the interval between phage introduction and bacterial clearance. This also allows for greater odds of treatment success without requiring precise knowledge of the causative bacteria, since a greater diversity of phage infection mechanisms in the cocktail extends the range of bacteria against which therapy will be effective. However, this creates further challenges, as phage cocktail design requires the correct diagnosis of the bacteria responsible for an infection, and identification of the appropriate phage to target them. This places phage therapy in the category of personalised medicine (Loh and Leptihn, 2020), as opposed to antibiotics which affect bacteria more broadly and hence do not generally require such a detailed screening process to be matched to the specific bacteria responsible for the infection.

1.4.3 Phage, *S. aureus*, and antibiotics interactions

Although an objective of phage therapy research is to design standalone phage treatments, ethical considerations mean that in ongoing trials, phage are systematically delivered alongside antibiotics (Brives and Pourraz, 2020). Phage may also be more widely found alongside antibiotics in the environment (Larsson, 2014). It is therefore essential to understand how phage and antibiotics interact with each other. Previous studies, mostly focused on other organisms than *S. aureus*, have found evidence of both synergism and antagonism between phage and antibiotics (Abedon, 2019; Li *et al.*, 2021). One example in *S. aureus* is (Rahman *et al.*, 2011), who showed that a *S. aureus* population exposed to both phage and rifampicin at 10 x MIC will be affected by a stronger decrease than when exposed to either alone. On the other hand, (Valério *et al.*, 2017) find that an *E. coli* phage population will not increase if the

bacteria are simultaneously exposed to tetracycline at 1 x MIC, suggesting an antagonistic effect of the antibiotic. There is therefore conflicted evidence on this topic. Previous experimental work suggests that timing and concentration may determine the joint effect of phage and antibiotics on bacteria, including *S. aureus*, although the precise nature and dynamics of this relationship are still unclear (Lopes, Pereira and Almeida, 2018; Berryhill *et al.*, 2021). Furthermore, this joint effect may depend on the type of antibiotic used, as bactericidal antibiotics can complement the action of phage whilst bacteriostatic antibiotics may prevent phage replication, as they inhibit the bacterial machinery required for phage replication, hence limiting the usefulness of phage (Payne and Jansen, 2003; Berryhill *et al.*, 2021).

1.5 Phage-mediated AMR gene transfer in *S. aureus* by generalised transduction

1.5.1 Transduction as a mechanism of horizontal gene transfer of AMR

1.5.1.1 Biology and types of HGT

As is the case with other organisms, genes spread in bacterial populations by “vertical gene transfer”, where they are inherited by progeny bacteria after the division of their parent, but also by “horizontal gene transfer” (HGT) (Hall, Brockhurst and Harrison, 2017). Here, I use the term HGT to refer to the capacity of bacteria to exchange genetic material between themselves, including bacterial chromosome genes and MGEs. The latter can persist in the bacteria by either existing independently of the core bacterial genome, or integrating into it, and can contain virulence as well as AMR genes, increasing bacterial pathogenicity (Ochman, Lawrence and Groisman, 2000; Thomas and Nielsen, 2005; von Wintersdorff *et al.*, 2016). There are three main mechanisms by which HGT can occur: conjugation, transformation and transduction.

In conjugation, bacteria form a conjugative bridge to directly exchange DNA (Ochman, Lawrence and Groisman, 2000). This mechanism requires the bacteria to be physically

located next to each other, which limits the speed at which genes can spread throughout a bacterial population. Conjugation of AMR genes is common in *Enterobacteriaceae* for example (Huddleston, 2014).

On the other hand, transformation designates the capacity for bacteria to uptake DNA from their surrounding environment, which can be present after other bacteria have lysed and released their own genetic material (Ochman, Lawrence and Groisman, 2000). In addition to facilitating intra-species HGT, this mechanism is likely an important method for inter-species gene exchange since it does not require the bacteria to achieve direct physical contact (von Wintersdorff *et al.*, 2016). Transformation was first identified in *Streptococcus pneumoniae* (Griffith, 1928), and transfer of AMR genes by this method was subsequently observed in other species such as *Neisseria* and *Bacillus* (von Wintersdorff *et al.*, 2016).

1.5.1.2 Specialised and generalised transduction

Transduction, which is the focus of this thesis, broadly describes the ability for phage to act as vectors of DNA, moving genes between bacteria; here, I specifically use the term transduction to describe the transfer of bacterial genes by phage (Griffiths *et al.*, 2000). Tied to the two life cycles of phage, there are two main types of transduction: generalised and specialised (Figure 1.1). Generalised transduction occurs during the lytic cycle, at the phage packaging step. It is the result of an error where the terminase accepts DNA without the correct *pac* or *cos* site, leading to the packaging of bacterial DNA instead of the phage genome. Generalised transduction can in theory lead to the packaging of any DNA present in the bacteria, including plasmids. On the other hand, specialised transduction is linked to the lysogenic cycle. This occurs when the prophage excises from the bacterial chromosome and accidentally picks up adjacent bacterial DNA. This type of transduction is therefore restricted to DNA adjacent to the phage insertion site in the bacterial chromosome. The resulting transducing phage will carry both phage and bacterial DNA.

Variations of generalised and specialised transduction have been identified in recent years. Auto-transduction for example describes a specific interaction involving lysogenic phage and transduction (Haaber *et al.*, 2016). The principle of auto-transduction is that bacteria which acquire a prophage will gain lysogenic immunity,

protecting them from lysis by phage of the same species. However, phage can still bind to these lysogenic bacteria and inject the DNA they contain. Therefore, these lysogenic bacteria can still benefit from transduction, accumulating new genes whilst being protected from lysis by the phage.

Another identified mechanism is lateral transduction, a variation of specialised transduction, where phage packaging occurs before a prophage is excised from the bacterial chromosome (Chen *et al.*, 2018). This has been suggested as leading to transfer of bacterial DNA located several thousands of kilobases downstream of the prophage, much further than theoretically possible by regular specialised transduction.

Overall, these studies highlight the many unknown aspects of transduction that still remain, finding instances of gene transfer mediated by bacteriophage which cannot be easily explained using our current biological understanding of this HGT mechanism.

1.5.1.3 Complex dynamics of transduction

The dynamics of transduction are unique compared to conjugation and transformation, in the sense that this mechanism relies on a vector (the phage) to actively package and carry the DNA between bacteria, and that it will occur in parallel to killing of the bacteria by these same vectors. This means that rates of transduction may vary depending on the characteristics and concentrations of both phage and bacteria. For example, *pac* phage are likely able to perform generalised transduction more frequently that *cos* phage, due to their less selective packaging mechanism (Casjens and Gilcrease, 2009). Transduction is also affected by complex factors which broadly apply to all HGT mechanisms, including conjugation and transformation. For example, transduction in *S. aureus* has been found to occur at a higher rate *in vivo* rather than *in vitro*, suggesting that environmental conditions likely play an important role in this process (McCarthy *et al.*, 2014). The action of the immune system or antibiotics can affect bacterial or phage prevalence, potentially further affecting predation and transduction dynamics (Banuelos *et al.*, 2021). Antibiotics present at sub-inhibitory concentrations may also increase the production of transducing phage independently of lytic phage via currently unknown mechanisms (Stanczak-Mrozek, Laing and Lindsay, 2017).

1.5.1.4 Importance of HGT in the context of AMR

Observations in the last decades suggest that instead of considering the spread of AMR as analogous to the spread of bacteria carrying genes encoding AMR mechanisms, we should study the spread of these genes in the bacterial population. All the HGT mechanisms described above have been shown as capable of spreading AMR genes (von Wintersdorff *et al.*, 2016). A novel AMR gene may initially arise in one bacterial population, and subsequently rapidly spread to other populations belonging to the same family via HGT. For example, the *mcr-1* gene conferring colistin resistance, first identified in *E. coli* and subsequently in other Enterobacteriaceae (*Klebsiella*, *Salmonella* etc.), initially integrated in a single transposon which then stabilised in various plasmids, allowing the gene to spread across various Enterobacteriaceae populations (Wang *et al.*, 2018). HGT can also help bacteria to efficiently acquire multidrug resistance, since two separate bacterial populations could each independently acquire a different, novel resistance, then share these with each other. HGT can allow antibiotic resistance to persist in bacterial populations in cases where it would otherwise become extinct in the absence of a selection pressure (Brockhurst and Harrison, 2021). HGT can also help to maintain the overall bacterial population diversity in microbial communities, by increasing stability when these communities are exposed to antibiotics (Coyte *et al.*, 2022). For example, if a resistance gene can move easily between bacteria and spread, it will be present in many bacteria with diverse genetic profiles, and would allow all of these to survive antibiotic exposure. On the other hand, a non-mobile resistance gene will only be associated with one genetic profile, hence only this profile would remain following antibiotic exposure, likely reducing the genetic diversity of the bacterial community (Coyte *et al.*, 2022).

1.5.2 The complex dynamics of generalised transduction of AMR in *S. aureus*

1.5.2.1 The importance of generalised transduction as a mechanism of HGT of AMR in *S. aureus*

The major mechanism by which HGT occurs in *S. aureus* is likely to be transduction (Lindsay, 2014). As explained above, transduction is mediated by phage, and these are extremely common amongst *S. aureus* populations, with clinical *S. aureus* strains found to almost always carry at least one prophage in their genome (Lindsay *et al.*, 2006; McCarthy, Witney and Lindsay, 2012; Lindsay, 2014). There is clear evidence that transduction can occur at a high rate in *S. aureus in vitro* and *in vivo* (McCarthy *et al.*, 2014). Inversely, conjugation is rare in *S. aureus*, as an analysis of *S. aureus* plasmids found that only 5% of these carry the necessary *tra* genes to be self-transmissible by conjugation (McCarthy and Lindsay, 2012). As for transformation, previous work indicates that it is unlikely to frequently transfer small DNA fragments like AMR genes, as the sigma H factor required for this process is only expressed by *S. aureus* under specific environmental conditions (Morikawa *et al.*, 2012). However, transformation may be important in the extremely rare cases where it allows to the transfer of large portions of *S. aureus* genome, including SCC*mec* elements (Robinson and Enright, 2004).

Of the two mechanisms of transduction, generalised transduction has a greater potential to substantially contribute to the spread of many different AMR genes in *S. aureus*, as it can lead to the transfer of any bacterial DNA including plasmids, major vectors of AMR genes (von Wintersdorff *et al.*, 2016). In fact, many plasmids in *S. aureus* are less than 45kb in length, which matches the packaging size of common *S. aureus* phage and further suggests that these non-self-transmissible plasmids are transferred by generalised transduction (Deghorain and Van Melderen, 2012; McCarthy and Lindsay, 2012). On the other hand, specialised transduction can only lead to the transfer of AMR genes adjacent to the prophage insertion site in the bacterial chromosome. Although we are currently lacking data to quantify this, generalised transduction may occur substantially in the human population, since at

least 50% of individuals colonised by *S. aureus* also carry phage capable of generalised transduction (Stanczak-Mrozek *et al.*, 2015).

Previous attempts to quantify transduction of AMR in *S. aureus* and *E. coli* have estimated frequencies of transducing phage per non-transducing phage between 10^{-5} to 10^{-11}, depending on the bacteria and phage used (Jiang and Paul, 1998; Mašlaňová *et al.*, 2016). Note that these values were obtained by generating a mixed population of lytic phage and an unknown amount of transducing phage carrying a marker gene, briefly exposing bacteria to this mixed phage population, rapidly neutralising all the phage to prevent bacterial killing, and counting how many bacteria gained the marker gene via transduction during this exposure. These experiments therefore did not measure the incidence of transduction over time, instead providing only point estimates for the frequency of transducing phage in a phage population.

1.5.2.2 The risk of transduction in the context of *S. aureus* phage therapy

One key recommendation for phage therapy is to select phage which have a high capacity to lyse the bacteria, and a low capacity to perform transduction (Verheust *et al.*, 2010; Jassim and Limoges, 2014). Otherwise, if the phage fail to clear all of the bacteria, by performing transduction they could spread bacterial genes encoding for AMR mechanisms, virulence factors, or other undesirable characteristics in the bacterial population, leaving the patient at a subsequent risk of a more severe infection, or non-responsive to antibiotic treatment. As phage therapy aims to use lytic phage only, this removes lysogeny and therefore the risk of specialised transduction, but generalised transduction remains. As generalised transduction is a mistake during the lytic cycle, we currently do not know how to prevent it from happening. Yet, the consequences of generalised transduction happening during phage therapy are unknown, and recent reviews have highlighted the worrying lack of research on this topic (Raj and Karunasagar, 2019; Hassan *et al.*, 2021). No estimates for potentially acceptable rates of generalised transduction have yet been established. To the best of my knowledge, the risk for transduction to occur is not currently explored in ongoing clinical trials of *S. aureus* phage therapy, possibly due to insufficient evidence regarding its consequences, and the complexity of measuring the impact of

transduction of AMR *in vivo*. In theory, even if transduction occurred rarely, a more widespread use of phage for treatment could facilitate frequent within-host evolution of multidrug-resistant bacteria.

1.5.2.3 The unknown impact of transduction when phage, *S. aureus*, and antibiotics are jointly present

As mentioned earlier in this Introduction, the dynamics of phage predation and antibiotics are already complex, yet to my knowledge the consequences of transduction of AMR genes in an environment where bacteria, phage, and antibiotics are all jointly present are unknown. In addition to decreasing bacterial prevalence in an environment and potentially increasing transduction rates at sub-inhibitory concentrations (Stanczak-Mrozek, Laing and Lindsay, 2017), antibiotics may act as an important selection pressure for AMR genes transferred by transduction. For example, phage could generate multidrug-resistant bacteria by transduction, which may then gain a selective advantage due to the antibiotics present in the same environment. Under these conditions, multidrug-resistant bacteria may be able to persist for prolonged periods of time, during which they could be transmitted to individuals or acquire further adaptations such as phage resistance or compensatory mutations to overcome fitness costs.

Overall, the appearance and persistence of multidrug-resistant bacteria may therefore depend on the frequency of transduction, the predator-prey relationship of phage and bacteria, and the effect of antibiotics, potentially acting in synergy with phage or inhibiting phage replication via inhibition of bacterial growth. These interactions are complex, requiring specific tools to be disentangled and to reveal the underlying dynamics of bacteria, phage and antibiotics which are invisible when only using experimental data.

1.6 Mathematical modelling of AMR gene transfer by generalised transduction

1.6.1 Mathematical modelling of AMR

1.6.1.1 Principles of mathematical modelling

Mathematical modelling is a tool commonly employed to simulate real-life processes, with model structure and parameter values informed by our understanding of the underlying characteristics of these processes, and by data when available. A model provides an environment in which the dynamics of these real-life processes can be studied with greater flexibility. The concept of modelling infectious diseases was originally proposed by Kermack and McKendrick, as a method to summarise simple epidemic dynamics in a few equations (Kermack and McKendrick, 1927). Since then, models have been used to study more complex disease dynamics such as spatial heterogeneity in transmission, or vector-borne pathogens (Anderson and May, 2010; Heesterbeek *et al.*, 2015). Modelling is a powerful tool to gain an understanding of the dynamics governing in a system which cannot be obtained using classical experimental methods alone, and to identify potential opportunities to affect these dynamics. For example, a model of disease spread can be used to explore the theoretical effect of a reduction in contact rate between susceptible and infectious individuals to reduce the number of new cases. Mathematical models are now often implemented via computer software, allowing the equations to be solved numerically by running simulations, instead of only analytically.

1.6.1.2 Complexity in mathematical models

In general, the population represented in mathematical models is separated into compartments, and the numbers of individuals in each compartment are tracked over time as they change. The interactions between compartments are generally represented using a density- or frequency-dependent approach. An example of a density-dependent interaction is predator-prey dynamics in Lotka-Volterra equations, where the increase in predator numbers and decrease in prey numbers are dependent on the density of predator, multiplied by the density of prey and by an interaction term

(Bacaër, 2011). In that case, an increase in either predator or prey numbers leads to an equivalent increase in the strength of their interaction. On the other hand, frequency-dependent interactions are often used in mathematical models of infectious diseases in humans. In that case, the number of new infections during a time period *t* is estimated by multiplying the number of susceptible individuals, their contact rate over the period *t*, and the proportion of infected people in the total population (rather than number of infected) (Keeling and Rohani, 2008).. This is more biologically accurate in this context, since the contact rate of an individual during the period *t* is independent of the total population size, and hence limits the number of new infections (McCallum, Barlow and Hone, 2001). The clearest examples to show the importance of frequency-dependent transmission are sexually-transmitted infections, such as human immunodeficiency virus infection, since transmission relies on sexual contact, and the distribution of sexual partners in humans is only weakly correlated with total population size (May and Anderson, 1987).

Models can include more than one population, as is the case with malaria models for example which often include both humans and mosquitoes (Anderson and May, 2010). The number of compartments can range from two (e.g. in an infectious disease model, one for healthy and one for infected individuals), up to one compartment for each single individual in the model, in which case we talk about “individual-based” or “agent-based” models. A model with fewer compartments will be less computationally expensive and easier to solve analytically, at the cost of more assumptions and simplifications in the representation of the system of interest. On the other hand, an agent-based model will allow for a greater representation of possible individual behaviours, and therefore increased biological accuracy with a reduced number of assumptions, at the cost of a higher computational cost and a difficulty to disentangle the underlying dynamics in the system of interest.

We then distinguish between two classes of models: “deterministic”, which always generate the same results for a given set of parameters, and “stochastic”, where events are random (Anderson and May, 2010). Deterministic models are often represented as a series of ordinary differential equations, which can be solved analytically (depending on the other sources of complexity in the model, such as number of compartments). Deterministic models allow the identification of key

thresholds in population numbers or parameter values which affect the outcome of a simulation, as well as potential equilibria, but do not account for the randomness which can play an important role in certain processes. For this reason, deterministic models are more appropriate when representing larger populations, where it is reasonable to summarise individual variability into a single, average behaviour at the entire population level. On the other hand, stochastic models can capture random events which are particularly relevant for smaller populations, such as the appearance of a new mutation in a bacterial population which provides no advantage nor cost, and may therefore disappear or persist simply by chance. However, stochastic models are harder to solve analytically, and have a higher computational cost. Note that, for greater flexibility, some components within a single model can be deterministic and others stochastic.

As there are multiple ways to design mathematical models, it is essential to identify the relevant underlying characteristics of the processes we are aiming to model. Models must be designed with the appropriate level of complexity, capturing the relevant properties of the system represented, whilst ensuring that these properties can still be parameterised and that analysis of the model remains feasible (both mathematically and computationally) (Brooks and Tobias, 1996). For example, bed occupancy in a single hospital is more accurately estimated when taking into consideration all the different patient movements between bed types (general ward, intensive care etc.), yet this same complexity is not appropriate when attempting to model bed occupancy across an entire country, due to lack of data and computational cost (Leclerc *et al.*, 2021).

1.6.1.3 Fitting models to data

The values of parameters in mathematical models are often informed by data. This is essential to ensure that the conditions modelled are realistic, and that we can gain a meaningful understanding of real-life processes through the model. Some parameter values can be measured directly, such as the contact rate between individuals which can be obtained via contact surveys (Mossong *et al.*, 2008). However, for other parameters this may not be feasible. For example, disease transmission during a contact between infectious and susceptible individuals cannot be directly observed, hence the corresponding parameter value (probability for disease transmission during

such a contact) cannot be directly measured. In such cases, parameter values can be estimated by fitting the model to data (Kirkeby *et al.*, 2017). By obtaining the incidence curve for cases of an infectious disease over time, we can test multiple possible values for the probability of transmission, and identify the ones which allow the model to replicate this curve as best as possible.

Bayesian methods are commonly used for model fitting, such as Markov chain Monte-Carlo (Gelman *et al.*, 2015). This involves running the model repeatedly, each time slightly changing the value of the parameters we are attempting to estimate, and calculating the likelihood that the data could have been generated from this model and with this set of parameter values. Over time, the algorithm will converge towards the values which are most likely to correspond to reality, and will generate distributions to summarise the likelihood of these values. The ability for different models to reproduce the same data can be compared through this approach, using metrics such as the Deviance Information Criteria (Spiegelhalter *et al.*, 2002). This rewards models for their ability to reproduce data, whilst penalising their complexity (e.g. penalising models with more parameters). This process aims to identify the most appropriate model to use, which can reproduce the dynamics of interest whilst maintaining a reasonable level of complexity.

1.6.1.4 Modelling AMR in humans

Mathematical modelling is a valuable tool to study AMR dynamics in the human population (Opatowski *et al.*, 2011; van Kleef *et al.*, 2013; Knight *et al.*, 2019; Niewiadomska *et al.*, 2019). In particular, it is commonly used to understand the spread of infection by antibiotic-resistant bacteria in human populations, and the effects of interventions to reduce this burden. For example, previous work has shown that antibiotic stewardship in the community can reduce AMR prevalence both in the community and in hospitals, as opposed to stewardship in hospital which will mostly reduce the prevalence of AMR in hospitals only (MacFadden *et al.*, 2019). This example highlights the differential impact of interventions against AMR depending on the setting.

Mathematical models have also been used to look at infections by antibiotic-resistant *S. aureus*, mostly focusing on MRSA (Niewiadomska *et al.*, 2019). These studies are particularly useful to reveal the potential impact of interventions against MRSA which may not otherwise be measurable, such as vaccines (Tekle *et al.*, 2012; Joice and Lipsitch, 2013). A previous modelling study was notably able to quantify the indirect impact of influenza vaccination on the burden of antibiotic-resistant *S. aureus*, via the resulting reduction in inappropriate antibiotic prescribing in cases of influenza infections (Chae *et al.*, 2020). Mathematical models can also reveal the dynamics of nosocomial transmission of MRSA, highlighting the importance of initial transmission from the community to the hospital, and suggesting interventions to prevent this such as the systematic identification and decolonisation of MRSA carriers upon admission (Pei *et al.*, 2018).

1.6.1.5 Epidemiological modelling of AMR

Since AMR is a global problem, it is essential to understand its distribution across space and time. This type of epidemiological analysis relies on using large datasets to monitor various trends related to AMR. For example, (Bruinsma *et al.*, 2004) used routinely collected antibiotic susceptibility results for *Streptococcus pneumoniae* to estimate and forecast trends in antibiotic resistance in Europe. Epidemiological analysis of such large datasets has also been used to estimate the relative impact of various factors affecting AMR, such as antibiotic consumption, governance, and healthcare infrastructure (Collignon *et al.*, 2018). However, these studies and others have highlighted a key challenge: the lack of appropriate data to inform model design and parameterisation (Birkegård *et al.*, 2018). Epidemiological analysis often relies on datasets collected from routine surveillance at a local level (e.g. hospital, such as the ECDC data (European Centre for Disease Prevention and Control, 2022)), which are then aggregated at a national or international level. The problem is that variability in how the information in these datasets is collected at these local levels can limit the usefulness of these aggregated datasets for analysis. If only isolates from severely ill patients are collected for example, this would bias the dataset towards showing higher rates of antibiotic resistance (Leclerc *et al.*, 2020; Catalán *et al.*, 2022). Acknowledging these potential sources of variability and biases in essential. In addition, such datasets are often not publicly available, or may simply not exist in resource-limited settings. There have been recent attempts to use modelling to overcome these limitations and

fill in gaps in AMR surveillance datasets, but this still requires assumptions to be made, and hence we still cannot be completely confident in the conclusions made by such analyses (Murray *et al.*, 2022).

1.6.2 Mathematical modelling to study HGT of AMR

1.6.2.1 Modelling bacteria dynamics

Models are useful at a human population level, but also at a smaller scale. The dynamics of bacterial populations are arguably as complex as human ones, and modelling has previously been used to gain greater insight into these (Vlazaki, Huber and Restif, 2019). For example, data generated in the lab can be used alone to show that specific events of interest happened (e.g. multidrug-resistant bacteria have appeared following exposure of a bacterial population to phage capable of transduction), but cannot easily reveal the complete dynamics behind these events (e.g. what is the rate at which phage and bacteria interacted to generate these multidrug-resistant bacteria), which is where modelling can help.

A relatively simple and common use of modelling bacterial dynamics is pharmacodynamic modelling, to study how bacteria grow and are affected by an antibiotic (Nielsen and Friberg, 2013). This often relies on a Hill function, shown in equation 1.1, which can be used to express the effect of an antibiotic on bacteria as a function of antibiotic concentration.

$$E(C) = E_{max} * C^H/(C^H + EC50^H) \qquad (1.1)$$

Here, the effect of the antibiotic on bacteria (*E(C)*) increases up to a maximum effect (E_{max}) as the concentration of antibiotic increases (*C*). The speed of this increase is controlled by the concentration at which the effect is half the maximum (*EC50*), and a Hill coefficient (*H*). This type of equation can be easily parameterised with a few experiments using predetermined concentrations of antibiotics (typically doubling from 0.25 to 32 mg/L), and can then be used to predict the effect of a wide, continuous

range of different concentrations. The flexibility of this equation means that it can successfully reproduce the saturating dynamics of antibiotic-mediated bacteria killing for different combinations of bacteria and antibiotics, with an example shown in Figure 1.2. This demonstrates the usefulness of mathematical models to infer trends from data generated in the lab, reveal the underlying dynamics of organisms and antibiotics of interest, and allow a faster exploration of alternative conditions, instead of having to rely on multiple, time-consuming experiments.

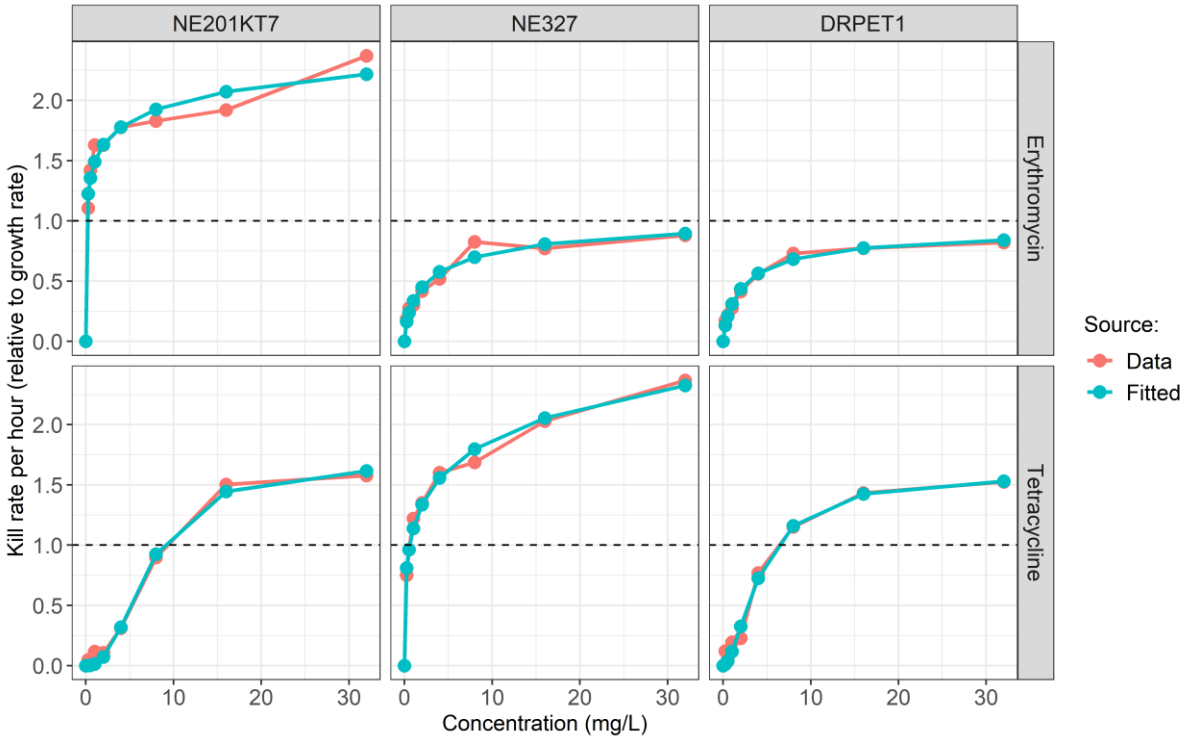


Figure 1.2: Measured effect of antibiotics on bacteria (pink) versus model-predicted effect after fitting a Hill equation (blue). All bacteria are *S. aureus*. NE201KT7 carries a *tetK* gene granting tetracycline resistance, NE327 carries an *ermB* gene granting erythromycin resistance, and DRPET1 carries both *tetK* and *ermB*. Figure reproduced from (Leclerc, Lindsay and Knight, 2022).

1.6.2.2 Modelling HGT of AMR

Mathematical models have also been used to study within-host bacterial dynamics during infection or colonisation of humans by resistant bacteria. These models generally only represent within-host AMR diversity as the co-existence of two bacterial

strains, one susceptible and one resistant to an antibiotic of interest (Davies *et al.*, 2019; Smith, Temime and Opatowski, 2021). New antibiotic resistant bacteria are often assumed to appear at a constant rate (corresponding to mutations), or at a rate which depends on antibiotic usage (Niewiadomska *et al.*, 2019). In reality however, as highlighted earlier in this Introduction, there is substantial AMR diversity in within-host populations, with multiple resistance genes circulating between bacteria via HGT. Mathematical models are likely to be valuable tools in the study of factors shaping within-host diversity and evolution (Metcalf *et al.*, 2015), yet the degree to which they capture the relevant and complex microbiological processes of HGT of AMR was previously unknown when this thesis commenced (Opatowski *et al.*, 2011). Clarifying this is essential, as models which do not include this complexity at the microbiological level may then inaccurately estimate the prevalence of AMR in bacterial population, in turn leading to an inaccurate estimate of AMR at the human level, and hence an incorrect evaluation of the impact of potential interventions to reduce the health burden of AMR (Spicknall *et al.*, 2013).

To address this, I conducted a systematic review of studies describing mathematical models of HGT of AMR (Leclerc, Lindsay and Knight, 2019). This systematic review is presented in the next Chapter of this thesis. In summary, I found that the majority of studies focused on conjugation in *E. coli*. In this review conducted in 2019, I only identified a single study which modelled transduction of AMR (Volkova *et al.*, 2014), and none which focused on *S. aureus*. Importantly, this lack of knowledge of HGT dynamics may be partly due to limited data available on HGT of AMR in within-host bacterial populations. Generating new data on HGT *in vivo* is difficult, as this requires longitudinal sampling of individuals, which limits the study sample size due to costs and practical challenges. Instead, it may be relevant to identify alternative, routinely collected data already available, to identify instances where HGT of AMR may be occurring *in vivo* and driving AMR evolution.

1.6.3 Mathematical modelling to study transduction of AMR

1.6.3.1 Modelling phage predation dynamics

To study the dynamics of transduction, it is firstly essential to consider the dynamics of phage predation, as the process of transduction relies on these. Mathematical models have been used to understand the complex predator-prey relationship of phage and bacteria; for example, by decreasing bacteria numbers now, phage are restricting their ability to replicate later, as they will have removed the organisms they need to replicate (Payne and Jansen, 2001). This type of work disentangling these interactions has been particularly used in the context of phage therapy, for example to show how the concentration and timing of phage presence can determine the success of phage therapy to clear a bacterial population (Cairns *et al.*, 2009).

A simple deterministic compartmental model of phage predation is shown in equations 1.2 and 1.3.

$$dB/dt = \mu * B - \beta * P * B \quad (1.2)$$

$$dP/dt = \beta * P * B * \delta - \beta * P * B - \gamma * P \quad (1.3)$$

In this system, bacteria (*B*) grow at a constant rate (*μ*), and phage (*P*) infect bacteria following a density-dependent process, the speed of which depends on the phage adsorption rate (*β*). Infected bacteria then burst, releasing more phage depending on the burst size (*δ*). Finally, free phage can decay in this system at a constant rate (*γ*). This central modelling structure has been adapted and applied to different environments, for example to quantify the synergistic bacterial-killing effect of antibiotics and phage (Rodriguez-Gonzalez *et al.*, 2020), or to highlight the important role of the immune system to guarantee bacterial eradication during phage therapy (Roach *et al.*, 2017).

1.6.3.2 Modelling transduction of AMR

As mentioned in my systematic review I originally identified only one study which attempted to model the dynamics of transduction of AMR (Volkova *et al.*, 2014). This model represented transduction in *E. coli* with great biological detail, with multiple phage binding and infection steps before a successful transduction event could occur.

The authors chose to represent the complete dynamics of a temperate phage, including the lytic and lysogenic cycles, and both generalised and specialised transduction. They concluded that, in *E. coli* and for their assumed conditions (representing the large intestine of cattle), a plasmid carrying an AMR gene was transferred 1000 times more frequently by conjugation than by transduction. However, the complexity in their model meant that they did not generate data to parameterise it, relying instead on assumptions and values estimated in previous studies. This is an important limitation, as combining these external sources requires the implicit assumption that all these parameter values are applicable to the system of interest, which may not always be true. Different independent studies are often not conducted following the same experimental protocol (bacteria growth conditions, plating techniques etc.). Even if different studies focused on the same species, they may have used different strains. As highlighted earlier in this Introduction with *S. aureus*, these strains may differ in their biological characteristics (growth rate, susceptibility to phage etc.). Finally, these limitations apply to the sources cited themselves, as some of these may also be modelling studies which again relied on combining multiple, previous sources to inform their model and estimate the parameter value being reused here.

After this systematic review was completed in 2019, two other studies modelling transduction were published. In (Fillol-Salom *et al.*, 2019), the authors focused on *S. aureus*. They used a detailed stochastic agent-based model to simulate phage-bacteria dynamics in a non-specific environment containing up to 10^4 bacteria, allowing for both lysis and lysogeny alongside generalised transduction of a single AMR gene, with a fitness cost. This study showed that phage and bacteria may benefit from lysogeny and transduction in the presence of antibiotics, as phage can persist for long time periods via lysogeny, and occasional transduction events increase the prevalence of the AMR gene in the bacterial population. Although the authors included a sensitivity analysis, and some *in vitro* work to generate hypotheses to study, their model parameter values were again either assumed or taken from multiple previous studies.

In (Arya *et al.*, 2020), the authors employed both deterministic and stochastic models to study the evolutionary implications of generalised transduction of AMR in a small *E. coli* population (168 bacteria) living in a non-specific environment. This model only

included the phage lytic cycle and generalised transduction, but allowed bacteria to become resistant to phage infection. The authors focused on a single AMR gene with a fitness cost, and examined the consequences of including the corresponding antibiotic in the environment. This study showed that stochasticity in phage-bacteria interactions, fitness costs for bacterial carriage of AMR, and fitness costs for phage resistance greatly determined if these very small populations of phage and bacteria could co-exist, and if AMR genes could be transferred by transduction. Once again however, parameter values were not derived from *in vitro* data generated as part of the study, and instead taken from previous studies or assumed.

In my project, I chose not to directly adapt the models or use the parameter values from these three previous studies (Volkova *et al.*, 2014; Fillol-Salom *et al.*, 2019; Arya *et al.*, 2020). These studies designed their models with a structural complexity that was not fully and robustly parameterised using data from a single set of *in vitro* conditions and environment, and therefore only provided a theoretical understanding of the dynamics of transduction. Instead, in this thesis I focus on capturing the key fundamental interactions underlying the dynamics of phage predation and generalised transduction, as the degree to which they are appropriately represented in these mathematical models is unclear (Leclerc, Lindsay and Knight, 2019). I believe that this can only be correctly captured using a mathematical model developed alongside *in vitro* experiments. This simultaneously allows me to be confident that I am including in my model all the relevant biological characteristics shaping the interaction between phage and bacteria in my experimental system, and that my model is robustly parameterised. Using further data analysis, I can then translate these findings from *in vitro* to *in vivo*, suggesting how transduction may shape AMR evolution in within-host bacterial populations.

1.7 Thesis aim and objectives

In summary, antimicrobial resistance in the major nosocomial pathogen *Staphylococcus aureus* represents a significant public health threat. The prevalence of infections by antibiotic-resistant *S. aureus* is linked to the prevalence of resistance genes in bacterial populations. In *S. aureus*, generalised transduction mediated by bacteriophage is a major process responsible for the horizontal gene transfer of AMR, affecting the prevalence of these genes. However, the dynamics of this generalised transduction, how to best represent them mathematically, and how these may ultimately affect the AMR diversity of within-host *S. aureus* populations are unclear. This research gap must be urgently addressed to ensure we are correctly understanding how AMR arises and spreads, and hence that we are designing effective interventions to reduce the health burden of infections by antibiotic-resistant bacteria. To do this, I combined laboratory, epidemiological data and mathematical modelling methods, overcoming some limitations of each of these individual disciplines and best utilising them to complement each other.

My first objective was to identify our current understanding of the dynamics of transduction of AMR, which I addressed by completing a systematic review of published literature on mathematical modelling of HGT of AMR. This allowed me to identify previous methodologies to inform my own framework, and suggest directions to further develop this field.

My second objective was to gain fundamental knowledge of the dynamics of phage predation and generalised transduction of AMR in *S. aureus*. To do this, I developed a joint experimental and modelling framework, where I first co-cultured single antibiotic-resistant *S. aureus* strains alongside phage, and observed the appearance of double antibiotic-resistant bacteria via transduction. I then used this data to build and parameterise several models representing this system, to test different predation interactions between phage and bacteria, and reveal the invisible underlying dynamics of generalised transduction.

My third objective was to identify how the impact of transduction on AMR evolution in *S. aureus* may vary when antibiotics are jointly present, which could occur either in the environment or during phage therapy. For this, I extended the framework developed

as part of my second objective to include pharmacodynamics of antibiotics, generated further *in vitro* data to parameterise this addition, and conducted simulations with varying timings and concentrations of phage and antibiotics.

My final objective was to understand how the findings from objectives two and three on transduction of AMR *in vitro* may translate to an *in vivo* setting, as we currently have a limited knowledge of how often these events may shape AMR within-host diversity, notably due to lack of longitudinal data. For this, I analysed 20 years of pseudonymised routinely-collected patient data from Great Ormond Street Hospital, with detailed information on the sensitivity of bacterial isolates to multiple antibiotics. This allowed me to test if I could detect within-host *S. aureus* diversity, and identify potential changes in diversity over time that may be due to HGT.

2 Mathematical modelling to study the horizontal transfer of antimicrobial resistance genes in bacteria: current state of the field and recommendations

2.1 Overview

As explained in the Introduction of this thesis, the appearance of new antibiotic resistant bacteria is often represented as occurring at a constant rate in mathematical models of AMR. In reality however, resistance genes can circulate between bacteria via HGT, which affects the overall prevalence of AMR in bacterial populations. The rate at which HGT occurs is affected by multiple factors (mechanism, environment, bacteria, presence of antibiotics etc.), and the extent to which mathematical models appropriately capture these dynamics was unknown before this thesis commenced. In this Chapter, I aimed to clarify the current state of this research field, as models which do not appropriately capture HGT complexity at the microbiological level may then inaccurately estimate the prevalence of AMR at that scale. This in turn can lead to an inaccurate estimate of AMR at the human level, and hence an incorrect evaluation of the impact of potential interventions to reduce the health burden of AMR.

To address this, I systematically reviewed studies describing mathematical models of HGT of AMR. I devised a list of 11 elements to extract from each study, including information on the mechanism of HGT represented (conjugation, transformation, or transduction), the bacterial species, and the source for model parameters (whether they were estimated from data generated as part of the same study, taken from previous studies, assumed, or a combination of these sources). The aim of this work was to broadly assess mathematical models of HGT of AMR, identify potential research gaps in this field, and particularly to search for previous models of transduction of AMR which may be useful to inform the work in this thesis.

This work has been published following peer-review in the Journal of the Royal Society Interface (Leclerc, Lindsay and Knight, 2019). The co-authors are Quentin J Leclerc, Jodi A Lindsay, and Gwenan M Knight. The version included below is the author accepted manuscript.

2.2 Research paper cover sheet

Please note that a cover sheet must be completed <u>for each</u> research paper included within a thesis.

<u>SECTION A – Student Details</u>

Student ID Number	1702140	**Title**	Mr
First Name(s)	Quentin		
Surname/Family Name	Leclerc		
Thesis Title	Interdisciplinary investigations of phage predation dynamics and generalised transduction of antimicrobial resistance in *Staphylococcus aureus*		
Primary Supervisor	Dr Gwenan Knight		

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

<u>SECTION B – Paper already published</u>

Where was the work published?	Journal of the Royal Society Interface		
When was the work published?	14/08/2019		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion	N/A		
Have you retained the copyright for the work?*	**Yes**	Was the work subject to academic peer review?	**Yes**

***Copyright retention: see point 5 of Open access licence to publish, available at https://royalsociety.org/journals/authors/author-guidelines/licence-to-publish/**

SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	
Please list the paper's authors in the intended authorship order:	
Stage of publication	Choose an item.

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I developed the search strategy, screened the titles and abstracts of the identified studies, evaluated the full texts of the included studies, and wrote the first draft of the manuscript. I then subsequently edited the manuscript.

SECTION E

Student Signature	[redacted]
Date	06/06/2022

Supervisor Signature	[redacted]
Date	06/06/2022

2.3 Abstract

Antimicrobial resistance (AMR) is one of the greatest public health challenges we are currently facing. To develop effective interventions against this, it is essential to understand the processes behind the spread of AMR. These are partly dependent on the dynamics of horizontal transfer of resistance genes between bacteria, which can occur by conjugation (direct contact), transformation (uptake from the environment) or transduction (mediated by bacteriophages). Mathematical modelling is a powerful tool to investigate the dynamics of AMR, however the extent of its use to study the horizontal transfer of AMR genes is currently unclear. In this systematic review, we searched for mathematical modelling studies which focused on horizontal transfer of AMR genes. We compared their aims and methods using a list of predetermined criteria, and utilised our results to assess the current state of this research field. Of the 43 studies we identified, most focused on the transfer of single genes by conjugation in *Escherichia coli* in culture, and its impact on the bacterial evolutionary dynamics. Our findings highlight the existence of an important research gap on the dynamics of transformation and transduction, and the overall public health implications of horizontal transfer of AMR genes. To further develop this field and improve our ability to control AMR, it is essential that we clarify the structural complexity required to study the dynamics of horizontal gene transfer, which will require cooperation between microbiologists and modellers.

2.4 Introduction

Antimicrobial resistance (AMR) is undeniably one of the greatest global public health challenges we are currently facing [1]. The recent discoveries on the spread of resistance genes for key antimicrobials such as NDM-1 for carbapenem resistance [2–4] suggest that to tackle this challenge, instead of only studying the spread of resistant bacteria, we must understand the processes by which individual resistance genes spread. The first is “vertical gene transfer”, where genes are passed from parent to progeny during bacterial replication. The second, which is our focus here, is “horizontal gene transfer” (HGT). This allows bacteria to acquire genetic material, including AMR genes, from their environment or other bacteria [5–7]. There are three mechanisms of

HGT. Firstly, “transformation” is the capacity of bacteria to intake genetic material from their environment. Secondly, “conjugation” occurs when two bacteria come into contact with each other and form a conjugative bridge, enabling direct exchange of genetic material. Finally, “transduction” occurs when a bacteriophage (a virus that can infect bacteria) replicates and packages a bacterial gene instead of its own genetic material, then acts as a vector and transfers this gene into another bacterium.

The consequences of HGT of AMR in a bacterial population are varied and can change depending on the setting that this process occurs in. Firstly, HGT can often be at the origin of new combinations of resistances to multiple antimicrobials in single bacteria strains [8]. This is amplified by the fact that HGT can occur both intra- and inter-species [9], therefore allowing for mixing between many different gene pools. Fortunately, these resistance mechanisms often impose a fitness cost which reduces the competitiveness of bacteria with AMR genes in settings where antibiotics are absent [10], thereby limiting the increase in the prevalence of these bacteria in the environment. Studying HGT of AMR can be further complicated by differences in transfer rates and importance of transfer mechanisms between bacterial species [11], with transformation for example being rare for *Staphylococcus aureus* [12] but common for *Neisseria gonorrhoea* [13], and by differences between rates estimated *in-vitro* and *in-vivo*, as was seen with transduction in *Staphylococcus aureus* [14] and conjugation in *Klebsiella pneumoniae* and *Escherichia coli* [15]. Lastly, HGT dynamics appear to vary depending on the presence or absence of antibiotics in the surrounding environment [16–20], therefore requiring studies to be conducted in multiple settings to fully capture this process.

It is essential to fully understand HGT of AMR since it can impact the overall transmission of AMR, and therefore the predicted effect of interventions against bacterial infections, to varying degrees depending on the setting. A most striking example of this is phage therapy, where bacteriophages are proposed as antimicrobials. A risk is that therapeutic phages could perform transduction and increase the proportion of bacteria in the patient that carry a resistance gene. In that case, if the phage therapy treatment fails to clear all the bacteria this could leave the patient at a higher risk of antimicrobial-resistant bacteria infection [21,22]. In addition to the aforementioned differences between bacterial species, HGT mechanisms

themselves are biologically complex. For example, the capacity to form a conjugative bridge generally requires the presence of a specific set of "*tra*" genes [23]. These can themselves be transferred, leading to an increase through time in the prevalence of bacteria that can perform conjugation. Transformation gene expression is extremely variable depending on the environmental conditions that bacteria are exposed to [6], therefore we cannot realistically assume that bacteria are able to perform transformation at all times. Finally, some phages can either undergo a "lytic cycle", where they immediately replicate upon infecting a bacterium, or a "lysogenic cycle", where they first integrate into the bacterial genome for a variable duration [12]. Consequently, transduction dynamics can be further complicated by the characteristics of the phage life cycle.

HGT is therefore complex in its dynamics, and studying these requires appropriate tools. Mathematical modelling is often used to study infectious disease processes [24]. It provides a simulation environment that can be informed by real-life data, in which dynamics can be disentangled and easily studied. Mathematical models can be split into "deterministic models", which always generate the same results for a given set of parameter values [24], and "stochastic models", which generate variability in their results using random events [24]. Mathematical modelling is already being used to study AMR dynamics and their public health implications [25,26]. For example, it has been employed to study within-host bacterial dynamics (i.e. the bacterial processes that occur during colonisation or infection of a host) and derive conclusions on patterns of AMR seen in the host population [27]. Consequently, it can provide novel insight into optimal strategies to combat AMR spread by analysing the effect that these have on the transmission dynamics [28]. However, existing models may not always capture the relevant and complex microbiological dynamics of HGT. In this systematic review, we aimed to find modelling studies which focus on HGT of AMR, to record their methods and research questions, and hence, to identify potential research gaps and areas for improvement in this field.

2.5 Methods

The methodology of our systematic review follows the recommended PRISMA guidelines [29].

2.5.1 Inclusion criteria

In order to be included in this review, studies had to fulfil all of the following criteria:

1) Study the horizontal transfer of genes between bacteria
2) The genes studied must explicitly be identified as genes encoding antimicrobial resistance
3) Use at least one dynamic population model. A model is "dynamic" if it tracks the changes in the number of bacteria belonging to various populations (e.g. antibiotic-resistant and susceptible bacteria) over time

2.5.2 Screening process

The entire screening process is summarised in Figure 2.1. We searched two databases on the 26th of April 2019 using the following terms:

- PubMed search: "(antimicrobial OR antibacterial OR antibiotic) resist* AND (horizontal transfer OR mobile genetic element OR plasmid OR transformation OR conjugation OR transduction OR phage) AND (math* OR dynamic*) model*", 171 results
- Web of Science search: "TS = ((antimicrobial OR antibacterial OR antibiotic) resist* AND (horizontal transfer OR mobile genetic element OR plasmid OR transformation OR conjugation OR transduction OR phage) AND (math* OR dynamic*) model*)", 185 results

After removal of duplicates, these combined searches yielded a list of 272 studies. Both QL and GK independently screened the titles and abstracts of all 272 studies. 54 studies were retained by both authors, and two more were discussed and retained after an additional screen of the methods due to uncertainty, leading to a total of 56 studies retained after the first screening step.

The full texts of these 56 studies were then screened by QL, leading to 34 studies being retained as relevant for this review. Finally, by screening the reference lists in these 34 studies, nine more were included, leading to a total of 43 studies to discuss in this review.

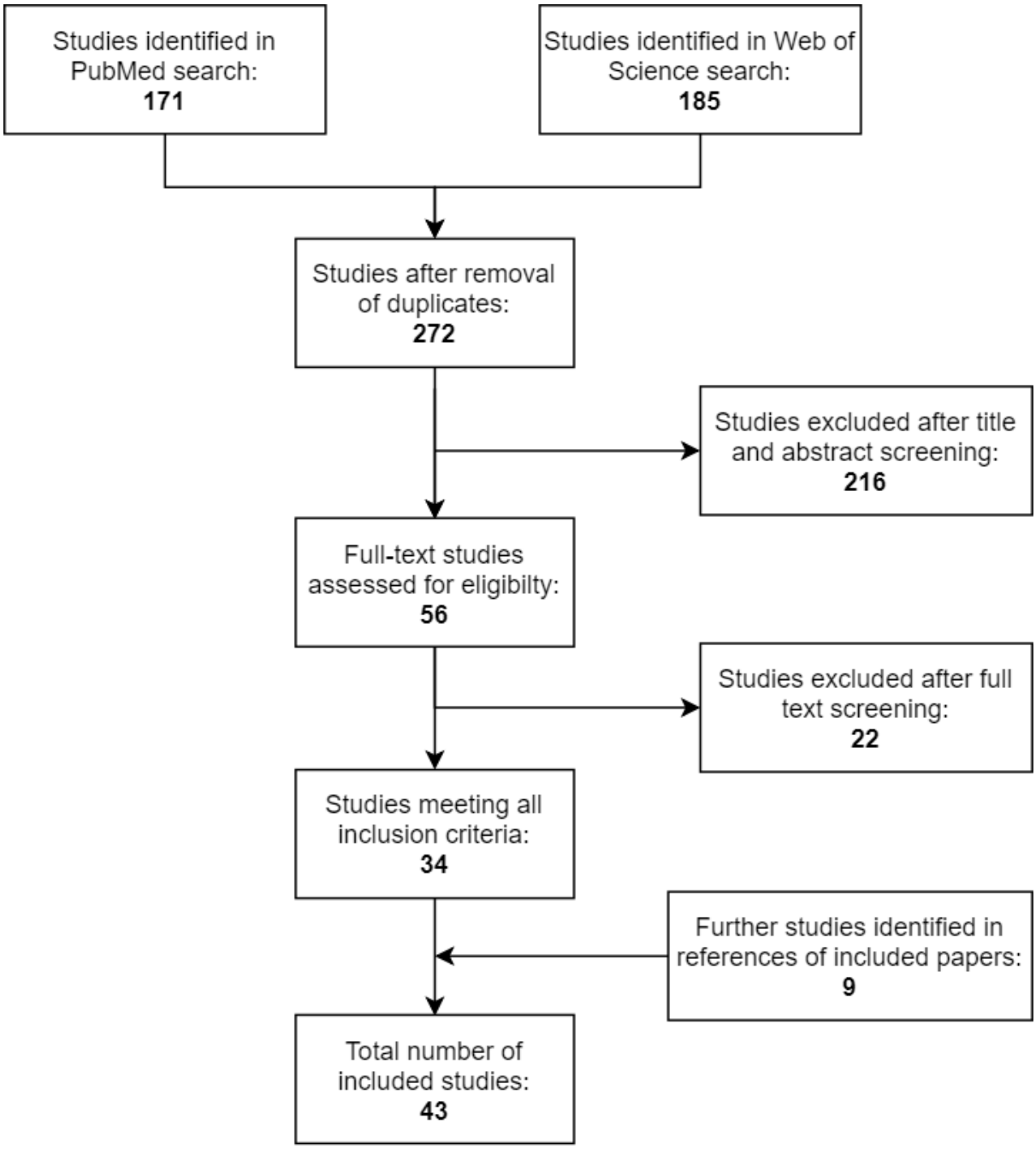


Figure 2.1: PRISMA flow diagram of the search and exclusion process.

2.5.3 Information extracted from the included studies

To maximise comparability between studies, we devised a list of 11 elements to extract from every study. These are summarised and explained in Table 2.1.

Table 2.1: Elements recorded from all included studies. Where no "Possible values" are given in the table, this indicates that the values were not restricted to a predetermined list.

RECORDED ELEMENT	SIGNIFICATION	POSSIBLE VALUES
Transfer mechanism	Biological mechanism of horizontal gene transfer modelled	"Conjugation" or "Transformation" or "Transduction"
Bacteria	Any species of bacteria explicitly modelled	-
Aim of the study	Whether the study looked at gene transfer to understand evolutionary trends seen in the bacterial population, or to understand its impact on public health, or both	"Evolutionary" or "Public Health" or "Both"
Bacterial environment	Any environment which contained bacteria in the model	-
Antibiotic effect considered	Whether one or more antibiotic(s) were present in the model(s)	"Yes" or "No"
Multiple resistances considered	Whether the model(s) tracked multiple resistance genes that could be transferred separately	"Yes" or "No"
Fitness cost of resistance considered	Whether the model(s) included a fitness cost for bacteria carrying a resistance gene	"Yes" or "No"
Source of model parameters	Whether the study also generated its own experimental data to support its parameter values, or chose values informed by previous studies (which could be experimental studies or not), or assumed values	"Experimental" and/or "External" and/or "Assumed"

Type of model	Whether the structure of the model(s) was deterministic, or stochastic, or both (if the study presented more than one model)	“Deterministic” or “Stochastic” or “Both”
Type of parameter values	If the model(s) structure was “Deterministic”, whether the parameter values were constant or were sampled from distributions before each model run	“Constant” or “Sampled”
Sensitivity analysis performed	Whether the study performed any type of sensitivity analysis of the effect of model parameter values on the results	“Yes” or “No”

Note that in our analysis, “Type of parameter values” and “Sensitivity analysis performed” are two independent criteria. We can therefore report that a study only uses “Constant” parameter values, yet still performs a sensitivity analysis. If a study is reported to have “Sampled” parameters, this means that the values of the parameters vary for each model run, and that this is represented in the main results, with figures showing model output with ranges instead of single lines for example. If a sensitivity analysis was performed, this means that the authors report conducting such a procedure to support their findings (e.g. to argue that their choice of “Constant” parameter values is a reasonable assumption, and does not significantly affect their results).

2.6 Results

The table showing all of the recorded elements from the 43 included studies can be found in the Supplementary Material of this paper (Supplementary Table 2.1).

Firstly, when looking at the transfer mechanism modelled by these studies, we observe that almost all exclusively focus on conjugation (40 out of 43) [30–69] (Figure 2.2). Of the remaining three, two focused on transformation [70,71], and one on transduction [72]. Additionally, more than a third of the studies (16/43) chose exclusively

Escherichia coli (*E. coli*) as the bacteria in which to model the transfer processes [30,34,36,41–46,52,53,59,64,66,68,72] (Figure 2.2). It is also worth noting that another third of the studies (15/43) do not model a specific organism, and instead indicate that they are looking at bacteria in general [31,32,37,38,48,51,54,56–58,61,62,65,67,69]. Finally, while eight studies applied their model to more than one bacterial species [33,35,39,40,47,49,60,63], only four of these modelled two strains of bacteria simultaneously and captured inter-species transfer of resistance genes [39,49,60,63].

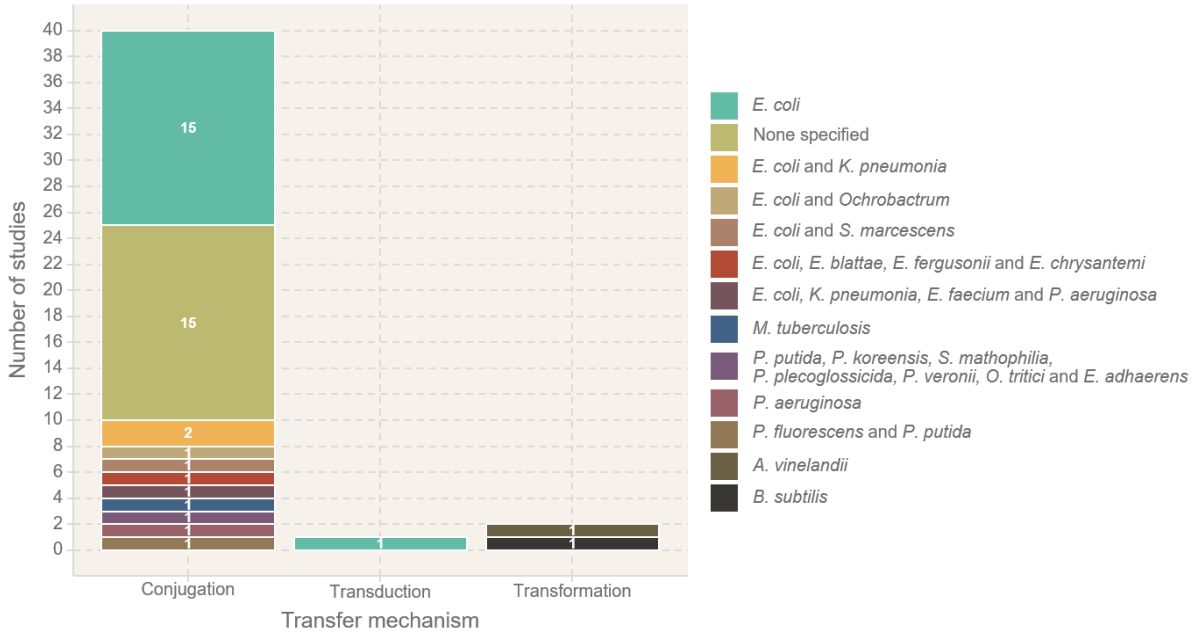


Figure 2.2: Transfer mechanisms and bacterial species modelled in the 43 studies included in our review.

In terms of the aims of these studies, all except eight [32,55,58,60,63–65,69] used modelling approaches exclusively to improve the understanding of bacterial evolutionary dynamics (Figure 2.3). This covered questions such as how the prevalence of resistance genes in the bacterial population changes over time (as in [34] for example), or how the rise of multi-drug resistant bacteria varied under different environmental conditions (as in [30] for example). Inversely, the remaining eight studies [32,55,58,60,63–65,69] attempted to place at least some of their results in a public health setting by, for example, quantifying the impact of transfer on the

incidence of multi-drug resistant bacteria infection in humans [32,69]. In accordance with this previous point, almost half of the studies (20/43) modelled bacteria exclusively in culture [33–42,47,49,50,52,53,58,59,66,70,71], and only seven modelled bacteria in humans [30,32,55,60,63,65,69] (Figure 2.3). In the remaining studies, seven did not specify an environment for their bacteria [31,48,56,57,61,62,67].

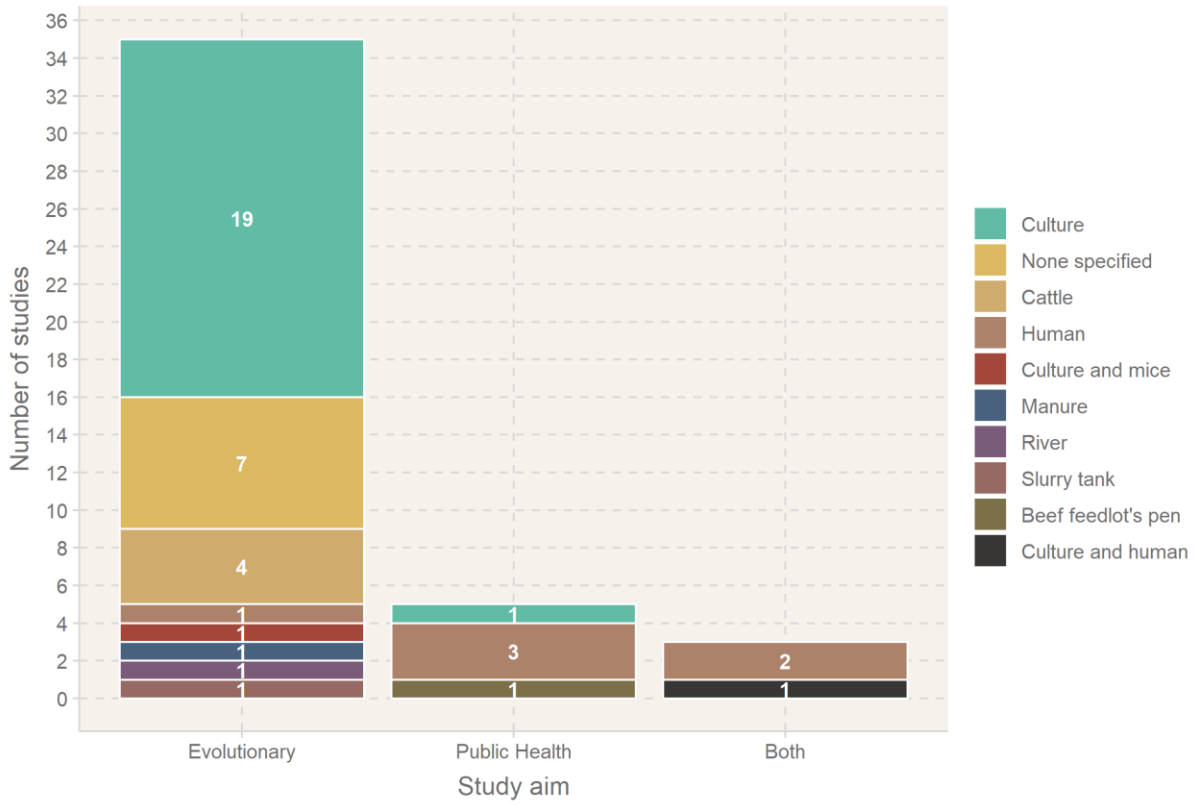


Figure 2.3: Aims and environments modelled in the 43 studies included in our review.

Almost all of the studies included a bacterial fitness cost for the carriage of a resistance gene in their models (Table 2.2), except for six [32,42,48,63,66,71]. On the other hand, despite the fact that in reality bacteria can acquire multiple AMR genes independently (i.e. the acquisition of each gene is a separate HGT event), only four studies included this feature [30,32,60,69] (Table 2.2). Lastly, it is important to note that almost half of the studies did not model the presence of antibiotics, and therefore did not consider the effect of antibiotics on transfer rates [33–36,39–42,47,52,53,59,63,66,68,71,72] (Table 2.2).

Table 2.2: Summary of the presence or absence of model characteristics in the 43 studies we reviewed.

	Include antibiotic effect	Include multiple AMR genes	Include fitness cost	Include sensitivity analysis
Yes	26	4	37	29
No	17	39	6	14

Almost half of these modelling studies (19/43) included their own experimental work to generate data and estimate at least some parameter values for their models [33–36,39–42,47,49,51–54,59,66,68,70,71] (Figure 2.4). On the other hand, more than half (23/43) chose to assume the values of at least some of their parameters, without explicitly citing any sources to support their choices, and a quarter (12/43) assumed the values of all of their parameters [31,32,37,38,65,67]. Finally, a third (15/43) used previous studies to obtain at least some of their parameter values. For these, except for three studies (two of which were each the direct follow-up of another one on the same topic [44,50], and one an analysis of data collected during an outbreak [63]), more than one previous study was taken to estimate the value of parameters, with a median number of studies of 8 and a maximum of 42.

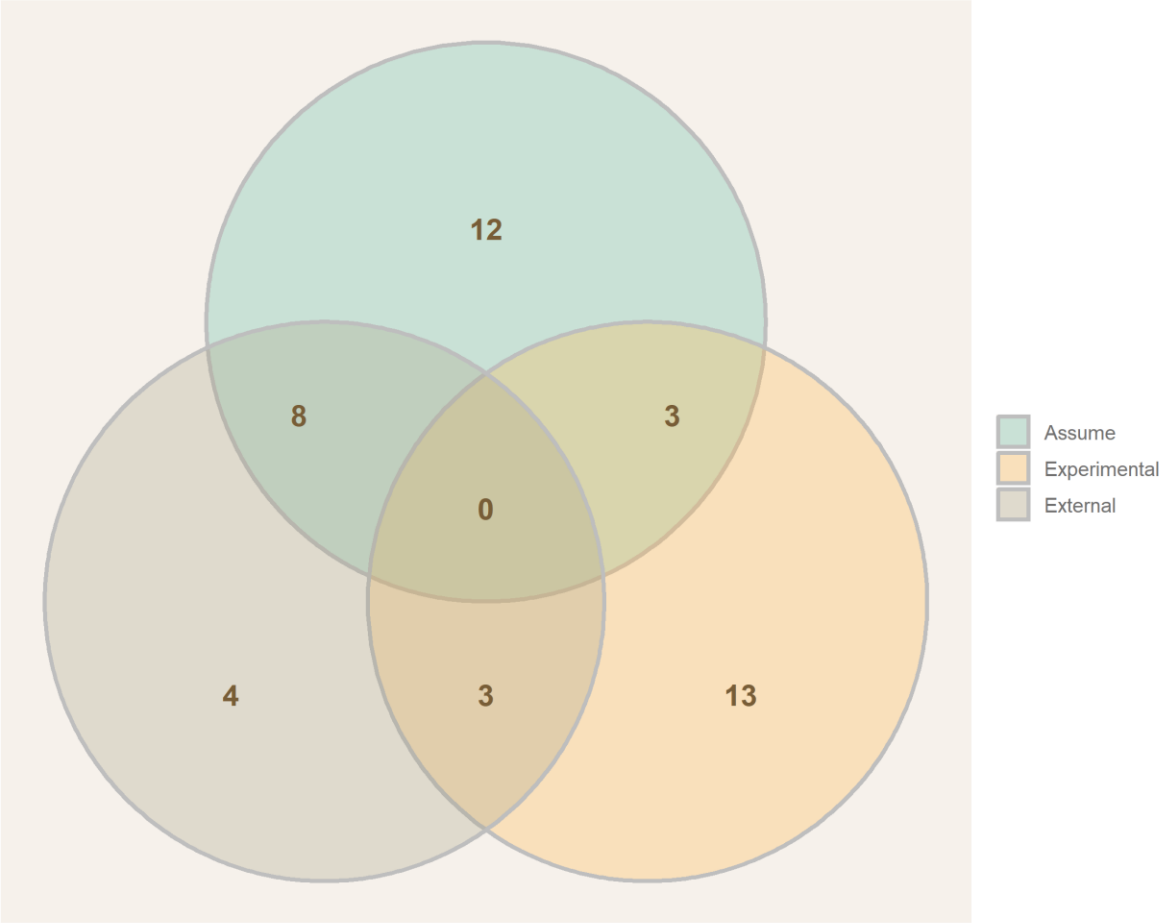


Figure 2.4: Sources of parameter values in the 43 studies included in our review. "Assume" (top, green): no clear reference is given to support the choice of parameter value; "Experimental" (right, orange): the study generated its own experimental data to support the choice of parameter value; "External" (left, brown): the study references a previous study to support the choice of parameter value. Studies in an overlap region used each of the corresponding methods at least once to estimate the value of their parameters.

Finally, more than three quarters of the studies (33/43) exclusively relied on deterministic models to obtain their results [30,32,34,36–40,42,43,45,47–51,53–56,58,59,61,63–69,71–73]. All of these deterministic models were composed of a set of ordinary differential equations to track the different sub-populations (susceptible bacteria, resistant bacteria etc.) through time. As for the ten studies which relied on stochastic models [31,33,35,41,44,52,57,60,62,70], most of these were agent-based models, where the bacteria were tracked individually [31,33,41,52,57,60], while the

remaining ones either used stochastic differential equations [44,62,70] or difference equations [35]. Out of the studies which exclusively used deterministic models, only eight acknowledge variability in the parameter values by running their model multiple times and sampling parameters from distributions instead of assuming them to be constant [32,38,43,46,56,64,65,72]. Nevertheless, most studies performed sensitivity analyses of the effect of their parameter values on their model results (Table 2.2). Overall, nine studies still relied solely on a deterministic model without either sampling their parameter values or performing sensitivity analyses [30,36,40,42,48,54,55,58,68]. We also noted that except for the one study on transduction [72], all the studies modelled transfer as a mass-action process. This assumes that the number of transfer events is determined by multiplying the number of bacteria that can receive the gene, the number of bacteria that can transfer the gene, and the rate at which transfer occurs. This is therefore generally written as some form of $\beta*S*R/N$, where β is a rate of transfer, S is the number of bacteria that can receive the resistance gene, R is the number of bacteria that can provide the resistance gene, and N is the total bacterial population in the system.

2.7 Discussion

We used a systematic literature review of mathematical models of horizontal gene transfer (HGT) to determine our current understanding of the dynamics of HGT of AMR. The first main observation from our results is that the majority of studies assessed only focus on HGT by conjugation (40 out of 43). The likely reason for this is the simplicity of conjugation dynamics. Effectively, these are comparable to infections transmitted upon contact, such as influenza, where established modelling exists using mass-action dynamics [24]. Consequently, modelling conjugation does not require much complexity to be added to these models. However, we know that transformation and transduction also contribute to HGT [7,14] and the lack of studies on these mechanisms is worrying.

Conjugation, transformation and transduction fundamentally differ in their biology, making it essential to study each of them in their own modelling framework; it is unknown whether models of conjugation could be directly applied to transformation

and transduction. When looking at the studies which attempted to model these two processes, we first see that the one which focused on transduction [72] attempted to place it in a complex setting, with the phage able to undergo both lytic and lysogenic cycle, and the possibility for some bacteria to be resistant to phage infection. Transduction is represented as a multi-step process in this model, as opposed to relying on a single rate. The phage must first successfully infect a bacterium, then pick up a resistance gene, before successfully transferring this gene to a different bacterium. This model aims to accurately represent most of the biological complexity of transduction, which necessarily requires many assumptions regarding parameter values. Further study of this trade-off would be greatly beneficial; it is currently unclear whether this complexity is required, at the cost of more assumptions, or if the process of transduction could be simplified and modelled using fewer parameters, which could be estimated from experimental data. The two studies which focused on transformation [70,71] applied similar mass-action dynamics to this process as what can be seen in models of conjugation. However, this approach assumes that the number of resistance genes available in the environment is equivalent to the number of bacteria carrying these genes. This is questionable, as we would only expect these genes to be available in the environment after the bacteria die and release their genetic material; while it is possible for bacteria to actively release their genetic material while still alive, the extent of this phenomenon is unclear [6]. Further exploration of this assumption, and perhaps redesigns of model structures for transformation would be of value.

E. coli is the most commonly studied model organism for bacteria in general [74]. Its rapid growth and consistent behaviour in *in-vitro* settings make it amenable to experimental work, including transfer studies, therefore its overwhelming presence as the organism of choice for studies modelling HGT of AMR genes is not a surprise. However, HGT is known to occur with varying rates in multiple bacterial species, consequently it is unlikely that the rates of transfer estimated by looking at *E. coli* are equally applicable to other bacterial species [7]. In addition, HGT of AMR is a process that can also occur between bacterial species [9,11], while most models here exclusively focused on *E. coli* alone. Some resistances in bacterial species are in fact thought to have been originally acquired following a gene transfer event with another

species, such as the *mecA* resistance gene in *Staphylococcus aureus* acquired from *S. fleurettii* [75].

Despite the fact that the carriage of an AMR gene often imposes a reduction in the growth rate of the bacteria [10], a few studies did not model this (6/43), but only one argued that this element could be ignored after fitting their model to experimental data [66]. However, this was once more only based on observations *in-vitro*, which are likely to differ from the *in-vivo* reality. Including a fitness cost, while requiring the estimation of an additional parameter, does not add any particular complexity to the model structure itself, effectively only requiring a reduced growth rate value for the bacteria carrying AMR genes as opposed to bacteria susceptible to the modelled antibiotic (as can be seen in [68] for example), and should therefore be included at least for sensitivity analyses. In addition, although it is understandable that the first models of HGT of AMR should focus on tracking single genes to understand the basic dynamics of this process, in reality many bacteria carry multiple AMR genes that can be transferred independently [8]. However, we only identified four studies in our review which included more than one independent AMR gene in their model [30,32,60,69]. 13 studies did model the transfer of multiple linked genes [34,35,66,68,70,40–42,47,49,53,55,59]; however in these cases a single HGT event causes the transfer of all of these genes, therefore there is little difference between the model structures of these 13 studies and those of other studies which modelled the transfer of single genes.

Many studies did not allow for the presence of an antibiotic in their model. However, antibiotics are likely to modify HGT dynamics by directly affecting transfer rates, as well as the survival of bacteria not carrying the AMR gene [16–20]. The former has been shown to occur for transduction in *S. aureus*, where the addition of antibiotics induced a higher proportion of transducing phage compared to lytic phage [76]. On the other hand, some studies correctly argue that it is equally important to understand the dynamics of HGT in the absence of antibiotics. Effectively, it is common for bacterial populations to rapidly transition between being exposed to antibiotics or not, with the most obvious example being individuals transiently consuming antibiotics. Consequently, understanding the dynamics of HGT of AMR both in the presence and absence of antibiotics is essential.

HGT of AMR has been studied in laboratory settings, consequently data around which models can be built have been generated and are available [7,77]. However, we note that, to the best of our knowledge, most data appear to focus on conjugation in *in-vitro* settings. The availability of experimental data on HGT of AMR by transformation or transduction, and on any of the three HGT mechanisms in more complex settings (such as *in-vivo*), is unclear. This should be investigated in future work to further refine the recommendations we make here, and identify where more data are needed to support the development of mathematical models. This is essential to understand which of the gaps we identify are due to theory outpacing data collection, and which are due to under-utilisation of available data. In any case, using these external data sources for purposes they were not originally designed for can require assumptions to be made in the model structure and parameters. In addition, it is essential to bear in mind how these data were originally collected, since for example combining sources which look at bacteria in multiple environments to derive parameters in a single environment-specific model is far from ideal. On the other hand, the fact that a quarter of the studies we reviewed (12/43) assumed all of their parameter values is worrying. While the purpose of some of these studies was to exclusively test a range of parameter values to identify conditions for a specific event to occur (e.g. AMR prevalence increases), the absence of any clear sources for the limits of these ranges is questionable. Looking at studies which determined their parameter values experimentally, we see that some of these also assume values such as the initial proportion of bacteria capable of performing transformation and the rate at which they can gain this ability [70], the bacterial growth rate and the conjugation rate [40], or the fitness cost of carrying an AMR gene and the rate at which such genes are lost by the bacteria [34]. Informing models with data is essential to ensure that they are accurate representations of reality, therefore, as stated above, we believe that further work is required to review the availability of data on HGT of AMR, and the methods that could be used to generate them when they are currently missing.

Regarding model structures, the majority of studies relied on deterministic models. To allow variability in the dynamics and therefore increased realism, studies more often chose to sample their parameter values, run their deterministic model, and repeat this process a number of times (as can be seen in [32,38,43,46,56,64,65,72]), a simpler alternative to developing new stochastic models. Acknowledging stochasticity when

looking at HGT is essential; HGT rates are typically low (estimates from studies in our review include for example $5.1*10^{-15}$(cells/mL)$^{-1}$hour^{-1} for conjugation [49] and 10^{-16}(cells/mL)$^{-1}$hour^{-1} for transformation [70]). These are therefore models of rare events which, by chance, might not always occur as expected, a feature which deterministic models fail to capture [24]. Sensitivity analysis is extremely important in any case since a small change in parameter value can lead to a greater change in the results. Despite this, nine studies exclusively relied on a deterministic model without sampling parameters or performing sensitivity analyses [30,36,40,42,48,54,55,58,68]. Interestingly, five of these nine studies also generated their own parameter values experimentally [36,40,42,54,68]. Although they capture variation when measuring the parameters experimentally, often providing distributions for their values, they then only retain fixed point estimates for their corresponding model parameter values instead of sampling them from these distributions, and only use these fixed estimates to derive their conclusions. Acknowledging variability in microbiological observations by specifying distributions rather than point estimates is essential, and this must be represented in the corresponding mathematical models.

This also raises the question of how to best represent these microbiological events in mathematical models. Effectively, almost all of the models here describe transfer as a mass-action process (42/43). However, as stated above this approach is acceptable for conjugation, but might not fully apply to transformation, where transfer depends on the density of DNA in the surrounding environment rather than the number of bacteria, and transduction, which follows vector-like dynamics with the phage acting as carriers of resistance genes between bacteria. Transformation dynamics might therefore be better represented by models of environmental transmission of infections (such as [78]), and transduction by models of vector-borne diseases (such as [79]), as opposed to mass-action models. The degree of modelling complexity required to accurately represent HGT is therefore unclear. This is also true for models designed to understand the public health implications of HGT of AMR, for which the level of detail required to represent within-host dynamics must be clarified. In addition, since transfer dynamics have thus far been mostly studied in bacterial culture, mostly “short” time-frames have been explored (hours or days), with long term dynamics remaining unclear despite our knowledge that even resistant bacteria can colonise us for weeks or months [80–82]. To best guide our public health policies with mathematical

modelling, we must first clarify the complexity of the process we are actually attempting to model, and the time required to fully capture its *in-vivo* dynamics.

This is the first attempt at providing an overview of existing mathematical modelling work on HGT of AMR. Our systematic review methods, with two individuals separately screening the titles and abstracts of candidate studies, allowed us to identify and bring together key studies on this topic. Using our list of comparison elements, we extracted and contrasted essential information between studies, overall allowing us to obtain a broad overview of the field and identify research gaps. However, our approach also has some limitations. Firstly, it was necessary for us to specify "(math* OR dynamic*) model*" rather than just "model*" in the search, since otherwise it would have returned results on experimental models (e.g. mice) as opposed to mathematical models. Effectively, repeating our search with "model*" instead of "(math* OR dynamic*) model*" yields 2,360 and 1,560 results on PubMed and Web of Science respectively, as opposed to our 171 and 185 results. The consequence of our choice however was that nine relevant studies were missed in the search, and were only identified by screening the references of already included studies. These nine studies were missed in the original literature search due to the absence of at least one of the search terms, with some studies for example referring to their models as "mass action models" instead of "mathematical models". In addition, we only searched for studies which modelled transfer of AMR genes, as opposed to HGT of any gene. This is firstly due to our specific research interest; horizontal transfer of AMR genes is an especially strong evolutionary driver for bacterial populations, compared to transfer of other genes. This is because AMR genes can be strongly selected for by environmental factors, such as the presence of antibiotics, while many other genes are often not subject to such selection pressures. In addition, AMR genes can be selected in more settings compared to other genes; for example, genes involved in immune evasion will only be selected for during infection of the host, while AMR genes can also be selected for during asymptomatic colonisation. The consequences of HGT of AMR in the bacterial population can therefore be greater than for other genes, which is why we believe it is important to study this process. Secondly, repeating the search without "(antimicrobial OR antibacterial OR antibiotic) resist*" yields 12,236 and 38,148 results on PubMed and Web of Science respectively, which would be too many to cover in a single systematic review. Nevertheless, this suggests that there are other studies

which model HGT more broadly. These could be a source of methodologies that could be applied to further develop the specific field of HGT of AMR modelling. In terms of the elements gathered from the studies to compare them, we were unable to extract any meaningful quantitative data (e.g. estimated gene transfer rates) common to all studies due to the high variability of study designs. This variability also prevented us from identifying common measures of study quality we could report aside from the presence or absence of sensitivity analysis.

Studying the effect of HGT of AMR on bacterial evolutionary dynamics is a necessary first step to understand the overall importance of this process. This has been the focus of the vast majority of the studies identified in this review, however the public health implications remain vastly unknown. This is related to the observation that the majority of studies model bacteria in an *in-vitro* setting; to understand the public health impact of HGT of AMR, it is essential to expand this to include other bacterial environments such as within humans and animals. In addition, important differences have been identified between transfer rates estimated *in-vitro* and *in-vivo*, with *in-vivo* transduction rates in *S. aureus* and conjugation rates in *K. pneumoniae* and *E. coli* for example being much higher than expected [14,15]. This difference in dynamics is attributable to the fact that *in-vitro* conditions fail to capture essential biological mechanisms influencing bacteria and therefore HGT [6,10]. Studying HGT *in-vitro* allows for a controlled environment to understand the basic dynamics of this process and the factors that might influence them (e.g. antibiotic exposure), and consequently offers a starting point to inform *in-vivo* models. We therefore recommend that future modelling studies should build upon the work of existing *in-vitro* studies to evaluate HGT of AMR in more complex scenarios, utilising parameter estimates from *in-vitro* studies as a baseline and refining them using data generated with *in-vivo* model organisms such as mice [68]. Due to the added complexity (e.g. immune system, simultaneous within-host and between-hosts dynamics, rapidly varying host exposure to antibiotics and therefore selection pressure on the bacteria etc.), this will require major extensions to existing models. However, we believe that this is necessary to truly assess the potential consequences of HGT of AMR on human well-being.

This systematic review allowed us to identify key research gaps on the dynamics of HGT of AMR. Firstly, we recommend that future studies should focus on developing models of transformation and transduction to determine the required complexity to represent these dynamics. Since these mechanisms fundamentally differ in their biological characteristics, this will likely require substantial, novel modelling work as opposed to the extension of existing models of conjugation. In parallel, since the basic dynamics of conjugation are already reasonably well understood, future studies on this mechanism should focus on other bacterial species than *E. coli*, preferably in a setting where inter-specific HGT and the movement of multiple, separate AMR genes can both be observed. This should be achievable simply by re-parameterisation or minor extension of existing models; the greatest challenge would be to generate new data on HGT in these currently unexplored settings. The optimal solution to address these research questions would be to design frameworks to study HGT of AMR that encompass both laboratory and modelling work; this would ensure that the data collected are appropriate for the modelling needs, and that the actual model is a good representation of the situation measured in the laboratory. We therefore believe that, to fully understand the complexity of both the biology and the dynamics of HGT, collaboration of both microbiologists and mathematical modellers would be the best strategy for future research on this topic, and that studies should attempt to generate both their own data and models to reduce the assumptions they require.

While exclusively microbiological approaches have successfully been used to identify when HGT occurs, combining these with modelling has allowed us to estimate rates at which these events occur, and to disentangle the finer temporal dynamics of this process. For example, some studies we identified in our review which combined microbiology and modelling work answered questions such as how changing the exposure of bacteria to antibiotics influences HGT rates [49], how a bacterium interacts in space with its neighbours to perform HGT [31], or how to adjust shaking speed to maximise contact between bacteria, and thus the rate of HGT, in a liquid culture [66]. Modelling also allows faster exploration of situations that could be harder to test using only microbiological methods, since an experiment where the bacteria need to grow for 24 hours in the lab could be completed in a few seconds using a mathematical model. Crucially, this requires the model to be an accurate representation of reality, which in turn requires it to be informed by microbiological

data to begin with. Our conclusion here is therefore not that either one of modelling or microbiology is superior to the other, but that both approaches complement each other. Consequently, we believe that close cooperation between these two fields would allow us to greatly improve our understanding of complex microbiological processes, such as HGT of AMR.

2.8 Conclusions

In this systematic review, we aimed to assess the current state of mathematical modelling as a tool to improve our understanding of horizontal gene transfer of antimicrobial resistance. From the 43 studies identified, we found that the majority focused on conjugation in *E. coli*, exploring evolutionary dynamics of HGT in culture. Whilst this provides a solid base for a key method of HGT, future work must also consider HGT by transformation and transduction which are also essential drivers of HGT in bacteria. Importantly for public health implications, only one bacterial species was considered in most models when we know that inter species transfer is responsible for many of our epidemic AMR clones and much of the work was fitted to data in the absence of antibiotic exposure. Crucially, to answer these questions we must first clarify the level of modelling complexity required to accurately represent HGT dynamics, as well as the availability and capacity to generate experimental data on these processes. This complex topic requires close collaboration between mathematical modellers and microbiologists in order to determine the full impact of these processes on our ability to control the public health threat posed by antimicrobial resistance.

2.9 References

1. World Health Organisation. 2015 Global Action Plan on Antimicrobial Resistance.
2. Kumarasamy KK *et al.* 2010 Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *Lancet Infect. Dis.* **10**, 597–602. (doi:10.1016/S1473-

3099(10)70143-2)

3. Woodford N, Johnson AP. 2013 Global spread of antibiotic resistance: the example of New Delhi metallo-β-lactamase (NDM)-mediated carbapenem resistance. *J. Med. Microbiol.* **62**, 499–513. (doi:10.1099/jmm.0.052555-0)
4. Cantón R, González-Alba JM, Galán JC. 2012 CTX-M Enzymes: Origin and Diffusion. *Front. Microbiol.* **3**, 110. (doi:10.3389/fmicb.2012.00110)
5. Ochman H, Lawrence JG, Groisman EA. 2000 Lateral gene transfer and the nature of bacterial innovation. *Nature* **405**, 299–304. (doi:10.1038/35012500)
6. Thomas CM, Nielsen KM. 2005 Mechanisms of and Barriers to, Horizontal Gene Transfer between Bacteria. *Nat. Rev. Microbiol.* **3**, 711–721. (doi:10.1038/nrmicro1234)
7. von Wintersdorff CJH, Penders J, van Niekerk JM, Mills ND, Majumder S, van Alphen LB, Savelkoul PHM, Wolffs PFG. 2016 Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer. *Front. Microbiol.* **7**, 173. (doi:10.3389/fmicb.2016.00173)
8. Tanwar J, Das S, Fatima Z, Hameed S. 2014 Multidrug resistance: an emerging crisis. *Interdiscip. Perspect. Infect. Dis.* **2014**, 541340. (doi:10.1155/2014/541340)
9. Naidoo J. 1984 Interspecific co-transfer of antibiotic resistance plasmids in staphylococci in vivo. *J. Hyg., Camb*. **93**.
10. Melnyk AH, Wong A, Kassen R. 2015 The fitness costs of antibiotic resistance mutations. *Evol. Appl.* **8**, 273–83. (doi:10.1111/eva.12196)
11. Watanabe T, Fukasawa T. 1961 Episome-mediated transfer of drug resistance in Enterobacteriaceae I. Transfer of resistance factors by conjugation.
12. Lindsay JA. 2014 Staphylococcus aureus genomics and the impact of horizontal gene transfer. *Int. J. Med. Microbiol.* **304**, 103–109. (doi:10.1016/J.IJMM.2013.11.010)
13. Hamilton HL, Dillard JP. 2006 Natural transformation of *Neisseria gonorrhoeae* : from DNA donation to homologous recombination. *Mol. Microbiol.* **59**, 376–385. (doi:10.1111/j.1365-2958.2005.04964.x)
14. McCarthy AJ, Loeffler A, Witney AA, Gould KA, Lloyd DH, Lindsay JA. 2014 Extensive horizontal gene transfer during Staphylococcus aureus co-colonization in vivo. *Genome Biol. Evol.* **6**, 2697–708. (doi:10.1093/gbe/evu214)
15. Gottig S, Gruber TM, Stecher B, Wichelhaus TA, Kempf VAJ. 2015 In Vivo

Horizontal Gene Transfer of the Carbapenemase OXA-48 During a Nosocomial Outbreak. *Clin. Infect. Dis.* **60**, 1808–1815. (doi:10.1093/cid/civ191)

16. Velkov VW. 1999 How environmental factors regulate mutagenesis and gene transfer in microorganisms.
17. Hastings PJ, Rosenberg SM, Slack A. 2004 Antibiotic-induced lateral transfer of antibiotic resistance. *Trends Microbiol.* **12**, 401–404. (doi:10.1016/J.TIM.2004.07.003)
18. Beaber JW, Hochhut B, Waldor MK. 2004 SOS response promotes horizontal dissemination of antibiotic resistance genes. *Nature* **427**, 72–74. (doi:10.1038/nature02241)
19. Maiques E, Ubeda C, Campoy S, Salvador N, Lasa I, Novick RP, Barbé J, Penadés JR. 2006 beta-lactam antibiotics induce the SOS response and horizontal transfer of virulence factors in Staphylococcus aureus. *J. Bacteriol.* **188**, 2726–9. (doi:10.1128/JB.188.7.2726-2729.2006)
20. Prudhomme M, Attaiech L, Sanchez G, Martin B, Claverys J-P. 2006 Antibiotic Stress Induces Genetic Transformability in the Human Pathogen Streptococcus pneumoniae. *Science (80-.).* **313**, 89–92. (doi:10.1126/SCIENCE.1127912)
21. Jassim SAA, Limoges RG. 2017 Bacteriophage and Antimicrobial Resistance. In *Bacteriophages: Practical Applications for Nature's Biocontrol*, pp. 19–57. Cham: Springer International Publishing. (doi:10.1007/978-3-319-54051-1_2)
22. Verheust C, Pauwels K, Mahillon J, Helinski DR, Herman P. 2010 Contained use of Bacteriophages: Risk Assessment and Biosafety Recommendations. *Appl. Biosaf.* **15**, 32–44. (doi:10.1177/153567601001500106)
23. Drlica K, Gennaro ML. 2001 Plasmids. *Encycl. Genet.* , 1485–1490. (doi:10.1006/RWGN.2001.1000)
24. Anderson RM, May RM. 1991 *Infectious diseases of humans : dynamics and control*. Oxford University Press.
25. Opatowski L, Guillemot D, Boëlle P-Y, Temime L. 2011 Contribution of mathematical modeling to the fight against bacterial antibiotic resistance. *Curr. Opin. Infect. Dis.* **24**, 279–287. (doi:10.1097/QCO.0b013e3283462362)
26. van Kleef E, Robotham J V, Jit M, Deeny SR, Edmunds WJ. 2013 Modelling the transmission of healthcare associated infections: a systematic review. *BMC Infect. Dis.* **13**, 294. (doi:10.1186/1471-2334-13-294)
27. Davies NG, Flasche S, Jit M, Atkins KE. 2019 Within-host dynamics shape

antibiotic resistance in commensal bacteria. *Nat. Ecol. Evol.* , 1. (doi:10.1038/s41559-018-0786-x)

28. van Kleef E, Luangasanatip N, Bonten MJ, Cooper BS. 2017 Why sensitive bacteria are resistant to hospital infection control. *Wellcome Open Res.* **2**, 16. (doi:10.12688/wellcomeopenres.11033.2)
29. Liberati A *et al.* 2009 The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate healthcare interventions: explanation and elaboration. *BMJ* **339**, b2700. (doi:10.1136/bmj.b2700)
30. D'Agata EMC, Dupont-Rouzeyrol M, Magal P, Olivier D, Ruan S. 2008 The Impact of Different Antibiotic Regimens on the Emergence of Antimicrobial-Resistant Bacteria. *PLoS One* **3**, e4036. (doi:10.1371/journal.pone.0004036)
31. Gehring R, Schumm P, Youssef M, Scoglio C. 2010 A network-based approach for resistance transmission in bacterial populations. *J. Theor. Biol.* **262**, 97–106. (doi:10.1016/j.jtbi.2009.09.002)
32. Obolski U, Hadany L. 2012 Implications of stress-induced genetic variation for minimizing multidrug resistance in bacteria. *BMC Med.* **10**, 89. (doi:10.1186/1741-7015-10-89)
33. Krone SM, Lu R, Fox R, Suzuki H, Top EM. 2007 Modelling the spatial dynamics of plasmid transfer and persistence. *Microbiology* **153**, 2803–16. (doi:10.1099/mic.0.2006/004531-0)
34. Lundquist PD, Levin' BR. 1986 Transitory derepression and the maintenance of conjugative plasmids. *Genetics*. **113**.
35. Ponciano JM, De Gelder L, Top EM, Joyce P. 2007 The population biology of bacterial plasmids: a hidden Markov model approach. *Genetics* **176**, 957–68. (doi:10.1534/genetics.106.061937)
36. Fischer EA, Dierikx CM, van Essen-Zandbergen A, van Roermund HJ, Mevius DJ, Stegeman A, Klinkenberg D. 2014 The IncI1 plasmid carrying the blaCTX-M-1 gene persists in in vitro culture of a Escherichia coli strain from broilers. *BMC Microbiol.* **14**, 77. (doi:10.1186/1471-2180-14-77)
37. Svara F, Rankin DJ. 2011 The evolution of plasmid-carried antibiotic resistance. *BMC Evol. Biol.* **11**, 130. (doi:10.1186/1471-2148-11-130)
38. Willms AR, Roughan PD, Heinemann JA. 2006 Static recipient cells as reservoirs of antibiotic resistance during antibiotic therapy. *Theor. Popul. Biol.* **70**, 436–51. (doi:10.1016/j.tpb.2006.04.001)

39. Hall JPJ, Wood AJ, Harrison E, Brockhurst MA. 2016 Source-sink plasmid transfer dynamics maintain gene mobility in soil bacterial communities. *Proc. Natl. Acad. Sci. U. S. A.* **113**, 8260–5. (doi:10.1073/pnas.1600974113)
40. Dionisio F, Matic I, Radman M, Rodrigues OR, Taddei F. 2002 Plasmids spread very fast in heterogeneous bacterial communities. *Genetics* **162**, 1525–32.
41. Zhong X, Droesch J, Fox R, Top EM, Krone SM. 2012 On the meaning and estimation of plasmid transfer rates for surface-associated and well-mixed bacterial populations. *J. Theor. Biol.* **294**, 144–152. (doi:10.1016/J.JTBI.2011.10.034)
42. Qiu Z *et al.* 2015 Effects of nano-TiO2 on antibiotic resistance transfer mediated by RP4 plasmid. *Nanotoxicology* **9**, 895–904. (doi:10.3109/17435390.2014.991429)
43. Cazer CL, Ducrot L, Volkova V V., Gröhn YT. 2017 Monte Carlo Simulations Suggest Current Chlortetracycline Drug-Residue Based Withdrawal Periods Would Not Control Antimicrobial Resistance Dissemination from Feedlot to Slaughterhouse. *Front. Microbiol.* **8**. (doi:10.3389/fmicb.2017.01753)
44. Volkova V V, Lu Z, Lanzas C, Scott HM, Gröhn YT. 2013 Modelling dynamics of plasmid-gene mediated antimicrobial resistance in enteric bacteria using stochastic differential equations. *Sci. Rep.* **3**, 2463. (doi:10.1038/srep02463)
45. Baker M, Hobman JL, Dodd CER, Ramsden SJ, Stekel DJ. 2016 Mathematical modelling of antimicrobial resistance in agricultural waste highlights importance of gene transfer rate. *FEMS Microbiol. Ecol.* **92**, fiw040. (doi:10.1093/femsec/fiw040)
46. Volkova V V, Lanzas C, Lu Z, Gröhn YT. 2012 Mathematical model of plasmid-mediated resistance to ceftiofur in commensal enteric Escherichia coli of cattle. *PLoS One* **7**, e36738. (doi:10.1371/journal.pone.0036738)
47. Kneis D, Hiltunen T, Hess S. 2019 A high-throughput approach to the culture-based estimation of plasmid transfer rates. *Plasmid* **101**, 28–34. (doi:10.1016/j.plasmid.2018.12.003)
48. Knopoff DA, Sanchez Sanso JM. 2017 A kinetic model for horizontal transfer and bacterial antibiotic resistance. *Int. J. Biomath.* **10**. (doi:10.1142/S1793524517500516)
49. Lopatkin AJ, Huang S, Smith RP, Srimani JK, Sysoeva TA, Bewick S, Karig DK, You L. 2016 Antibiotics as a selective driver for conjugation dynamics. *Nat.*

Microbiol. **1**, 16044. (doi:10.1038/nmicrobiol.2016.44)

50. Peña-Miller R, Rodríguez-González R, MacLean RC, San Millan A. 2015 Evaluating the effect of horizontal transmission on the stability of plasmids under different selection regimes. *Mob. Genet. Elements* **5**, 29–33. (doi:10.1080/2159256X.2015.1045115)
51. Heuer H, Focks A, Lamshoeft M, Smalla K, Matthies M, Spiteller M. 2008 Fate of sulfadiazine administered to pigs and its quantitative effect on the dynamics of bacterial resistance genes in manure and manured soil. *Soil Biol. Biochem.* **40**, 1892–1900. (doi:10.1016/j.soilbio.2008.03.014)
52. Freese PD, Korolev KS, Jimenez JI, Chen IA. 2014 Genetic Drift Suppresses Bacterial Conjugation in Spatially Structured Populations. *Biophys. J.* **106**, 944–954. (doi:10.1016/j.bpj.2014.01.012)
53. Simonsen L, Gordon DM, Stewart FM, Levin BR. 1990 Estimating the rate of plasmid transfer: an end-point method. *J. Gen. Microbiol.* **136**, 2319–2325. (doi:10.1099/00221287-136-11-2319)
54. Gothwal R, Thatikonda S. 2018 Mathematical model for the transport of fluoroquinolone and its resistant bacteria in aquatic environment. *Environ. Sci. Pollut. Res. Int.* **25**, 20439–20452. (doi:10.1007/s11356-017-9848-x)
55. Ibargueen-Mondragon E, Romero-Leiton JP, Esteva L, Mariela Burbano-Rosero E. 2016 Mathematical modeling of bacterial resistance to antibiotics by mutations and plasmids. *J. Biol. Syst.* **24**, 129–146. (doi:10.1142/S0218339016500078)
56. Zwanzig M, Harrison E, Brockhurst MA, Hall JPJ, Berendonk TU, Berger U. 2019 Mobile Compensatory Mutations Promote Plasmid Survival. *mSystems* **4**. (doi:10.1128/mSystems.00186-18)
57. Connelly BD, Zaman L, McKinley PK, Ofria C. 2011 Modeling the Evolutionary Dynamics of Plasmids in Spatial Populations. In *GECCO-2011: Proceedings of the 13th Annual Genetic and Evolutionary Computation conference* (ed N Krasnogor), pp. 227–233. 1515 BROADWAY, NEW YORK, NY 10036-9998 USA: ASSOC COMPUTING MACHINERY.
58. Khan A, Imran M. 2018 Optimal dosing strategies against susceptible and resistant bacteria. *J. Biol. Syst.* **26**, 41–58. (doi:10.1142/S0218339018500031)
59. Malwade A, Nguyen A, Sadat-Mousavi P, Ingalls BP. 2017 Predictive Modeling of a Batch Filter Mating Process. *Front. Microbiol.* **8**.

(doi:10.3389/fmicb.2017.00461)

60. Campos M *et al.* 2019 Simulating Multilevel Dynamics of Antimicrobial Resistance in a Membrane Computing Model. *MBio* **10**, e02460-18. (doi:10.1128/mBio.02460-18)
61. Xu S, Yang J, Yin C, Zhao X. 2018 The dominance of bacterial genotypes leads to susceptibility variations under sublethal antibiotic pressure. *Future Microbiol.* **13**, 165–185. (doi:10.2217/fmb-2017-0070)
62. Raz Y, Tannenbaum E. 2010 The influence of horizontal gene transfer on the mean fitness of unicellular populations in static environments. *Genetics* **185**, 327–337. (doi:10.1534/genetics.109.113613)
63. Haverkate MR, Dautzenberg MJD, Ossewaarde TJM, van der Zee A, den Hollander JG, Troelstra A, Bonten MJM, Bootsma MCJ. 2015 Within-Host and Population Transmission of bla(OXA-48) in K. pneumoniae and E. coli. *PLoS One* **10**. (doi:10.1371/journal.pone.0140960)
64. Volkova V V, Lu Z, Lanzas C, Grohn YT. 2013 Evaluating targets for control of plasmid-mediated antimicrobial resistance in enteric commensals of beef cattle: a modelling approach. *Epidemiol. Infect.* **141**, 2294–312. (doi:10.1017/S0950268812002993)
65. Webb GF, D'Agata EMC, Magal P, Ruan S. 2005 A model of antibiotic-resistant bacterial epidemics in hospitals. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 13343–8. (doi:10.1073/pnas.0504053102)
66. Zhong X, Krol JEJE, Top EM, Krone SM. 2010 Accounting for mating pair formation in plasmid population dynamics. *J. Theor. Biol.* **262**, 711–719. (doi:10.1016/j.jtbi.2009.10.013)
67. Stewart FM, Levin BR. 1977 The Population Biology of Bacterial Plasmids: A PRIORI Conditions for the Existence of Conjugationally Transmitted Factors. *Genetics* **87**, 209–28.
68. Freter R, Freter RR, Brickner H. 1983 Experimental and mathematical models of Escherichia coli plasmid transfer in vitro and in vivo. *Infect. Immun.* **39**, 60–84.
69. Gomes ALC, Galagan JE, Segrè D. 2013 Resource competition may lead to effective treatment of antibiotic resistant infections. *PLoS One* **8**, e80775. (doi:10.1371/journal.pone.0080775)
70. Johnsen PJ, Dubnau D, Levin BR. 2009 Episodic selection and the maintenance

of competence and natural transformation in Bacillus subtilis. *Genetics* **181**, 1521–33. (doi:10.1534/genetics.108.099523)

71. Lu N, Massoudieh A, Liang X, Kamai T, Zilles JL, Nguyen TH, Ginn TR. 2015 A kinetic model of gene transfer via natural transformation of Azotobacter vinelandii. *Environ. Sci. Res. Technol.* **1**, 363–374. (doi:10.1039/c5ew00023h)
72. Volkova V V, Lu Z, Besser T, Gröhn YT. 2014 Modeling the infection dynamics of bacteriophages in enteric Escherichia coli: estimating the contribution of transduction to antimicrobial gene spread. *Appl. Environ. Microbiol.* **80**, 4350–62. (doi:10.1128/AEM.00446-14)
73. Volkova V V., Lanzas C, Lu Z, Gröhn YT. 2012 Mathematical Model of Plasmid-Mediated Resistance to Ceftiofur in Commensal Enteric Escherichia coli of Cattle. *PLoS One* **7**, e36738. (doi:10.1371/journal.pone.0036738)
74. Cooper GM, Hausman RE. 2015 *The Cell: A Molecular Approach, Seventh Edition*. See https://www.sinauer.com/media/wysiwyg/samples/TheCell7e_Brochure.pdf.
75. Tsubakishita S, Kuwahara-Arai K, Sasaki T, Hiramatsu K. 2010 Origin and molecular evolution of the determinant of methicillin resistance in staphylococci. *Antimicrob. Agents Chemother.* **54**, 4352–9. (doi:10.1128/AAC.00356-10)
76. Stanczak-Mrozek KI, Laing KG, Lindsay JA. 2017 Resistance gene transfer: induction of transducing phage by sub-inhibitory concentrations of antimicrobials is not correlated to induction of lytic phage. *J. Antimicrob. Chemother.* **72**, 1624. (doi:10.1093/JAC/DKX056)
77. Lerminiaux NA, Cameron ADS. 2019 Horizontal transfer of antibiotic resistance genes in clinical environments. *Can. J. Microbiol.* **65**, 34–44. (doi:10.1139/cjm-2018-0275)
78. Breban R, Drake JM, Stallknecht DE, Rohani P. 2009 The Role of Environmental Transmission in Recurrent Avian Influenza Epidemics. *PLoS Comput. Biol.* **5**, e1000346. (doi:10.1371/journal.pcbi.1000346)
79. Day T. 2002 Virulence evolution via host exploitation and toxin production in spore-producing pathogens. *Ecol. Lett.* **5**, 471–476. (doi:10.1046/j.1461-0248.2002.00342.x)
80. Haverkate MR, Derde LPG, Brun-Buisson C, Bonten MJM, Bootsma MCJ. 2014 Duration of colonization with antimicrobial-resistant bacteria after ICU discharge. *Intensive Care Med.* **40**, 564–571. (doi:10.1007/s00134-014-3225-8)

81. Haverkate MR, Weiner S, Lolans K, Moore NM, Weinstein RA, Bonten MJM, Hayden MK, Bootsma MCJ. 2016 Duration of Colonization With Klebsiella pneumoniae Carbapenemase-Producing Bacteria at Long-Term Acute Care Hospitals in Chicago, Illinois. *Open forum Infect. Dis.* **3**, ofw178. (doi:10.1093/ofid/ofw178)
82. O'Fallon E, Gautam S, D'Agata EMC. 2009 Colonization with Multidrug-Resistant Gram-Negative Bacteria: Prolonged Duration and Frequent Cocolonization. *Clin. Infect. Dis.* **48**, 1375–1381. (doi:10.1086/598194)

3 Growth-dependent predation and generalised transduction of antimicrobial resistance by bacteriophage

3.1 Overview

The systematic review I conducted, presented in Chapter 2, highlighted an important lack of knowledge on the dynamics of transduction of AMR, and how to best represent them mathematically. This research gap is particularly worrying in the case of *S. aureus*, as transduction is the main mechanism of HGT in these bacteria, and existing evidence suggests that AMR genes may frequently move within *S. aureus* populations. The three studies modelling transduction published before this thesis explored these dynamics in complex settings, aiming to obtain a theoretical understanding of this process. In this Chapter, I instead aimed to gain a fundamental understanding of the core dynamics of generalised transduction in *S. aureus*, as well as the dynamics of phage predation which must be considered since generalised transduction is directly dependent on these.

I designed this work with an interdisciplinary approach, combining lab work and mathematical modelling. I first conducted *in vitro* experiments, where I co-cultured single antibiotic-resistant strains of *S. aureus* alongside phage capable of generalised transduction, with hourly counts of bacteria and phage concentrations in the co-culture over 24h. Via generalised transduction, double antibiotic-resistant bacteria appeared, whilst bacteria simultaneously grew by replication, and phage multiplied via lytic infection. Lysogeny and specialised transduction were not detected in this system. The advantage of these well-defined experimental conditions is that I was able to design a mathematical model with a structure mirroring the *in vitro* environment. I then considered several interaction terms to represent phage predation, based on elements suggested in previous studies, yet never combined. This included linear versus saturated predation, and a link between bacterial growth and either or both phage adsorption rate and burst size. By fitting the model to the data, I was then able to

identify the underlying biological mechanisms behind phage predation and quantify generalised transduction dynamics in *S. aureus*.

This work has been published following peer-review in mSystems (Leclerc *et al.*, 2022). The co-authors are Quentin J Leclerc, Jacob Wildfire, Arya Gupta, Jodi A Lindsay, and Gwenan M Knight. The version included below is the author accepted manuscript, with some changes following examiner corrections which will be submitted as a correction to the published article. Importantly, the manuscript only presents the final *in vitro* results, and therefore does not reflect the initial experimental work conducted to design the protocol, and the work necessary to optimise the experimental conditions and replicate results consistently. Overall, the experimental work presented in this Chapter was conducted over a period of 21 months (February 2019 to October 2020), although this included a 4 months disruption due to the COVID-19 pandemic.

3.2 Research paper cover sheet

Please note that a cover sheet must be completed <u>for each</u> research paper included within a thesis.

<u>SECTION A – Student Details</u>

Student ID Number	1702140	**Title**	Mr
First Name(s)	Quentin		
Surname/Family Name	Leclerc		
Thesis Title	Interdisciplinary investigations of phage predation dynamics and generalised transduction of antimicrobial resistance in *Staphylococcus aureus*		
Primary Supervisor	Dr Gwenan Knight		

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

<u>SECTION B – Paper already published</u>

Where was the work published?	mSystems		
When was the work published?	21/03/2022		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion	N/A		
Have you retained the copyright for the work?*	**Yes**	Was the work subject to academic peer review?	**Yes**

***Copyright retention: see mSystems FAQ available here https://journals.asm.org/journal/msystems/faq**

SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	
Please list the paper's authors in the intended authorship order:	
Stage of publication	Choose an item.

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I designed the in vitro experiments, and conducted them to generate the data in this paper. I designed the mathematical models, coded them, fitted them to the data, and ran simulations to generate the results presented in this paper. I then wrote the first draft of this manuscript, and subsequently edited it. Jacob Wildfire generated the in vitro data for Figure 5c.

SECTION E

Student Signature	[redacted]
Date	06/06/2022

Supervisor Signature	[redacted]
Date	06/06/2022

3.3 Abstract and Author Summary

3.3.1 Abstract

Bacteriophage (“phage”) are both predators and evolutionary drivers for bacteria, notably contributing to the spread of antimicrobial resistance (AMR) genes by generalised transduction. Our current understanding of this complex relationship is limited. We used an interdisciplinary approach to quantify how these interacting dynamics can lead to the evolution of multi-drug resistant bacteria. We co-cultured two strains of Methicillin-resistant *Staphylococcus aureus*, each harbouring a different antibiotic resistance gene, with generalized transducing phage. After a growth phase of 8h, bacteria and phage surprisingly coexisted at a stable equilibrium in our culture, the level of which was dependent on the starting concentration of phage. We detected double-resistant bacteria as early as 7h, indicating that transduction of AMR genes had occurred. We developed multiple mathematical models of the bacteria and phage relationship, and found that phage-bacteria dynamics were best captured by a model in which phage burst size decreases as the bacterial population reaches stationary phase, and where phage predation is saturated at high concentrations. We estimated that one in every 10^8 new phage generated was a transducing phage carrying an AMR gene, and that double-resistant bacteria were always predominantly generated by transduction rather than by growth. Our results suggest a shift in how we understand and model phage-bacteria dynamics. Although rates of generalised transduction could be interpreted as too rare to be significant, they are sufficient in our system to consistently lead to the evolution of multi-drug resistant bacteria. Currently, the potential of phage to contribute to the growing burden of AMR is likely underestimated.

3.3.2 Author Summary

Bacteriophage (phage), viruses that can infect and kill bacteria, are being investigated through phage therapy as a potential solution to the threat of antimicrobial resistance (AMR). In reality, however, phage are also natural drivers of bacterial evolution by transduction when they accidentally carry nonphage DNA between bacteria. Using laboratory work and mathematical models, we show that transduction leads to

evolution of multidrug-resistant bacteria in less than 8 h and that phage production decreases when bacterial growth decreases, allowing bacteria and phage to coexist at stable equilibria. The joint dynamics of phage predation and transduction lead to complex interactions with bacteria, which must be clarified to prevent phage from contributing to the spread of AMR.

3.4 Introduction

Bacteriophage (or “phage”) are major bacteria predators and the most abundant biological entities on the planet [1]. However, phage are also natural drivers of bacterial evolution through horizontal gene transfer by “transduction” [2,3]. Antimicrobial resistance (AMR) genes can be transferred by transduction at high rates, both *in vitro* and *in vivo* [4–6], meaning that phage may be substantially contributing to the rapidly increasing global public health threat of AMR [7]. However, our understanding of these joint dynamics of predation and transduction and how to best represent them is limited.

There are two main types of transduction; here, we focus on “generalised transduction”, which occurs during the phage lytic cycle, when non-phage genome DNA is mistakenly packaged in a new phage particle (Figure 3.1). The resulting transducing phage released upon lysis can then inject this genetic material into another bacterium. The second type of transduction, specialised transduction, relies on lysogeny, during which sections of bacterial DNA adjacent to the prophage integration site may be transferred upon excision of the prophage [8,9]. Generalised transduction is currently often dismissed as too rare to be significant, yet it is likely a substantial contributor to AMR spread as it is a common mechanism for the transfer of plasmids, major vectors of AMR genes [2]. There are currently no estimates or work quantifying rates of transduction of AMR genes under various conditions. Previous reviews have highlighted the necessity to further investigate the potential impact of transduction in the context of phage therapy, where phage are used as antimicrobial agents against bacteria [10–13].

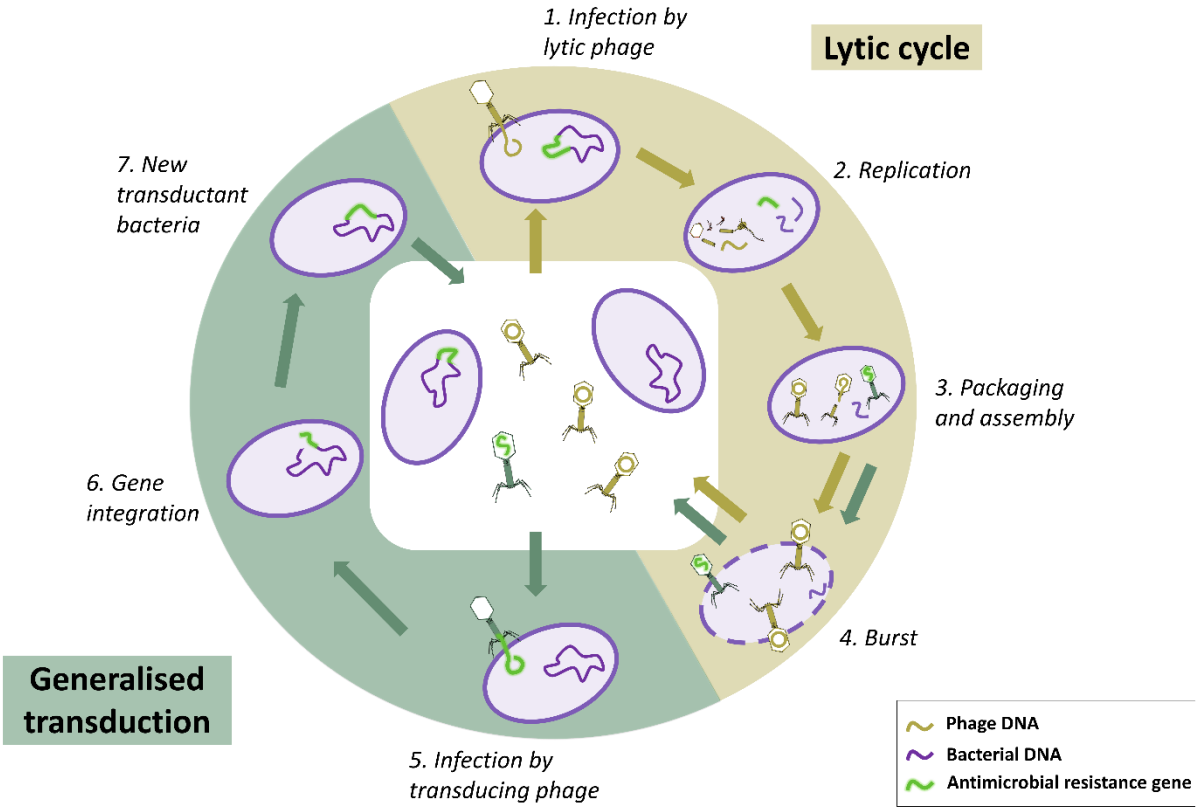


Figure 3.1: Phage lytic cycle and generalised transduction. In this environment, only some bacteria carry an antimicrobial resistance (AMR) gene (shown in green). The lytic cycle starts when a lytic phage infects a bacterium by binding and injecting its DNA (1). Phage molecules degrade bacterial DNA and utilise bacterial resources to create new phage components and replicate (2). These components are then assembled to form new phage particles (3). At this stage, bacterial DNA leftover in the cell can be packaged by mistake instead of phage DNA, which creates a transducing phage and starts the process of generalised transduction. In our example, the transducing phage carries the AMR gene. After a latent period of typically several minutes, the phage trigger lysis of the bacterium, bursting it and releasing the phage (4). The transducing phage can infect another bacterium, binding and injecting the AMR gene it is carrying (5). If this gene is successfully integrated into the bacterial chromosome (6), this creates a new transductant bacterium carrying this AMR gene (7). Note that the transduced bacterial DNA could also be a plasmid, in which case it would circularise instead of integrating into the chromosome of the transductant bacterium. Not to scale.

Mathematical models have been used to gain insights into phage predation dynamics which cannot be obtained solely with experimental work, such as rates of predation and optimal conditions for phage to clear bacteria [14]. Such models typically assume a density-dependent interaction, with new phage infections calculated as the number of susceptible bacteria, multiplied by the number of phage and an adsorption constant [14–16]. This approach has limitations, as density-dependent models have failed to predict equilibria observed in some *in vitro* conditions between phage and *E. coli* [17]. Moreover, phage and bacterial replication are likely to be linked, as they both rely on the bacterial machinery; phage predation may slow as bacteria reach stationary phase [14,17–23]. However, this is a feature which is not commonly included in mathematical models of phage-bacteria dynamics [14]. Finally, models often only rely on data of phage-bacteria interactions measured once per day, or for a few hours [17–19,24]. A current lack of detailed data means that capturing these underlying dynamics which occur in less than an hour has not yet been possible.

To the best of our knowledge, only three modelling studies have included transduction of AMR genes [25–27]. All three modelled complex environments, including resistance to phage, antibiotics, and both lytic and lysogenic cycles. This complexity, combined with the fact that these studies were not paired with complementary *in vitro* or *in vivo* data, means that they relied on assumptions and previously published estimates, instead of parameter values derived from a single environment and set of conditions. This limits the wider reliability of conclusions made using these models [12].

In this article, we investigate the dual nature of phage dynamics using the clinically relevant bacteria Methicillin-resistant *Staphylococcus aureus* (MRSA) [28]. Transduction is the main mechanism of horizontal gene transfer driving evolution for these bacteria [29], and phage therapy is currently being investigated to treat MRSA infections [30,31]. We aim to clarify the joint dynamics of predation and generalised transduction between MRSA and phage by generating novel *in vitro* data, identifying biologically plausible hypotheses which may explain the dynamics seen, and developing mathematical models to test the validity of these hypotheses in our system.

3.5 Results

3.5.1 Transduction and phage predation dynamics *in vitro*

We focused on two laboratory strains of *Staphylococcus aureus*, each resistant to either erythromycin (and referred to as B_E) or tetracycline (B_T). In our experimental conditions, the antimicrobial resistance (AMR) genes can only be transferred between bacteria by generalised transduction mediated by exogenous phage. Transduction of either AMR gene to the other strain will result in the formation of double-resistant progeny (referred to as B_{ET}).

We conducted a co-culture with only the two single-resistant strains and exogenous phage (P_L) capable of generalised transduction. We detected double-resistant progeny (B_{ET}) as early as 7h in our co-cultures, indicating that transfer of AMR genes by generalised transduction had occurred (Figure 3.2). B_{ET} numbers remained below 100 cfu/mL after 24h, but were consistently generated in each of our experimental replicates. Colonies of double-resistant progeny were screened using polymerase chain reaction (PCR) to confirm that they contained both resistance genes *ermB* and *tetK*, and had not instead gained resistance to either antibiotic by mutation (Supplementary Figure 3.1).

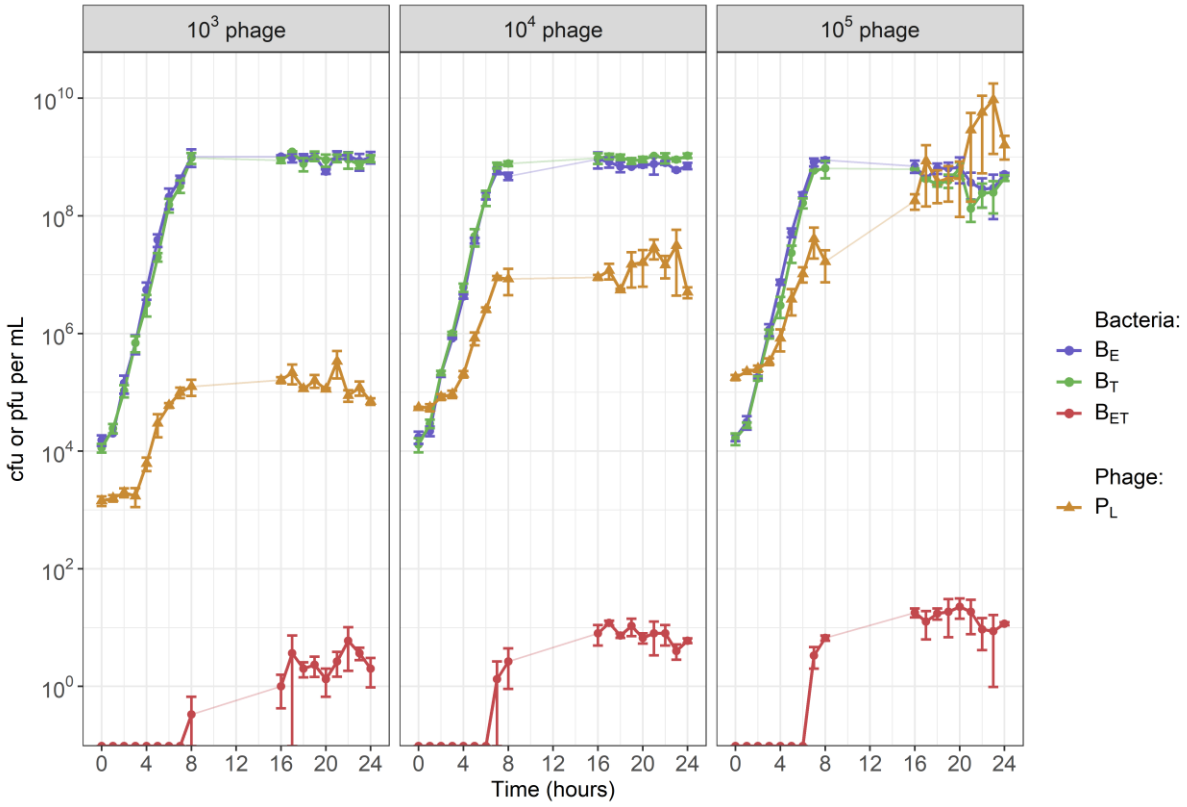


Figure 3.2: The starting concentration of exogenous phage 80α affected the equilibrium values of phage and bacteria in our co-cultures. The starting concentration of both single-resistant *S. aureus* parent strains (B_E to erythromycin & B_T to tetracycline) was 10^4 colony-forming units (cfu) per mL. Each panel shows the results with a different starting concentration of exogenous phage (P_L): either 10^3, 10^4 or 10^5 plaque-forming units (pfu) per mL. We detected double-resistant progeny (B_{ET}) as early as 7h, indicating that transduction occurred rapidly. Error bars indicate mean +/- standard error, from 3 experimental replicates. There is no data for the time period 9h-15h.

The starting concentration of exogenous phage affected whether phage and bacteria were able to reach an equilibrium and co-exist without increasing or decreasing in our co-cultures (Figure 3.2). With a starting concentration of either 10^3 or 10^4 pfu/mL (equivalent to multiplicities of infection of 0.1 or 1, defined as starting ratio of phage to bacteria [32]), lytic phage reached a steady-state after 8h (at approximately 10^5 pfu/mL for a starting concentration of 10^3, and 10^7 pfu/mL for 10^4). In both cases, bacteria replicated for 8h before reaching a steady-state around 10^9 cfu/mL, similar to what was seen in the absence of exogenous phage (Supplementary Figure 3.2). With a

starting phage concentration of 10^5 pfu/mL (multiplicity of infection of 10), we did not see an equilibrium between phage and bacteria, as phage numbers kept increasing up to 10^{10} pfu/mL by 24h, and bacteria numbers started decreasing after 20h. The datasets are shown overlaid in Supplementary Figure 3.3.

3.5.2 Absence of lysogeny in our co-culture

The phage we used in our experiments is 80α, a well-known generalised transducing phage. It has also been reported as a temperate phage, which means that it may undergo lysogeny and integrate in the bacterial chromosome as a prophage [33]. This would grant lysogenic immunity to the bacteria, preventing further lytic infection by 80α, and potentially explaining why bacteria and phage densities reached steady-states in our experiments (Figure 3.2).

To investigate whether this was a potential mechanism, we initiated co-cultures either with stock bacteria, or bacteria exposed to phage during a previous co-culture. We did not see any difference in phage and bacteria numbers after 24h regardless of whether or not the bacteria had been previously exposed to phage, suggesting that lysogenic immunity, or any other mechanism of phage resistance (e.g. surface modification), has not been substantially gained by bacteria over 24h (Figure 3.3a).

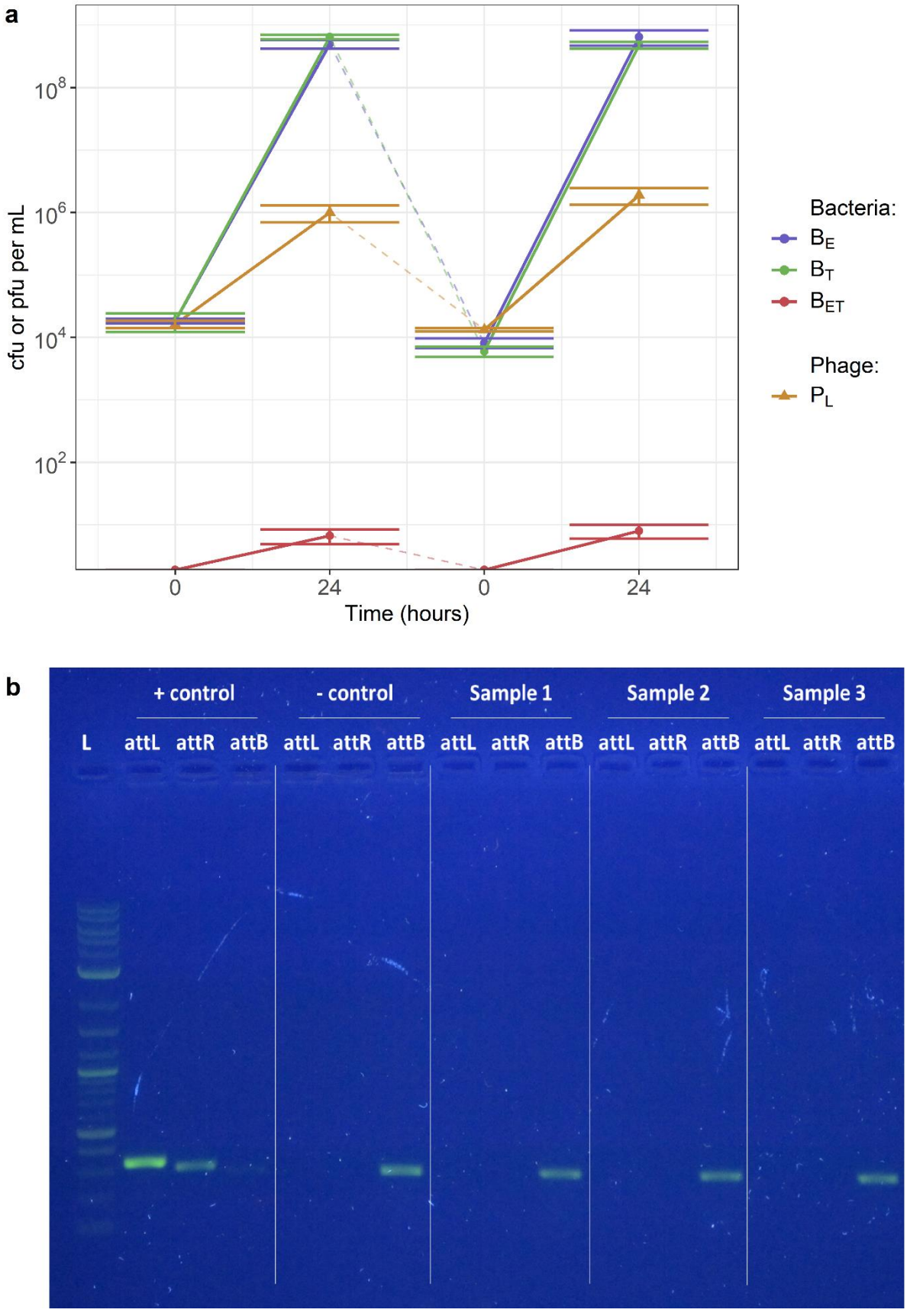


Figure 3.3: 80α lysogeny does not occur at a detectable level in our co-culture.

(a) Co-cultures with bacteria not exposed or previously exposed to phage. The

starting concentration of both single-resistant *S. aureus* parent strains (B_E to erythromycin & B_T to tetracycline) was 10^4 colony-forming units (cfu) per mL, and the starting concentration of exogenous phage 80α (P_L) was 10^4 plaque-forming units (pfu) per mL. Double-resistant progeny (B_{ET}) are generated by transduction. The initial co-culture was diluted in fresh media after 24h, to form a new co-culture with bacteria previously exposed to phage. Phage were added in the new co-culture to reach a concentration of 10^4 pfu/mL. Error bars indicate mean +/- standard error, from 3 experimental replicates. **(b) Confirmation of absence of detectable lysogeny by polymerase-chain reaction.** DNA was extracted from the co-cultures after 24h. *S. aureus* RN4220 strains lysogenic and non-lysogenic for 80α were used as positive and negative controls. L: ladder; *attL*: left prophage junction; *attR*: right prophage junction; *attB*: bacterial insertion site. Detection of *attL* and *attR* indicates that prophage are present in the DNA, while detection of *attB* indicates the presence of bacteria not lysogenic for 80α.

In addition, we extracted DNA from 1mL of co-cultures after 24h, and conducted PCRs targeting the prophage junctions (*attL* and *attR*) and bacterial insertion site (*attB*) with a positive control of a strain lysogenic for 80α. This DNA extraction from population samples and PCR protocol mean that the detection limit for our protocol is a frequency of at least 3.3×10^{-8} lysogenic per non-lysogenic bacteria after 24h of co-culture (see Materials and Methods for details). Only the intact bacterial insertion site was detected in our samples, indicating an absence of prophage in our bacteria above this detection limit (Figure 3.3b).

Another concern linked to lysogeny we must address is that, if lysogeny did occur, the movement of the resistance genes *tetK* and *ermB* could have occurred by specialised instead of generalised transduction. However, this is unlikely to be the case in our system since specialised transduction can only lead to transfer of genes adjacent to the integrated prophage [8,9]. This adjacency limitation also applies to lateral transduction, a type of specialised transduction reported for 80α leading to higher rates of transfer for DNA located downstream of the insertion site [34]. This condition of proximity to the insertion site is not met in our system. The tetracycline resistance marker *tetK* is located on a plasmid, where 80α cannot integrate, thus preventing

specialised and lateral transduction. As for the erythromycin resistance marker *ermB*, the distance between the location of this gene on the chromosome (position 2,126,759bp [35]) and the 80α integration site (next to the *rpmF* gene [33], position 1,122,198bp [35]) suggests specialised and lateral transduction are unlikely.

Overall, these results suggest that after 24h the frequency of lysogenic per non-lysogenic bacteria is less than 3.3×10^{-8} in our co-culture, and hence it is reasonable to exclude any dynamics relating to lysogeny and specialised or lateral transduction in our analysis and model below. Therefore, phage lysis and generalised transduction are likely the main mechanisms shaping phage-bacteria interactions in our co-culture.

3.5.3 Bacterial growth estimates in the absence of exogenous phage

When grown together in the absence of exogenous phage, single and double resistant bacteria replicated exponentially and reached stationary phase after 8h at approximately 2×10^9 colony-forming units (cfu) per mL (Supplementary Figure 3.2).

B_E did not show a significant fitness cost relative to B_T over 24h of growth (mean relative fitness 1.02, sd 0.03). The double-resistant progeny B_{ET} did not show a significant fitness cost relative to either single-resistant parent strain (mean relative fitness to B_E: 0.96, sd 0.06; mean relative fitness to B_T: 0.98, sd 0.03).

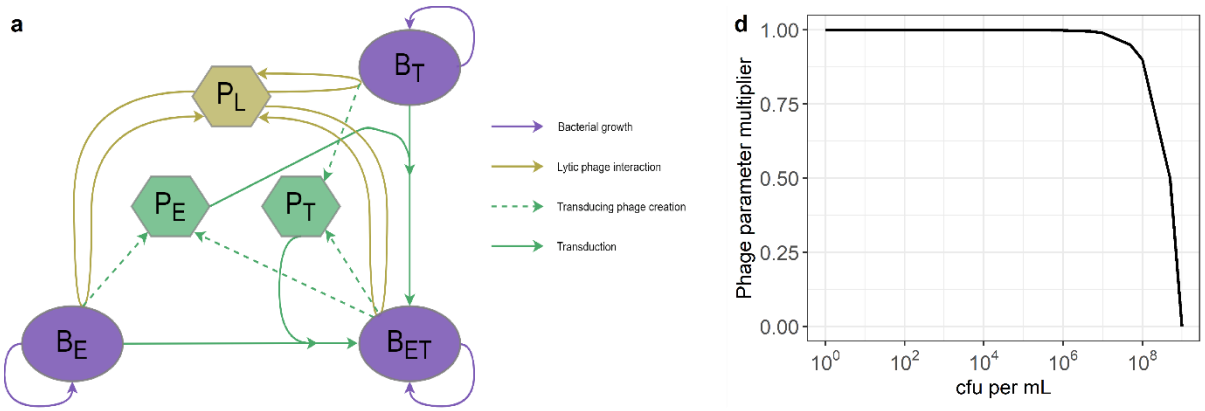
We obtained maximum growth rate estimates by fitting a logistic growth model to the *in vitro* data. The median estimated maximum growth rates were 1.61 for B_E (95% credible interval 1.59-1.63), 1.51 for B_T (1.49-1.53) and 1.44 for B_{ET} (1.42-1.47), with a total carrying capacity of 2.76×10^9 cfu/mL (2.61×10^9 - 2.98×10^9).

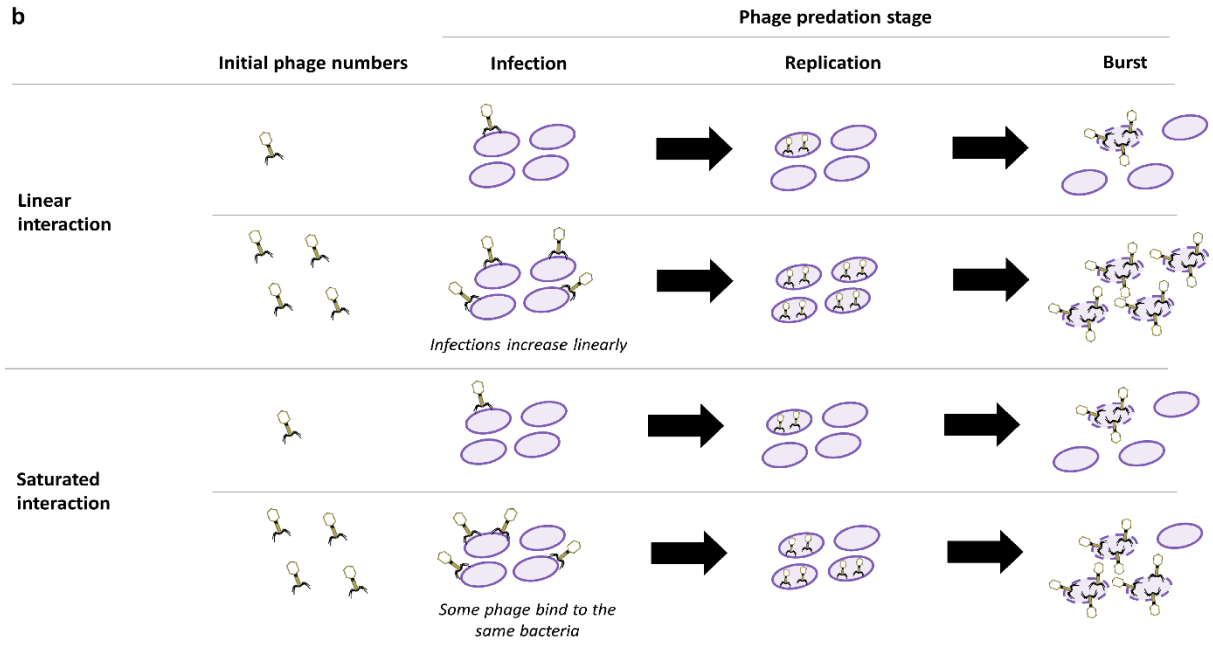
3.5.4 Investigation of possible phage-bacteria interactions using a flexible modelling framework

3.5.4.1 Model structure

We designed a mathematical model to reproduce the *in vitro* phage-bacteria dynamics, including generalised transduction of resistance genes. During our experiment, our co-culture contained up to three strains of bacteria: the two single-resistant parents (B_E, B_T) and the double-resistant progeny (B_{ET}). Although we were only able to count lytic phage (P_L), based on the biology of generalised transduction (Figure 3.1) we know that there were also transducing phage carrying either the erythromycin resistance gene (P_E), or the tetracycline resistance gene (P_T). Since we did not detect any evidence of 80α lysogeny in our co-culture after 24h, we did not include this feature in the model. The corresponding model diagram is shown in Figure 3.4a. The complete model equations can be found in Methods.

Using this modelling framework, we explored a combination of different phage-bacteria interactions, described below (Figure 3.4b-c). By fitting the models to our experimental data, we could rule out certain interactions and suggest the best model to reproduce the phage-bacteria dynamics seen *in vitro*.


a
B_T
P_L
P_E
P_T
B_E
B_{ET}
Bacterial growth
Lytic phage interaction
Transducing phage creation
Transduction
d
Phage parameter multiplier
1.00
0.75
0.50
0.25
0.00
10^0
10^2
10^4
10^6
10^8
cfu per mL


b
Phage predation stage
Initial phage numbers
Infection
Replication
Burst
Linear interaction
Infections increase linearly
Saturated interaction
Some phage bind to the same bacteria

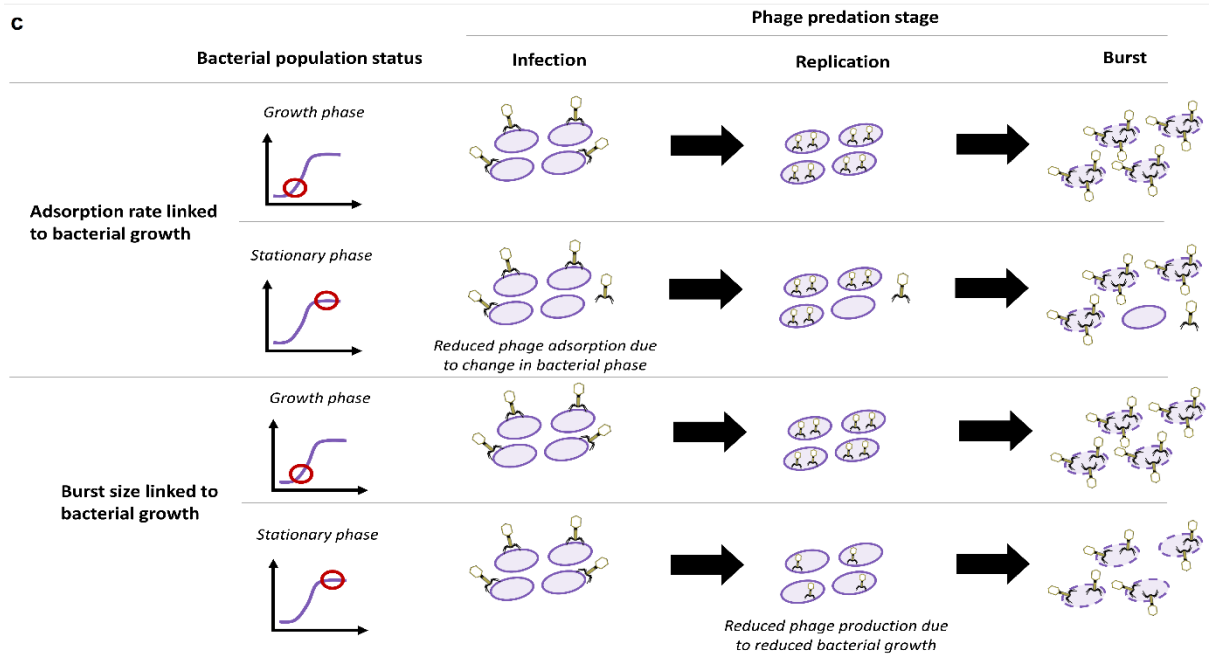

c
Phage predation stage
Bacterial population status
Infection
Replication
Burst
Adsorption rate linked to bacterial growth
Growth phase
Stationary phase
Reduced phage adsorption due to change in bacterial phase
Burst size linked to bacterial growth
Growth phase
Stationary phase
Reduced phage production due to reduced bacterial growth

Figure 3.4: Phage predation and generalised transduction model diagram, and different phage-bacteria interactions considered. (**a) Model diagram.** Each bacteria strain (B_E resistant to erythromycin, B_T resistant to tetracycline, or B_{ET} resistant to both) can replicate (purple). The lytic phage (P_L) multiply by infecting a bacterium and bursting it to release new phage (gold). This process can create transducing phage (P_E or P_T) carrying a resistance gene (*ermB* or *tetK* respectively) taken from the infected bacterium (green). These transducing phage can then generate new double resistant progeny (B_{ET}) by infecting the bacteria strain carrying the other resistance gene (green). **(b) Phage predation in the model is either linear or saturated.** With a linear interaction, the number of infections scales linearly with the number of phage and bacteria (top). A saturated interaction illustrates that at high phage concentrations, multiple phage may bind to the same bacterium, hence limiting the rate at which bacteria are lysed by phage (bottom). **(c) Phage predation in the model can decrease as bacterial growth decreases.** A change in bacterial growth phase can affect surface receptors, leading to a reduced phage adsorption rate (top). Since phage replication relies on bacterial processes, a reduced bacterial growth can translate into a reduced phage burst size (bottom). **(d) Proposed function linking phage predation parameters to bacterial growth.** This shows the multiplier applied to decrease phage parameters as the bacterial population increases towards carrying capacity, equivalent to a decrease in bacterial growth. Here, the carrying capacity is 2.76×10^9 colony-forming units (cfu)/mL, estimated from our data.

3.5.4.2 First phage-bacteria interaction: linear versus saturated phage predation

The most common approach to model phage-bacteria dynamics is to assume that phage predation is density-dependent and linear [14]. This means that, over one time step, the number of phage infecting bacteria and the number of bacteria infected by phage are both equal to the product of the number of bacteria (B), phage (P), and a constant phage adsorption rate (β), as shown in equation (3.1).

$$B * P * \beta \qquad (3.1)$$

This interaction implies that the number of new infections scales linearly with the number of phage and bacteria (Figure 3.4b). Therefore, even if we keep a constant number of phage, increasing bacteria numbers always leads to a linear increase in the estimated number of new infections. Although this simplification is useful and holds for a range of values, it has been suggested that it is not biologically realistic for high concentrations of phage, since multiple phage may bind to the same bacterium, leading to a sublinear increase in the number of infections [17].

To overcome these issues, we consider an alternative interaction, where phage predation is saturated [36]. This accounts for the fact that one phage does not necessarily always lead to one infection. For example, multiple phage may bind to the same bacterium [32] (Figure 3.4b). The number of new infections is restricted with the use of a Hill function, as shown in equation (3.2).

$$B * P * \frac{\beta}{(1+\frac{P}{P50})} \qquad (3.2)$$

with *P50* corresponding to the phage concentration at half saturation, where the adsorption rate is equal to half the maximum. This heuristic approach was originally proposed in [36], as it captured well the dynamics of lytic phage and bacteria.

3.5.4.3 Equilibrium analysis for the density-dependent model with linear phage predation

Despite this being a common method to represent phage-bacteria interactions in mathematical models, previous analyses have suggested that a density-dependent interaction alone cannot capture the equilibrium levels we and others have seen [18,37]. We explore this in the context of our own *in vitro* data using equilibrium analyses.

Assuming that transduction and the phage latent period are negligible, which a simplified model representing phage predation as a density-dependent process is shown in equations (3.3) and (3.4).

$$\frac{d\mathrm{B}}{dt} = \mu_{max} * B * (1 - \frac{B}{B_{max}}) - B * P * \beta \qquad (3.3)$$

$$\frac{d\mathrm{P}}{dt} = B * P * \beta * \delta' - \gamma * P \qquad (3.4)$$

Where μ_{max} is the maximum bacterial growth rate, B_{max} is the carrying capacity, β is the phage adsorption rate, γ is the phage decay rate, and δ is the phage burst size, with δ' equal to δ - 1. To solve for equilibrium (i.e. $\frac{d\mathrm{B}}{dt} = \frac{d\mathrm{P}}{dt} = 0$), equations (3.3) and (3.4) can be rewritten as equations (3.5) and (3.6).

$$\mu_{max} * B * (1 - \frac{B}{B_{max}}) - B * P * \beta = 0 \qquad (3.5)$$

$$B * P * \beta * \delta' - \gamma * P = 0 \qquad (3.6)$$

Since we are interested in an equilibrium with the condition that there are still bacteria and phage in the environment (i.e. B≠0 and P≠0), we can divide equations (3.5) and (3.6) by B and P respectively to obtain equations (3.7) and (3.8). These must hold true for there to be a non-zero bacteria and phage population at equilibrium.

$$\mu_{max} * (1 - \frac{B}{B_{max}}) - P * \beta = 0 \qquad (3.7)$$

$$B * \beta * \delta' - \gamma = 0 \qquad (3.8)$$

We then obtain equations (3.9) and (3.10) by rearranging (3.7) and (3.8) to give expressions for P and B at equilibrium.

$$\mathrm{P} = \frac{\mu_{max}}{\beta} * (1 - \frac{B}{B_{max}}) \qquad (3.9)$$

$$B = \frac{\gamma}{\beta * \delta'} \qquad (3.10)$$

In our experiment with a starting phage concentration of 10^4 plaque-forming units (pfu)/mL, after 24h the bacteria concentration was approximately 10^9 colony-forming units (cfu)/mL, and the phage concentration was 10^5 pfu/mL. If we replace the corresponding terms in equations (3.9) and (3.10) with these values, alongside the carrying capacity (2.8 x 10^9) and average of the growth rates estimated (1.52), we obtain equations (3.11) and (3.12).

$$10^5 = \frac{1.52}{\beta} * (1 - \frac{10^9}{2.8*10^9}) \quad (3.11)$$

$$10^9 = \frac{\gamma}{\beta * \delta'} \quad (3.12)$$

Rearrangement of equation (3.11) leads to a solution for phage adsorption (*β*) (equation (3.13)).

$$\beta = 9.77 * 10^{-6} \approx 10^{-5} \quad (3.13)$$

Substituting this into equation (3.12) leads to a value for phage decay rate (*γ*) (equation (3.14)).

$$\gamma = 10^9 * 10^{-5} * \delta' \approx 10^4 * \delta' \quad (3.14)$$

Giving rise to the condition that the phage decay rate *γ* must be approximately 10^4 times greater than the burst size *δ'*. This is not biologically plausible, since phage decay rates are typically several orders of magnitude lower, and not greater, than burst size. For example, phage decay rates are estimated to be at most 0.1 $phage^{-1}.h^{-1}$ [44], while an estimate for the burst size of 80α is 40 [39].

Even though these analyses rely on a simplified set of equations, using realistic parameter values we have shown that a non-zero equilibrium, as we have seen *in vitro,* cannot be reproduced using models with only a density-dependent interaction. Note that the inclusion of the phage latent period in these equations would not change the outcome of the equilibrium analysis, as this would only briefly delay the increase in phage numbers following bacterial infection (by approximately 40mins with 80α [39]), and not otherwise restrict phage predation. Instead, phage-bacteria co-existence may be explained by variations in phage predation parameters depending on bacterial resources availability, or bacterial growth rate [14,17–22]. However, to the best of our knowledge a simple mathematical expression linking phage predation to bacterial growth has not yet been developed.

3.5.4.4 Second phage-bacteria interaction: dependence of phage predation on bacterial growth

Here, we consider that a decrease in bacterial growth as bacteria reach stationary phase could firstly affect the phage adsorption rate ***β***, due to changes in receptors on bacterial surfaces, which affect opportunities for phage to bind (Figure 3.4c). Secondly, this could affect phage production, and thus burst size ***δ***, as phage replication relies on bacterial processes and may decrease when bacterial growth slows down (Figure 3.4c). Using a single phage predation multiplier, with the same principle of logistic growth applied to bacteria, we allow either or both ***β*** and ***δ*** to decrease as bacterial growth decreases in our model (equations (3.15) and (3.16)).

$$\beta = \beta_{max} * \left(1 - \frac{B}{B_{max}}\right) \qquad (3.15)$$

$$\delta = \delta_{max} * \left(1 - \frac{B}{B_{max}}\right) \qquad (3.16)$$

These equations imply that, as bacterial population size increases towards carrying capacity (B_{max}), phage parameters will be reduced (Figure 3.4d).

3.5.5 Identification of the best-fitting phage-bacteria interactions to reproduce the *in vitro* dynamics

Overall, we considered 6 different models, with either linear or saturated phage predation, and with either or both the phage adsorption rate and burst size linked to bacterial growth. Note that we did not include a phage decay rate in these models, as this did not affect the dynamics of the system over 24h, for a wide range of decay rates (Supplementary Figure 3.4), and this value systematically tended towards 0 when included in the model fitting process.

All models successfully reproduced the trends seen *in vitro* when the phage were started at either 10^3 and 10^4 pfu/mL (Figure 3.5a-b). However, only the two models where only phage burst size decreases as the bacteria population approaches carrying capacity were able to reproduce the increase in phage numbers seen in the

later hours of the 10^5 pfu/mL dataset, despite all models having been fitted to this dataset (Figure 3.5a-b). This was confirmed by calculating the average Deviance Information Criteria (DIC) value for the models, which favours best-fitting models while penalising more complex models (i.e. with more parameters) [38]. The two models where only phage burst size decreases as the bacteria population approaches carrying capacity had the lowest DIC values, indicating that they were the better-fitting models (Table 3.1).

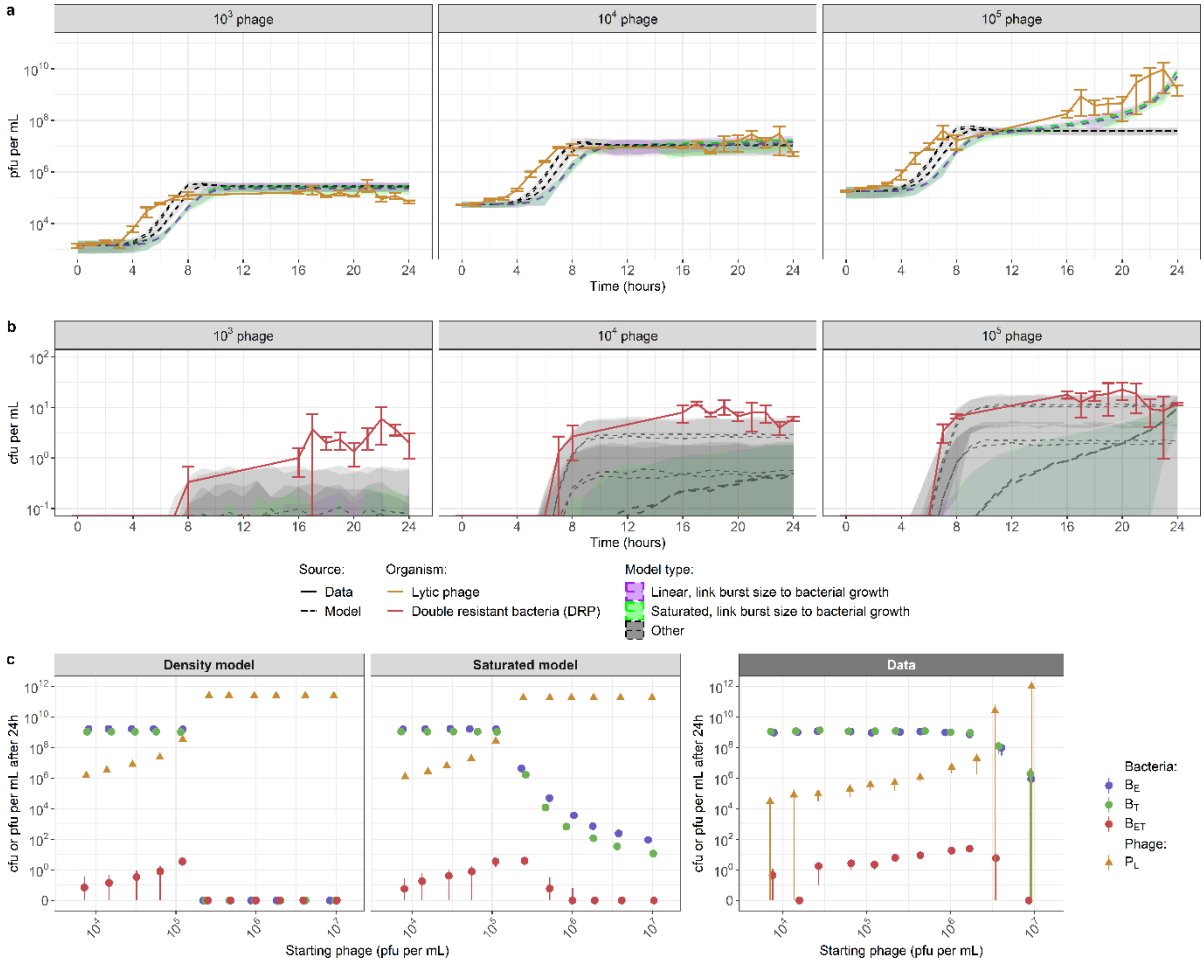


Figure 3.5: Accuracy of the best-fitted models to reproduce *in vitro* phage-bacteria dynamics. (a-b) The models with only phage burst size linked to bacterial growth are the most accurate to reproduce *in vitro* trends in lytic phage (a) and double resistant bacteria (b) numbers, starting from a bacteria concentration of 10^4 cfu/mL and varying phage concentrations. All models (dashed lines) can reproduce the trends seen *in vitro* when phage are started at 10^3 or 10^4 pfu/mL (data in solid lines), but only the models with just the phage burst size linked to bacterial growth (coloured model output) can reproduce the trend seen when

phage are started at 10^5 pfu/mL. Other models (grey) either only have the phage adsorption rate linked to bacterial growth, or both the phage adsorption rate and burst size. Models are fitted to the 10^3 and 10^5 data, and tested with the 10^4 data. Parameter values used are the median fitted values (Table 1). Shaded areas indicate standard deviation generated from Poisson resampling of model results. Error bars for the data (solid lines) indicate mean +/- standard error, from 3 experimental replicates. **(c) When further testing fitted model dynamics starting from 10^6 cfu/mL bacteria and varying phage concentrations, the model with linear phage predation incorrectly predicts bacterial extinction, while the model with saturated predation reproduces the trend, but not the exact values of the 24h data.** In the co-culture used to generate the data, each single-resistant parent strain (B_E and B_T) is added at a starting concentration of 10^6 cfu/mL, and no double-resistant progeny (B_{ET}) are initially present. The starting concentration of lytic phage (P_L) varies (x axis). Points indicate mean results, and are each slightly shifted horizontally to facilitate viewing. Error bars indicate either mean +/- standard deviation for the models (left/centre panels), or mean +/- standard error for the data (right panel). Parameter values used are the median fitted values (Table 3.1).

Table 3.1: Estimated parameter values from fitting to *in vitro* data. Values show median and 95% credible intervals for posterior distributions. Parameter units are indicated in parentheses. Fitting was performed using the Markov chain Monte Carlo Metropolis–Hastings algorithm and the data from the co-culture with a starting bacterial concentration of 10^4 cfu/ml and phage concentration of 10^3 and 10^5 pfu/ml. DIC: Deviance Information Criteria. A smaller DIC indicates better model fit. DIC values are relative to the smallest DIC calculated, which is for the frequency-dependent model with only burst size linked to bacterial growth (line 5, parameters in bold).

Interaction type	**Adsorption rate linked to growth**	**Burst size linked to growth**	**Adsorption rate β (phage^{-1} bacteria^{-1} hour^{-1})**	**Burst size δ (phage)**	**Transducing phage proportion α (proportion of burst size)**	**Phage latent period τ (hour)**	**Phage concentration at half-saturation $P50$ (phage)**	**DIC**
Linear	**Yes**	**No**	4.5×10^{-9} (4.1×10^{-9} ; 5.0×10^{-9})	12 (10 ; 14)	3.1×10^{-8} (1.5×10^{-8} ; 5.8×10^{-8})	0.64 (0.55 ; 0.73)	N/A	1104
	No	**Yes**	1.6×10^{-10} (1.5×10^{-10} ; 1.7×10^{-10})	79 (72 ; 86)	1.4×10^{-8} (1.1×10^{-8} ; 1.7×10^{-8})	0.65 (0.62 ; 0.69)	N/A	995
	Yes	**Yes**	4.3×10^{-9} (3.9×10^{-9} ; 4.6×10^{-9})	43 (37 ; 49)	1.2×10^{-8} (6.4×10^{-9} ; 2.3×10^{-8})	0.93 (0.86 ; 0.99)	N/A	774
Saturated	**Yes**	**No**	3.3×10^{-9} (1.8×10^{-9} ; 5.6×10^{-9})	14 (11 ; 21)	2.5×10^{-7} (1.2×10^{-7} ; 5.5×10^{-7})	0.67 (0.60 ; 0.78)	5.1×10^{10} (2.8×10^{9} ; 9.7×10^{10})	631
	No	**Yes**	**2.3×10^{-10} (2.1×10^{-10} ; 2.7×10^{-10})**	**50 (43 ; 54)**	**1.2×10^{-8} (1.1×10^{-9} ; 1.3×10^{-8})**	**0.60 (0.60 ; 0.61)**	**1.2×10^{10} (1.0×10^{10} ; 1.3×10^{10})**	**0**
	Yes	**Yes**	2.6×10^{-9} (1.9×10^{-9} ; 3.4×10^{-9})	36 (28 ; 43)	1.4×10^{-7} (9.21×10^{-8} ; 2.2×10^{-7})	0.75 (0.63 ; 0.80)	5.1×10^{10} (3.6×10^{9} ; 9.8×10^{10})	385

Our initial experiments considered the dynamics over 24h for varying phage starting concentrations. To test the ability of our model to recreate the dynamics under changing bacterial levels, we replicated our transduction co-culture experiments with starting concentrations of 10^6 cfu/mL bacteria instead of 10^4 cfu/mL, varying the starting phage concentration between 10^4 and 10^6 pfu/mL, and measuring bacteria and phage numbers after 24h of co-culture. We then used the estimated parameter values (Table 3.1) to try to reproduce these 24h numbers of bacteria and phage.

Increasing the starting phage concentration led to an increase in the number of phage after 24h (Figure 3.5c). For a starting phage concentration between 10^4 and 10^6 pfu/mL, increasing starting phage numbers did not affect single-resistant parents B_E and B_T numbers after 24h, but led to a progressive increase in double-resistant progeny B_{ET} numbers. Increasing starting phage numbers above 10^6 pfu/mL caused bacteria numbers after 24h to decrease.

Using the estimated parameter values (Table 3.1) with the model where only burst size is linked to bacterial growth, we see that the model with linear phage predation cannot reproduce these dynamics as it predicts that bacteria become extinct rapidly (Figure 3.5c). The model with saturated predation is able to reproduce these trends, but fails to recreate the exact same numbers of phage and bacteria, predicting a decline in bacterial levels when the starting phage concentration increases above 10^5 pfu/mL, a lower threshold than seen in the data (Figure 3.5c). The same overall trends are seen for the models where only the adsorption rate is linked to bacterial growth, or both adsorption rate and burst size (Supplementary Figure 3.5).

3.5.6 Analysis of phage predation and transduction dynamics

Parameter estimates for our best-fitting model (with a saturated phage predation and a link between phage burst size and bacterial growth only) suggest that the adsorption rate is 2.3×10^{-10} (95% credible interval: 2.1×10^{-10} - 2.7×10^{-10}) which is the smallest estimate from the models (Table 3.1). On the other hand, the estimated burst size is 50 (43 - 54) phage, similar to a previous *in vitro* estimate for 80α of 40 [39]. However,

due to the decrease in burst size when bacteria are in stationary phase, we expect that this number would change depending on the conditions under which it is measured (Figure 3.6a). Finally, the estimated latent period of 0.60h (0.60 - 0.61) is slightly shorter than a previous in vitro estimate of 0.67h [39]. Regarding the other models, we note some biologically unlikely parameter estimates which further suggest that these models are inappropriate, such as the low burst size for the models with only the adsorption rate linked to bacterial growth (12 (10 - 14) and 14 (11 - 21)), or the high latent period for the models with both adsorption rate and burst size linked to bacterial growth (0.93 (0.86 - 0.99) and 0.75 (0.63 - 0.80)) (Table 3.1).

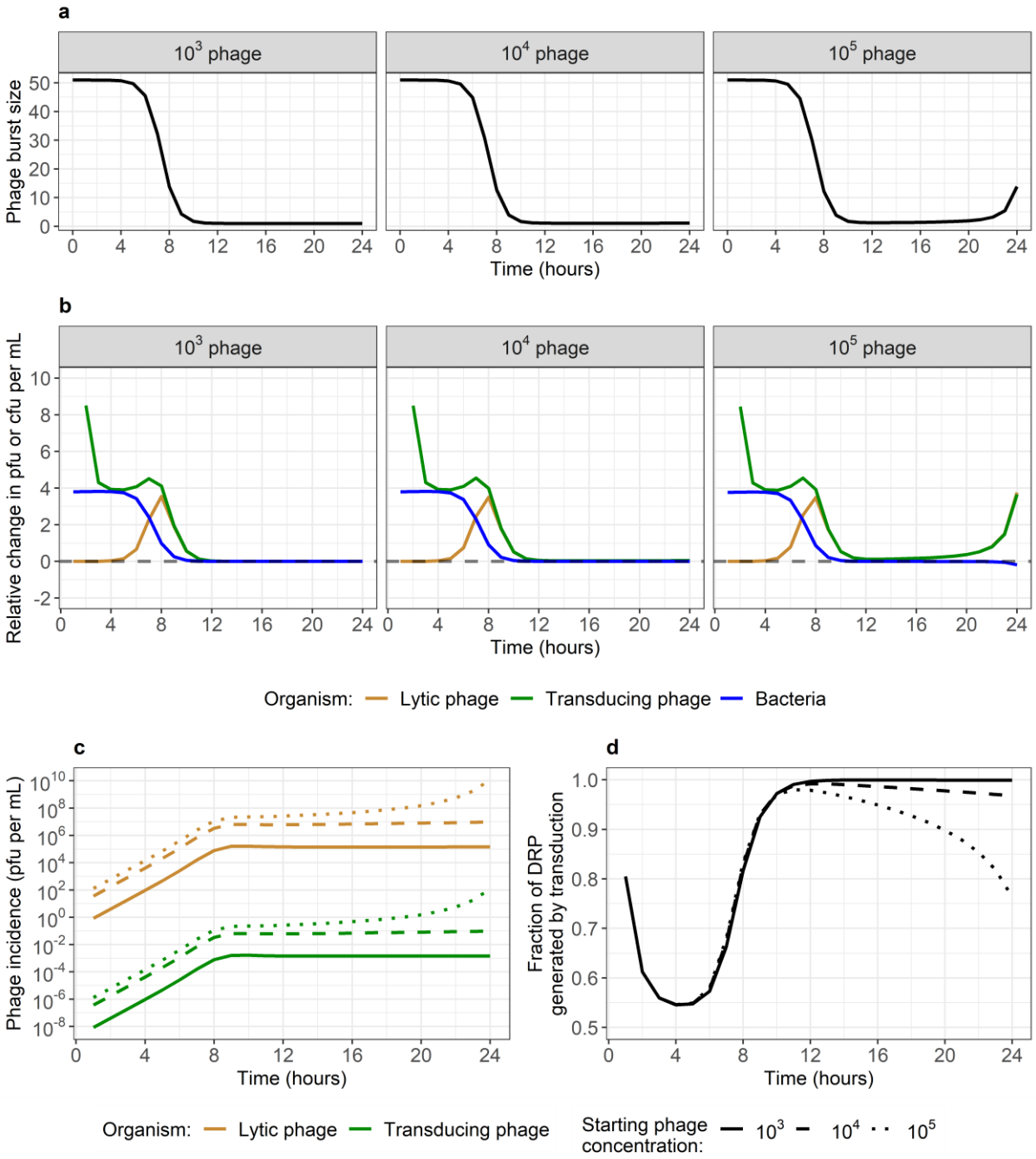


Figure 3.6: Underlying phage and bacteria dynamics generated by the best-fitting model with saturated phage predation and burst size linked to bacterial growth. Model parameters are the median estimates from model fitting (Table 3.1). **(a) Phage burst size over time, by starting phage concentration.** As bacteria reach stationary phase after 8h, phage burst size decreases. In the 10^5 dataset, we see that burst size is predicted to increase again after 20h. This is due to bacterial numbers decreasing as bacteria are being lysed by phage. **(b) Relative change in phage and bacteria numbers over time, by starting phage concentration.** The number of new phage generated at each time step increases (positive value) until bacteria reach

stationary phase around 8h. This applies to lytic and transducing phage. In the 10^5 dataset, phage keep increasing after 10h, eventually causing a decrease in bacterial numbers (negative value), which translates into a further acceleration in the increase in phage numbers due to the increased burst size (Figure 5a). After 8h, the relative changes in lytic and transducing phage numbers are identical. **(c) Incidence of lytic (gold) and transducing (green) phage over time, by starting phage concentration (linetype).** For any dataset and time-point, there is approximately 1 new transducing phage generated for each 10^8 new lytic phage. **(d) Fraction of double-resistant progeny (DRP) generated by transduction each hour over time, by starting phage concentration (linetype).** DRP generation always occurs predominantly by transduction, rather than by growth of already existing DRP. Note that the time at which DRP are first generated varies by starting phage concentration.

We used our best-fitting model to reproduce our *in vitro* data (Figure 3.2) and uncover the underlying phage-bacteria dynamics. Due to the link between phage burst size and bacterial growth, burst size decreases as bacteria reach carrying capacity after 8h (Figure 3.6a-b). This is reflected in the relative change in phage numbers, which tends towards 0 after 8h (Figure 3.6b). After this point, phage incidence remains stable for the 10^3 and 10^4 pfu/mL dataset, but starts increasing again significantly after 20h for the 10^5 pfu/mL dataset as bacteria numbers start decreasing due to phage predation, allowing burst size to increase again (Figure 3.6a-c).

We estimate that for every 10^8 new lytic phage released during burst, there was approximately one transducing phage carrying an antibiotic resistance gene (Table 3.1, Figure 3.6c). Note that new double-resistant progeny (DRP) can either be generated by transduction, or by replication of already existing DRP. Using the model, we found that DRP were always predominantly generated by transduction rather than by growth (Figure 3.6d). This is because after 4h bacterial growth rate starts decreasing as the total bacteria population approaches carrying capacity (Figure 3.6b&d).

3.6 Discussion

3.6.1 Results in context

We observed rapid *in vitro* horizontal gene transfer of antimicrobial resistance (AMR) by generalised transduction in *Staphylococcus aureus*, alongside equilibria in phage and bacteria numbers which varied depending on the starting number of phage. The most accurate mathematical model to replicate phage-bacteria dynamics, including generalised transduction, represented phage predation as a saturated process, and linked phage burst size to bacterial growth. To the best of our knowledge, these two elements have both been suggested previously [17,18,36], yet never combined.

Density-dependent models with linear phage predation have been compared to data at less fine time scales (e.g. daily time points) or over smaller time periods (e.g. less than 8h), where they were able to reproduce *in vitro* values from experiments in chemostats, and have been helpful to improve our basic understanding of phage-bacteria dynamics [14–16]. However, here we show that this type of interaction is not able to reproduce finer hourly dynamics, and does not perform consistently when varying concentrations of starting phage and bacteria. Using this, alongside a critique of the mathematical implications of this process, we argue that linear phage predation is not a biologically accurate representation of phage predation, as it fails to reproduce these dynamics at high numbers of phage and bacteria, which would correspond to scenarios potentially seen during phage therapy.

Our work adds to the growing body of evidence that phage predation depends on bacterial growth [14,17–23]. This has implications for antibiotic-phage combination therapy, as it suggests that bacteriostatic antibiotics, which prevent bacterial growth, could reduce phage predation. This effect has been previously seen in *S. aureus* [40]. In the environment, including in persistent infections, bacteria spend most of their time in stationary phase [41]. This suggests that bacteria and phage may be able to co-exist for prolonged periods of time in a broad range of settings, without the phage systematically eradicating the bacteria. Under such conditions, phage may mediate horizontal gene transfer by transduction between bacteria at relatively low levels, but for prolonged periods of time. This may be particularly relevant for *S. aureus*, since

approximately 20% of humans are colonised asymptomatically by this bacterium at any given time [42], and at least 50% of these carriers may also carry phage capable of generalised transduction [43], suggesting a constant background evolution rate for *S. aureus* in the human population. Combined with environmental exposure to antibiotics which acts as a selective pressure, this may contribute to the risk of multidrug-resistant bacteria evolution.

3.6.2 Strengths and limitations

Our experimental design is both a strength and a limitation of our study. Since we jointly designed the experiments and models, we are confident that we have included in our mathematical model all the organisms and interactions present *in vitro*. We are therefore confident in the conclusions on model structure, which is generalizable to other systems. Saturated phage predation is biologically plausible for lytic phage in general, a link between phage predation and bacterial growth has been seen in other systems [14,17–23], and our model includes the relevant biological characteristics of generalised transduction [2,3], requiring a transducing phage to first be generated before the transfer of the AMR gene to another bacterium can occur. In addition, our equations for phage-bacteria interaction can be directly applied to systems containing more strains of phage and bacteria than in our study.

However, the usage of such a specific experimental system with two bacterial strains of the same genetic background and one phage limits the generalisability of our parameter values, as these will likely vary for different bacteria and phage. Growth conditions will likely also differ between the *in vitro* environment studied here, and *in vivo* conditions. Here, our model assumes that phage do not decay, that bacteria do not become resistant to phage, and that their growth is only limited by carrying capacity and not nutrients, as they are observed in a rich medium for 24h only, but over longer periods of time it may be necessary to revisit these assumptions, by including for example a finite concentration of resources which are consumed during growth [44]. The role of the immune system may have to be considered *in vivo*, as this could impact both the numbers of phage and bacteria [45,46], and our model could be extended to include this. We assumed that the proportion of transducing phage created was

independent of the gene being transduced (*ermB*, on the bacterial chromosome, or *tetK*, on a plasmid). This was supported by preliminary work (see Material and Methods), but should be further investigated to improve our understanding of the factors that can facilitate or prevent transduction of different genes. Finally, our model does not include lysogeny and specialised transduction, and would therefore need to be extended with additional compartments for lysogenic bacteria to represent these dynamics. To answer all of these questions, future work should investigate both phage predation and transduction dynamics over longer time periods, with different strains of bacteria and phage.

All our models captured certain aspects of the trends seen *in vitro*, but also underestimated phage numbers between 5-7h by up to 20 times. This is likely a consequence of our experimental design. To count lytic phage, we centrifuged and filtered the co-culture to remove bacteria. This could have caused the premature burst of some phage-infected bacteria, artificially increasing the numbers of phage we then counted [47]. Since the period between 5-7h is when phage infections are highest (Figure 3.6b), this is why we would see such a large discrepancy at this stage. We also note that the models with only phage burst size linked to bacterial growth underestimated the number of double-resistant progeny (DRP). This small difference (up to 10 cfu/mL) is likely due to our choice of using a deterministic model. This type of model is useful for our purpose of fitting to *in vitro* data and analysing the underlying dynamics here, but mathematically allows for fractions of bacteria to exist, instead of just whole numbers. Future analyses using a stochastic model would better capture random effects, which can have an important impact at low numbers.

Multiplicity of infection (MOI, starting ratio of phage to bacteria) is a commonly used metric to present results of experiments with these organisms [32]. With a starting concentration of 10^4 bacteria per mL, we were able to fit our model to the dynamics for two MOI (0.1 and 10), and replicate those of a third (1). However, when trying to use the same model for these same three MOI, but with a starting bacterial concentration to 10^6, we found differences between our model and values seen after 24h. This indicates that MOI is not appropriate to summarise all the complexity of the underlying phage-bacteria dynamics. Future experimental studies should express

their results as a function of their starting concentration of phage and bacteria, not just MOI.

In any case, the failure of our model to replicate 24h values with a different starting bacteria concentrations shows that, whilst we have reduced the model structure uncertainty, we are still not fully capturing the phage-bacteria interaction. Currently, our model predicts that, for a starting concentration of 10^6 bacteria, a starting concentration of 10^5 phage or more will be enough to cause a decrease in bacterial numbers after 24h, while our data shows that the starting concentration of phage must be higher than 10^6 for this to happen. *In vitro*, it is likely that slower bacterial growth simultaneously affects the phage adsorption rate, latent period and burst size, each to varying extents [14,17–23]. This would explain why we would need a higher starting concentration of phage for a higher starting concentration of bacteria, to exert a strong enough predation pressure before bacteria reach stationary phase, causing a reduction in phage predation. However, here we have only made the first step in this process, having linked the burst size linearly to the bacterial growth rate, instead of trying to link different phage predation parameters to bacterial growth using different functions. These complexities need to be explored further, supported by i*n vitro* work measuring phage predation parameters at various time points. In *S. aureus*, wall teichoic acid (WTA) is the phage receptor [48,49]. Lack of WTA glycosylation has been shown to induce phage resistance [50], and changes in WTA structure at different growth phases may be possible, since one of the genes involved in its synthesis is repressed by a quorum sensing system [51]. However, to the best of our knowledge this has not yet been investigated, although changes in WTA are unlikely to occur at high rates due to the resulting fitness costs [52].

3.6.3 Implications

Despite being recognised as a major mechanism of horizontal gene transfer, thus far there have been limited mathematical modelling studies on the dynamics of transduction of AMR [12]. Using our model, we are able to estimate numbers of transducing phage which we cannot count *in vitro*, and see that approximately 1 generalised transducing phage is generated per 10^8 lytic phage, consistent with previous estimates [53,54]. Here, we show that this number, which may seem

insignificant, is enough to consistently lead to the successful horizontal gene transfer of AMR, resulting in DRP after only 7h from phage addition, substantially less than the usual duration of antibiotic treatment. We also show that transduction is the dominant mechanism to create new DRP throughout the entire experiment, rather than growth of existing DRP. This echoes the conclusions of previously published work on the importance of transduction, including *in vivo* experiments and with other *Staphylococcus* species [4,5,29,55].

Our findings suggest that transduction is currently under-emphasised in the exploration of phage-bacteria dynamics. Future studies on this topic should not assume that transduction can be dismissed by default, but instead investigate whether it is relevant in their system. This requires further *in vitro* and *in vivo* monitoring to identify scenarios where transduction plays a significant role in the transfer of AMR genes, likely depending on the environment, and characteristics of the bacteria and phage present. This will require new experimental designs, since counting phage numbers can be difficult, notably with clinical strains of bacteria. This should also be investigated in the presence of antibiotics, where the importance of selection enters, increasing the fitness of the small numbers of DRP generated by transduction.

Our results confirm that generalised transduction can consistently lead to the spread of AMR genes, yet to the best of our knowledge there have not been any attempts to evaluate the potential consequences of this process during phage therapy. Unlike specialised transduction, likely not relevant in the context of phage therapy as temperate phage would not be used for this purpose, generalised transduction is by definition a mistake during the lytic cycle, therefore difficult to prevent [8,9]. As phage therapy is generally administered alongside antibiotics [56], and we know that patients can be colonised and infected with strains carrying different resistance genes [42], a potential risk is for multidrug-resistant strains to be generated by transduction, and then selected for by these antibiotics. These new strains could in turn be transmitted to other individuals, or gain resistance to phage infection, which would lead to a worse treatment outcome for the patient. Echoing recommendations from previous reviews [10–12], we suggest that future studies of phage therapy should acknowledge the risk of generalised transduction, and evaluate the impact of this on *in vivo* bacterial evolution during therapy.

3.6.4 Conclusion

The joint dynamics of phage predation and transduction lead to complex interactions with bacteria. These dynamics must be clarified, to correctly evaluate the extent to which phage contribute to the global spread of AMR. We must also understand these dynamics in the context of phage therapy, as transduction may lead to worse health outcomes in patients if phage contribute to spreading AMR instead of overcoming it. Current modelling research that ignores transduction may underestimate AMR development in various systems. Interdisciplinary work will be essential to answer these urgent public health questions in the near future.

3.7 Material and Methods

All analyses were conducted using the statistical software R [57]. The underlying code and data are available in a GitHub repository: https://github.com/qleclerc/mrsa_phage_dynamics.

3.7.1 Experimental methods

3.7.1.1 Strains and phage used

The *Staphylococcus aureus* parent strains used for our transduction experiment were obtained from the Nebraska Transposon Mutant Library [35]. These were strain NE327, carrying the *ermB* gene encoding erythromycin resistance and knocking out the φ3 integrase gene, and strain NE201KT7, a modified NE201 strain with a kanamycin resistance cassette instead of the *ermB* gene knocking out the φ2 integrase gene, and a pT181 plasmid carrying the *tetK* gene encoding tetracycline resistance [58]. Previous work estimated that the pT181 copy number was 20 in *S. aureus* [59], however this old number may not be applicable to our specific NE201KT7 strain, and there is ongoing whole-genome sequencing work in our group to generate an updated estimate. Growing these strains together in identical conditions as our co-culture below, but without the addition of exogenous phage, does not lead to detectable horizontal gene transfer (HGT; data not shown). To enable HGT, exogenous 80α phage was used, a

well-characterised temperate phage of *S. aureus* capable of generalised transduction [33]. To count lytic phage, *S. aureus* strain RN4220 was used, a restriction deficient strain highly susceptible to phage infection [60].

3.7.1.2 Transduction co-culture protocol

Pre-cultures of NE327 and NE201KT7 were prepared separately in 50mL conical tubes with 10mL of Brain Heart Infusion Broth (BHIB, Sigma, UK), and incubated overnight in a shaking water bath (37°C, 90rpm). The optical densities of the pre-cultures were checked at 625nm the next day to confirm growth. The pre-cultures were diluted in phosphate-buffered saline (PBS), and added to a glass bottle of fresh BHIB to reach the desired starting concentration in colony forming units per mL (cfu/mL) for each strain, forming a master mix for the co-culture. $CaCl_2$ was added at a concentration of 10mM to the master mix. Phage 80α stock was diluted in phage buffer (50 mM Tris-HCl pH 7.8, 1 mM MgSO4, 4 mM CaCl2 and 1 g/L gelatin; Sigma–Aldrich), and added to the master mix to reach the desired starting concentration in plaque forming units per mL (pfu/mL). Ten 50mL conical tubes were prepared (one co-culture tube for each timepoint, from 0 to 8h and 16 to 24h), each with 10mL from the master mix. Each co-culture tube was then incubated in a shaking water bath (37°C, 90rpm) for the corresponding duration.

Bacteria counts for each timepoint were obtained by diluting the co-culture in PBS before plating 50µL on selective agar, either plain Brain Heart Infusion Agar (BHIA, Sigma, UK), BHIA with erythromycin (Sigma, UK) at 10mg/L, BHIA with tetracycline (Sigma, UK) at 5mg/L, or BHIA with both erythromycin and tetracycline (10mg/L and 5mg/L respectfully). Note we plated 500µL instead of 50 on the plates with both antibiotics, to increase the sensitivity of the assay. This allowed distinction between each parent strain, resistant to either erythromycin or tetracycline, and the double resistant progeny (DRP) generated by transduction. Plates were then incubated at 37°C for 24h, or 48h for plates containing both antibiotics. Colonies were counted on the plates to derive the cfu/mL in the co-culture for that timepoint.

Previous work in our lab conducted by Lorna Chapman found that there was no substantial difference in the direction of transfer between NE327 and NE201KT7. This was done by screening DRP for kanamycin resistance, with the assumption that DRP

also resistant to kanamycin would be from the NE201KT7 background, as they would possess the kanamycin resistance cassette. On the other hand, kanamycin-susceptible DRP would be from the NE327 background. 16 DRP were screened, with 10 resistant to kanamycin and 6 susceptible. Here, and in the model below, we therefore assume that transfer occurs at equal frequency between NE327 and NE201KT7.

Lytic phage counts for each timepoint were obtained using the agar overlay technique [61]. Briefly, the co-culture was centrifuged at 4000rpm for 15 minutes, filtered twice with 10µm filters, and diluted in Nutrient Broth No. 2 (NB2, ThermoFisher Scientific, UK). 15mL conical tubes were prepared with 300µl of RN4220 grown overnight in NB2, and $CaCl_2$ at a concentration of 10mM. 200µl of diluted phage were added, and the tubes were left to rest on the bench for 30 minutes. The contents of the tubes were then mixed with 7mL of phage top agar, and poured on phage agar plates. Phage agar was prepared using NB2, supplemented with agar (Sigma, UK) at 3.5g/L for top agar and 7g/L for plates. The plates were incubated overnight at 37°C. Clear spots in the bacterial lawn were counted to derive the pfu/mL in the co-culture for that timepoint.

3.7.1.3 Relative fitness

Relative fitness was calculated using data from co-cultures of NE327, NE201KT7, and DRP, in the absence of phage. For each pair of strains, we estimated relative fitness *W* using Equation 3.17.

$$W = \frac{ln[\frac{S1(24)}{S1(0)}]}{ln[\frac{S2(24)}{S2(0)}]} \qquad (3.17)$$

Where S1(*t*) and S2(*t*) represent the number of bacteria (in cfu/mL) from the chosen strains 1 and 2, at times *t* = 0 or 24 hours.

3.7.1.4 Polymerase chain reaction protocols

To confirm that DRP contained both the *ermB* and *tetK* genes, primers ermBF (5′-CGTAACTGCCATTGAAATAGACC-3′), ermBR (5′-AGCAAACTCGTATTCCACGA-3′), tetKF (5′-ATCTGCTGCATTCCCTTCAC-3′), and tetKR (5′-

GCAAACTCATTCCAGAAGCA-3′) were used. Strains NE327 (only containing *ermB*) and NE201KT7 (only containing *tetK*) were used as positive and negative controls.

To confirm that 80α lysogeny did not occur in our co-culture, we applied a previously published method [33] and used a combination of four primers: SaRpmF (5′-GACTGAATGCCCAAACTGTG-3′) in the *S. aureus rpmF* gene, SMT178 (5'-GGCTGGGAATTAATGGAAGATG-3′) in the 80α integrase, SaSirH (5′-TTAAGTAGCATCGTTGCATTCG-3′) in the *S. aureus sirH* gene, and SMT179 (5′-GAGTCCTGTTTGCGAATTAGG-3′) in the 80α ORF73 region. SaRpmF and SMT178 were used to amplify the left prophage junction (*attL*), SaSirH and SMT179 to amplify the right junction (*attR*), and SaRpmF and SaSirH to amplify the bacterial insertion site (*attB*). RN4220 was used as a negative control for lysogeny, and JP8488, an RN4220 strain lysogenic for 80α, was used as a positive control (obtained from José Penadés and Nuria Quiles, Imperial College London).

All PCRs were conducted using OneTaq Hot Start Quick-Load 2X Master-mix, following the manufacturer's protocol. Tested samples were homogenised in 20µl nuclease-free water, except for samples used to test for lysogeny which were generated by DNA extraction and therefore already suspended in nuclease-free water (see below). 1.5µl of each suspension was used as template for a total reaction volume of 25µl.

3.7.1.5 DNA extraction protocol

To prepare samples for PCR to detect lysogeny, we extracted DNA from a 1mL sample of our NE327, NE210KT7 and 80α co-culture after 24h (approximately 10^9 bacteria) using the bacterial genomic DNA purification kit PurElute (Edge Biosystems), supplemented with 2.5µl of lysostaphin (10mg/mL, Sigma–Aldrich) [43].

Since the final DNA suspension was in 50µl of nuclease free water, and we used 1.5µl of this suspension as a template for the PCR and conducted three experimental replicates, this is equivalent to saying that we tested DNA from approximately 9×10^7 bacteria ($10^9 \times (1.5/50) \times 3 = 9 \times 10^7$). Using a Binomial probability density function and assuming a 100% PCR specificity, we estimate that the probability for a false negative result (i.e. that the PCR results are negative yet that the true number of

lysogenic bacteria is greater than 0) exceeds 5% only if the frequency of lysogenic bacteria in our sample is lower than 3.3×10^{-8}. We therefore consider that the detection limit of our protocol is a frequency of 3.3×10^{-8} lysogenic per non-lysogenic bacteria after 24h of our co-culture. This means that, in our system, we would be able to detect lysogenic bacteria if there were more than ($3.3 \times 10^{-8} \times 10^{9} = 33$) 33 lysogenic bacteria in 1mL of our co-culture after 24h.

3.7.2 Mathematical modelling methods

3.7.2.1 General model structure

We designed a deterministic, compartmental model to replicate our experimental conditions. We included 6 populations: B_E (corresponding to ery-resistant NE327), B_T (tet-resistant NE201KT7), B_{ET} (double resistant progeny, DRP), P_L (lytic phage), P_E (phage transducing *ermB*) and P_T (phage transducing *tetK*). Their interactions are represented in Figure 3.2.

Bacteria from each strain θ ($\theta \in \{E, T, ET\}$) can multiply at each time step t following logistic growth at rate μ_θ, with a maximum value $\mu_{max\theta}$ which declines as the total bacteria population N (= $B_E + B_T + B_{ET}$) approaches carrying capacity N_{max}.

$$\mu_\theta = \mu_{max_\theta} * (1 - \frac{N}{N_{max}}) \qquad (3.18)$$

At each time step t, lytic phage (P_L) infect bacteria according to the function $F(P_L)$, replicate, and burst out from the bacteria with a burst size $\delta + 1$ after a latent period τ. During phage replication, a proportion α of new phage are transducing phage. The nature of the transducing phage (P_E or P_T) depends on the bacteria being infected (e.g. B_E bacteria can only lead to P_E phage). Then, these transducing phage (P_E or P_T) infect bacteria according to $F(P_E)$ and $F(P_T)$. If they successfully infect a bacterium carrying the other resistance gene (e.g. P_E phage infecting a B_T bacterium), this creates double resistant progeny (B_{ET}). The complete model equations can be found below.

$$\frac{dB_E}{dt} = \mu_E * B_E - B_E * F(P_L) - B_E * F(P_T) \quad (3.19)$$

{Change in B_E = growth of B_E – infections by P_L – infections by P_T}

$$\frac{dB_T}{dt} = \mu_T * B_T - B_T * F(P_L) - B_T * F(P_E) \quad (3.20)$$

{Change in B_T = growth of B_T – infections by P_L – infections by P_E}

$$\frac{dB_{ET}}{dt} = \mu_{ET} * B_{ET} - B_{ET} * F(P_L) + B_T * F(P_E) + B_E * F(P_T) \quad (3.21)$$

{Change in B_{ET} = growth of B_{ET} – infections by P_L + infections of B_T by P_E + infections of B_E by P_T}

$$\frac{dP_L}{dt} = [(B_E + B_T) * F(P_L)](t - \tau) * \delta * (1 - \alpha) + [B_{ET} * F(P_L)](t - \tau) * \delta * (1 - 2 * \alpha) - N * F(P_L) \quad (3.22)$$

{Change in P_L = new P_L phage – P_L phage infecting bacteria}

$$\frac{dP_E}{dt} = [(B_E + B_{ET}) * F(P_L)](t - \tau) * \delta * \alpha - N * F(P_E) \quad (3.23)$$

{Change in P_E = new P_E phage – P_E phage infecting bacteria}

$$\frac{dP_T}{dt} = [(B_T + B_{ET}) * F(P_L)](t - \tau) * \delta * \alpha - N * F(P_T) \quad (3.24)$$

{Change in P_T = new P_T phage – P_T phage infecting bacteria}

Some parameters (*τ, α*) are constant, while others (μ_E, μ_T, μ_{ET},, *δ*) and the function $F(P_\theta)$ ($\theta \in$ {L, E, T}) can change at each time step and depending on the specified interaction mechanism. Note that since B_{ET} can give rise to both types of transducing phage P_E and P_T, as it carries the two resistance genes as opposed to B_E and B_T which only carry one, we assume that transducing phage carrying an AMR gene are twice as likely to be produced by B_{ET} than by B_E or B_T, hence the multiplication of *α* by 2 in Equation 3.22.

3.7.2.2 Phage predation function

Over one time step, both the number of phage P_θ ($\theta \in$ {L, E, T}) infecting bacteria B_ε ($\varepsilon \in$ {E, T, ET}) and the number of bacteria infected by phage are equal to

$$B_\varepsilon * F(P_\theta) \quad (3.25)$$

In our equations for linear phage predation, the phage adsorption rate *β* is constant, hence $F(P_\theta)$ is equal to:

$$F(P_\theta) = P_\theta * \beta \quad (3.26)$$

On the other hand, the saturated phage predation equation limits the number of phage infecting bacteria over one time step at higher phage concentrations according to a Hill function. Equations (3.26) then becomes:

$$F(P_\theta) = P_\theta * \frac{\beta}{(1+\frac{P_\theta}{P50})} \qquad (3.27)$$

With *P50* corresponding to the phage concentration at which the adsorption rate is equal to half the maximum value. We assume that the adsorption and saturation process is the same regardless of whether the phage is lytic or transducing, hence we estimate only a single set of *β* and *P50* parameters which we apply to all phage.

3.7.2.3 Link between bacterial growth and phage predation

We consider that reduced bacterial growth can lead to decreased phage predation, through reduced adsorption (*β*) and/or burst size (*δ*). Equations (3.28) and (3.29) allow these parameters to decrease as bacterial growth decreases, using the same principle of logistic growth as seen in equation (3.18).

$$\beta = \beta_{max} * \left(1 - \frac{N}{N_{max}}\right) \qquad (3.28)$$

$$\delta = \delta_{max} * \left(1 - \frac{N}{N_{max}}\right) \qquad (3.29)$$

If we do not link these parameters to bacterial growth, we assign them their maximum values.

$$\beta = \beta_{max} \qquad (3.30)$$

$$\delta = \delta_{max} \qquad (3.31)$$

3.7.2.4 Model fitting

We fit our model to the *in vitro* data using the Markov chain Monte Carlo Metropolis–Hastings algorithm. For every iteration, this algorithm slightly changes the parameter values, runs the model, assesses the resulting model fit to the data, and accepts or

rejects these new parameter values based on whether the model fit is better or worse than with the previous set of values. We run the algorithm with two chains, and once convergence has been reached (determined using the Gelman-Rubin diagnostic, once the multivariate potential scale reduction factor is less than 1.2 [62]), we generate 50,000 samples from the posterior distributions for each parameter.

In a first instance, we used our growth co-culture data, where phage are absent, to calibrate the bacterial growth rate parameters $\mu_{max\theta}$ for each bacteria strain θ ($\theta \in$ {E, T, ET}), as well as the carrying capacity N_{max} using a simple logistic growth model (equation (3.32)). All other parameters related to phage predation were set to 0.

$$\frac{dB_\theta}{dt} = \mu_{max_\theta} * B_\theta * \left(1 - \frac{B_\theta}{N_{max}}\right) \qquad (3.32)$$

The phage predation parameters (τ, α, β_{max}, δ_{max}) were jointly estimated by fitting to the phage and double resistant bacteria numbers from the transduction co-culture data. We fitted to the transduction co-culture datasets with starting phage concentrations of 10^3 and 10^5 pfu/mL, and tested whether the estimated parameters could reproduce the dynamics seen with the starting phage concentration of 10^4 pfu/mL. Convergence and posterior distribution plots for our best-fitting model are shown in Supplementary Figure 3.6.

To mirror our experimental sampling variation, *in vitro* data points were scaled down to be between 1 and 100 before fitting, with the same correction applied to the corresponding model-predicted value for the same timepoint generated using a set of parameter values Θ. For example, if at 1h there are 1.4 x 10^4 phage *in vitro*, this is scaled down to 14, and if the corresponding model value is 5.3 x 10^6, this is scaled down by the same magnitude (i.e. 10^3), resulting in a value of 5300. We then calculated the log-likelihood of the *in vitro* data point (Y) being observed in a Poisson distribution, with the corresponding model data point (X) as a mean, hence assuming *Y ~ Poisson(X)*. In our example with Y = 14 and X = 5300, we would calculate the log-likelihood of observing 14 phage from a Poisson distribution with a mean of 5300. This process is repeated separately for each data point and corresponding model point

generated using the set of parameter values Θ, then all the log-likelihoods are summed to obtain a single value, representing the log-likelihood of Θ.

Previous research estimated that the latent period for 80α in *S. aureus* was approximately 40mins (0.67h), and that the burst size was approximately 40 phage per bacterium [39]. Since this study did not provide error values for these point estimates, we assumed the standard deviation and chose the following informative priors for these parameters: $\tau \sim Normal(0.67, 0.07)$ (95% confidence interval: 0.53-0.81) and $\delta_{max} \sim Normal(40, 7)$ (95% confidence interval: 54-26). Due to a lack of available data, we used uninformative priors for the remaining parameters: $\alpha \sim Uniform(0, 1)$ and $\beta_{max} \sim Uniform(0, 1)$.

3.8 References

1. Clokie, M. R. J., Millard, A. D., Letarov, A. V. & Heaphy, S. Phages in nature. *Bacteriophage* **1**, 31–45 (2011).
2. von Wintersdorff, C. J. H. *et al.* Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer. *Front. Microbiol.* **7**, (2016).
3. Fernández, L., Rodríguez, A. & García, P. Phage or foe: an insight into the impact of viral predation on microbial communities. *ISME J.* **12**, 1171–1179 (2018).
4. McCarthy, A. J. *et al.* Extensive Horizontal Gene Transfer during Staphylococcus aureus Co-colonization In Vivo. *Genome Biol. Evol.* **6**, 2697–2708 (2014).
5. Haaber, J., Penadés, J. R. & Ingmer, H. Transfer of Antibiotic Resistance in Staphylococcus aureus. *Trends Microbiol.* **25**, 893–905 (2017).
6. Balcázar, J. L. How do bacteriophages promote antibiotic resistance in the environment? *Clin. Microbiol. Infect.* **24**, 447–449 (2018).
7. Laxminarayan, R. *et al.* Antibiotic resistance—the need for global solutions. *Lancet Infect. Dis.* **13**, 1057–1098 (2013).
8. Verheust, C., Pauwels, K., Mahillon, J., Helinski, D. R. & Herman, P. Contained use of Bacteriophages: Risk Assessment and Biosafety Recommendations. *Appl. Biosaf.* **15**, 32–44 (2010).
9. Jassim, S. A. A. & Limoges, R. G. Natural solution to antibiotic resistance:

bacteriophages ‘The Living Drugs’. *World J. Microbiol. Biotechnol.* **30**, 2153–2170 (2014).

10. Hassan, A. Y., Lin, J. T., Ricker, N. & Anany, H. The Age of Phage: Friend or Foe in the New Dawn of Therapeutic and Biocontrol Applications? *Pharmaceuticals* **14**, 199 (2021).
11. Raj, J. R. M. & Karunasagar, I. Phages amid antimicrobial resistance. *Crit. Rev. Microbiol.* **45**, 701–711 (2019).
12. Leclerc, Q. J., Lindsay, J. A. & Knight, G. M. Mathematical modelling to study the horizontal transfer of antimicrobial resistance genes in bacteria: current state of the field and recommendations. *J. R. Soc. Interface* **16**, 20190260 (2019).
13. Levin, B. R. & Bull, J. J. Population and evolutionary dynamics of phage therapy. *Nat. Rev. Microbiol.* **2**, 166–173 (2004).
14. Krysiak-Baltyn, K., Martin, G. J. O., Stickland, A. D., Scales, P. J. & Gras, S. L. Computational models of populations of bacteria and lytic phage. *Crit. Rev. Microbiol.* **42**, 942–968 (2016).
15. Campbell, A. Conditions for the Existence of Bacteriophage. *Evolution* **15**, 153–165 (1961).
16. Levin, B. R., Stewart, F. M. & Chao, L. Resource-Limited Growth, Competition, and Predation: A Model and Experimental Studies with Bacteria and Bacteriophage. *Am. Nat.* **111**, 3–24 (1977).
17. Weld, R. J., Butts, C. & Heinemann, J. A. Models of phage growth and their applicability to phage therapy. *J. Theor. Biol.* **227**, 1–11 (2004).
18. Schrag, S. J. & Mittler, J. E. Host-Parasite Coexistence: The Role of Spatial Refuges in Stabilizing Bacteria-Phage Interactions. *Am. Nat.* **148**, 348–377 (1996).
19. Santos, S. B., Carvalho, C., Azeredo, J. & Ferreira, E. C. Population Dynamics of a Salmonella Lytic Phage and Its Host: Implications of the Host Bacterial Growth Rate in Modelling. *PLOS ONE* **9**, e102507 (2014).
20. Kokjohn, T. A. & Sayler, G. S. Y. Attachment and replication of Pseudomonas aeruginosa bacteriophages under conditions simulating aquatic environments. *Microbiology* **137**, 661–666 (1991).
21. Hadas, H., Einav, M., Fishov, I. & Zaritsky, A. Bacteriophage T4 Development Depends on the Physiology of its Host Escherichia Coli. *Microbiology* **143**, 179–185 (1997).
22. Middelboe, M. Bacterial Growth Rate and Marine Virus–Host Dynamics. *Microb.*

Ecol. **40**, 114–124 (2000).

23. Nabergoj, D., Modic, P. & Podgornik, A. Effect of bacterial growth rate on bacteriophage population growth rate. *MicrobiologyOpen* **7**, e00558 (2018).
24. Buckling, A. & Rainey, P. B. . Antagonistic coevolution between a bacterium and a bacteriophage. *Proc. R. Soc. Lond. B Biol. Sci.* **269**, 931–936 (2002).
25. Volkova, V. V., Lu, Z., Besser, T. & Gröhn, Y. T. Modeling the Infection Dynamics of Bacteriophages in Enteric Escherichia coli: Estimating the Contribution of Transduction to Antimicrobial Gene Spread. *Appl. Environ. Microbiol.* **80**, 4350–4362 (2014).
26. Arya, S. *et al.* A generalised model for generalised transduction: the importance of co-evolution and stochasticity in phage mediated antimicrobial resistance transfer. *FEMS Microbiol. Ecol.* **96**, (2020).
27. Fillol-Salom, A. *et al.* Bacteriophages benefit from generalized transduction. *PLOS Pathog.* **15**, e1007888 (2019).
28. Cassini, A. *et al.* Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. *Lancet Infect. Dis.* **19**, 56–66 (2019).
29. Lindsay, J. A. Staphylococcus aureus genomics and the impact of horizontal gene transfer. *Int. J. Med. Microbiol.* **304**, 103–109 (2014).
30. Kifelew, L. G. *et al.* Efficacy of phage cocktail AB-SA01 therapy in diabetic mouse wound infections caused by multidrug-resistant Staphylococcus aureus. *BMC Microbiol.* **20**, 204 (2020).
31. Petrovic Fabijan, A. *et al.* Safety of bacteriophage therapy in severe Staphylococcus aureus infection. *Nat. Microbiol.* **5**, 465–472 (2020).
32. Abedon, S. T. & Katsaounis, T. I. Basic Phage Mathematics. in *Bacteriophages: Methods and Protocols, Volume 3* (eds. Clokie, M. R. J., Kropinski, A. M. & Lavigne, R.) 3–30 (Springer, 2018). doi:10.1007/978-1-4939-7343-9_1.
33. Christie, G. E. *et al.* The complete genomes of Staphylococcus aureus bacteriophages 80 and 80α—Implications for the specificity of SaPI mobilization. *Virology* **407**, 381–390 (2010).
34. Chen, J., Quiles-Puchalt, N., Chiang, Y. N., Bacigalupe, R., Fillol-Salom, A., Chee, M. S., Fitzgerald, J. R., Penadés, J.R. Genome hypermobility by lateral transduction. *Science*. **362**, 207-212 (2018).

35. Fey, P. D. *et al.* A Genetic Resource for Rapid and Comprehensive Phenotype Screening of Nonessential Staphylococcus aureus Genes. *mBio* **4**, (2013).
36. Roach, D. R., Leung, C. Y., Henry, M., Morello, E., Singh, D., Di Santo, J. P., Weitz, J. S. & Debarbieux, L.. Synergy between the host immune system and bacteriophage is essential for successful phage therapy against an acute respiratory pathogen. *Cell host & microbe* **22**, 38–47 (2017).
37. Eriksen, R. S., Mitarai, N. & Sneppen, K. Sustainability of spatially distributed bacteria-phage systems. *Sci. Rep.* **10**, 3154 (2020).
38. Spiegelhalter, D. J., Best, N. G., Carlin, B. P. & Linde, A. V. D. Bayesian measures of model complexity and fit. *J. R. Stat. Soc. Ser. B Stat. Methodol.* **64**, 583–639 (2002).
39. Sjöström, J.-E., Lindberg, M. & Philipson, L. Transfection of Staphylococcus aureus with Bacteriophage Deoxyribonucleic Acid. *J. Bacteriol.* **109**, 285–291 (1972).
40. Berryhill, B. A., Huseby, D. L., McCall, I. C., Hughes, D. & Levin, B. R. Evaluating the potential efficacy and limitations of a phage for joint antibiotic and phage therapy of Staphylococcus aureus infections. *Proc. Natl. Acad. Sci.* **118**, (2021).
41. Gefen, O., Fridman, O., Ronin, I. & Balaban, N. Q. Direct observation of single stationary-phase bacteria reveals a surprisingly long period of constant protein production activity. *Proc. Natl. Acad. Sci.* **111**, 556–561 (2014).
42. Heijer, C. D. den *et al.* Prevalence and resistance of commensal Staphylococcus aureus, including meticillin-resistant S aureus, in nine European countries: a cross-sectional study. *Lancet Infect. Dis.* **13**, 409–415 (2013).
43. Stanczak-Mrozek, K. I. *et al.* Within-host diversity of MRSA antimicrobial resistances. *J. Antimicrob. Chemother.* **70**, 2191–2198 (2015).
44. Suttle, C. A. & Chen, F. Mechanisms and Rates of Decay of Marine Viruses in Seawater. *Appl. Environ. Microbiol.* **58**, 3721–3729 (1992).
45. Banuelos, S. *et al*. Investigating the Impact of Combination Phage and Antibiotic Therapy: A Modeling Study. in *Using Mathematics to Understand Biological Complexity: From Cells to Populations*. (ed. Segal, R., Shtylla, B. & Sindi S.) 111-134 (Springer, 2021).
46. Rodriguez-Gonzalez, R. A., Leung, C. Y., Chan, B. K., Turner, P. E. & Weitz, J. S. Quantitative Models of Phage-Antibiotic Combination Therapy. *mSystems* **5**, (2020).

47. Peterson, B. W., Sharma, P. K., van der Mei, H. C. & Busscher, H. J. Bacterial Cell Surface Damage Due to Centrifugal Compaction. *Appl. Environ. Microbiol.* **78**, 120–125 (2012).
48. Chatterjee, A. N. Use of Bacteriophage-resistant Mutants to Study the Nature of the Bacteriophage Receptor Site of Staphylococcus aureus. *J. Bacteriol*. **98**, 519–527 (1969).
49. Park, J. T., Shaw, D. R. D., Chatterjee, A. N., Mirelman, D. & Wu, T. Mutants of Staphylococci with Altered Cell Walls. *Ann. N. Y. Acad*. Sci. **236**, 54–62 (1974).
50. Winstel, V., Xia, G. & Peschel, A. Pathways and roles of wall teichoic acid glycosylation in Staphylococcus aureus. *Int. J. Med. Microbiol*. **304**, 215–221 (2014).
51. Xia, G., Kohler, T. & Peschel, A. The wall teichoic acid and lipoteichoic acid polymers of Staphylococcus aureus. *Int. J. Med. Microbiol*. **300**, 148–154 (2010).
52. van Dalen, R., Peschel, A. & van Sorge, N.M. Wall teichoic acid in Staphylococcus aureus host interaction. *Trends in microbiology*, **28**, 985-998 (2020).
53. Mašlaňová, I., Stříbná, S., Doškař, J. & Pantůček, R. Efficient plasmid transduction to Staphylococcus aureus strains insensitive to the lytic action of transducing phage. *FEMS Microbiol. Lett.* **363**, (2016).
54. Jiang, S. C. & Paul, J. H. Gene Transfer by Transduction in the Marine Environment. *Appl. Environ. Microbiol.* **64**, 2780–2787 (1998).
55. Fišarová, L. *et al.* Staphylococcus epidermidis Phages Transduce Antimicrobial Resistance Plasmids and Mobilize Chromosomal Islands. *mSphere* **6**, (2021).
56. Brives, C. & Pourraz, J. Phage therapy as a potential solution in the fight against AMR: obstacles and possible futures. *Palgrave Communications* **6**, 1-11 (2020).
57. R Core Team. *R: A Language and Environment for Statistical Computing*. (R Foundation for Statistical Computing, 2020).
58. Khan, S. A. & Novick, R. P. Complete nucleotide sequence of pT181, a tetracycline-resistance plasmid from Staphylococcus aureus. *Plasmid* **10**, 251–259 (1983).
59. Novick, R.P., Adler, G.K., Projan, S.J., Carleton, S., Highlander, S.K., Gruss, A., Khan, S.A. & Iordanescu, S. Control of pT181 replication I. The pT181 copy control function acts by inhibiting the synthesis of a replication protein. *The EMBO journal* **3**, 2399-2405 (1984).
60. Veiga, H. & Pinho, M. G. Inactivation of the SauI Type I Restriction-Modification

System Is Not Sufficient To Generate Staphylococcus aureus Strains Capable of Efficiently Accepting Foreign DNA. *Appl. Environ. Microbiol.* **75**, 3034–3038 (2009).

61. Adams, M. H. *Bacteriophages.* (Interscience Publishers, 1959).
62. Gelman, A. & Rubin, D. B. Inference from Iterative Simulation Using Multiple Sequences. *Stat. Sci.* **7**, 457–472 (1992).

4 Modelling the joint dynamics of bacteriophage and antibiotics: killers and drivers of resistance evolution

4.1 Overview

Through the interdisciplinary approach presented in Chapter 3, I obtained a fundamental understanding of phage predation and generalised transduction dynamics in *S. aureus*, and designed a mathematical model supported by *in vitro* data to explore these processes. The next step was to examine the effect of antibiotics on phage-bacteria dynamics, since in reality these may often be present alongside *S. aureus* and their phage. Evidence on the joint killing effect of phage and antibiotics on bacteria is conflicted, and the role of transduction as a potential driver of antibiotic-resistance in environments where antibiotics, phage and bacteria are all present is unknown. In this Chapter, I generated further *in vitro* data and extended the mathematical model developed in Chapter 3 to identify conditions where phage and antibiotics either remove bacteria synergistically, or drive AMR evolution.

This Chapter relied again on an interdisciplinary approach, since using experimental methods alone would not be sufficient to disentangle the interactions between antibiotics, phage, and bacteria. Instead, by generating further *in vitro* data to parameterise the effect of antibiotics on *S. aureus*, I was then able to use this extended model to explore a wide range of scenarios, with varying phage and antibiotic presence timing and concentration. This approach allowed me to identify conditions under which the action of the phage complements the antibiotic, leading to a faster decline in the bacterial population, and conditions where, due to the antibiotics restricting bacterial growth and hence phage growth, double antibiotic-resistant bacteria are generated via transduction, not killed by phage, and then selected for by the antibiotics.

At the time of writing, this work is available as a preprint (Leclerc, Lindsay and Knight, 2022), and is being revised for resubmission to PLoS Computational Biology following reviewer comments obtained on 25/05/2022. The co-authors are Quentin J Leclerc,

Jodi A Lindsay, and Gwenan M Knight. The version included below is the preprint, with some minor changes in response to reviewer comments.

4.2 Research paper cover sheet

Please note that a cover sheet must be completed <u>for each</u> research paper included within a thesis.

<u>SECTION A – Student Details</u>

Student ID Number	1702140	**Title**	Mr
First Name(s)	Quentin		
Surname/Family Name	Leclerc		
Thesis Title	Interdisciplinary investigations of phage predation dynamics and generalised transduction of antimicrobial resistance in *Staphylococcus aureus*		
Primary Supervisor	Dr Gwenan Knight		

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

<u>SECTION B – Paper already published</u>

Where was the work published?			
When was the work published?			
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion			
Have you retained the copyright for the work?*	Choose an item.	Was the work subject to academic peer review?	Choose an item.

*If yes, please attach evidence of retention. If no, or if the work is being included in its published format, please attach evidence of permission from the copyright holder (publisher or other author) to include this work.

SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	PLoS Computational Biology
Please list the paper's authors in the intended authorship order:	Quentin J Leclerc, Jodi A Lindsay, Gwenan M Knight
Stage of publication	**Undergoing revision**

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I designed and conducted the *in vitro* experiments. I designed, coded, and fitted the mathematical model. I ran all the simulations to generate the results presented in this paper. I wrote the first draft of this manuscript, and subsequently edited it.

SECTION E

Student Signature	[redacted]
Date	06/06/2022

Supervisor Signature	[redacted]
Date	06/06/2022

4.3 Abstract and Author Summary

4.3.1 Abstract

Bacteriophage (phage) are bacterial predators that can also spread antimicrobial resistance (AMR) genes between bacteria by generalised transduction. Phage are often present alongside antibiotics in the environment, yet evidence of their joint killing effect on bacteria is conflicted, and the dynamics of transduction in such systems are unknown. Here, we combine *in vitro* data and mathematical modelling to identify conditions where phage and antibiotics act to remove bacteria synergistically, or drive AMR evolution.

We adapt a published model of phage-bacteria dynamics, including transduction, to add the pharmacodynamics of erythromycin and tetracycline, parameterised from new *in vitro* data. We simulate a system where two strains of *Staphylococcus aureus* are present at stationary phase, each carrying either an erythromycin or tetracycline resistance gene, and where multidrug-resistant bacteria can be generated by transduction only. We determine rates of bacterial clearance and multidrug-resistant bacteria appearance, when either or both antibiotics and phage are present at varying timings and concentrations.

Although phage and antibiotics act in synergy to kill bacteria, by reducing bacterial growth antibiotics reduce phage production. A low concentration of phage introduced shortly after antibiotics fails to replicate and exert a strong killing pressure on bacteria, instead generating multidrug-resistant bacteria by transduction which are then selected for by the antibiotics. Multidrug-resistant bacteria numbers were highest when antibiotics and phage were introduced simultaneously.

The interaction between phage and antibiotics leads to a trade-off between a slower clearing rate of bacteria (if antibiotics are added before phage), and a higher risk of multidrug resistance evolution (if phage are added before antibiotics), exacerbated by low concentrations of phage or antibiotics. Our results form hypotheses to guide future experimental and clinical work on the impact of phage on AMR evolution, notably for studies of phage therapy which should investigate varying timings and concentrations of phage and antibiotics.

4.3.2 Author Summary

Bacteriophage (“phage”) are viruses that can infect and kill bacteria, but also natural drivers of antimicrobial resistance (AMR) evolution by transduction, when they accidentally carry non-phage DNA between bacteria, including AMR genes. Phage are often present alongside antibiotics in the environment and in humans, yet the joint dynamics of phage, antibiotics, and bacteria are unclear. Using laboratory work and mathematical modelling, we show for the first time that depending on timing and concentration, phage and antibiotics can either work together to kill bacteria faster, or phage can generate multidrug-resistant bacteria by transduction which are then selected for by antibiotics. This may be particularly important in the context of phage therapy, where phage are used to treat bacterial infections, often alongside antibiotics. Our conclusions highlight the urgent need for clinical and laboratory work to quantify the currently unknown contribution of phage to AMR evolution in humans. Otherwise, we may be missing opportunities to reduce the global public health burden of AMR.

4.4 Introduction

Bacteriophage (phage) are major bacterial predators and the most common organisms on the planet [1]. Phage are often present alongside antibiotics, naturally or in the context of antibacterial treatment [2–4], yet reports on their combined effect on bacteria are conflicting. Some previous studies showed that phage and antibiotics work synergistically to clear bacteria [5–7], while others have demonstrated that antibiotics reduce phage production [8,9]. This is further complicated by the fact that phage are also major drivers of bacterial evolution via horizontal gene transfer by transduction [10,11], which can notably contribute to antimicrobial resistance (AMR) spread [12,13]. If multidrug-resistant bacteria are generated by transduction, antibiotics present in the same environment may act as a selective pressure to increase their prevalence, yet to our knowledge the dynamics of transduction have not yet been investigated in such systems [14].

There are two types of transduction: specialised and generalised [15,16]. Specialised transduction occurs when a prophage accidentally picks up adjacent bacterial DNA

upon excision from the bacterial chromosome, at the end of the lysogenic cycle. On the other hand, generalised transduction occurs during phage replication, when non-phage DNA is packaged by mistake in a new phage particle. The resulting transducing phage is released upon bacterial lysis, and injects this DNA in another bacterium. Generalised transduction is likely the most important type of transduction in the context of AMR, as it can lead to the horizontal transfer of any genetic material contained in a bacterium (including plasmids, major vectors of AMR genes) [10].

A major bacterial pathogen often exposed to phage is *Staphylococcus aureus*, which at any given time is colonising approximately 20% of humans [17]. Previous work suggests that all *S. aureus* carry integrated prophage [18], and at least 50% of individuals colonised by *S. aureus* also carry free phage capable of generalised transduction [19], the main mechanism of horizontal gene transfer for *S. aureus* [18]. This is particularly relevant for methicillin-resistant *S. aureus* (MRSA), a group of *S. aureus* present in both the ESKAPE list and the World Health Organization priority list of antibiotic-resistant bacteria due to its large clinical burden [20–22]. *In vitro*, the generation rate of generalised transducing MRSA phage carrying an AMR gene has been estimated to be approximately one per 10^8 new phage produced, sufficient to consistently generate bacteria resistant to multiple antibiotics in less than 24h [23], and *in vivo* transduction rates are likely to be even higher [24].

Understanding the dynamics of bacteria, antibiotics, phage and transduction is especially important in the context of phage therapy, which aims to use phage as antibacterial agents, generally in combination with antibiotics [4]. Phage therapy is currently investigated as a solution to counter the threat of AMR, with ongoing clinical trials against MRSA infection [6,25–29]. Phage therapy guidelines recommend that only phage with a limited ability to perform transduction should be used [15,30,31], yet to our knowledge there is currently no technique to prevent phage from accomplishing generalised transduction, which is fundamentally a mispackaging and thus a biological error similar to a mutation. Hence, as previous reviews have highlighted, it is essential to explore the importance of this mechanism, and identify conditions under which it could affect the outcome of therapy and lead to multidrug resistance evolution [14,30–32].

In this study, we aim to investigate the potential combined effect of antibiotics and phage capable of generalised transduction on bacteria. Making conclusions about this multidimensional chequerboard space of potential combinations and timings is difficult when using data from time-consuming, single-scenario, *in vitro* experiments. Instead, we generate hypotheses to guide future experimental work by simulating these conditions. We adapt a previously published mathematical model of phage-bacteria dynamics, including generalised transduction, to incorporate the effect of antibiotics on bacteria [23]. This model is parameterised using *in vitro* data from the same environment and set of conditions as when it was originally developed [23], making it a reliable tool to infer the dynamics governing this system.

We hypothesise that, depending on the timing and concentration at which they are added, phage and antibiotics can either act to eradicate bacteria synergistically, or to create and select for multidrug-resistant bacteria. We explore this in our model and generate guidelines to minimise the risk of generating double-antibiotic-resistant *S. aureus* when two single-resistant strains are exposed to antibiotics and phage, whilst maximising bacterial eradication.

4.5 Materials and Methods

4.5.1 Laboratory methods

4.5.1.1 Bacterial strains and phage

Two *Staphylococcus aureus* strains were obtained from the Nebraska transposon library in the MRSA USA300 background [33]. These were NE327, with the *ermB* gene conferring resistance to erythromycin, and NE201KT7, a modified NE201 strain with a kanamycin resistance cassette replacing the *ermB* gene and a plasmid carrying the *tetK* gene conferring resistance to tetracycline. Horizontal gene transfer can only happen between these two strains via generalised transduction. When co-cultured with 80α phage, these give rise to double-resistant progeny (DRP) bacteria resistant to both erythromycin and tetracycline [23]. In the experiments conducted here, we used the double-resistant strain DRPET1, a DRP strain with a NE327 background

generated during previous NE327, NE201KT7 and 80α co-cultures, containing both *ermB* and *tetK* genes [23]. Note that although 80α is a temperate phage, we have previously shown that lysogeny does not occur at a detectable level in our experiments, with a lysogenic frequency detection limit of 3.3×10^{-8} per non-lysogenic bacteria after 24h of co-culture, and that specialised transduction is unlikely to be responsible for horizontal gene transfer, due to the location of *tetK* on a plasmid and a ~1Mbp distance between *ermB* and the 80α integration site on the bacterial chromosome [23]. Hence the only interactions we consider between phage and bacteria in our environment are lysis and generalised transduction.

4.5.1.2 Time-kill curves

The growth conditions are identical to the ones previously used to generate data on lysis and transduction with these bacteria and phage [23]. Pre-cultures of each *S. aureus* bacterial strain (NE327, NE201KT7 and DRPET1) were separately generated overnight in 50mL conical tubes containing 10mL of brain-heart infusion broth (BHIB, Sigma, UK). Unless otherwise stated, liquid cultures were incubated in a warm shaking water bath (37°C, 90 rpm). Each pre-culture was then diluted in phosphate-buffered saline (PBS) and mixed with 10mL of fresh BHIB in a 50mL conical tube to reach a starting concentration of 10^4 colony-forming units (cfu)/mL. The new culture was incubated for 2h to allow the bacteria to reach log-growth phase, following standard protocol for time-kill experiments [34]. Erythromycin or tetracycline was then added to the culture, at a concentration of either 0 (control), 0.25, 0.5, 1, 2, 4, 8, 16, or 32 mg/L. At 0, 1, 2, 3, 4, 6 and 24h after antibiotic addition, 30µl were sampled from the incubated culture, diluted in PBS, and plated on plain brain-heart infusion agar. The plates were incubated overnight at 37°C. Colonies on the plates were then counted to derive the concentration of bacteria in cfu/mL at the corresponding time point.

The experiment was repeated 3 times for each strain (NE327, NE201KT7 and DRPET1) and each antibiotic (erythromycin and tetracycline).

4.5.1.3 Minimum inhibitory concentration

We measured the minimum inhibitory concentrations (MIC) of erythromycin and tetracycline for NE327, NE201KT7 and DRPET1 by microbroth dilution [35]. Briefly,

pre-cultures of each strain were generated overnight in Mueller-Hinton broth (MHB-II, Sigma, UK). Antibiotic stocks were generated in 50% ethanol at concentrations doubling from 0.25 to 256 mg/L, and 10µL of each dilution were added to separate wells on a 96-well plate. The overnight pre-cultures were diluted to a concentration of 10^5 cfu/mL, and 90µL were mixed with each antibiotic dilution in the 96-well plate. The plate was incubated overnight at 37°C, and the MIC were then determined by eye, identifying the lowest concentration of antibiotic which did not allow bacterial growth (i.e. the contents of the well were not turbid).

4.5.2 Modelling methods

All analyses were conducted in the R statistical analysis software [36]. The underlying code and data are available in a GitHub repository: https://github.com/qleclerc/phage_antibiotic_dynamics.

4.5.2.1 Mathematical model

We adapted a previously published mathematical model of phage-bacteria dynamics, including generalised transduction [23]. This model was previously parameterised using the same bacterial and phage strains as in this study. Our three *S. aureus* strains are represented: B_E (erythromycin-resistant, corresponding to *in vitro* strain NE327), B_T (tetracycline-resistant, corresponding to NE201KT7), and B_{ET} (resistant to both erythromycin and tetracycline, corresponding to double-resistant bacteria) (Figure 4.1). The model also contains 80α lytic phage (P_L), which can give rise to transducing phage carrying either the erythromycin (P_E) or tetracycline (P_T) resistance gene. The transducing phage can give rise to B_{ET} bacteria via generalised transduction (Figure 4.1).

We added two compartments to the model to track the concentration of antibiotics: C_E for erythromycin, and C_T for tetracycline. The concentrations are expressed in mg/L. We can simulate the addition of a chosen concentration of antibiotic at any given time, and the antibiotics decay at a constant rate γ_E and γ_T for erythromycin and tetracycline, respectively (Figure 4.1, Equations 4.1-2). A similar constant rate γ_P is used for phage decay (Equations 4.7-9). Note that these decay rates are all set to 0 in our analysis,

as we were not able to parameterise these using our *in vitro* data, but we explore the effect of changing their values between biologically plausible limits in the sensitivity analysis (see Results).

$$\frac{dC_E}{dt} = -\gamma_E * C_E \qquad (4.1)$$

$$\frac{dC_T}{dt} = -\gamma_T * C_T \qquad (4.2)$$

The antibiotic concentrations are then used to calculate $\varepsilon_{i,j}$, the effect of each antibiotic *i* (i ∈ {E, T}) on each bacterial strain *j* (*j* ∈ {E, T, ET}), according to a pharmacodynamic relationship parameterised through Hill equations [34,37] (Equation 4.3). This commonly used function calculates the effect of an antibiotic using four parameters: the maximum effect ($\varepsilon^{max}_{i,j}$), current concentration (C_i), half maximal effective concentration ($EC50_{i,j}$), and a Hill coefficient ($H_{i,j}$). Note that $\varepsilon^{max}_{i,j}$ is relative to the maximum growth rate of the corresponding strain *j* (μ^{max}_j, see below and Equation 4.10).

$$\varepsilon_{i,j} = \mu_j^{max} * \varepsilon_{i,j}^{max} * \frac{C_i^{(H_{i,j})}}{EC50_{i,j}^{(H_{i,j})} + C_i^{(H_{i,j})}} \qquad (4.3)$$

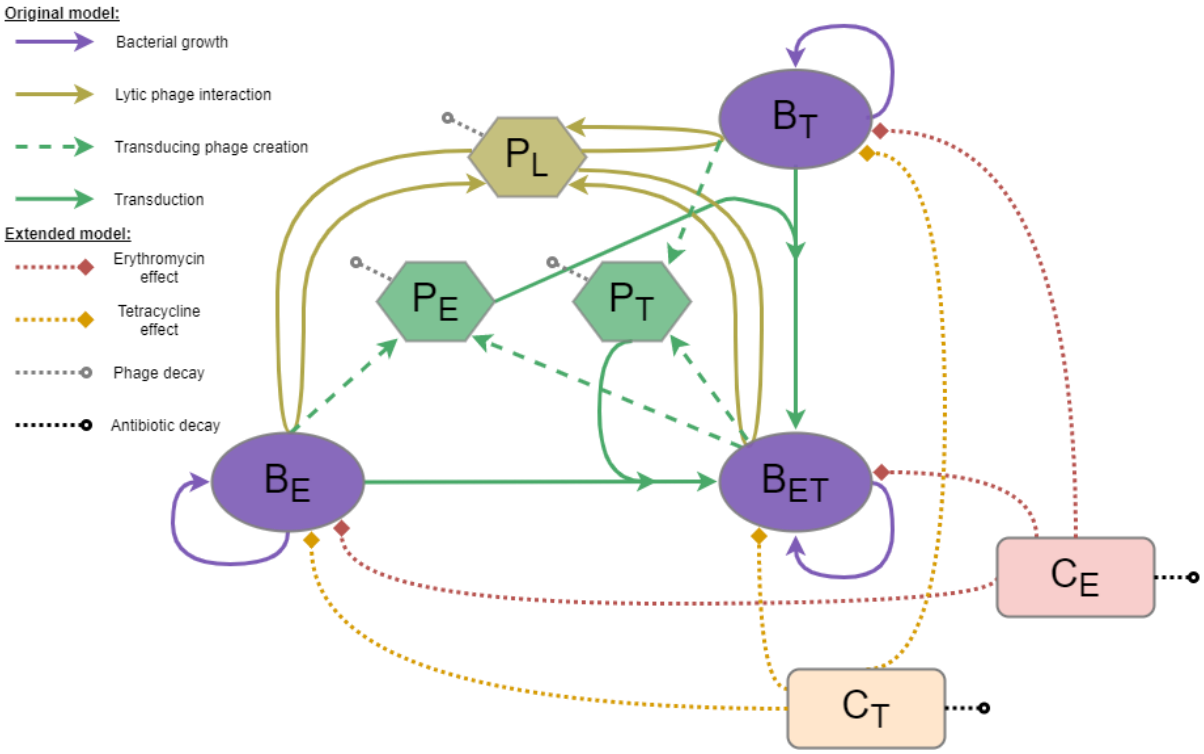


Figure 4.1: Mathematical model diagram. This model is an extension of our original model presented in [23], with the inclusion of antibiotic effects. Each bacteria strain (B_E resistant to erythromycin, B_T resistant to tetracycline, or B_{ET} resistant to both) can replicate (purple). The lytic phage (P_L) multiply by infecting a bacterium and bursting it to release new phage (gold). This process can create transducing phage (P_E or P_T) carrying a resistance gene (*ermB* or *tetK* respectively) taken from the infected bacterium (dashed, green). These transducing phage can then generate new double-resistant progeny (B_{ET}) by infecting the bacteria strain carrying the other resistance gene (solid, green). The antibiotics, erythromycin (C_E) and tetracycline (C_T), decrease the growth rate of each bacteria strain to varying extents, depending on their concentration and the resistance level of the strain (dotted, red and orange). Phage and antibiotics can decay at a fixed rate (dotted, grey and black).

The complete model equations can be found below.

$$\frac{dB_E}{dt} = \mu_E * B_E - B_E * F(P_L) - B_E * F(P_T) - (\varepsilon_{E,E} + \varepsilon_{T,E}) * B_E \quad (4.4)$$

{Change in B_E = growth of B_E – infections by P_L – infections by P_T - ery killing - tet killing}

$$\frac{dB_T}{dt} = \mu_T * B_T - B_T * F(P_L) - B_T * F(P_E) - (\varepsilon_{E,T} + \varepsilon_{T,T}) * B_T \quad (4.5)$$

{Change in B_T = growth of B_T – infections by P_L – infections by P_E - ery killing - tet killing}

$$\frac{dB_{ET}}{dt} = \mu_{ET} * B_{ET} - B_{ET} * F(P_L) + B_T * F(P_E) + B_E * F(P_T) - (\varepsilon_{E,ET} + \varepsilon_{T,ET}) * B_{ET} \quad (4.6)$$

{Change in B_{ET} = growth of B_{ET} – infections by P_L + infections of B_T by P_E + infections of B_E by P_T - ery killing - tet killing}

$$\frac{dP_L}{dt} = [F(P_L) * B_E](t - \tau) * \delta_E * (1 - \alpha) +$$

$$[F(P_L) * B_T](t - \tau) * \delta_T * (1 - \alpha) +$$

$$[F(P_L) * B_{ET}](t-\tau) * \delta_{ET} * (1 - 2 * \alpha\ ) \ -$$

$$\lambda * P_L - \gamma_P * P_L \qquad (4.7)$$

{Change in P_L = new P_L phage from B_E + new P_L phage from B_T + new P_L phage from B_{ET} –

P_L phage infecting bacteria – P_L decay}

$$\frac{dP_E}{dt} = [F(P_L) * B_E](t-\tau) * \delta_E * \alpha +$$

$$[F(P_L) * B_{ET}](t-\tau) * \delta_{ET} * \alpha -$$

$$\lambda * P_E - \gamma_P * P_E \qquad (4.8)$$

{Change in P_E = new P_E phage from B_E + new P_E phage from B_{ET} –

P_E phage infecting bacteria – P_E decay}

$$\frac{dP_T}{dt} = [F(P_L) * B_T](t-\tau) * \delta_T * \alpha +$$

$$[F(P_L) * B_{ET}](t-\tau) * \delta_{ET} * \alpha -$$

$$\lambda * P_T - \gamma_P * P_T \qquad (4.9)$$

{Change in P_T = new P_T phage from B_T + new P_T phage from B_{ET} –

P_T phage infecting bacteria – P_T decay}

The bacterial growth rate μ_θ ($\theta \in \{E, T, ET\}$, $N = B_E+B_T+B_{ET}$) is modelled using logistic growth, with a maximum growth rate of μ^{max}_θ and carrying capacity N^{max}.

$$\mu_\theta = \mu_\theta^{max} * (1 - \frac{N}{N^{max}}) \qquad (4.10)$$

Phage predation is modelled as a saturated process. Previous work has suggested that this interaction is more biologically realistic than the more commonly used linear

interaction, as it accounts for multiple phage binding to the same bacterium at high phage concentration, leading to a sublinear increase in predation [23]. This is represented using a Hill equation, with $F(P_\theta)$ ($\theta \in \{L, E, T\}$) equal to

$$F(P_\theta) = P_\theta * \frac{\beta}{(1+\frac{P_\theta}{P50})} \qquad (4.11)$$

with β representing the maximum rate of successful phage adsorption leading to bacterial lysis and $P50$ corresponding to the phage concentration at half saturation, where the adsorption rate is equal to half the maximum. The latent period τ corresponds to the delay between bacterial infection and burst, and the transduction probability α corresponds to the proportion of new phage released upon burst which are transducing phage carrying the erythromycin or tetracycline resistance gene.

In the model, phage burst size decreases as bacterial growth decreases. This happens as bacteria enter stationary phase when the population approaches carrying capacity N^{max} (as demonstrated previously [23,38,39]), but here the additional effect of antibiotics must be included. To capture this, we use the same effective scaling as the bacterial growth rate (Equations 4.10 and 4.4-4.6) with inclusion of the relative effect of antibiotics $\varepsilon_{E,\theta}$ and $\varepsilon_{T,\theta}$ in the phage burst size estimation. Since antibiotics can kill bacteria, leading to a net negative growth rate, we limit the multiplier value to 0 as a minimum, to prevent a negative burst size. The phage burst size δ_θ for each bacteria strain θ ($\theta \in \{E, T, ET\}$) is then calculated as a fraction of the maximum phage burst size δ^{max} (Equation 4.13).

$$\delta_\theta = \delta^{max} * max\left(0, 1 - \frac{N}{N^{max}} - \frac{\varepsilon_{E,\theta}}{\mu_\theta^{max}} - \frac{\varepsilon_{T,\theta}}{\mu_\theta^{max}}\right) \qquad (4.13)$$

4.5.2.2 Parameter estimation

Parameters for bacterial growth and phage predation were originally obtained by fitting our model to *in vitro* data for the same bacteria and phage strains as in this study [23]. Parameters for antibiotic effect were obtained in two steps using the least squares methods for model fitting, which aims to minimise the squared difference between data and model output, calculated for n points as

$$\sum_{i=1}^{n}(d_i - m_i)^2 \qquad (4.14)$$

with d_i representing data points and m_i representing model points.

Firstly, the reduction in bacterial growth caused by a specific concentration of antibiotic (ɛ́) was obtained by fitting a deterministic growth model (Equation 4.15) to the bacterial concentration B over time, estimated as the mean of three *in vitro* replicates.

$$\frac{d\mathrm{B}}{dt} = \mu * (1 - \frac{B}{N^{max}}) * \mathrm{B} - \acute{\varepsilon} * B \qquad (4.15)$$

ɛ́ was then scaled to growth (ε = ɛ́/μ), to represent the relative impact of the antibiotic on bacterial growth rather than an absolute value. Since we obtained growth curve time series data for 8 concentrations for each strain, we generated 8 estimates of antibiotic effect for each strain.

Secondly, the three parameters of the Hill equation [34,37] (maximum effect ε^{max}, Hill coefficient *H*, and half maximum effective concentration *EC50*), which calculates the antibiotic effect as a function of concentration, were estimated by fitting Equation 4.3 to the 8 antibiotic effects for each strain.

4.5.3 Model scenarios considered

4.5.3.1 Antibiotics alone

We start with an environment containing both single-resistant strains at stationary phase (10^9 cfu/mL). This mirrors the within-host diversity we could expect to see during bacterial infections [19], and the fact that bacteria most often live at stationary phase in the environment [40]. We first investigate the effect of the presence of either or both erythromycin and tetracycline at concentrations of 1 mg/L, similar to antibiotic concentrations measured *in vivo* during treatment [41,42].

4.5.3.2 Antibiotic and phage

We then consider scenarios where phage are present. We first artificially inactivate transduction, by setting the corresponding model parameter to 0. We add 10^9 pfu/mL of phage, similar to a concentration that could be added during phage therapy, and equivalent to a multiplicity of infection (ratio of phage to bacteria) of 1, similar to what could be found naturally in the environment [1]. We consider scenarios where the phage are present alone, alongside only one antibiotic, or alongside both antibiotics. Phage and antibiotics are assumed to be introduced concurrently at the start, and the simulations run for 24h.

We then repeat these scenarios, but with transduction enabled to levels that were previously observed *in vitro*, with approximately 1 transducing phage carrying an AMR gene generated for each 10^8 new lytic phage [23].

We repeat the analyses above with either single-resistant strain present at 10^9 cfu/mL, and the other at 10^6 cfu/mL (0.1%). This could correspond to a scenario where bacterial diversity is underestimated, with only one type of resistance detected [19].

4.5.3.3 Antibiotic and phage level and timing variation

To further investigate the scenario where transduction is enabled and both single-resistant bacterial strains have a starting concentration of 10^9 cfu/mL, we vary the timing of introduction for antibiotic and phage, with up to 24h delay between their respective additions, as well as varying the concentration of antibiotics between 0.25, 0.5, 1 and 2 mg/L, and phage between 10^7, 10^8, 10^9, 10^{10} pfu/mL, chosen to reflect realistic ranges [1,41,42]. We run these simulations for 48h after the introduction of antibiotics or phage (whichever is added first).

4.5.3.4 Phage and bacteria parameters variation

Finally, we vary the probability for phage to perform generalised transduction to identify threshold values which dictate whether multidrug-resistant bacteria appear above detectable levels within 24h. To explore the sensitivity of our results to the parameter values estimated from this single environment, we also conduct a partial rank correlation to assess the effect of varying phage predation parameters

(adsorption rate, phage concentration at half saturation, latent period, burst size), bacteria growth rates, and antibiotic and phage decay on total bacteria remaining after 48h, and maximum double-resistant bacteria generated. This allows us to generalise our conclusions as these parameters likely vary for different combinations of phage, antibiotics and bacteria. The ranges evaluated are shown in Table 4.1, and are either derived from our initial parameterisation of the model [23], or from other studies [43,44].

4.6 Results

4.6.1 *In vitro* pharmacodynamics of erythromycin and tetracycline

Erythromycin at all concentrations caused a decrease in erythromycin-sensitive NE201KT7 numbers over 6h, but only slowed down growth for erythromycin-resistant NE327 and double-resistant DRPET1, even at 32 mg/L (Figure 4.2). Tetracycline caused a decrease in bacterial numbers over 6h at concentrations greater than 0.5 mg/L for tetracycline-sensitive NE327, 8 mg/L for tetracycline-resistant NE201KT7, and 4 mg/L for DRPET1 (Figure 4.2). As a comparison, the minimum inhibitory concentrations (MICs) of erythromycin measured by microbroth dilution were 0.25, >256 and >256 mg/L for NE201KT7, NE327 and DRPET1 respectively. For tetracycline, the MICs were 32, 0.25 and 32 mg/L for NE201KT7, NE327 and DRPET1 respectively.

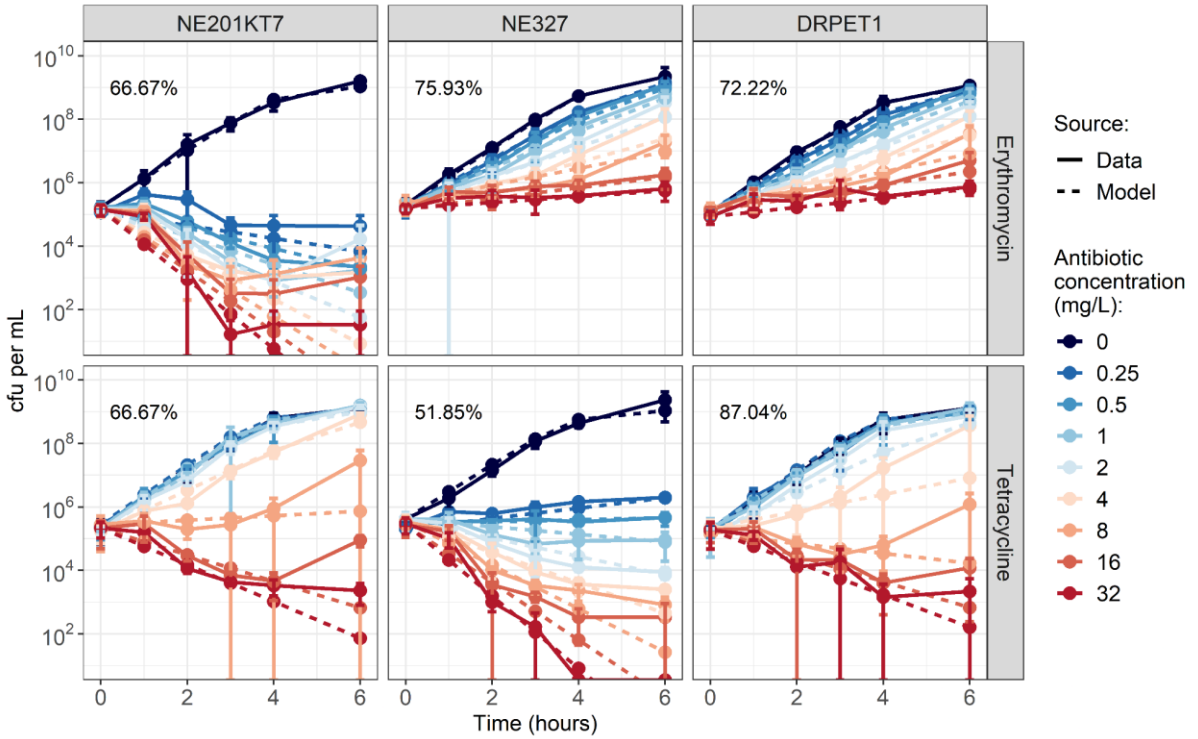


Figure 4.2: Growth curves of NE201KT7 (tetracycline-resistant, left), NE327 (erythromycin-resistant, middle) and DRPET1 (double-resistant, right), exposed to varying concentrations of erythromycin (top) or tetracycline (bottom). Solid lines show *in vitro* data, with error bars indicating mean +/- standard deviation, from 3 replicates. Dashed lines show model output after fitting. Values indicate the percentage of model points that fall within the range of the corresponding *in vitro* data point +/- standard deviation. cfu: colony-forming units. Note that cfu per mL are shown on a log-scale.

The Hill equations fitted well to the effect of varying concentrations of antibiotics on the bacteria (Figure 4.2, Supplementary Figure 4.1). The corresponding parameter values are presented in Table 4.1. The antibiotic effect curves for the DRPET1 are similar to the one for NE201KT7 for tetracycline and NE327 for erythromycin (Supplementary Figure 4.1), which was expected since DRPET1 contains both antibiotic-resistance genes from NE201KT7 (*tetK*) and NE327 (*ermB*).

Table 4.1: Model parameter values. Parameters with no units are dimensionless. All estimates were obtained by fitting the model to *in vitro* data, except those marked with a * which are assumed.

Name (unit)		Symbol	Estimate	Range for sensitivity analysis	Reference
Bacterial growth parameters	Carrying capacity (bacteria.mL^{-1})	N^{max}	2.76 x 10^{9}	-	[23]
	B_E growth rate (h^{-1})	μ^{max}_{E}	1.61	1.59 - 1.63	
	B_T growth rate (h^{-1})	μ^{max}_{T}	1.51	1.49 - 1.53	
	B_{ET} growth rate (h^{-1})	μ^{max}_{ET}	1.44	1.42 - 1.47	
Phage parameters	Phage adsorption rate (phage^{-1}.bacteria^{-1}.h^{-1})	β	2.3 x 10^{-10}	2.1 x 10^{-10} - 2.7 x 10^{-10}	
	Phage concentration at half saturation (phage.mL^{-1})	P50	1.19 x 10^{10}	1.02 x 10^{10} - 1.29 x 10^{10}	
	Phage burst size (phage)	δ^{max}	50	43 - 54	
	Phage latent period (h)	τ	0.60	0.60 - 0.61	
	Transduction probability (phage^{-1})	α	1.19 x 10^{-8}	1.11 x 10^{-8} - 1.31 x 10^{-8}	
	Phage decay rate (h^{-1})	γ_P	0*	0 - 0.1	[43]
Antibiotic parameters	Erythromycin decay rate (h^{-1})	γ_E	0*	0 - 0.1	[44]
	Tetracycline decay rate (h^{-1})	γ_T	0*	0 - 0.1	
Effect of erythromycin on B_E	Max effect (h^{-1})	$\varepsilon^{max}_{E,E}$	1.10	-	This study
	Half maximal effective concentration (mg.L^{-1})	$EC50_{E,E}$	3.42	-	
	Hill coefficient	$H_{E,E}$	0.67	-	
Effect of erythromycin on B_T	Max effect (h^{-1})	$\varepsilon^{max}_{E,T}$	3.91	-	
	Half maximal effective concentration (mg.L^{-1})	$EC50_{E,T}$	9.26	-	
	Hill coefficient	$H_{E,T}$	0.22	-	
Effect of	Max effect (h^{-1})	$\varepsilon^{max}_{E,ET}$	0.95	-	

erythromycin on B_{ET}	Half maximal effective concentration ($mg.L^{-1}$)	$EC50_{E,ET}$	2.47	-
	Hill coefficient	$H_{E,ET}$	0.79	-
Effect of tetracycline on B_E	Max effect (h^{-1})	$\varepsilon^{max}_{T,E}$	5.40	-
	Half maximal effective concentration ($mg.L^{-1}$)	$EC50_{T,E}$	81.43	-
	Hill coefficient	$H_{T,E}$	0.30	-
Effect of tetracycline on B_T	Max effect (h^{-1})	$\varepsilon^{max}_{T,T}$	1.66	-
	Half maximal effective concentration ($mg.L^{-1}$)	$EC50_{T,T}$	7.27	-
	Hill coefficient	$H_{T,T}$	2.41	-
Effect of tetracycline on B_{ET}	Max effect (h^{-1})	$\varepsilon^{max}_{T,ET}$	1.58	-
	Half maximal effective concentration ($mg.L^{-1}$)	$EC50_{T,ET}$	4.42	-
	Hill coefficient	$H_{T,ET}$	1.70	-

4.6.2 Model-predicted antibacterial effect of concurrent erythromycin, tetracycline and bacteriophage presence

When simulating the dynamics of two single-resistant *S. aureus* strains in our mathematical model, as expected, the presence of only one antibiotic at 1 mg/L (4 x MIC for susceptible strains) leads to a decrease in the susceptible strain, while the resistant strain does not decrease (Figure 4.3a, top row). On the other hand, the presence of both antibiotics, or of only phage without transduction, causes a decrease in both bacterial strains (Figure 4.3a, top and middle rows). The presence of phage and one antibiotic leads to a decrease in both bacterial strains, with the antibiotic-susceptible strain decreasing faster (Figure 4.3a, middle row). Out of all the conditions shown here, the presence of phage and both antibiotics leads to the fastest decrease in both bacterial strains (Figure 4.3b). For comparison, to replicate the combined effect of 10^9 pfu/mL of phage, 1 mg/L of erythromycin, and 1 mg/L of tetracycline, we would

need 1.94mg/L of erythromycin and 1.08 mg/L of tetracycline in the absence of phage (Supplementary Figure 4.2).

Transduction does not appear to affect the antibacterial activity of antibiotics and phage when phage are either present alone, alongside erythromycin, or alongside tetracycline (Figure 4.3a, bottom row). Double-resistant progeny bacteria (B_{ET}) appear, but only reach a maximum concentration of 30 cfu/mL, and do not remain higher than 1 cfu/mL for more than 8h (Figure 4.3a, bottom row). However, when both erythromycin and tetracycline are present alongside phage capable of transduction, there is a steady increase in the number of B_{ET} throughout 24h, reaching 80 cfu/mL after 24h (Figure 4.3a, bottom row & Figure 4.3b). It is important to note here that when both antibiotics are present, phage numbers do not increase throughout the 24h period, regardless of transduction ability (Figure 4.3a, middle and bottom rows & Figure 4.3b, right).

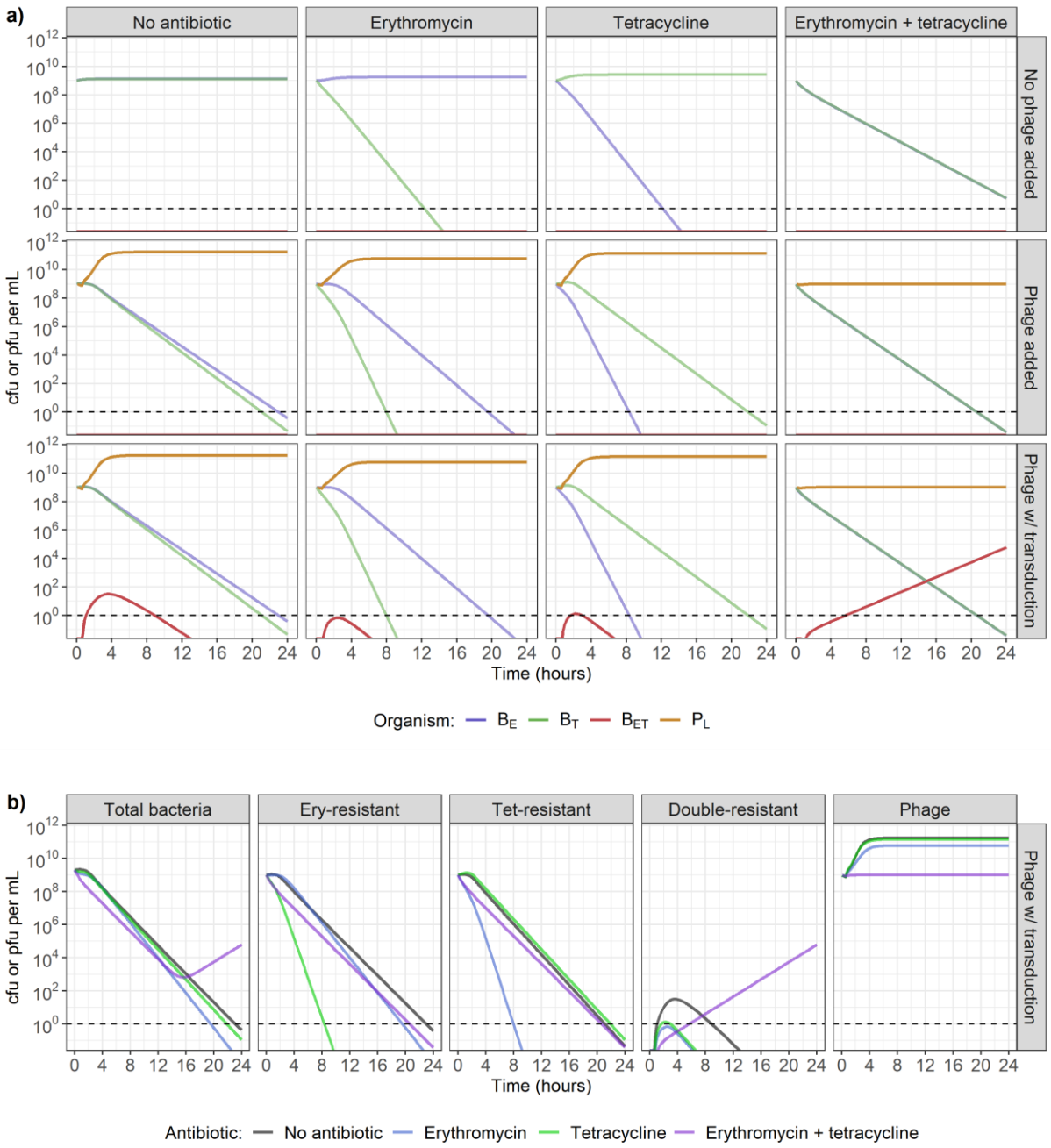


Figure 4.3: a) Model-predicted dynamics with two single-resistant strains starting at carrying capacity (10^9 colony-forming units (cfu)/mL), in the presence of no antibiotics (1st column), erythromycin only (2nd column), tetracycline only (3rd column), or both erythromycin and tetracycline (4th column), combined with either no phage (top row), phage incapable of transduction (middle row), or phage capable of generalised transduction (bottom row). The starting strains are either erythromycin-resistant (B_E) or tetracycline-resistant (B_T). Antibiotics and/or phage (P_L) are present at the start of the simulation, at concentrations of 1 mg/L (4 x MIC for susceptible strains) and 10^9 plaque-forming units (pfu)/mL respectively.

Double-resistant bacteria (B_{ET}) can be initially generated by generalised transduction only, and then by replication of existing B_{ET}. Dashed line indicates the detection threshold of 1 cfu or pfu/mL. **b) Change in bacteria (single-resistant to erythromycin, single-resistant to tetracycline, or double-resistant) and phage numbers depending on the antibiotic exposure, in the presence of phage capable of generalised transduction.** Dashed line indicates the detection threshold of 1 cfu or pfu/mL.

With a reduced starting concentration of either *S. aureus* strain to 10^6 cfu/mL, while the other remains at 10^9 cfu/mL, the conclusions are similar as described above, regardless of which strain is in the minority. Figure 4.4 shows the scenario where tetracycline-resistant bacteria are in minority, and Supplementary Figure 4.3 shows the scenario where erythromycin-resistant bacteria are in minority. When erythromycin, tetracycline, and phage capable of generalised transduction are all present, B_{ET} are still generated, although they do not reach a concentration higher than 1 cfu/mL within 24h, instead of 11h above (Figure 4.4, Figure 4.3).

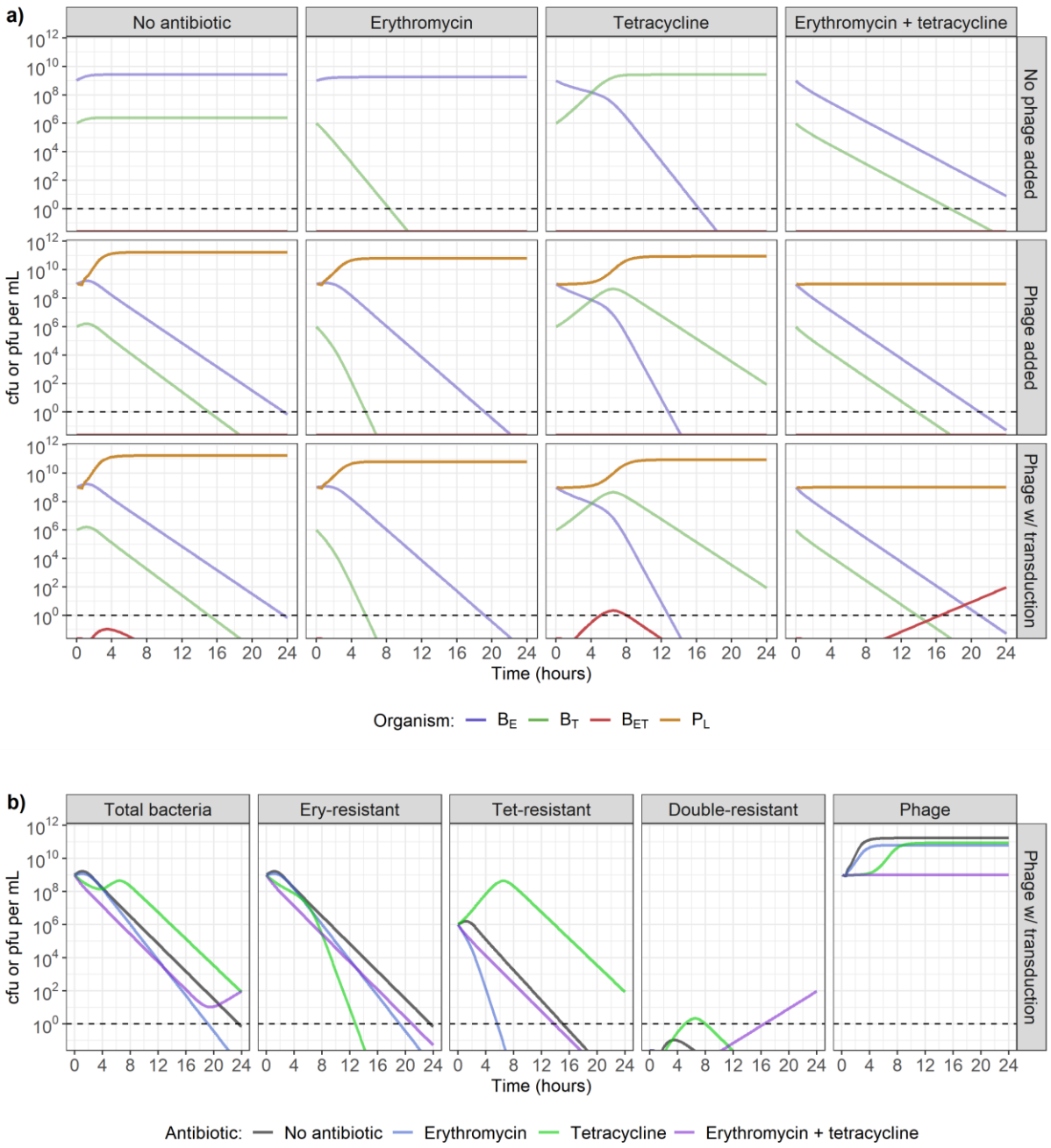


Figure 4.4: a) Model-predicted dynamics with one single-resistant strains starting at carrying capacity (10^9 colony-forming units (cfu)/mL) and the second in minority (10^6 cfu/mL), in the presence of no antibiotics (1st column), erythromycin only (2nd column), tetracycline only (3rd column), or both erythromycin and tetracycline (4th column), combined with either no phage (top row), phage incapable of transduction (middle row), or phage capable of generalised transduction (bottom row). Erythromycin-resistant bacteria (B_E) are initially present at a concentration of 10^9 cfu/mL, and tetracycline-resistant bacteria (B_T) at 10^6 cfu/mL. Antibiotics and/or phage (P_L) are present at the start of the

simulation, at concentrations of 1 mg/L (4 x MIC for susceptible strains) and 10^9 plaque-forming units (pfu)/mL respectively. Double-resistant bacteria (B_{ET}) can initially be generated by generalised transduction only, and then by replication of existing B_{ET}. Dashed line indicates the detection threshold of 1 cfu or pfu/mL. **b) Change in bacteria (single-resistant to erythromycin, single-resistant to tetracycline, or double-resistant) and phage numbers depending on the antibiotic exposure, in the presence of phage capable of generalised transduction.** Dashed line indicates the detection threshold of 1 cfu or pfu/mL.

4.6.3 Effect of variation in antibiotic and phage timing and concentration on bacterial populations

Under conditions where both erythromycin and tetracycline are present, the timing and concentration of antibiotics and phage capable of generalised transduction drives substantial variation in dynamics, with failure to clear all bacteria after 48h being a possibility (Figure 4.5a-b, top row).

With a phage concentration of 10^9 cfu/mL and for any antibiotics concentration between 0.25 and 2 mg/L (Figure 4.5a), the optimal conditions to clear bacteria within 48h are when phage are initially present, and antibiotics are introduced at least 2h later (Figure 4.5a, top row). However, this systematically leads to B_{ET} appearance, with a peak concentration of up to 30 cfu/mL (Figure 4.5a, middle row), and a presence time (hours when cfu/mL > 1) of up to 8h (Figure 4.5a, bottom row). The presence of antibiotics at the same time or shortly before phage leads to failure to clear bacteria within 48h (up to 5.5 x 10^7 cfu/mL remaining after 48h - top row), and substantial B_{ET} appearance (maximum concentration up to 6 x 10^7 cfu/mL - middle row; presence time up to 47h - bottom row). Regardless of timings, the addition of at least 2 mg/L of antibiotics guarantees bacterial clearance within 48h (top row, lightest green line), and no detectable B_{ET} if the antibiotics are added at the same time as or before the phage (bottom row, lightest green line).

When keeping the antibiotic concentrations at 1 mg/L, but varying the phage concentration, the impact of the delay between phage and antibiotic presence on the

bacterial population is strongly dependent on phage concentration (Figure 4.5b). We again see that bacteria are not cleared within 48h if antibiotics are present at the same time as or shortly before a concentration of phage between 10^7 and 10^9 pfu/mL (Figure 4.5b, top row). However, we now note that this also occurs if antibiotics are introduced after a concentration of phage between 10^7 or 10^8 pfu/mL (Figure 4.5b, top row). Regardless of timing, the presence of a phage concentration of 10^{10} pfu/mL guarantees bacterial clearance within 48h (top row, lightest blue line), but leads to B_{ET} if the antibiotics are introduced after the phage (maximum concentration up to 30 cfu/mL - middle row, red line; presence time up to 7h - bottom row, lightest blue line).

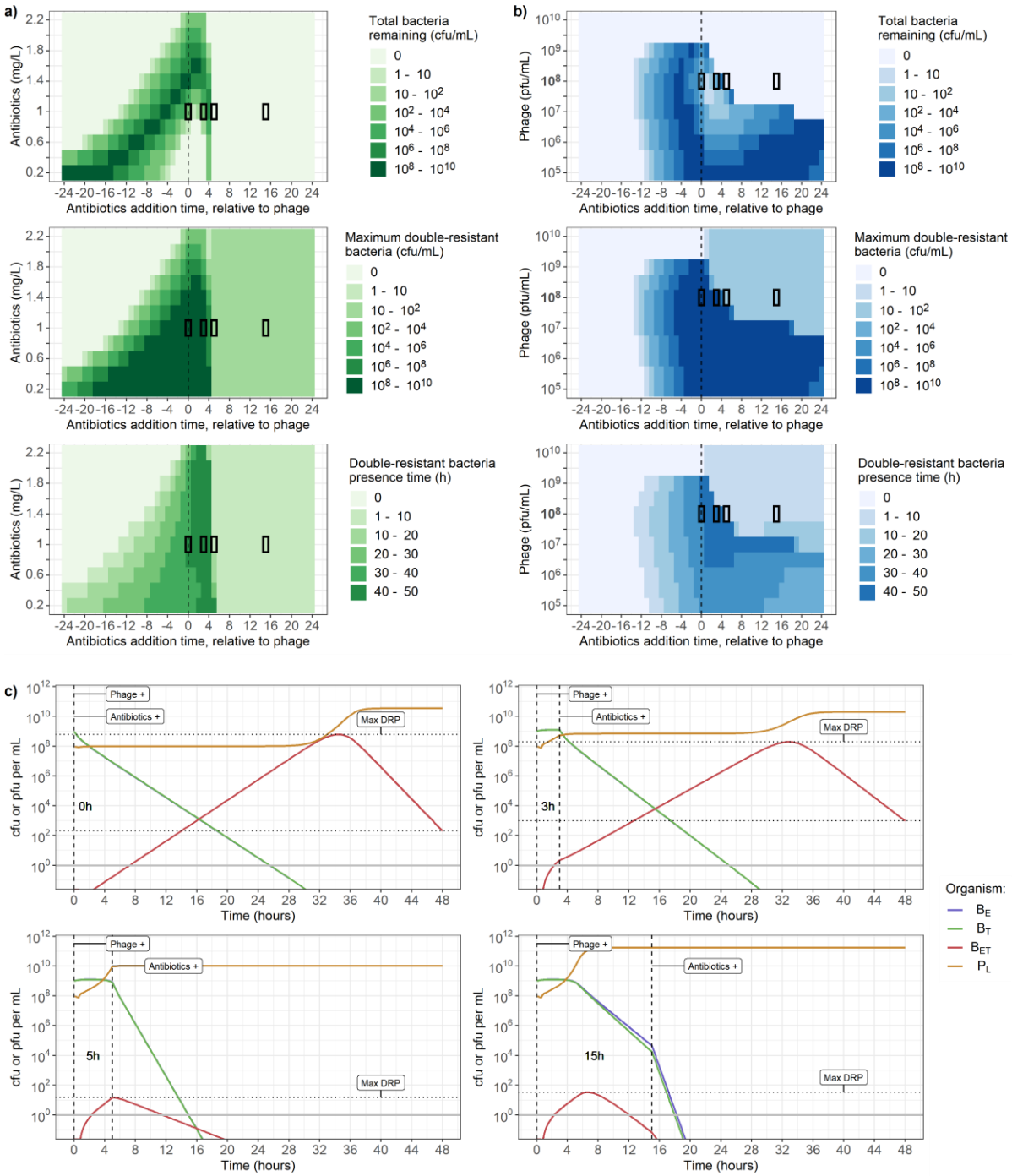


Figure 4.5: a-b) Varying timing (x-axis) and dose of antibiotic and phage (y-axis) affects total bacterial count after 48h (top), maximum concentration of double-resistant bacteria (B_{ET}) (middle), and time when the concentration of B_{ET} is greater than 1 colony-forming unit (cfu) per mL (bottom). a) Adding 10^8 plaque-forming units (pfu) per mL of phage, and between 0.2 and 2.2 mg/L of both erythromycin and tetracycline. **b)** Adding 1 mg/L of both erythromycin and tetracycline, and between 10^5 and 10^{10} pfu/mL of phage. The x-axis indicates the time when antibiotics were added, relative to when phage were added. For example, the value

“4” indicates that phage were present at the start of the simulation, and antibiotics were introduced 4h later. The segments with black borders correspond to the dynamics shown in c). **c) Phage and bacteria dynamics over 48h for 4 conditions taken from panel b.** In all 4 conditions, indicated by the black rectangles, phage are initially present at a concentration of 10^8 pfu/mL, while erythromycin and tetracycline are both introduced at concentrations of 1 mg/L after either 0h, 3h, 5h or 15h, stated on the plots, with the timing indicated by the vertical dashed lines. Horizontal dotted lines indicate bacteria remaining after 48h (corresponding to the top row of a-b) and maximum double-resistant bacteria (B_{ET}) concentration (middle row of a-b). Solid line indicates the detection threshold of 1 cfu or pfu/mL. The concentrations of single-resistant bacteria (B_E, blue, and B_T, green) overlap and cannot be distinguished.

To investigate the dynamics behind these results, we selected four conditions from Figure 4.5b (points indicated by the 4 shapes) with varying antibiotic addition times and plotted the underlying phage and bacteria dynamics over 48h for each (Figure 4.5c). In all four conditions the starting concentrations are 10^8 pfu/mL for phage and 1 mg/L for antibiotics, but antibiotics are introduced either 0h (circle), 3h (square), 5h (star) or 15h (triangle) after phage. These plots show that if phage are increasing, they stop immediately following antibiotic addition (Figure 4.5c, star and square). If antibiotics are added too soon after phage (Figure 4.5c - circle, square), phage do not reach a high enough number to exert a sufficient killing pressure on bacteria. In that case B_{ET}, which are not substantially affected by either antibiotic, replicate faster than they are killed by phage. After more than 35h, the B_{ET} population reaches a sufficiently high number such that the phage population increases, and the resulting pressure is enough to lead to a net negative bacterial growth rate. If antibiotics are added 15h or later after phage (Figure 4.5b, Figure 4.5c - triangle), B_{ET} generation will not change, as during this period they will have already arisen by transduction and been removed by phage predation. The presence of antibiotics 5h after phage is optimal to ensure the lowest maximum number of B_{ET} (Figure 4.5c - star, compare horizontal dotted lines). This timing allows phage to initially increase to a concentration of almost 5 x 10^9, sufficiently high to exert a strong killing pressure on bacteria, while the added effect of antibiotics prevents further B_{ET} generation by decreasing the single-resistant strains.

These results also apply to a scenario where one of the two bacterial strains is in the minority (starting concentration of 10^6 instead of 10^9 cfu/mL), regardless of which strain is in the minority (Supplementary Figures 4.4 & 4.5). However, B_{ET} peak, presence time, and total bacteria remaining after 48h decrease faster with a higher dose of antibiotic or phage, or with a higher delay between phage and antibiotics, suggesting that phage and antibiotics are able to exert a greater killing pressure and generate fewer B_{ET} when one strain is in the minority.

4.6.4 Effect of variation in phage and bacteria parameters on multidrug resistance evolution

Our results above rely on parameters estimated from phage and bacteria interactions *in vitro*, but these may vary depending on the bacteria, phage, and environment. We can explore these different conditions using our model to quantify the dynamics under varying values for parameters governing phage-bacteria interactions (see Table 1 for the ranges used) to determine whether our results would hold.

We first examine the impact of varying the transduction probability on our results (corresponding to the probability that a transducing phage carrying an AMR gene is released instead of a lytic phage during bacterial burst) as this is a vital and yet poorly quantified parameter. When 10^9 cfu/mL of each single-resistant strain are simultaneously exposed to 10^9 pfu/mL of phage and 1 mg/L of erythromycin and tetracycline, varying the transduction probability between 10^{-10} and 10^{-6} leads to a similar log-fold increase in double-resistant bacteria numbers (B_{ET}, Figure 4.6a). Decreasing the probability only delays the appearance of B_{ET} in the model, and does not prevent it. However, a probability lower than 10^{-11} may prevent the appearance of B_{ET} in reality, since the single-resistant strains become almost undetectable (< 1 cfu/mL) before the B_{ET} become detectable. If antibiotics are added more than 10h after phage, B_{ET} will have already started declining due to phage predation, hence the antibiotics only contribute to further increasing the decline in bacterial numbers (Figure 4.5, Figure 4.6b). Under these conditions, a transduction probability lower than 10^{-9} is necessary to prevent B_{ET} from increasing past the detection threshold (1 cfu/mL)

(Figure 4.6b). If antibiotics are introduced 10h before phage, the resulting decline in single-resistant bacteria prevents any B_{ET} from reaching a detectable level before single-resistant bacteria are eradicated (< 1 cfu/mL), even with the highest transduction probability of 10^{-6} (Figure 4.6c).

Looking at how changes in other phage and bacteria parameters may affect our results, partial rank correlation shows that an increase in phage predation either through an increase in phage adsorption rate (β), phage concentration at half saturation (P50) or phage burst size (δ^{max}) correlates with a decrease in maximum B_{ET} detected over 48h (Figure 4.6d, blue). For example, the correlation coefficient of -0.95 between β and maximum B_{ET} implies that a 100% increase in adsorption rate is correlated with a 95% decrease in maximum double-resistant bacteria detected over 48h. An increase in these parameters is also correlated with a decrease in bacteria remaining after 48h (Figure 4.6d, red). An increase in latent period (τ), equivalent to a decrease in predation since phage will take longer before lysing the bacteria, is weakly correlated with an increase in maximum B_{ET}, but is not substantially correlated with bacteria remaining after 48h. Finally, in this partial rank correlation analysis the transduction probability α was not significantly correlated with either bacteria numbers remaining after 48h or maximum B_{ET}, likely since the range investigated was too small (Table 1).

Although we were not able to parameterise our model for antibiotic decay, our 24h time-kill curves suggest that antibiotics no longer decrease bacterial numbers after 24h (Supplementary Figure 4.6). Although decay is only one possible explanation for this effect (see Discussion), we have chosen to include it in our partial rank correlation analysis, as the inclusion of this effect in the model did not require substantial modifications such as additional compartments. Phage may also be affected by decay in the environment, therefore we also included this parameter. An increase in phage or antibiotic decay (γ_P, γ_E, γ_T) is correlated with an increase in maximum B_{ET} (Figure 4.6d, blue), but unexpectedly with a decrease in bacteria remaining after 48h (Figure 4.6d, red). This is explained by the fact that such an increase leads to a weakened killing pressure on B_{ET}, which are able to increase faster. This translates to a shorter time before the phage population is able to increase again due to enough bacteria being available for predation, and thus a shorter time before B_{ET} start decreasing due

to phage killing (Supplementary Figure 4.7). Finally, single-resistant bacterial growth rates (μ^{max}_E, μ^{max}_T) are not substantially correlated with either maximum B_{ET} or remaining bacteria, while double-resistant growth rate (μ^{max}_{ET}) is only weakly positively correlated with maximum B_{ET}, and negatively with remaining bacteria.

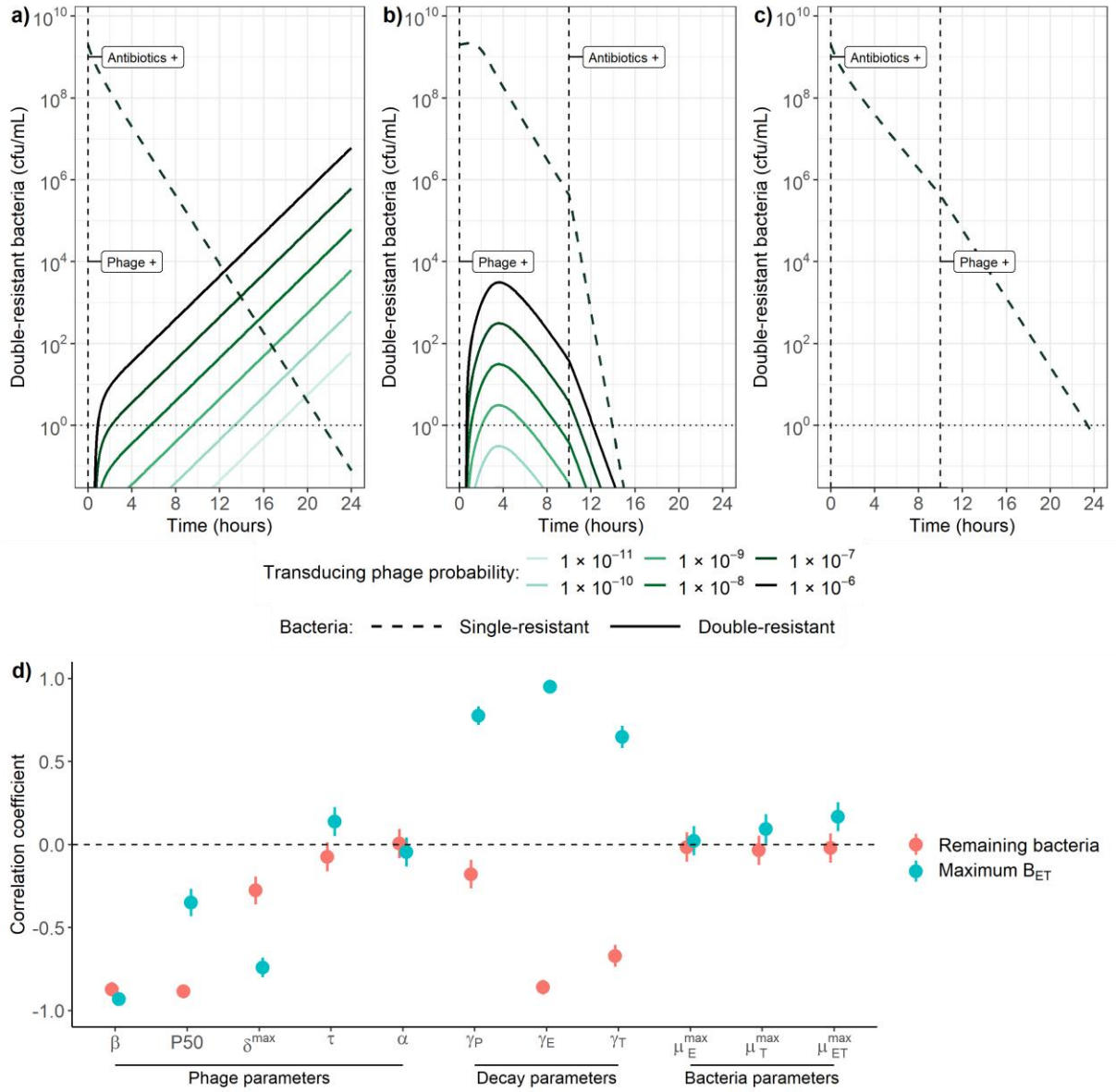


Figure 4.6: Sensitivity of phage-bacteria dynamics to changes in model parameters. Effect of varying the transduction probability between 10^{-11} and 10^{-6} when a) antibiotics and phage are present at the start of the simulation, b) phage are present at the start, antibiotics are introduced 10h later, and c) antibiotics are present at the start, phage are added 10h later. Transduction

probability is defined as the probability that a transducing phage carrying an AMR gene is released instead of a lytic phage during bacterial burst. The dashed lines for single-resistant bacteria overlap and cannot be distinguished. Vertical dashed lines indicate timing of addition of antibiotics or phage. Cfu/mL: colony-forming units per mL. **d) Partial rank correlation between model parameters, and remaining bacteria after 48h (pink) or maximum double-resistant bacteria (B_{ET}) concentration (blue).** Information on the parameter ranges investigated can be found in Table 4.1. β: adsorption rate, P50: phage concentration at half saturation, δ^{max}: burst size, τ: latent period, α: transduction probability, γ_P: phage decay, γ_E: erythromycin decay, γ_T: tetracycline decay, μ^{max}_E: B_E growth rate, μ^{max}_T: B_T growth rate, μ^{max}_{ET}: B_{ET} growth rate.

4.7 Discussion

4.7.1 Summary of results

In this work, we reconcile the existing literature suggesting either that phage and antibiotics can synergistically kill bacteria, or that antibiotics reduce the efficacy of phage predation, whilst also considering the joint effect of phage and antibiotics on antimicrobial resistance (AMR) evolution. We showed that although phage replication is limited in the presence of antibiotics, which negatively affect bacterial growth, phage are still able to exert a strong killing pressure on bacteria to complement the action of antibiotics. Under such conditions, the concentration and timing of antibiotics and phage are essential: phage introduced after antibiotics at a low concentration may not be able to replicate and hence not contribute to killing the bacterial population. Phage and antibiotics can drive AMR evolution when phage generate multidrug-resistant bacteria by transduction, and antibiotics act as a selection pressure. This is again exacerbated by the reduction in phage replication caused by antibiotics: a low concentration of phage capable of transduction introduced after antibiotics will not exert a strong killing pressure on bacteria, and instead generate multidrug-resistant bacteria at a background rate which are then selected for by the antibiotics.

4.7.2 Optimal conditions to clear bacteria and minimise AMR evolution

The best conditions to guarantee bacterial eradication within 48h whilst minimising the risk of multidrug-resistant bacteria evolution in our system are when antibiotics are present before phage, and either when erythromycin and tetracycline are present at a concentration of at least 2 mg/L (8 x MIC for susceptible strains), or phage at a concentration of at least 10^{10} pfu/mL (Figure 4.5a-b). Multidrug-resistant bacteria generation is also restricted if phage are introduced at least 10h after antibiotics (Figure 4.5a-b). This may be particularly relevant in the context of antibacterial treatment, further discussed below. The worst outcome in which antibiotics and phage fail to rapidly clear all bacteria and instead drive AMR evolution is a combination of a low antibiotic dose (< 1 mg/L) with a low phage dose (< 10^9 pfu/ml), introduced around the same time (Figure 4.5a-b). Unfortunately, this may correspond to values seen in natural environments where phage are commonly found and antibiotics are residually present due to pollution [2,3].

4.7.3 Importance of transduction in the environment and during phage therapy

Our results highlight the necessity to better understand the role of transduction in AMR spread and evolution, and not assume by default that it is too rare to be relevant compared to other mechanisms of horizontal gene transfer such as conjugation and transformation. We found that multidrug resistance evolution remained possible even at the lowest probability we considered here of a transducing phage carrying an AMR gene being released instead of a lytic phage during burst (1 transducing phage per 10^{11} lytic phage). Additionally, in this work we assumed that transduction rates are constant, but previous research has shown that sub-MIC antibiotic exposure can lead to an increase in transducing phage, but not lytic ones [45]. Hence, our results may still underestimate the relative impact of transduction versus predation by phage when antibiotics are present. However, our findings are encouraging for conditions under which AMR evolution is limited, since even with a high risk of transduction (1 transducing phage per 10^6 lytic phage), if phage are only introduced to an environment

more than 10h after antibiotics, multidrug-resistant bacteria do not reach detectable levels before single-resistant strains are eradicated (Figure 4.6c).

To the best of our knowledge, our model is the first to consider the potential consequences of transduction in a system where concentrations of bacteria, phage, and antibiotic are similar to those we may see during phage therapy. We echo the conclusions from previous studies which highlighted that the timing of antibiotics and phage introduction during phage therapy can affect the rate at which bacteria are cleared [6–9], but extend these to show that timing may also impact the risk for multidrug-resistant bacteria to be generated. Here, we suggest that, although both sequential treatments can ultimately lead to bacterial eradication, the timing leads to a trade-off between a slower clearing rate of bacteria (if antibiotics are added before phage), and a higher risk of multidrug resistance evolution (if phage are added before antibiotics). Future studies and clinical trials of phage therapy should investigate varying timings of phage and antibiotics, instead of only investigating their simultaneous application, and consider the risk of transduction during treatment.

In any case, our ability to measure the impact of transduction as a driver of AMR evolution *in vivo* is currently limited since individuals are not routinely screened for phage. A first step to measure this despite the limitation may be to investigate evidence for within-patient changes in the resistance profile of *S. aureus* isolates, as these would likely be caused by transduction [18]. In the case of antibiotic treatment, the natural presence of phage capable of transduction may explain instances of treatment failure, if these generate multidrug-resistant strains which are then selected for by the antibiotics. Future studies monitoring therapeutic outcomes of antibacterial treatment in patients where phage are also detected will be essential to better understand how our findings translate to *in vivo* settings.

4.7.4 Limitations

The major limitation of our work is the deterministic nature of our model. While it does not account for stochastic events which would play a large role when bacterial numbers are low, the deterministic model is useful for analysis purposes, as it

represents the average scenario we would observe. In reality, we would likely see either bacterial clearance or unexpected increases at low numbers of bacteria. We only generated model predictions for up to 48h, as our parameter values were obtained using data from experiments over 24h. Beyond this time, bacteria and phage may be affected negatively due to resource depletion, depending on the environment.

Our model does not include some dynamics which may be present *in vivo*, as we do not currently have robust data available to parameterise these features, and would instead have had to rely on assumptions or previously estimated parameter values from different settings. Firstly, bacteria *in vivo* may be present at lower concentrations and display a lower growth rate than in the *in vitro* conditions studied here [40]. The corresponding model parameter values (carrying capacity and maximum growth rate) could be adjusted to better capture these conditions, but this would require further growth data. We have not included the effect of the immune system, which may limit the number of multidrug-resistant bacteria generated as it could suppress both bacteria and phage populations *in vivo* [46,47]. If the model was extended to include the immune system, it would also have to consider potential detrimental effects of large doses of phage and antibiotics, which would restrict these concentrations to prevent side effects *in vivo* [48,49]. In addition, we assume that all the bacteria in our environment are equally susceptible to phage infection, and have not considered the possibility for any further evolution (e.g. adaptation mutation, resistance to phage). This could include the evolution of antibiotic tolerance in bacteria, which could be an alternative explanation to the lack of antibiotic effect we observed after 24h (Supplementary Figure 4.6) instead of antibiotic decay and is currently being investigated in our research group. Such evolution may allow multidrug-resistant bacteria to overcome any fitness cost and persist for longer durations in the environment, hence further highlighting the necessity to minimise the risk of their initial appearance.

Although we varied the concentration of antibiotics in our results, we have consistently added erythromycin and tetracycline in equal amounts. Our model would allow us to change this, yet we have chosen not to for simplicity and because the antibacterial effect curves look similar for these two antibiotics in our setting (Supplementary Figure 4.1). However, for other antibiotics it may be necessary to revisit this assumption and

investigate concentrations which may better reflect those to which bacteria are exposed to in the environment or during antibacterial treatment. In addition, while we observed the effect of two antibiotics with two contrasting mechanisms of resistance in our strains (target modification for erythromycin, and efflux pump for tetracycline), this work should be repeated using antibiotics with different resistance mechanisms. For example, if the mechanism involves active degradation of the antibiotic, this resulting antibiotic decay may affect the dynamics of our system.

4.7.5 Generalisability

Our model is extensively parameterised using data from a single phage and three *S. aureus* strains, making it a robust tool to study the dynamics of these organisms, as it relies on a minimum number of assumptions [23]. However, the parameters we have estimated (adsorption rate, phage concentration at half saturation, burst size, latent period and transduction probability) will likely vary depending on the phage, bacteria, and environment studied. Our sensitivity analysis shows that the model outputs are reasonable with alternative parameter values, predicting for example that phage with a higher predation capacity (higher adsorption rate, phage concentration at half saturation or burst size, or lower latent period) would clear more bacteria within 48h, and reduce the maximum number of multidrug-resistant bacteria generated. This model has been developed as part of an interdisciplinary project alongside *in vitro* experiments, hence it could be easily re-parameterised using data for other strains of bacteria and phage showing similar dynamics of lysis and generalised transduction. The structure of the model is generalisable to other systems of generalised transducing phage and bacteria, as it captures the relevant biological characteristics of phage predation and generalised transduction [23].

4.7.6 The unique dynamics of phage, bacteria, and antibiotics

We suggest that transduction and the effect of antibiotics should be considered in the context of the previously described unique dynamics of phage and bacteria. These imply that phage must first reach a certain concentration (previously referred to as

“inundation threshold”) before they can offset bacterial growth and decrease the bacterial population, and bacteria must first reach a certain concentration (“proliferation threshold”) before the phage population can increase [9]. Generalised transduction and antibiotics affect the size of the bacterial population, and therefore how phage interact with bacteria to clear them and generate multidrug-resistant bacteria. Thus, multidrug-resistant bacteria are able to increase in our model if phage are initially present at a concentration lower than the inundation threshold (Figure 4.5c). This also explains our counterintuitive observation that higher decay rates may lead to less bacteria remaining after 48h (Figure 4.6d), as this would allow bacteria to reach the proliferation threshold sooner, and therefore allow phage to increase up to the inundation threshold sooner (Supplementary Figure 4.7). Importantly, our results similarly suggest that higher decay rates for antibiotics present alongside phage would reduce bacteria remaining after 48h, at the cost of a higher peak concentration of multidrug-resistant bacteria, since this decay would allow bacteria to reach the proliferation threshold sooner (Figure 4.6c, Supplementary Figure 4.7). This knowledge may be further useful in the context of phage therapy, to select the antibiotics that will be given alongside phage [44].

4.7.7 Conclusions

Our results demonstrate the complex interactions between phage and antibiotics to kill bacteria synergistically, and drive the evolution of AMR. We suggest this interaction leads to a trade-off between a slower clearing rate of bacteria (if antibiotics are added before phage), and a higher risk of multidrug resistance evolution (if phage are added before antibiotics), further exacerbated by low concentrations of either phage or antibiotics. Interdisciplinary frameworks such as ours combining *in vitro* data and mathematical models are key to understanding both fundamental AMR evolution, and new interventions like phage therapy or screening for phage in patients. Our conclusions form hypotheses to guide future experimental and clinical work, notably for studies of phage therapy which should consider the risk for multidrug resistance evolution by transduction, and investigate varying timings and concentrations of phage and antibiotics instead of only their simultaneous use.

4.8 References

1. Clokie MRJ, Millard AD, Letarov AV, Heaphy S. Phages in nature. Bacteriophage. 2011;1: 31–45. doi:10.4161/bact.1.1.14942

2. Batinovic S, Wassef F, Knowler SA, Rice DTF, Stanton CR, Rose J, et al. Bacteriophages in Natural and Artificial Environments. Pathogens. 2019;8: 100. doi:10.3390/pathogens8030100

3. Larsson DGJ. Pollution from drug manufacturing: review and perspectives. Philos Trans R Soc B Biol Sci. 2014;369: 20130571. doi:10.1098/rstb.2013.0571

4. Brives C, Pourraz J. Phage therapy as a potential solution in the fight against AMR: obstacles and possible futures. Palgrave Commun. 2020;6: 1–11. doi:10.1057/s41599-020-0478-4

5. Li X, He Y, Wang Z, Wei J, Hu T, Si J, et al. A combination therapy of Phages and Antibiotics: Two is better than one. Int J Biol Sci. 2021;17: 3573–3582. doi:10.7150/ijbs.60551

6. Berryhill BA, Huseby DL, McCall IC, Hughes D, Levin BR. Evaluating the potential efficacy and limitations of a phage for joint antibiotic and phage therapy of Staphylococcus aureus infections. Proc Natl Acad Sci. 2021;118. doi:10.1073/pnas.2008007118

7. Abedon ST. Phage-Antibiotic Combination Treatments: Antagonistic Impacts of Antibiotics on the Pharmacodynamics of Phage Therapy? Antibiotics. 2019;8: 182. doi:10.3390/antibiotics8040182

8. Lopes A, Pereira C, Almeida A. Sequential Combined Effect of Phages and Antibiotics on the Inactivation of Escherichia coli. Microorganisms. 2018;6: 125. doi:10.3390/microorganisms6040125

9. Payne RJH, Jansen VAA. Understanding Bacteriophage Therapy as a Density-dependent Kinetic Process. J Theor Biol. 2001;208: 37–48. doi:10.1006/jtbi.2000.2198

10. von Wintersdorff CJH, Penders J, van Niekerk JM, Mills ND, Majumder S, van Alphen LB, et al. Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer. Front Microbiol. 2016;7. doi:10.3389/fmicb.2016.00173

11. Fernández L, Rodríguez A, García P. Phage or foe: an insight into the impact of viral predation on microbial communities. ISME J. 2018;12: 1171–1179. doi:10.1038/s41396-018-0049-5

12. Balcázar JL. How do bacteriophages promote antibiotic resistance in the environment? Clin Microbiol Infect. 2018;24: 447–449. doi:10.1016/j.cmi.2017.10.010

13. Haaber J, Penadés JR, Ingmer H. Transfer of Antibiotic Resistance in Staphylococcus aureus. Trends Microbiol. 2017;25: 893–905. doi:10.1016/j.tim.2017.05.011

14. Leclerc QJ, Lindsay JA, Knight GM. Mathematical modelling to study the horizontal transfer of antimicrobial resistance genes in bacteria: current state of the field and recommendations. J R Soc Interface. 2019;16: 20190260. doi:10.1098/rsif.2019.0260

15. Verheust C, Pauwels K, Mahillon J, Helinski DR, Herman P. Contained use of Bacteriophages: Risk Assessment and Biosafety Recommendations. Appl Biosaf. 2010;15: 32–44. doi:10.1177/153567601001500106

16. Jassim SAA, Limoges RG. Natural solution to antibiotic resistance: bacteriophages 'The Living Drugs.' World J Microbiol Biotechnol. 2014;30: 2153–2170. doi:10.1007/s11274-014-1655-7

17. Heijer CD den, Bijnen EM van, Paget WJ, Pringle M, Goossens H, Bruggeman CA, et al. Prevalence and resistance of commensal Staphylococcus aureus, including meticillin-resistant S aureus, in nine European countries: a cross-sectional study. Lancet Infect Dis. 2013;13: 409–415. doi:10.1016/S1473-3099(13)70036-7

18. Lindsay JA. Staphylococcus aureus genomics and the impact of horizontal gene transfer. Int J Med Microbiol. 2014;304: 103–109. doi:10.1016/j.ijmm.2013.11.010

19. Stanczak-Mrozek KI, Manne A, Knight GM, Gould K, Witney AA, Lindsay JA. Within-host diversity of MRSA antimicrobial resistances. J Antimicrob Chemother. 2015;70: 2191–2198. doi:10.1093/jac/dkv119

20. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, et al. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. Lancet Infect Dis. 2018;18: 318–327. doi:10.1016/S1473-3099(17)30753-3

21. Rice LB. Federal Funding for the Study of Antimicrobial Resistance in Nosocomial Pathogens: No ESKAPE. J Infect Dis. 2008;197: 1079–1081. doi:10.1086/533452

22. Cassini A, Högberg LD, Plachouras D, Quattrocchi A, Hoxha A, Simonsen GS, et al. Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. Lancet Infect Dis. 2019;19: 56–66. doi:10.1016/S1473-3099(18)30605-4

23. Leclerc QJ, Wildfire J, Gupta A, Lindsay JA, Knight GM. Growth-Dependent Predation and Generalized Transduction of Antimicrobial Resistance by Bacteriophage. mSystems. 2022;0: e00135-22. doi:10.1128/msystems.00135-22

24. McCarthy AJ, Loeffler A, Witney AA, Gould KA, Lloyd DH, Lindsay JA. Extensive Horizontal Gene Transfer during Staphylococcus aureus Co-colonization In Vivo. Genome Biol Evol. 2014;6: 2697–2708. doi:10.1093/gbe/evu214

25. Fish R, Kutter E, Wheat G, Blasdel B, Kutateladze M, Kuhl S. Bacteriophage treatment of intransigent diabetic toe ulcers: a case series. J Wound Care. 2016;25: S27–S33. doi:10.12968/jowc.2016.25.Sup7.S27

26. Lehman SM, Mearns G, Rankin D, Cole RA, Smrekar F, Branston SD, et al. Design and Preclinical Development of a Phage Product for the Treatment of Antibiotic-Resistant Staphylococcus aureus Infections. Viruses. 2019;11: 88. doi:10.3390/v11010088

27. Ngassam-Tchamba C, Duprez JN, Fergestad M, De Visscher A, L'Abee-Lund T, De Vliegher S, et al. In vitro and in vivo assessment of phage therapy against Staphylococcus aureus causing bovine mastitis. J Glob Antimicrob Resist. 2020;22: 762–770. doi:10.1016/j.jgar.2020.06.020

28. Ooi ML, Drilling AJ, Morales S, Fong S, Moraitis S, Macias-Valle L, et al. Safety and Tolerability of Bacteriophage Therapy for Chronic Rhinosinusitis Due to Staphylococcus aureus. JAMA Otolaryngol Neck Surg. 2019;145: 723–729. doi:10.1001/jamaoto.2019.1191

29. Petrovic Fabijan A, Lin RCY, Ho J, Maddocks S, Ben Zakour NL, Iredell JR. Safety of bacteriophage therapy in severe Staphylococcus aureus infection. Nat Microbiol. 2020;5: 465–472. doi:10.1038/s41564-019-0634-z

30. Raj JRM, Karunasagar I. Phages amid antimicrobial resistance. Crit Rev Microbiol. 2019;45: 701–711. doi:10.1080/1040841X.2019.1691973

31. Hassan AY, Lin JT, Ricker N, Anany H. The Age of Phage: Friend or Foe in the New Dawn of Therapeutic and Biocontrol Applications? Pharmaceuticals. 2021;14: 199. doi:10.3390/ph14030199

32. Rodríguez-Rubio L, Jofre J, Muniesa M. Is Genetic Mobilization Considered When Using Bacteriophages in Antimicrobial Therapy? Antibiotics. 2017;6: 32. doi:10.3390/antibiotics6040032

33. Fey PD, Endres JL, Yajjala VK, Widhelm TJ, Boissy RJ, Bose JL, et al. A Genetic Resource for Rapid and Comprehensive Phenotype Screening of Nonessential Staphylococcus aureus Genes. mBio. 2013;4. doi:10.1128/mBio.00537-12

34. Mouton JW, Vinks AA. Pharmacokinetic/Pharmacodynamic Modelling of Antibacterials In Vitro and In Vivo Using Bacterial Growth and Kill Kinetics. Clin Pharmacokinet. 2005;44: 201–210. doi:10.2165/00003088-200544020-00005

35. Wayne P, Clinical and Laboratory Standards Institute. CSLI document M07-A9. 9th ed. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard. 9th ed. CSLI; 2012.

36. R Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2020. Available: https://www.R-project.org/

37. Regoes RR, Wiuff C, Zappala RM, Garner KN, Baquero F, Levin BR. Pharmacodynamic Functions: a Multiparameter Approach to the Design of Antibiotic Treatment Regimens. Antimicrob Agents Chemother. 2004;48: 3670–3676. doi:10.1128/AAC.48.10.3670-3676.2004

38. Weld RJ, Butts C, Heinemann JA. Models of phage growth and their applicability to phage therapy. J Theor Biol. 2004;227: 1–11. Available: https://www.sciencedirect.com/science/article/pii/S0022519303002625?via%3Dihub

39. Schrag SJ, Mittler JE. Host-Parasite Coexistence: The Role of Spatial Refuges in Stabilizing Bacteria-Phage Interactions. Am Nat. 1996;148: 348–377. doi:10.1086/285929

40. Gefen O, Fridman O, Ronin I, Balaban NQ. Direct observation of single stationary-phase bacteria reveals a surprisingly long period of constant protein production activity. Proc Natl Acad Sci. 2014;111: 556–561. doi:10.1073/pnas.1314114111

41. Krasniqi S, Matzneller P, Kinzig M, Sörgel F, Hüttner S, Lackner E, et al. Blood, Tissue, and Intracellular Concentrations of Erythromycin and Its Metabolite Anhydroerythromycin during and after Therapy. Antimicrob Agents Chemother. 2012;56: 1059–1064. doi:10.1128/AAC.05490-11

42. Somogyi AA, Bochner F, Hetzel D, Williams DB. Evaluation of the Intestinal Absorption of Erythromycin in Man: Absolute Bioavailability and Comparison with Enteric Coated Erythromycin. Pharm Res. 1995;12: 149–154. doi:10.1023/A:1016215510223

43. Heldal M, Bratbak G. Production and decay of viruses in aquatic environments. Mar Ecol Prog Ser. 1991;72: 205–212. Available: https://www.jstor.org/stable/24825594

44. Levin BR, Udekwu KI. Population Dynamics of Antibiotic Treatment: a Mathematical Model and Hypotheses for Time-Kill and Continuous-Culture Experiments. Antimicrob Agents Chemother. 2010;54: 3414–3426. doi:10.1128/AAC.00381-10

45. Stanczak-Mrozek KI, Laing KG, Lindsay JA. Resistance gene transfer: induction of transducing phage by sub-inhibitory concentrations of antimicrobials is not correlated to induction of lytic phage. J Antimicrob Chemother. 2017;72: 1624–1631. doi:10.1093/jac/dkx056

46. Banuelos S, Gulbudak H, Horn MA, Huang Q, Nandi A, Ryu H, et al. Investigating the Impact of Combination Phage and Antibiotic Therapy: A Modeling Study. In: Segal R, Shtylla B, Sindi S, editors. Using Mathematics to Understand Biological Complexity: From Cells to Populations. Cham: Springer International Publishing; 2021. pp. 111–134. doi:10.1007/978-3-030-57129-0_6

47. Rodriguez-Gonzalez RA, Leung CY, Chan BK, Turner PE, Weitz JS. Quantitative Models of Phage-Antibiotic Combination Therapy. mSystems. 2020;5: e00756-19. doi:10.1128/mSystems.00756-19

48. Liu D, Van Belleghem JD, de Vries CR, Burgener E, Chen Q, Manasherob R, et al. The Safety and Toxicity of Phage Therapy: A Review of Animal and Clinical Studies. Viruses. 2021;13: 1268. doi:10.3390/v13071268

49. Imani S, Buscher H, Marriott D, Gentili S, Sandaradura I. Too much of a good thing: a retrospective study of β-lactam concentration–toxicity relationships. J Antimicrob Chemother. 2017;72: 2891–2897. doi:10.1093/jac/dkx209

5 Examining the potential of routinely collected hospital data to reveal within-host diversity and dynamics of antimicrobial resistance in *Staphylococcus aureus*

5.1 Overview

Through the work conducted in the previous Chapters, I explored the dynamics of phage predation and generalised transduction of AMR in *S. aureus*. Although this was informed by *in vitro* data, I began to explore the potential *in vivo* implications of these dynamics by simulating conditions we might expect to see in within-host bacterial populations, with phage, antibiotics and bacteria jointly present. My next objective was to find evidence that transduction shapes *in vivo* AMR evolution in *S. aureus*, which I aimed to do by obtaining data on within-host *S. aureus* AMR diversity, and how this diversity changes over time.

Previous experiments in gnotobiotic piglets showed that phage can generate substantial within-host *S. aureus* diversity via transduction, but these experiments cannot be conducted in humans for ethical reasons. Instead, studies which attempted to quantify within-host *S. aureus* diversity in humans have relied on detailed genomic data, sampled at one time-point from a relatively small number of individuals (10-1000), and focused mostly on MRSA. Although these studies identified frequent genotypic diversity in *S. aureus* within-host populations, there is currently a knowledge gap regarding how often this diversity may occur in larger populations, and how this diversity may change over time, with bacteria gaining and losing resistance genes. Routinely collected data is a major source of epidemiological information, allowing the observation of AMR trends over large scales. However, this type of data has never been used previously to examine within-host *S. aureus* AMR diversity. Here, I hypothesised that due to the frequent occurrence of genotypic diversity in *S. aureus* populations, I could detect this diversity using routinely collected phenotypic data from a hospital diagnostics laboratory, by comparing isolates from multiple samples of the

same patients. Frequent changes in diversity may be partly driven by movement of genes via transduction, further highlighting the importance of this mechanism to drive AMR evolution *in vivo*.

In this Chapter, I used pseudonymised routinely collected patient data obtained from Great Ormond Street Hospital in London, UK. By analysing the antibiograms in this large dataset, which includes more than 20,000 patients and 70,000 *S. aureus* isolates collected over 20 years, I identified evidence of within-host AMR phenotypic diversity and changes in this diversity which may be caused by horizontal gene transfer. I also examined *S. aureus* AMR trends at the hospital level, highlighting variability over time, with implications for epidemiological analyses of AMR which may be using this type of routinely collected data.

This work represents an ongoing collaboration with clinicians from Great Ormond Street Hospital, and is not yet published or publicly available as a preprint. This Chapter is presented in a research paper style for consistent formatting across the thesis. We obtained a Collaboration Agreement between LSHTM and GOSH to download the data from GOSH on 10/03/2022. We have discussed preliminary analysis in April and May 2022 with the following collaborators at GOSH: Dr Louis Grandjean (consultant in paediatric infectious diseases), Dr Helen Dunn (consultant nurse infection prevention & control, and director of infection prevention & control), and Dr James Hatcher (consultant in microbiology and virology).

5.2 Abstract

Staphylococcus aureus populations in individuals can be diverse, composed of several subpopulations of bacteria carrying different antimicrobial resistance (AMR) genes. The structure of these populations may be flexible, with the prevalence of these resistances changing over time. Understanding this within-host diversity of AMR and how it changes over time is essential as this will affect the prevalence of infections by antibiotic-resistant bacteria, particularly as individuals are more likely to be infected by the strains of *S. aureus* they are colonised with. Previous studies examining within-host diversity have mostly sampled relatively small groups of patients at one time point only, separating isolates based on the resistance genes they carry, which may not always translate to clinically-relevant differences in phenotypic resistances. As these previous studies frequently identified AMR diversity in *S. aureus*, we hypothesised that we could also detect such diversity using exclusively phenotypic data. Here, we used antibiograms from pseudonymised data routinely collected from more than 20,000 patients and 70,000 isolates over 20 years at Great Ormond Street Hospital (GOSH, London, UK) to estimate the AMR phenotypic diversity in within-host *S. aureus* populations, and how this changes over time. We also aimed to use data on hospital admission and antibiotic exposure to suggest explanations for changes in diversity, including hospital practices, antibiotic selection pressures, between-host transmission, and within-host horizontal gene transfer.

Although the incidence of *S. aureus* isolates at GOSH remained constant over time, the proportion of isolates resistant to different antibiotics varied, with methicillin-resistant (MRSA) isolates consistently carrying more resistances than methicillin-susceptible (MSSA) isolates. 45.61% of all patients had more than one isolate recorded in the dataset. 2.00% of all patients in the dataset had at some point both MSSA and MRSA isolates detected on the same day, and 4.28% of all patients carried at some point multiple MSSA or MRSA isolates with different antibiograms on the same day (i.e. diverse populations). Although MRSA within-host populations were more likely to be diverse than MSSA, when this occurred the estimated phenotypic diversity was similar between MRSA and MSSA populations (2-3 unique antibiograms detected, 1-3 differences between antibiograms). This detected within-host diversity changed over time, with changes from detection of MSSA only to MRSA (0.35% of all

patients), and changes in the antibiograms reported (2.32% of all patients). These changes were equally likely to represent increases in resistance or susceptibility to antibiotics, and could not be fully explained by concurrent antibiotic consumption or between-patient transmission, suggesting instead that horizontal gene transfer may play an important role in shaping within-host AMR evolution.

S. aureus AMR phenotypic diversity within-host can be detected using antibiograms from routinely collected hospital data. This detected diversity changes over time, which may impact the success of infection treatment. Sampling strategies in diagnostic labs are not currently designed to fully capture this diversity, limiting our ability to use historical routine surveillance data for this purpose, and hence to broadly study within-host AMR evolution. Future work with longitudinal sampling of patients should be conducted to understand the relative importance of antibiotic use, between-patient transmission, and horizontal gene transfer to drive within-host evolution of AMR, and identify opportunities to reduce AMR prevalence.

5.3 Introduction

Antimicrobial resistance (AMR) represents a major public health threat, complicating the treatment of infections and leading to severe long term health consequences for patients [1]. A population of a single species of bacteria in a single environment (e.g. in a human host) can be composed of diverse subpopulations, carrying different antibiotic resistance genes [2]. Understanding this within-host bacterial diversity is essential, both during bacterial colonisation and infection, as it will shape the prevalence of AMR [3,4]. AMR diversity is of particular interest in *Staphylococcus aureus*, which are both commensal bacteria colonising the nose of 20% of the human population [5], and major nosocomial pathogens responsible for skin and blood infections [6]. *S. aureus* carry many antibiotic resistance genes on mobile genetic elements (MGEs) [7]. The presence of these MGEs is highly variable, leading to substantial diversity in *S. aureus* populations [8–12]. We currently have a limited understanding of how common *S. aureus* AMR within-host diversity is, how it changes over time, and how this may impact the treatment of infections, as previous studies

have mostly focused on genotypic diversity, quantified in a limited number of individuals at a single time point.

When talking about *S. aureus* diversity, it's first important to distinguish between methicillin-susceptible and -resistant *S. aureus* (MSSA and MRSA). While MSSA isolates tend to only be resistant to penicillin, MRSA carry an SCC*mec* cassette which grants them broad resistance to all beta-lactam antibiotics [13]. Current evidence suggests that SCC*mec* movement is rare in *S. aureus* isolates [14–16]. Hence, detection of MRSA in a patient is generally attributed to acquisition of MRSA from an external source, as opposed to gain of SCC*mec* by MSSA already present in the patient. This also applies to MSSA, which is generally attributed to acquisition from an external source, as opposed to loss of the SCC*mec* element in MRSA already present. In addition to SCC*mec*, MRSA isolates generally carry more resistance genes than MSSA, which leads us to expect more unique combinations of resistances in MRSA than MSSA subpopulations [17]. The risk of MRSA differs between groups of individuals, with MRSA incidence found to be higher in non-white patients [18]. Other risk factors for MRSA infection include previous antibiotic use, admission to an intensive care unit, and prolonged hospitalisation [19].

Previous studies found that within-host *S. aureus* diversity is common, both in terms of dual MSSA-MRSA carriage and variations in the unique combination of antibiotic resistances and susceptibilities displayed by bacteria [8–12]. This included estimates of 21% of patients carrying both MRSA and MSSA at the same time, and between 6.6% to 30% of patient carrying multiple subpopulations. However, these studies mostly focused on diversity within MRSA populations only, and sampled a limited number of individuals (10-1000), at one time point only. In addition, they relied on the presence or absence of resistance genes (genotypic data) to quantify diversity. While this captures the full spectrum of diversity accurately, the presence of a resistance gene may not always correlate with actual resistance displayed by bacteria (phenotypic resistance), as seen in multiple bacteria including *S. aureus* [20,21]. Measured diversity in phenotypic resistances is likely to be lower than genotypic, as bacteria of the same species displaying resistance to the same antibiotics will be considered identical, even if the resistance genes they carry are different. However, phenotypic resistance is more relevant clinically, as this corresponds to what is

generally measured in the context of bacterial infections, and what matters to determine treatment success.

In hospitals, samples are routinely collected from patients for various monitoring and clinical purposes, and analysed in diagnostic laboratories to identify any bacteria present. Antibiograms summarising the phenotypic resistances of these bacteria may also be generated, to determine which antibiotic should be used to treat an infection caused by these bacteria. This type of routine surveillance is the main source for epidemiological data, as the alternative to conduct new trials to collect specific data is more expensive, time-consuming, and does not allow for retrospective analysis of past trends. Routinely collected data is extremely useful for analysis of resistance trends over time, both retrospectively and for forecasting purposes, to estimate the impact of interventions against AMR [22], and to identify factors contributing to the spread of AMR [23]. If routinely collected data could be used to detect AMR phenotypic diversity, this would substantially improve our ability to monitor this diversity at a local scale, understand its potential causes and implications, and tailor local interventions to address this rapidly changing problem. To the best of our knowledge however, the feasibility of this analysis using data already available is currently unknown.

In this study, we search for evidence of phenotypic AMR diversity in within-host *S. aureus* populations using 20 years of routinely collected pseudonymised data from Great Ormond Street Hospital (GOSH) in London, UK. This hospital specialises in paediatric care, receiving between 30,000 and 40,000 inpatients per year, and has more than 383 beds spread out across 39 wards (including 44 beds in three intensive care units) [24,25]. GOSH possesses a unique processing system for their routinely collected data, managed by the Digital Research, Informatics and Virtual Environments unit (DRIVE). This system enables easy access to a wide range of pseudonymised data on patients, notably dates of admittance to the hospital, wards occupied, antibiotics prescribed, isolates detected from samples, and the antibiotic resistances and susceptibilities of these isolates. As multiple samples are often routinely collected repeatedly for patients, and antibiograms generated for several isolates detected, this data could allow us to see potential changes in the phenotypic resistances detected in within-host *S. aureus* populations.

However, appropriate usage of this routine data requires an understanding of how it was collected, and how this might have changed over time. For example, if a dataset only contains information on isolates collected from severely ill patients, this might bias the data towards reporting higher rates of resistance than reality [26]. Therefore, it's first necessary to examine trends in antibiotic resistance at the hospital level over time, and any changes in policy or testing which occurred and might have impacted these trends. In addition, it's important to bear in mind that this routinely collected data was not collected for the purpose we are interested in here. From a clinical perspective, the aim of analysing bacteria in patient samples is to detect any resistances present, not necessarily to identify all the different subpopulations. At GOSH, patient samples are generally plated on selective agar to identify different bacterial species, then a small (typically less than 3) number of colonies on these plates are collected, subcultured, and tested for resistance to antibiotics via disk diffusion. Guidelines only state that representative colonies should be subcultured, therefore multiple colonies are separately subcultured only when they are visually different on the plate (e.g. different sizes or colours) [27]. We therefore expect that our estimates of diversity using this routinely collected data will be lower than those in previous studies which collected data specifically for this purpose.

Although our primary objective is to determine whether we can detect diversity and changes in this diversity over time in patients using this routinely collected dataset, our secondary objective is to explore potential explanations for these changes which we may be able to identify using this same data. We expect that antibiotic consumption in patients will drive many of the changes in detected diversity, as a subpopulation of resistant bacteria may only be initially present in minority in a patient and not detected, but may increase and become dominant following antibiotic exposure [28]. As information on antibiotic usage is included in our routinely collected dataset, we expect to see events where resistance is only detected following antibiotic exposure. Nosocomial transmission between patients may explain other changes in detected diversity, if a patient acquires a new subpopulation of bacteria from another patient present in the same ward, at the same time. However, this is known to occur only rarely with *S. aureus*, as most patients testing positive for MRSA in hospitals acquired the bacteria before hospitalisation, as opposed to during hospitalisation [29–31]. We

may still be able to detect such rare events using our dataset, by comparing *S. aureus* isolates collected from patients who stayed in the same ward at the same time.

An important possible explanation for any changes in diversity we may detect is frequent horizontal gene transfer between bacteria, since many AMR genes in *S. aureus* are carried on MGEs [7]. Transduction, mediated by bacteriophage, is likely the dominant mechanism of gene transfer in *S. aureus*, and can consistently lead to multidrug resistance *in vitro*, even in the absence of antibiotics to act as a selection pressure [9,32–34]. Previous work to estimate rates of gene movement *in vivo* in gnotobiotic piglets found that *S. aureus* strains frequently gained MGEs via transduction, but also lost them equally rapidly, leading to changes in detected diversity in just a few hours [35]. Note that a detected loss of resistance can either happen as an MGE carrying this resistance gene is randomly segregated during bacterial replication and lost in some daughter cells [36], or if the subpopulation carrying the resistance gene decreases and no longer becomes detectable, either due to stochastic events or a fitness cost resulting from this resistance [37]. To the best of our knowledge, this shuffling of genes over short periods of time in *S. aureus* has never been measured in humans, yet is likely to happen frequently since approximately 20% of individuals are asymptomatically colonised by *S. aureus* at any given time, and phage capable of transduction are present in at least 50% of these individuals [5,9]. In addition to resistance genes moving, some resistances in *S. aureus* arise via mutations, such as ciprofloxacin or rifampicin resistance [38]. Overall, these genetic events may change the prevalence of different subpopulations over time, leading to changes in detected diversity. In that case, we would expect to see many changes in resistances known to be carried on MGEs, known to arise via mutations, or known to impose a fitness cost on the bacteria.

In this work, we aim to explore the potential of routinely collected data to observe *S. aureus* within-host diversity over a large scale, with more than 20,000 patients and 70,000 isolates included in our analysis. At the hospital level, we hypothesise that there is substantial variation over time in the proportion of isolates resistant to various antibiotics. This variation may be linked to changes in testing strategy, with important implications regarding analyses using this type of routinely collected data to derive epidemiologically-meaningful trends in AMR. Despite of the challenges highlighted

above leading us to expect that phenotypic resistances diversity will be rarer than genotypic, we hypothesise that we can still detect diversity and changes in this diversity over time relying exclusively on this routinely collected data, since substantial genotypic diversity has previously been reported in *S. aureus*. Finally, we aim to explore possible explanations for this diversity, again using exclusively this routinely collected dataset.

5.4 Methods

All the analyses and data processing were conducted in the R software [39]. The raw datasets are the property of GOSH and cannot be shared publicly. The processed datasets and the code to run the analyses presented in this paper are publicly available in a GitHub repository (https://github.com/gleclerc/gosh_mrsa).

5.4.1 Ethics approval

Ethics approval for this study was obtained both from GOSH (under ethical approval 17/LO/0008 for use of routine GOSH data for research), and the London School of Hygiene & Tropical Medicine (reference 26692).

5.4.2 Data processing

We accessed pseudonymised routinely collected data from all patients at GOSH who tested positive for *S. aureus* infection or colonisation at any point between 01/02/2000 and 30/11/2021, as determined by the GOSH diagnostic laboratory. Each isolate was assigned a unique identification number. We included all isolates labelled "Staphylococcus aureus" or "Methicillin-Resistant Staphylococcus aureus". We excluded isolates labelled "Staphylococcus sp." (41 isolates), as this may have included *Staphylococcus* species other than *S. aureus.* For each isolate, we had access to information on the date that the sample was collected, the sample source (wound, urine etc.), and antibiogram data. The antibiogram data listed isolates as resistant to an antibiotic with the label "R", or susceptible with the letter "S".

Although some isolates were already labelled as “Methicillin-Resistant Staphylococcus aureus” (MRSA), we manually applied this label to any isolate resistant to either cefoxitin, oxacillin, or flucloxacillin [40]. All other isolates were assumed to be methicillin-susceptible (MSSA), including 10,031 isolates with no susceptibility information recorded for any of these three antibiotics.

We then obtained routinely collected data for patients from whom these *S. aureus* isolates had been sampled. The resulting dataset included both in- and outpatients, and each patient was assigned a unique identification number. The routinely collected data included information on ethnicity for each patient (16 unique ethnicities). We regrouped these ethnicities in three categories: “White British” (which only included the “White British” ethnicity), “Other groups” (all other ethnicities recorded), and “None reported” (patients with no recorded ethnicity, labelled “Prefer Not To Say” or blank, 3,932 patients).

The routinely collected data also included information on dates when patients visited the hospital, and information on any hospital admission (for each ward occupied by the patient: start date, end date, ward name). Note that a single hospitalisation period was defined as a stay in a single ward. If a patient moved from one ward to another on the same day, this was counted as a new hospitalisation in the data.

Finally, the dataset included information on any antibiotics received by patients (date and time when each antibiotic was received and name of antibiotic). We further extended this information by matching the antibiotics to their class (e.g. methicillin was matched to the “penicillin” class) using information available online [41] (see Supplementary Table 5.1 for the matching information).

5.4.3 Statistical analyses

Linear regression was used to test if the number of isolates per patient followed a consistent trend over time (years) and significantly differed depending on the type of isolate (MRSA or MSSA). Linear regression was used to test if the number of antibiotic resistances detected in isolates increased linearly with the number of susceptibility

tests conducted for those isolates. Linear regression was used to test if the number of susceptibility tests conducted and resistances detected per isolate at the hospital level followed a consistent trend over time (years) and significantly differed depending on the type of isolate (MRSA or MSSA).

Spearman's correlation was used to test the correlation between the proportion of isolates resistant to different antibiotics over time. The association between colonising or non-colonising isolates and absence of any susceptibility test conducted was estimated using a Chi-square test. For this analysis, we considered that colonising isolates were only those originating from nose or throat swabs, as these are the sites most likely to correspond to colonisations rather than infections [42]. The association between antibiotic exposure and changes in diversity was estimated using a Chi-square test.

5.4.4 Within-patient diversity

5.4.4.1 MRSA and MSSA diversity

For each patient, we chronologically ordered the isolates collected, and identified instances where the type of isolate changed from MSSA to MRSA or vice versa. We then considered the recorded date when each isolate was collected. If a patient had at least one MRSA and one MSSA isolate detected on the same day, we considered that this indicated within-host methicillin resistance diversity in their *S. aureus* population.

We then focused on patients for whom MSSA and MRSA isolates were detected on different days, and notably on events where an MSSA isolate was initially identified, followed later by an MRSA isolate. We further filtered these events, keeping only those that occurred within a single hospitalisation period. These were identified if both the dates of the initial MSSA isolate and the follow-up MRSA isolate were within the time interval of a single hospitalisation period for the corresponding patient, defined by the recorded start and end dates of that hospitalisation period in the data, and if the MRSA

isolate was detected more than 2 days after the MSSA, a commonly used threshold to distinguish from already present MRSA versus acquisition [43].

In the events found to occur in a single hospitalisation period, potential nosocomial transmission of MRSA was identified by considering the ward in which the patient was located when the MRSA-positive swab was collected, and checking if any positive MRSA swab had been reported for any other patient present in that same ward at the same time as the patient, in the interval between the previous MSSA-positive only swab collected for the patient and the MRSA-positive swab.

Finally, to identify changes within a single hospitalisation period which may have been induced by antibiotic selection, we noted if patients had been exposed to any antibiotic in the interval between the MSSA-positive and MRSA-positive swab.

5.4.4.2 Detected phenotypic resistances diversity

We then searched for within-host diversity in detected phenotypic resistances. For this, we compared MRSA and MSSA isolates separately. We removed 10,029 isolates which did not have any antibiotic susceptibility test result recorded. Antibiograms of chronologically subsequent isolates within patients were compared, and we noted a difference if the antibiograms of these isolates differed by at least one susceptibility reported (i.e. the value for at least one antibiotic changed from susceptible to resistant, or vice-versa). Note that we do not consider that a change in an antibiotic susceptibility occurred if only one of the isolates had a recorded result for that antibiotic, and the other had not been tested for that antibiotic.

The same filtering methodology was used as described above to identify differences in antibiograms which occurred on the same day, on different days, and within a single hospitalisation period.

To identify changes within single hospitalisation periods which may have been induced by antibiotic selection, we noted if i) the patient was exposed to any antibiotic in the interval between two differing antibiograms, and ii) if the patient was exposed to an antibiotic of the same class as that of the change in resistance.

5.5 Results

5.5.1 Antibiotic resistance trends in *S. aureus* isolates

5.5.1.1 Incidence of MSSA and MRSA isolates

Altogether, we obtained information on 72,207 unique *S. aureus* isolates (51,020 MSSA, 21,187 MRSA) from 22,206 unique patients at Great Ormond Street Hospital (GOSH) between 01/02/2000 and 30/11/2021. Of these patients, 18,700 (84.21%) only ever tested positive for MSSA, 2,429 (10.94%) only for MRSA, and 1,077 (4.85%) tested positive for both MSSA and MRSA (although not necessarily at the same time). The isolates came from a range of sources (nose, wound, blood etc.), and therefore represented both colonising and infecting isolates.

Although the total number of *S. aureus* isolates did not increase or decrease over time (Figure 5.1a), we note a progressive increase in the proportion of MRSA isolates over time, with a peak in January 2018 (50% of *S. aureus* isolates, Figure 5.1b). The number of *S. aureus* isolates decreased sharply in April 2020, aligned with the first UK lockdown during the COVID-19 pandemic (Figure 5.1a). Since then, the total number of *S. aureus* isolates has increased back to pre-2020 levels, and the proportion of MRSA isolates has stabilised around 30% (Figure 5.1a-b).

These values are not directly equivalent to the number and proportion of individual patients testing positive for MRSA and/or MSSA, since multiple isolates are frequently recorded per patient (Figure 5.1c). More MRSA isolates were always obtained per patient than MSSA (regression coefficient = 1.91, p value < 0.001), and this sampling frequency varied negligibly over time (coeff. = -0.00009, p < 0.001) (Figure 5.1c), hence this does not explain variations in the number of MRSA and MSSA isolates seen at the hospital level. To identify a possible explanation, following recommendations from GOSH consultants and previous work which found that MRSA incidence was higher in non-white patients [18], we observed patient ethnicities over time and found that the increase in proportion of MRSA isolates aligned with an increase in the proportion of patients with an ethnicity other than “White British” admitted to GOSH (Supplementary Figure 5.1).

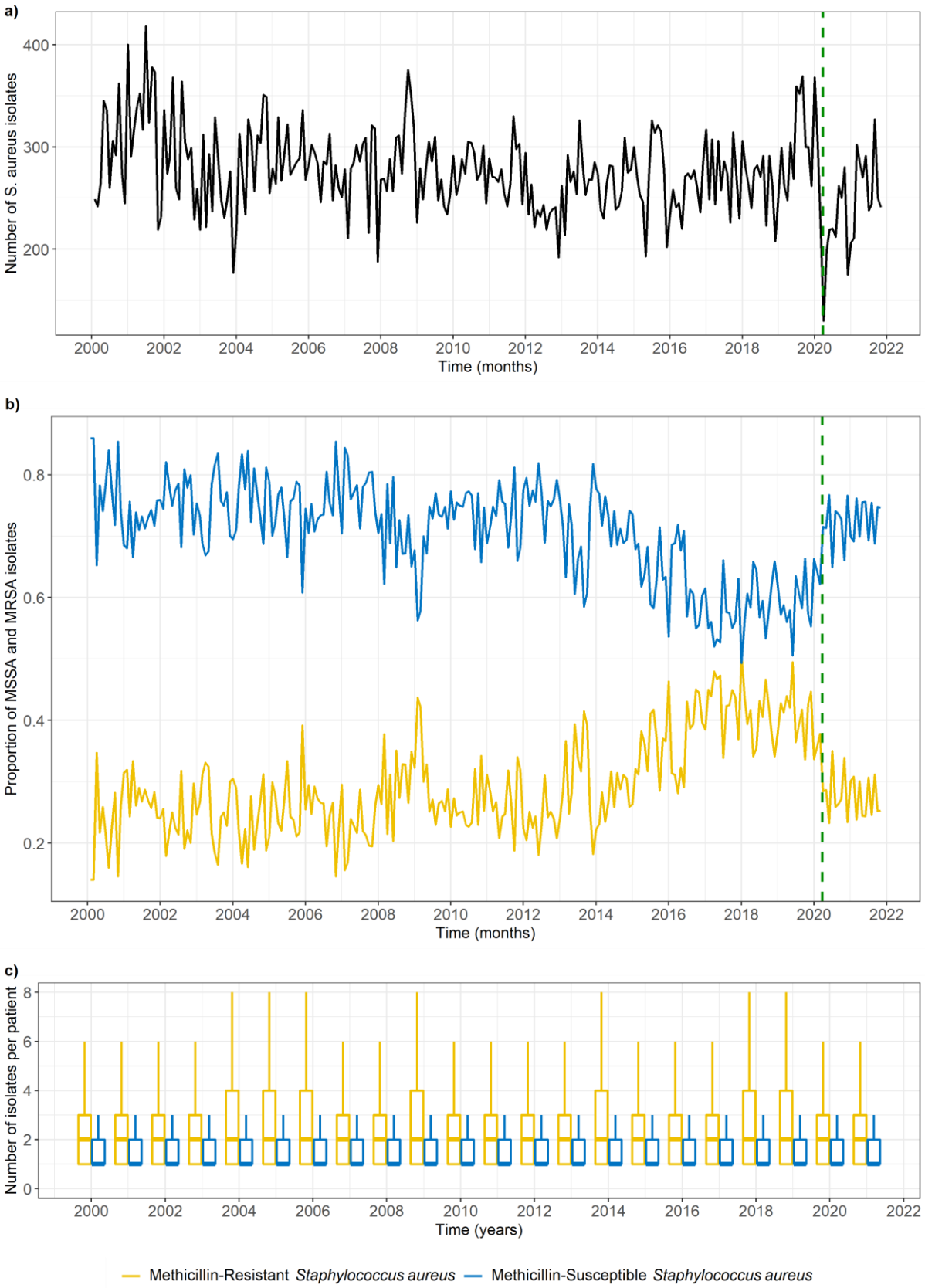


Figure 5.1: Trends in methicillin-resistant (MRSA) and -susceptible (MSSA) *Staphylococcus aureus* isolates at Great Ormond Street Hospital. a) Incidence of *S. aureus* isolates. b) Proportion of *S. aureus* isolates which are MRSA or

MSSA. Isolates are grouped by calendar month of date of isolation. Vertical green dashed line shows the date when lockdown began in the UK during the first wave of the COVID-19 pandemic (29/03/20). **c) Number of MRSA or MSSA isolates per patient per year.** Bold lines show the median, box limits show the interquartile range, line limits show the largest value within 1.5 interquartile range above the 75th percentile and smallest value within 1.5 interquartile range below the 25th percentile. Outliers are not shown on the figure.

5.5.1.2 Overview of antibiotic susceptibility testing and resistance detection

We obtained antibiogram information for 51 unique antibiotics or combinations of antibiotics (e.g. joint amikacin and flucloxacillin resistance) for our *S. aureus* isolates. The number of antibiotic resistances detected was higher in MRSA than MSSA isolates (coeff. = 2.79, $p < 0.001$) and increased slightly with the number of susceptibility tests conducted (coeff. = 0.12, $p < 0.001$), yet this linear correlation does not explain all the variability seen in the number of resistances detected ($R^2 = 0.572$) (Figure 5.2a).

Until 2011, more susceptibility tests were conducted for MRSA than for MSSA isolates (Figure 5.2b, coeff. = 4.04, $p < 0.001$). After 2010, the number of susceptibility tests for MRSA isolates decreased, with a smaller difference compared to MSSA isolates (Figure 5.2b, coeff. = 1.78, $p < 0.001$). This explains the points on the dashed line in Figure 5.2a, which correspond mostly to isolates from 2011 onwards with 4-5 susceptibility tests conducted, and an equal amount of resistances detected (Supplementary Figure 5.2). The number of antibiotic resistances detected in isolates did not substantially change over time (Figure 5.2c, coeff. = 0.0002, $p < 0.001$), but was always higher in MRSA than in MSSA isolates (Figure 5.2c, coeff. = 2.86, $p < 0.001$). Resistances in MRSA isolates were often correlated with each other, while resistances in MSSA isolates were generally independent (Supplementary Figures 5.3 and 5.4). The most common antibiotic susceptibility tests conducted across the entire time period were similar between MRSA and MSSA isolates (Figure 5.3), although MRSA were more frequently resistant, consistent with Figure 5.2c.

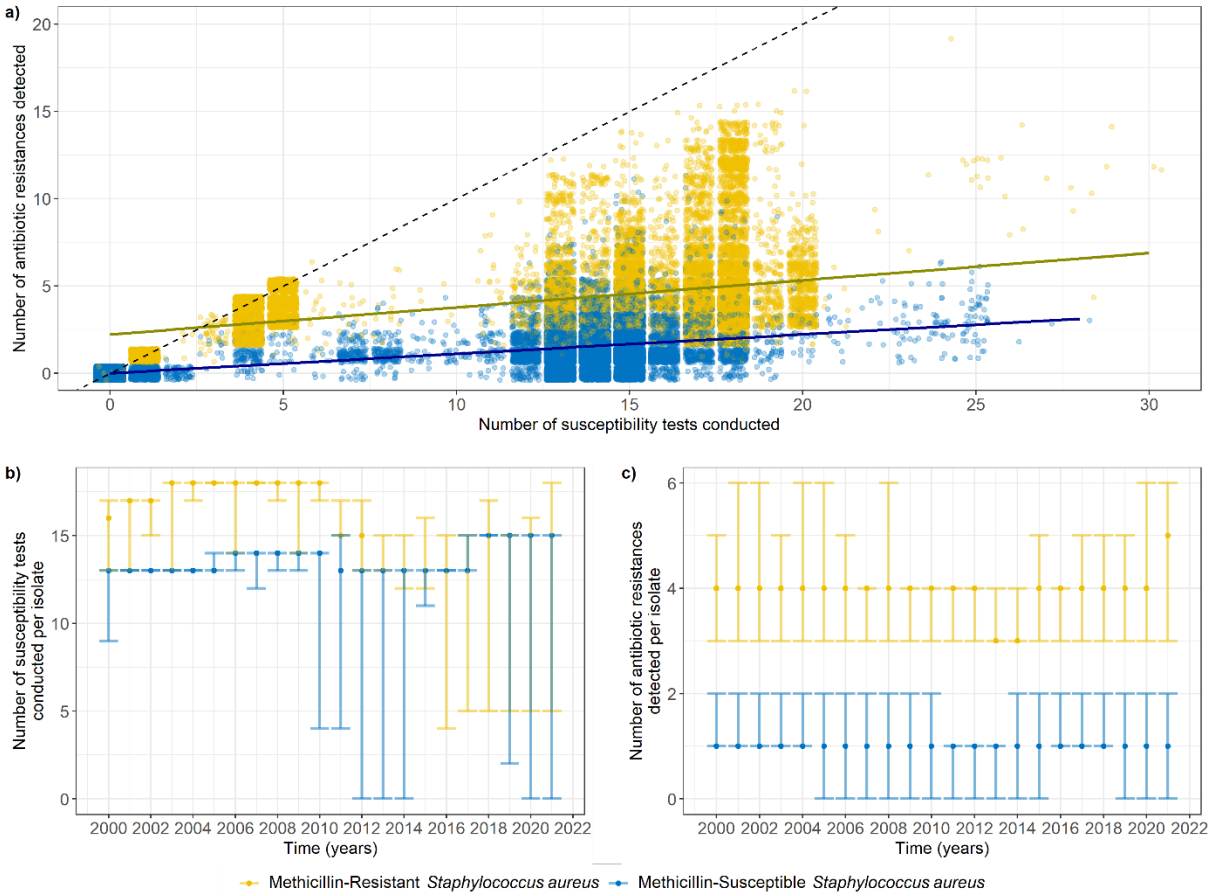


Figure 5.2: Numbers of susceptibility tests and antibiotic resistances in *S. aureus* isolates. a) Number of susceptibility tests conducted versus antibiotic resistances detected for each *S. aureus* isolate in our dataset. Each point corresponds to an isolate in the dataset, with the position of the points jittered on the figure to prevent the overlap of isolates with the same number of susceptibility tests and antibiotic resistances detected. The dashed line shows the maximum number of resistances that can be detected (equal to the number of susceptibility tests conducted). The blue and yellow solid lines show the linear regression trendline between these variables for MSSA and MRSA isolates respectively. **b) Median number of susceptibility tests conducted per isolate, by year.** Error bars show the 25th and 75th quantiles (note these can overlap). **c) Median number of antibiotic resistances detected per isolate, by year.** Error bars show the interquartile range.

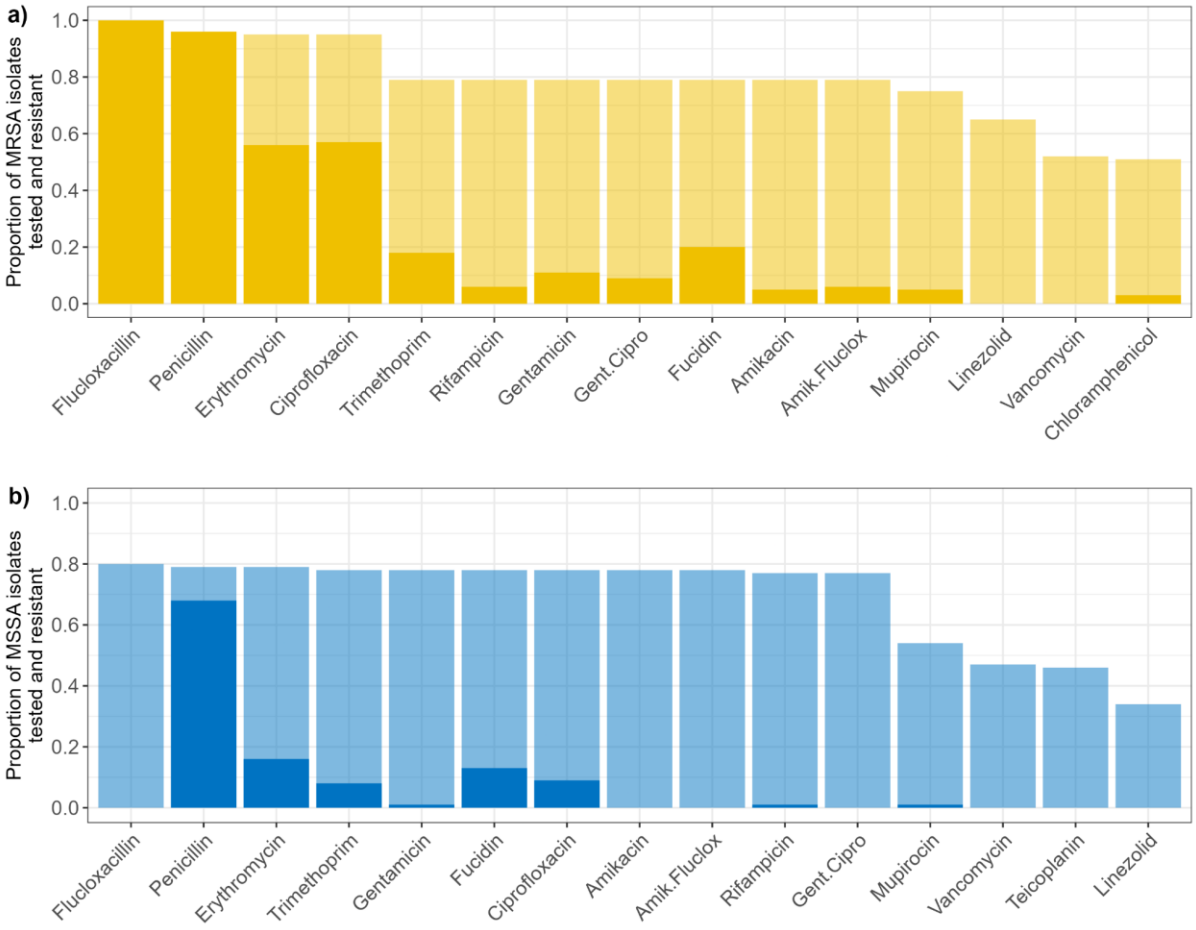


Figure 5.3: Fifteen most common antibiotic susceptibility tests conducted in methicillin-resistant (a) and -susceptible (b) *S. aureus* isolates, across the entire dataset. Transparent bars indicate the proportion of isolates that were tested for the corresponding antibiotic, and solid bars indicate the proportion of isolates that were resistant. Note that flucloxacillin is the indicator for MRSA, and that we would expect most MSSA isolates to be resistant to penicillin.

Interestingly, no susceptibility tests were recorded for 10,029/72,207 (13.89%) isolates. Removing these isolates removes 5,064/22,206 (22.80%) patients from our dataset, suggesting that these isolates are not only from repeated samples in patients, where only one isolate would have been tested for antibiotic susceptibility. The majority of these isolates were taken from nose or throat swabs (75.01%), likely corresponding to colonisation events, as opposed to wound or blood samples, likely corresponding to infections. This is higher than the percentage of isolates with at least one susceptibility test result reported which were taken from nose or throat swabs

(18.97%), indicating that colonising isolates are less likely to be tested for antibiotic susceptibility than infecting isolates (X^2 = 13,837, degrees of freedom = 1, p < 0.001).

5.5.1.3 Trends in antibiotic resistances detected in *S. aureus* isolates

We then observed trends in the proportion of *S. aureus* isolates tested for an antibiotic which were found to be resistant to that antibiotic. Only 12 of the 51 antibiotics in the dataset showed a substantial change at any point in time in that value, with changes not always consistent between MRSA and MSSA populations (see Supplementary Figure 5.5 for all antibiotics).

We found that the proportions of MRSA isolates resistant to amikacin (alone or joint with flucloxacillin), gentamicin (alone or joint with ciprofloxacin), and rifampicin were correlated, with simultaneous peaks in 2005 and 2007 (Figure 5.4a, Supplementary Figure 5.3). For both MRSA and MSSA isolates, ciprofloxacin resistance showed an overall decreasing trend since 2007 (more pronounced for MRSA), mupirocin resistance sharply decreased in 2004-2006, and clindamycin resistance increased since 2017 (Figure 5.4b). Erythromycin resistance showed a decreasing trend for MRSA isolates, but an increasing one for MSSA isolates (Figure 5.4c). Trimethoprim resistance sharply declined for MSSA isolates in 2002, but remained stable for MRSA isolates (Figure 5.4c). Cotrimoxazole resistance is practically only reported between 2010 and 2011 at low levels, while fosfomycin resistance is only reported between 2015 and 2018 at high levels (Figure 5.4d).

Figure 5.4: Change in proportion of *S. aureus* isolates resistant to antibiotics over time, out of those tested for resistance to the corresponding antibiotic. These were subjectively grouped in four categories to facilitate visualisation. **a) Strongly correlated antibiotic resistances.** Amik.Fluclox: joint amikacin and flucloxacillin resistance, Gent.Cipro: joint gentamicin and ciprofloxacin resistance. **b) Antibiotic resistances with similar trends between MRSA and MSSA isolates. c) Antibiotic resistances with differing trends between MRSA and MSSA isolates. d) Antibiotic resistances detected over a short time period only.** Missing lines indicate that no susceptibility testing for the corresponding antibiotic was conducted at that time.

5.5.1.4 Trends in antibiotic susceptibility testing of *S. aureus* isolates

We then checked if changes in resistances detected could be explained by changes in susceptibility testing frequency. Only 11 of the 51 antibiotics in the dataset showed a substantial change in the number of susceptibility tests conducted at any point in time (Figure 5.5, see Supplementary Figure 5.6 for all antibiotics). Mupirocin was the only antibiotic for which we could see a probable link between the proportion of isolates tested, and the proportion resistant. Mupirocin testing was always common in MRSA, but only became common for MSSA in 2007 (Figure 5.5a), which may explain the sharp decrease in the proportion of MSSA isolates found to be resistant to mupirocin in 2007 (Figure 5.4b).

The short intervals where cotrimoxazole and fosfomycin resistance were detected align with periods where testing for these antibiotics was conducted (Figure 5.4d, Figure 5.5b). Clindamycin and cefoxitin testing began in 2017, alongside more consistent cotrimoxazole testing (Figure 5.5c). Chloramphenicol and tetracycline testing were always at low levels for MSSA isolates, but decreased for MRSA isolates from 75% to 25% between 2012 and 2015 (Figure 5.5d).

Linezolid testing began in 2003, but was mostly reserved for MRSA isolates until 2011, after which the proportion of MSSA isolates tested for linezolid increased to the level of MRSA isolates (Figure 5.5e). Interestingly, this increase in 2011 aligned with a decrease in testing for syncercid (quinupristin and dalfopristin), teicoplanin and vancomycin, which affected both MRSA and MSSA isolates (Figure 5.5e). These decreases, combined with the decrease in chloramphenicol and tetracycline mentioned above, explained the lower median number of tested resistances for MRSA isolates seen in Figure 5.2b.

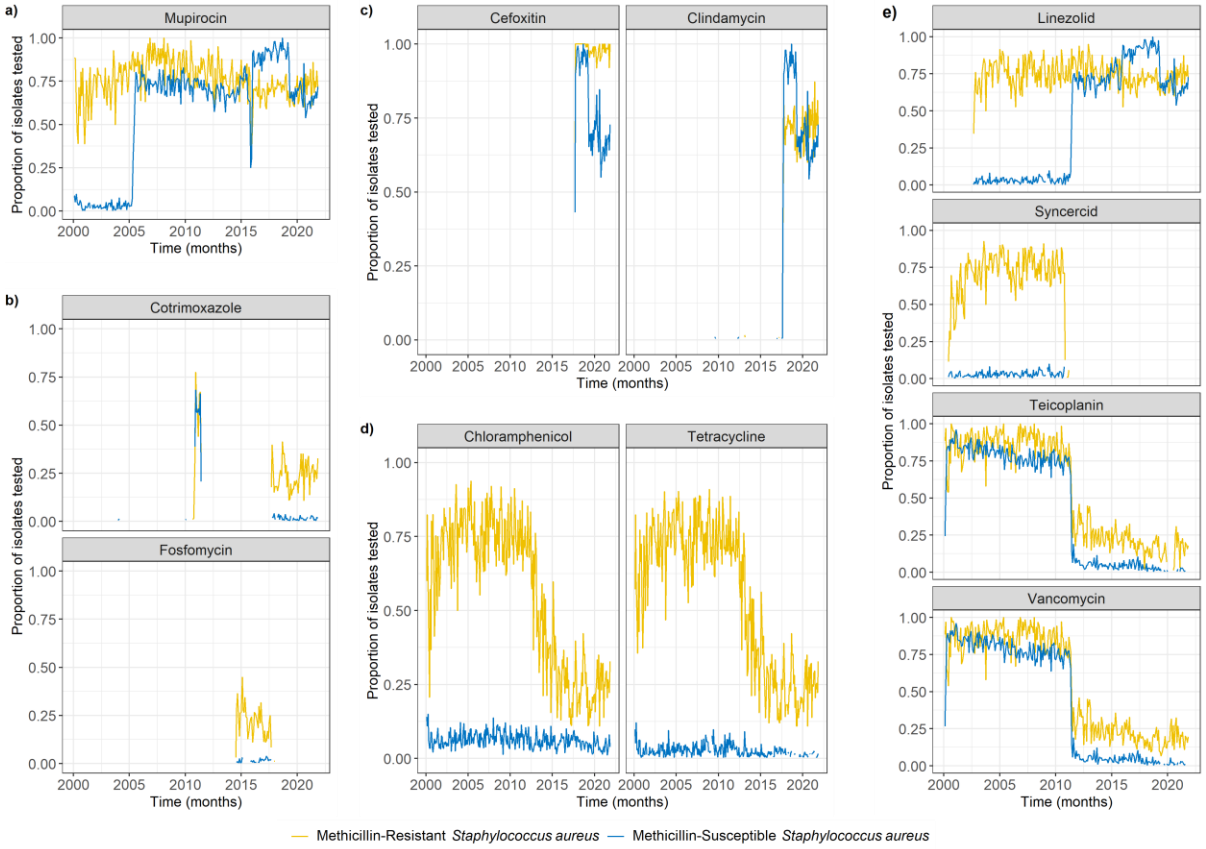


Figure 5.5: Change in proportion of *S. aureus* isolates tested for different antibiotic susceptibilities over time. These were subjectively grouped in five categories to facilitate visualisation. **a) Antibiotic with a change in susceptibility testing potentially responsible for a change in resistance detected. b) Antibiotics for which susceptibility testing was conducted over a short time period only. c) Antibiotics for which susceptibility testing began in 2017. d) Antibiotics with a decrease in susceptibility testing in MRSA. e) Antibiotics with aligned changes in susceptibility testing in 2011.** Missing lines indicate that no susceptibility testing for the corresponding antibiotic was conducted at that time.

5.5.2 Within-host *S. aureus* diversity

Across the entire dataset, 10,128/22,206 (45.61%) patients had more than one isolate recorded, allowing us to explore diversity. Note that we cannot control for sampling intensity in this study, since the dataset does not contain information on negative samples, hence we cannot distinguish between a patient with only one positive sample, and a patient with one positive sample and one or more negative samples.

We considered diversity in two stages. Firstly, we focused on patients with both MRSA and MSSA detected on the same day. Then, we searched for patients for which multiple, different antibiograms were recorded on the same day, indicating phenotypic resistances diversity. A summary of our filtering process to identify diversity is presented in Figure 5.6.

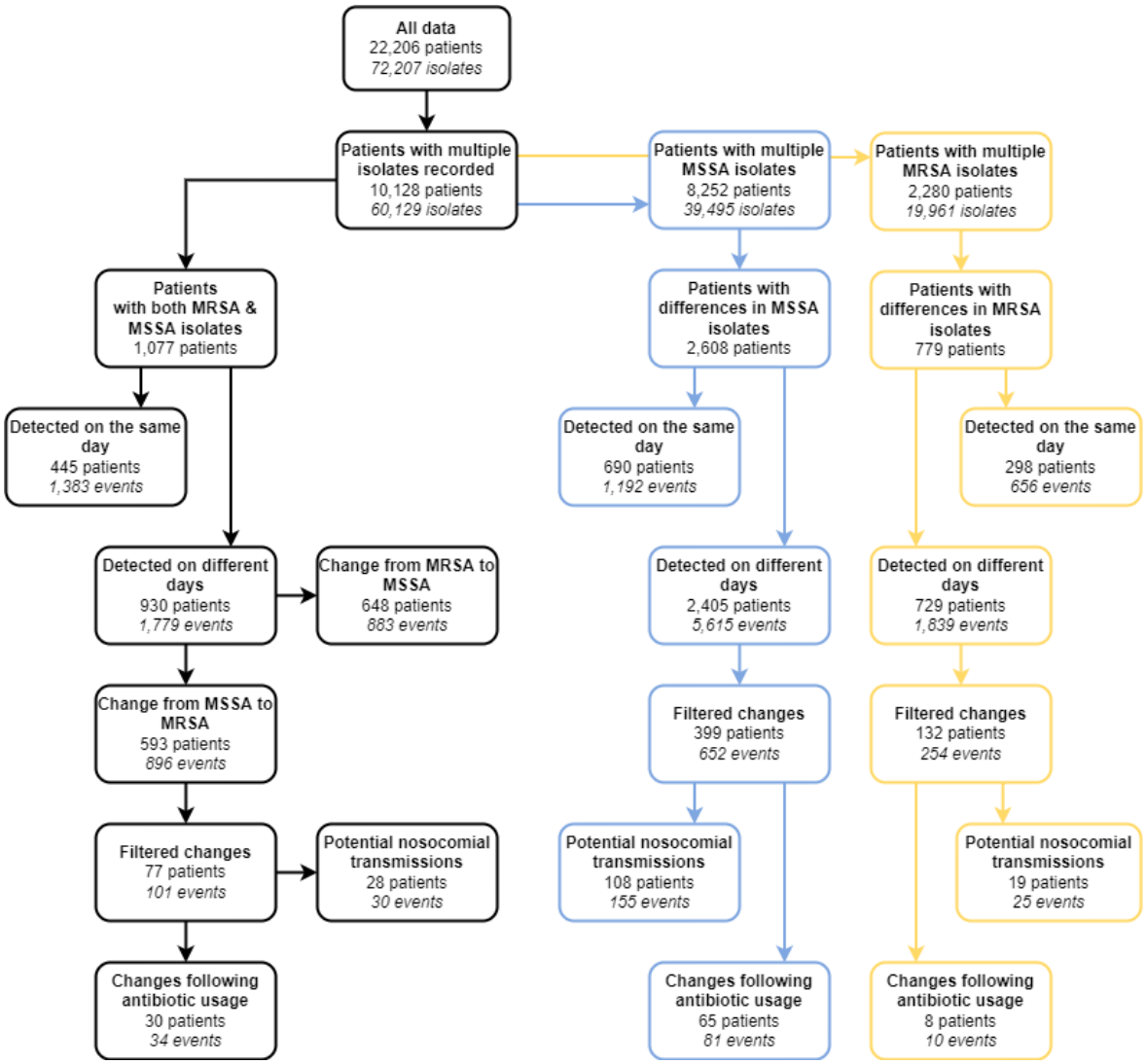


Figure 5.6: Data filtering process to identify within-host AMR phenotypic diversity. Arrows indicate the categories which are subsets of each other. Filtering for methicillin-susceptible *S.* aureus (MSSA) is shown in blue, and in yellow for methicillin-resistant (MRSA). A difference in MMSA or MRSA isolates corresponds to at least one difference in the antibiograms of these isolates. Categories may not be mutually exclusive (e.g. a patient could have both multiple MRSA isolates and multiple MSSA isolates recorded). Filtered changes: changes where both isolates where sampled during a single hospitalisation period, with at least 3 days between the isolates.

5.5.2.1 MRSA and MSSA diversity

We identified 1,077/22,206 (4.85%) patients for which both MRSA and MSSA isolates were reported at any point in time. In 445 of these patients (41.32%, 2.00% of all patients), MRSA and MSSA isolates were detected on the same day, and in most cases (84.24%) from the same sample source (e.g. nose), suggesting within-host diversity. The proportion of all patients positive for *S. aureus* who had within-host MRSA and MSSA diversity varied annually between 0.038 (2002) and 0.008 (2014) (Figure 5.7a).

5.5.2.2 Detected phenotypic resistances diversity

We separately analysed patients with more than one MSSA or more than one MRSA isolate recorded on the same day, comparing the isolates to identify within-host diversity in phenotypic resistances, defined as isolates with different antibiograms (regardless of the number of differences between antibiograms). In total, we identified 950 unique patients (4.28% of all patients) with multiple unique MSSA or MRSA antibiograms recorded on the same day. These included 690 patients (3.49% of patients ever tested positive for MSSA, 3.11% of all patients) with within-host diversity in the phenotypic resistances of their MSSA populations, and 298 patients (8.50% of patients ever tested positive for MRSA, 1.34% of all patients) with diversity in their MRSA populations. The isolates with different antibiograms were generally sampled from the same source (63.80%).

The percentage of patients with phenotypic resistances diversity was greater for patients with MRSA than for patients with MSSA (Figure 5.7b). In 90% of instances where diversity in within-patient populations was found, the number of unique antibiograms recorded was 2 (Figure 5.7c), and in 90% of instances less than 4 resistances differed between the antibiograms (Figure 5.7d). Surprisingly, these values did not substantially differ between MRSA and MSSA populations (Figure 5.7c-d). The most common differing resistance between MRSA isolates was erythromycin, and other differences were relatively homogeneously distributed amongst several antibiotics (Figure 5.7e). On the other hand, differences in MSSA isolates occurred predominantly in five antibiotics only: fucidin, penicillin, erythromycin, ciprofloxacin, and trimethoprim (Figure 5.7f).

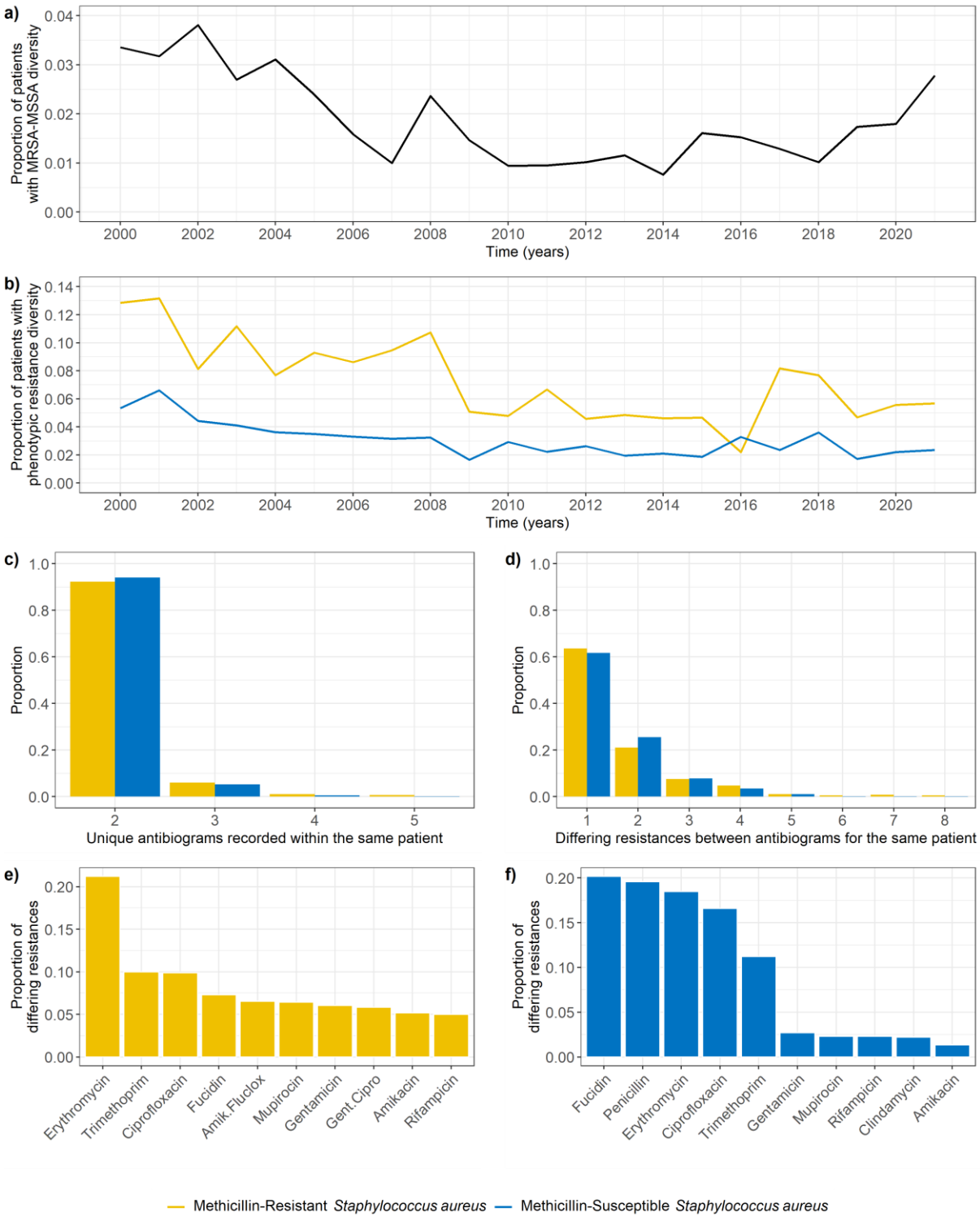


Figure 5.7: Within-host *S. aureus* phenotypic diversity detected in single patients on the same day. a) Annual proportion of patients for which both MRSA and MSSA isolates were detected on the same day, out of all patients positive for *S. aureus* in that same period. b) Annual proportion of patients for which diverse MRSA (yellow) or MSSA (blue) populations were detected on the same

day, out of all patients positive for MRSA or MSSA in that same period. c) Number of unique antibiograms recorded for patients with diverse MRSA or MSSA populations on the same day. d) Number of differing resistances between antibiograms recorded for patients with diverse MRSA or MSSA populations on the same day. e) Most common differing resistances between MRSA isolates detected within the same patient on the same day. f) Most common differing resistances between MSSA isolates detected within the same patient on the same day.

5.5.3 Changes in within-host *S. aureus* diversity

5.5.3.1 Potential nosocomial acquisition of MRSA

In the 1,077 patients for which both MRSA and MSSA isolates were reported, after excluding the cases where these were identified on the same day, we identified 883 events (648 patients) where an MRSA isolate was first detected, followed later by an MSSA isolate, and 896 events (593 patients) where an MSSA isolate was first detected, followed later by an MRSA isolate.

We further focused on changes from MSSA to MRSA, which represent more clinically worrying events. We excluded 723 events where the change did not happen within a single hospitalisation period, and 72 events where the MRSA isolate was detected within 2 days of the MSSA isolate, as this threshold is commonly used to distinguish between community- and nosocomial-acquired MRSA, and reduces the risk that we are capturing diversity present at baseline instead of a true change [43]. This left 101 events (77 patients). The median delay between the detection of the MSSA and MRSA isolates was 8 days (Figure 5.8a), and almost all delays were shorter than 30 days. As a comparison, the distribution of delays between any two subsequent isolates recorded for patients in a single hospitalisation (excluding delays shorter than 2 days) more frequently included delays between 30 and 120 days (Figure 5.8b). Patients in this group had substantially higher lengths of stay in ward (median: 72 days, interquartile range: 23-188) compared to the lengths of stay of all patients in our dataset (median: 6 days, IQR: 4-13, excluding stays shorter than 2 days).

We identified 34 changes from MSSA to MRSA in 30 patients which were preceded by exposure to any antibiotic. Compared to events filtered in the same way, but where the change was from MRSA to MSSA, we found no statistically significant link between the proportion of patients who were exposed to any antibiotic and the type of change after exposure (0.34 for MSSA to MRSA events, 0.28 for MRSA to MSSA; $X^2 = 0.54$, degrees of freedom = 1, $p = 0.46$).

We identified 30 potential nosocomial acquisitions of MRSA, in 28 patients, with an incidence varying over the years (Figure 5.8c). These were defined as events where a patient initially only positive for MSSA shared a ward with a patient positive for MRSA before themselves testing positive for MRSA.

Interestingly, this left 51 events (38 patients) for which we cannot easily explain the incidence of MRSA following the identification of MSSA only. These are events where the change happened within the same hospitalisation period, but where no MRSA isolate was detected in the same ward immediately before the change, and no antibiotic exposure was recorded for the patients preceding the change.

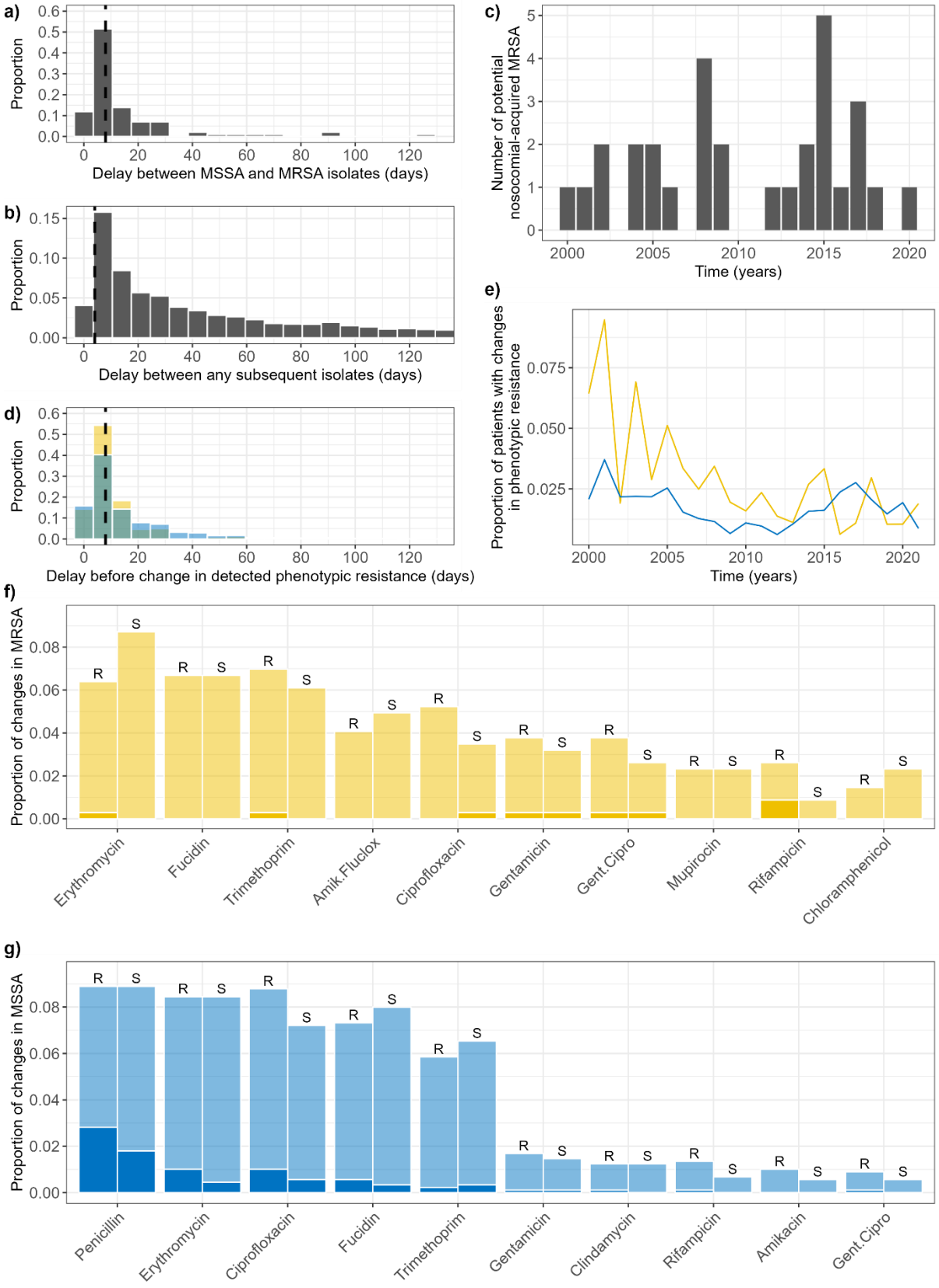


Figure 5.8: Changes in within-host *S. aureus* phenotypic diversity over time. a) Delay between detection of an MSSA and MRSA isolate in patients where the change happened within a single hospitalisation period. The dashed line shows

the median delay (8 days). Bin size is 7 days. Delays smaller than 2 days are not considered in this analysis. **b) Delay between detection of any two subsequent isolates in patients.** The dashed line shows the median delay (4 days). Bin size is 7 days. Delays smaller than 2 days are not considered in this analysis. We excluded 8,744 delays (32.49%) greater than 130 days from the figure for ease of comparison. **c) Potential MRSA nosocomial transmission events.** These are events from panel a) where an MRSA isolate was identified in another patient in the same ward immediately before the change. **d) Delay between detection of two isolates with a different antibiogram.** The dashed line shows the median delay (8 days). Bin size is 7 days. Delays smaller than 2 days are not considered in this analysis. We excluded 7 delays greater than 130 days from the figure. **e) Proportion of patients with differences in the antibiograms of their subsequent isolates over time.** MSSA and MRSA isolates are separated. **f) Most common changes detected in within-host MRSA populations. g) Most common changes detected in within-host MSSA populations.** Proportion of changes shown by antibiotic, type of change (R: susceptible to resistant, S: resistant to susceptible), and exposure to an antibiotic of the same class anytime between the original isolate, and the changed isolate (transparent: no, solid: yes). Amik.fluclox: joint amikacin and flucloxacillin resistance, gent.cipro: joint gentamicin and ciprofloxacin resistance.

5.5.3.2 Changes in detected phenotypic resistances diversity

We applied the same filtering process to identify events where the phenotypic resistances diversity in MSSA or MRSA populations within-host changed, defined as differences in subsequent antibiograms recorded for single patients over time (regardless of the number of differences between the antibiograms). We only included changes which occurred within a single hospitalisation period, with a delay between the two differing antibiograms greater than 2 days.

We identified a total of 906 events in 516 unique patients (2.32% of all patients). These included 652 events in 399 patients (2.02% of patients ever tested positive for MSSA, 1.80% of all patients) where the antibiograms of MSSA populations changed, and 254 events in 132 patients (3.76% of patients ever tested positive for MRSA, 0.59% of all

patients) where the antibiograms of MRSA populations changed. The median delay between differing antibiograms was 8 days (Figure 5.8d). These events occurred more frequently in patients with MRSA than with MSSA, although this varied over the years (Figure 5.8e). In 19.88% of all these events, the identification of a new subpopulation in a patient was immediately preceded by this patient being in the same ward as another patient positive for this subpopulation.

We found some overlap between the changes in resistances most commonly detected (Figure 5.8f-g) and the antibiotic susceptibility tests most commonly conducted (Figure 5.3). These changes almost equally represented gains and losses of resistance, regardless of the antibiotic (Figure 5.8f-g). In total, only 10.04% of all the changes we detected were preceded by patient exposure to an antibiotic of the same class as that of the change in the interval between the two differing antibiograms (Figure 5.8f-g). Interestingly, we noted several changes from resistance to susceptibility following antibiotic exposure (Figure 5.8f-g). The frequency of change detected for antibiotics did not align with the proportion of all patients exposed to antibiotics of the same class (Supplementary Figure 5.7).

We found no evidence for a causative link between antibiotic exposure and any change in resistance, as in fact the proportion of all patients exposed to antibiotics who had a change detected (0.08) was slightly lower than those who were not exposed to antibiotics and still had a change detected (0.10; $X^2 = 34.95$, degrees of freedom = 1, $p < 0.001$). Resistance genes for all of the antibiotics for which changes were most commonly detected can be found on MGEs in *S. aureus* (see [7] for a complete list of MGEs carrying antibiotic resistance genes in *S. aureus*), except for ciprofloxacin and rifampicin, which are resistances occurring via mutations [38].

Overall, we identified 678 events (74.83%) where a change in phenotypic diversity occurred without previous exposure to antibiotics, nor apparent between-patient transmission.

5.6 Discussion

5.6.1 Summary of results

Analysis of more than 70,000 isolates collected routinely for 20 years from 20,000 patients at Great Ormond Street Hospital (GOSH) revealed that the proportions of *S. aureus* resistant to different antibiotics varied over time. Susceptibility testing strategies changed at various points, which affected rates of antibiotic resistance. As multiple isolates were frequently recorded per patient, this data also revealed that *S. aureus* populations within-host are frequently diverse, with patients carrying both MRSA and MSSA bacteria, and carrying diverse MRSA and MSSA populations. Thanks to the longitudinal aspect of this dataset, with multiple antibiograms generated per patient, we found that the phenotypic resistances detected in patients can change over time. These changes could occur even within single hospitalisation periods, and equally included gains and losses of antibiotic resistance. Although some of these changes may be due to limitations in our ability to fully detect within-patient diversity in the diagnostic laboratory, they suggest that there is a frequent shuffling of antibiotic resistances in *S. aureus* populations.

5.6.2 Trends at the hospital level

We identified multiple ways in which antibiotic resistance and susceptibility testing can vary at the hospital level. Mupirocin is an example where the proportion of isolates resistant to this antibiotic decreased as a consequence of a substantial increase in the proportion of isolates tested for susceptibility to this. This is a well-known type of bias whereby, if testing is only conducted for the minority of patients more severely ill, this may lead to an over-representation of resistant isolates [26].

Importantly, the decrease in ciprofloxacin resistance demonstrates that policies to reduce antibiotic usage can successfully lead to a decrease in resistance over time. In 2006, a UK-wide policy was introduced to control the incidence of *Clostridium difficile* infections by limiting fluoroquinolone usage in all hospitals [44,45]. This aligns with the start of the decrease in ciprofloxacin resistance measured in our *S. aureus*

isolates, particularly for MRSA isolates as 90% were resistant to ciprofloxacin before 2007, down to 40% now (Figure 5.4). We are currently in the process of obtaining complete antibiotic usage data from GOSH to confirm the association between decrease in fluoroquinolone usage and ciprofloxacin resistance.

The large time period covered by the dataset allowed us to see slow changes in tetracycline and teicoplanin testing, and in erythromycin resistance, which would otherwise be missed if the analysis only included shorter time periods of even a couple of years. We also noted amikacin, gentamicin and rifampicin as examples of rare but highly correlated resistances. To the best of our knowledge, although joint amikacin and gentamicin resistance is well-known as both are aminoglycoside antibiotics [46,47], the biological mechanism explaining the correlation with rifampicin resistance is unknown.

Discussions with clinicians from GOSH were required to shed light on some of these trends. Fosfomycin was pointed out as an example where testing was conducted to assess if a new antibiotic may be useful to treat infections, as it was licensed in the UK in 2015, which aligns with the brief period where susceptibility testing was conducted. As almost all *S. aureus* isolates were found to be resistant, routine susceptibility testing for fosfomycin was rapidly halted, as this did not bring any additional useful clinical information. The short testing period for cotrimoxazole in 2011 on the other hand was linked to the investigation of an outbreak, where the causative bacterial strain was unusually resistant to this antibiotic.

During these discussions, it was also suggested that a link may exist between the increase in MRSA isolates between 2014 and 2020, and the ethnicity of patients admitted at GOSH, since non-white individuals are at higher risk of carrying MRSA [18]. We did find that increases in the proportion of patients with an ethnicity other than “White British” were aligned with increases in the proportion of *S. aureus* isolates which were MRSA, suggesting that this link does exist in our data. In addition, since patients from outside the UK were no longer admitted to GOSH during the first COVID-19 lockdown in March 2020, this may also explain the decrease in MRSA isolates seen at that time.

The comparison between the number of susceptibility tests conducted per isolates and number of resistances detected suggests that the testing strategy at GOSH is potentially optimal, in the sense that relatively few isolates are under-tested (corresponding to isolates with few susceptibility tests conducted, and an equal number of resistance detected) or over-tested (isolates with many susceptibility tests conducted, but much less resistances detected). To the best of our knowledge, this type of analysis is not commonly reported, and may be useful to determine if a wide range of antibiotic resistances is captured in routine surveillance datasets. The multiple changes in testing strategy we identified in 2011 were confirmed by the GOSH staff as being linked to a change in the head of infection prevention and control. We were also informed that following identification of MRSA in a patient, it is common to swab multiple other potential colonisation sites to identify the presence of *S. aureus*. However, any isolate subsequently identified through these additional swabs would not undergo complete antibiotic susceptibility testing. This explains the higher number of isolates since 2011 with fewer antibiotic susceptibility testing conducted, as well as the 10,029 isolates in our data with no antibiotic susceptibility results.

Overall, our analysis showed the multitude of factors which might affect AMR trends in routinely collected data. All of these factors should be considered in studies which attempt to reuse these datasets for epidemiological purposes, as they may introduce several types of biases. Crucially, some of these changes could only be understood following discussions with the clinicians at GOSH, highlighting the importance of continuous collaboration at all stages of the data analysis. Improved sharing of information on data collection to accompany the public release of these routinely collected datasets will be beneficial to the scientific community more broadly.

5.6.3 Within-host diversity

We found that within-host MRSA populations were more likely to be diverse compared to MSSA populations. This could be explained by more MRSA samples being taken per patient than MSSA, consistent with UK testing guidelines, and hence increasing the probability to detect diverse subpopulations [40]. However, this prevalence of diversity changed over time whilst the number of isolates recorded per patient did not,

suggesting there are other currently unknown factors affecting diversity over time. Surprisingly, when diversity was detected, the number of differences was similar regardless of whether they occurred in MSSA or MRSA populations (two unique antibiograms detected, with less than four differences between antibiograms). As MRSA isolates carry more resistances, we expected to see both i) more instances where diversity was detected than in MSSA, which we did, and ii) a greater amount of diversity, which we did not. This could firstly be due to detection limits, as sampling in the diagnostic laboratory relying on plating may not be powerful enough to detect profiles representing only small proportions of the total *S. aureus* population [10] (further discussed in the Limitations and Strength section). Alternatively, the previous study of *S. aureus* diversity in piglets found evidence of simultaneously high rates of gain and loss of MGEs in *S. aureus*, which may explain why not more diversity is detected here, as some subpopulations of *S. aureus* carrying different resistance genes may only be present at small proportions and therefore not detected [35].

Our estimates for diversity are lower than those reported in previous studies. Whilst we report 2.00% of all patients ever detected positive for *S. aureus* carried both MSSA and MRSA simultaneously at some point, a previous study estimated this value to be 21% in children sampled before surgery [11]. This same study identified multiple *S. aureus* genotypes in 30% of patients, while our estimate of diversity (defined as detection of multiple MSSA or MRSA isolates with different antibiograms on the same day in a patient with at least one MSSA or MRSA isolate detected) was 3.49% for patients with MSSA detected, and 8.50% for patients with MRSA detected. On the other hand, a second study found that 6.6% of individuals colonised by *S. aureus* carried more than one strain, defined using pulsed field gel electrophoresis [12]. However, the definitions and measures of diversity varied between these studies and ours. Finally, a study in patients colonised with MRSA found that 24% were colonised by more than one phenotypically distinct isolate [9]. Interestingly, this study identified a maximum of three distinct isolates in a single patient, and two in the remaining eight patients with diversity. This is consistent with our result that, in patients with diversity, we generally detected only two MSSA or MRSA subpopulation (i.e. with only two unique antibiograms), although we were able to detect up to five unique antibiograms within a single patient on the same day (Figure 5.7c).

These differences may be explained by our use of antibiotic susceptibility data to determine phenotypic resistances diversity, as opposed to these previous studies which used genotypic data. Whilst the genotypic data captures more diversity, from a clinical perspective phenotypic resistance is more meaningful, as this is what will ultimately determine treatment success or failure. In addition, while these studies were specifically designed to capture as much diversity as possible, we relied on data routinely collected for clinical purposes (further discussed in the Limitations and Strength section). On the other hand, these previous studies may have also overestimated diversity, by sampling a small number of patients belonging to specific groups at higher risk of severe infections (surgery patients or drug users).

5.6.4 Changes in within-host diversity over time

We identified 101 events (77 patients) which we believe correspond to patients acquiring MRSA during their hospitalisation period. These are events where patients initially tested positive for MSSA only, then at least 3 days later for MRSA, within the same hospitalisation period. Of these 101 events, 30 (29.70%, 28 patients) represented probable instances of nosocomial-acquired MRSA. These are events where the patient who acquired MRSA was in the same ward as another patient who was positive for MRSA immediately before the new MRSA-positive sample was reported. This likely represents a lower estimate of nosocomial-acquired MRSA, since we have not considered instances where patients initially tested negative for any *S. aureus* initially, and subsequently positive for MRSA. Arguably, this distinction means we are more confident that the events we have identified are true nosocomial-acquired MRSA, since in cases where nothing was initially detected we cannot be as confident that testing was properly conducted, and there is therefore a higher chance that MRSA was already present initially but not detected. However, we are left with 71 MRSA acquisition events (70.30%, 55 patients) where the patients did not share a ward with any other patient positive for MRSA immediately before the acquisition was reported. In such cases, the patients may have acquired MRSA from a source not monitored in our data, such as healthcare workers or environmental surfaces, or these events may represent instances where MRSA was incorrectly undetected in the first sample. While this again suggests that our number of nosocomial acquired MRSA may be an

underestimate, our results still indicate that we can identify at least some of such events using routinely collected data only.

To the best of our knowledge, this work is the first attempt to estimate changes in *S. aureus* AMR within-host diversity over time in a large routinely collected dataset with more than 20,000 hospital patients. We identified 906 events (652 in MSSA, 254 in MRSA, 516 unique patients in total, 2.32% of all patients) where a change in at least one phenotypic resistance was detected within a single hospitalisation period, with a delay between the two differing isolates greater than 2 days. We considered several hypotheses to explain these changes. Firstly, these may be due to limitations in the data, further discussed in the next section. The second possibility is selection for resistance following antibiotic exposure, where a subpopulation resistant to an antibiotic was initially present in minority and not detected, but became dominant following exposure to that antibiotic, and was detected in a subsequent swab. However, we only identified evidence supporting this for 10.04% of all changes, and we have not found that patients with a change in resistance were significantly more likely to have been exposed in hospital to antibiotics of the same class as that of the change than patients with no change. Furthermore, some detected losses in resistance counterintuitively occurred following exposure to antibiotics belonging to the same class. Since some resistances are co-located on plasmids, antibiotic consumption selecting for one resistance may have incidentally selected for other resistances present on the same plasmid [7], but we could not estimate this here due to lack of genetic data.

The third explanation is acquisition of new subpopulations from an external source, which we tried to rule out by focusing on changes which occurred within single hospitalisation periods only, reducing the risk of patients having acquired new subpopulations from a variety of external sources. We found that between-patient transmission in the hospital does not explain all our changes detected, with only 180 events (19.88%) where the detected changes in phenotypic resistances occurred after the patients shared a ward with another patient for which an isolate with the same antibiogram had previously been detected. Movement of single MGEs carrying antibiotic resistance between patients is possible, but a previous study which measured this using detailed genomic data from more than 2,000 isolates of various

bacteria species over 18 months only found a single instance where this may have occurred [48].

The majority of changes in phenotypic diversity (678 events, 74.83%) could not be explained by either antibiotic exposure or between-patient transmission. We therefore consider a fourth explanation to be frequent gain and loss of antibiotic resistance genes in within-host *S. aureus* populations. This type of frequent shuffling was previously seen *in vivo* in gnotobiotic piglets, and could explain changes for some resistances which are known to be located on MGEs [35]. However, we also saw changes in resistance to ciprofloxacin and rifampicin, which are gained via mutations instead of acquisition of an MGE [38]. We also failed to see changes in resistances we might have expected to move frequently, such as clindamycin, tetracycline, and chloramphenicol, which are present on many MGEs in *S. aureus* [7]. This may indicate varying rates of gain and loss for different genes. The varying rates of loss may be due to varying fitness costs between resistances. For example, a subpopulation of *S. aureus* resistant to rifampicin resistance may not persist long in absence of antibiotic selection pressure, due to the high fitness cost of this resistance [49]. As for rates of gain, we suggest that transduction (horizontal gene transfer mediated by bacteriophage) may play a substantial role this process, as it is known to be the major mechanism for horizontal gene transfer in *S. aureus* [9,32–34]. Importantly, generalised transduction is a type of transduction where any bacterial gene may be transferred by the phage, not only MGEs. In addition, previous work suggested that different genes may not be transferred at the same rate by this process, which may explain the variations we have seen here [34,35].

Overall, we found evidence of changes in diversity over time, but cannot explain these changes using a single hypothesis. These changes may be due to a combination of factors occurring at variable rates in the population (e.g. transfer of resistance genes by transduction, selection by antibiotics etc.).

5.6.5 Limitations and strengths

The major limitation of our work is that all the antibiotic resistances and susceptibilities for our isolates were determined routinely in the GOSH diagnostics laboratory. The sampling strategy has therefore not been designed to fully capture diversity in within-host *S. aureus* populations. Microbiologists at GOSH have confirmed that, although efforts are made to test multiple colonies from those initially grown after plating the patient sample, this process has never been audited, and that generally only 2-3 colonies are selected for subculture and antibiotic susceptibility testing. Previous work has shown that different strains may coexist at various proportions within a single *S. aureus* population, and that strains in minority may be missed if less than 18 colonies are sampled, much more than routinely done [9]. Crucially, this means that some of the changes we report may simply represent the variability of sampling in the laboratory. Consider an example where a patient is colonised by both erythromycin-resistant and -susceptible *S. aureus*. If only part of this diversity is detected, then it is possible that in a first sample only the resistant subpopulation is identified, whilst in a second sample only the susceptible subpopulation is identified, despite no real change in the within-host *S. aureus* population structure having occurred between the two samples. Although we tried to account for this by restricting our analysis to changes in samples collected at least 3 days apart, this limitation is an inevitable consequence of our attempt to use routinely collected diagnostics laboratory data.

In addition, not all isolates identified in the diagnostic lab are tested for all antibiotics. Here, we did not consider that a change from no test to resistant or susceptible was a valid change in resistance, which means that we may potentially have missed some changes. This suggests that our results for diversity are underestimated, which we also find by comparing our numbers to those of previous studies, as highlighted above. However, this limitation likely applies less to our results on MSSA-MRSA diversity, as we can confidently assume that detection of MSSA and MRSA is always more accurate than detection of phenotypic resistances. This is notably due to the policy of screening all patients upon admission to the hospital for *S. aureus* colonisation, and the systematic and accurate testing for MRSA as part of this process.

Susceptibility testing in the diagnostics laboratory is conducted using disk diffusion or gradient methods. As these methods rely on breakpoints to classify isolates as susceptible or resistant, there may be some subjectivity in the classification. However, through discussions with clinical microbiologists at GOSH we confirmed that all the antibiotics were equally likely to be affected by this subjectivity, hence this potential bias is homogeneously distributed in our data. This suggests that differences in the frequency of diversity seen between antibiotic resistances are real (Figure 5.7e-f).

Despite the previous points which show the limitations of our analysis, we argue that phenotypic resistances are more meaningful to compare than the presence or absence of resistance genes, as previous studies have done [9]. This is because phenotypic resistance is what truly matters with regards to the health burden of infections by antibiotic resistant pathogens. Genotypic traits of antibiotic resistance may not always translate to phenotypic resistance, as the expression of antibiotic resistance genes is affected by other genes and environmental conditions [20]. In that sense, the fact that we are still able to see evidence of diversity in *S. aureus* populations using phenotypic data only is a strong argument to support the importance of this diversity, and that it is likely to have consequences on the health burden of infections. In the eventuality that the sampling limitations listed above are really responsible for all the changes in phenotypic resistances we detected, that would clearly indicate that the testing strategy in diagnostics laboratories frequently misses resistant subpopulations in patient samples. This would have important implications for treatment, as failure to identify resistance may lead to inappropriate antibiotic choices, treatment failures, and worse health outcomes for patients.

To the best of our knowledge, this is the first time that such a large dataset, with more than 70,000 isolates taken from 20,000 patients over 20 years, is used to search for evidence of AMR diversity in *S. aureus*. This large sample size is a key strength of our analysis, notably due to its longitudinal nature. In addition, our dataset included both colonising and invasive isolates. This allowed us to capture a more complete picture of diversity, as opposed to previous studies which typically focused only on one of the two types, and only on MRSA subpopulations. However, as GOSH specialises in paediatric care, the patients in our analysis belong to a group which may not be representative of the entire population. *S. aureus* infections are typically less severe

in children than adults, with substantially lower mortality [50]. *S. aureus* colonisation may be more prevalent in children [51], yet the proportion of children with MRSA versus MSSA is likely similar as in adults [52]. This limits the generalisability of our diversity estimates.

5.6.6 Implications and next steps

Our results illustrate the many ways in which datasets of routinely collected information in hospital can contain changes in antibiotic resistance detected over time that do not correspond to true epidemiological trends, but rather are linked to changes in testing strategy. As this type of data is frequently used in secondary analyses to derive epidemiological trends in AMR prevalence, future studies should bear this important limitation in mind. Discussions with hospital staff should be encouraged, as they may provide explanations for unexpected changes in reported rates of AMR at the local level. This includes consultants who know which antibiotics are used to treat patients, infection control staff who are aware of the testing policies and events which might have changed these over time (e.g. outbreaks), and clinical microbiologists who can provide information on how susceptibility testing is performed in the diagnostics laboratory.

We have shown here the value of routinely collected data both from a clinical perspective, to understand how hospital policies can affect AMR over time (e.g. linked decrease in fluoroquinolone usage and ciprofloxacin resistance), but also from a microbiology perspective, to reveal AMR diversity within-host. This was only made possible thanks to the framework developed by GOSH to store and make this data easily accessible for research purposes. Other healthcare institutions should develop systems like the GOSH DRIVE to record and analyse routinely collected data, as this may benefit these institutions directly, and allow new analyses to further improve our understanding of AMR.

Building upon the previous point, this work should be repeated in other hospitals. *S. aureus* incidence can vary substantially within and between countries, and population structures such as dominant lineages vary geographically [54]. Expanding the settings

in which this analysis is conducted is therefore essential. This would also allow the study of different populations than here, including adults to overcome the generalisability limitation of our work which focused on children. Building upon existing evidence [55], there is ongoing work to investigate how the burden of AMR varies between age groups, hence this may provide further relevant insights by revealing how within-host diversity changes over the lifetime of individuals.

Although we found evidence of changes in detected diversity over time, our exploration of potential explanations for these changes was limited by the nature of the data. To explain these changes, further studies with longitudinal sampling of patients should be conducted. These studies could include measurements of both genotypic and phenotypic AMR diversity, to understand how these values are linked. For example, the correlation between several resistances we observed in MRSA (Supplementary Figure 5.3) could be explained by linkage of multiple resistance genes on the same mobile genetic elements, or reflect the distribution of resistance profiles between different clonal complexes [56]. In addition, linked to the importance of transduction we have mentioned and the role it may play in diversity, these studies could test patient samples for the presence of phage, as was done previously at a small scale [9]. These further analyses should also examine the link between diversity and patient outcomes, to determine for example if patients with more diversity are at higher risk of infections by multidrug-resistant bacteria, which we did not do here due to the high number of variables potentially affecting this link which we could not disentangle using the data available to us alone. We are currently in the process of obtaining routinely collected genomic information for our *S. aureus* isolates and wider antibiotic usage data across all patients in the hospital, which we will use to refine our analyses.

AMR diversity is likely relevant for other clinically important pathogens than *S. aureus* [57]. Examples include *Streptococcus pneumoniae* [58] *Pseudomonas aeruginosa* [59], and recently in *Klebsiella pneumoniae* [60]. In a first instance, our analysis could easily be repeated using the same routinely collected dataset from GOSH, looking at different bacterial species. To control the important public health threat of AMR, we must understand how it arises, and design efficient interventions preventing this. Understanding within-host AMR diversity in clinically relevant pathogens is therefore

essential, as changes in this diversity are directly linked to the frequency of AMR evolution.

5.6.7 Conclusion

In conclusion, we have shown that routinely collected data can be used to reveal within-host AMR diversity in *S. aureus*. Unlike previous studies, this work focused on detected phenotypic diversity, as this is more clinically relevant. In addition, to the best of our knowledge this is the first time that evidence of diversity changing over time was obtained at such a large scale. Changes in detected diversity are likely affected by multiple factors, including antibiotic selection pressures, between-host transmission, and frequent gain and loss of resistance genes in bacteria. We notably suggest that generalised transduction may play an important role in the shuffling of resistances in *in vivo S. aureus* populations. Future studies with longitudinal sampling of patients should be conducted to identify the relative importance of these factors, clarify the implication of this diversity on patient health, and identify opportunities to reduce AMR prevalence.

5.7 References

1. World Health Organization. Global action plan on antimicrobial resistance. Geneva: World Health Organization; 2015. Available: https://apps.who.int/iris/handle/10665/193736

2. Cottalorda A, Dahyot S, Soares A, Alexandre K, Zorgniotti I, Etienne M, et al. Phenotypic and genotypic within-host diversity of Pseudomonas aeruginosa urinary isolates. Sci Rep. 2022;12: 5421. doi:10.1038/s41598-022-09234-5

3. Didelot X, Walker AS, Peto TE, Crook DW, Wilson DJ. Within-host evolution of bacterial pathogens. Nat Rev Microbiol. 2016;14: 150–162. doi:10.1038/nrmicro.2015.13

4. Davies NG, Flasche S, Jit M, Atkins KE. Within-host dynamics shape antibiotic resistance in commensal bacteria. Nat Ecol Evol. 2019;3: 440–449. doi:10.1038/s41559-018-0786-x

5. den Heijer CDJ, van Bijnen EME, Paget WJ, Pringle M, Goossens H, Bruggeman CA, et al. Prevalence and resistance of commensal Staphylococcus aureus, including meticillin-resistant S aureus, in nine European countries: a cross-sectional study. Lancet Infect Dis. 2013;13: 409–415. doi:10.1016/S1473-3099(13)70036-7

6. O'Gara JP. Into the storm: Chasing the opportunistic pathogen Staphylococcus aureus from skin colonisation to life-threatening infections. Environ Microbiol. 2017;19: 3823–3833. doi:10.1111/1462-2920.13833

7. Haaber J, Penadés JR, Ingmer H. Transfer of Antibiotic Resistance in Staphylococcus aureus. Trends Microbiol. 2017;25: 893–905. doi:10.1016/j.tim.2017.05.011

8. Knight GM, Budd EL, Whitney L, Thornley A, Al-Ghusein H, Planche T, et al. Shift in dominant hospital-associated methicillin-resistant Staphylococcus aureus (HA-MRSA) clones over time. J Antimicrob Chemother. 2012;67: 2514–2522. doi:10.1093/jac/dks245

9. Stanczak-Mrozek KI, Manne A, Knight GM, Gould K, Witney AA, Lindsay JA. Within-host diversity of MRSA antimicrobial resistances. J Antimicrob Chemother. 2015;70: 2191–2198. doi:10.1093/jac/dkv119

10. Muenks CE, Hogan PG, Wang JW, Eisenstein KA, Burnham C-AD, Fritz SA. Diversity of Staphylococcus aureus strains colonizing various niches of the human body. J Infect. 2016;72: 698–705. doi:10.1016/j.jinf.2016.03.015

11. Mongkolrattanothai K, Gray BM, Mankin P, Stanfill AB, Pearl RH, Wallace LJ, et al. Simultaneous carriage of multiple genotypes of Staphylococcus aureus in children. J Med Microbiol. 2011;60: 317–322. doi:10.1099/jmm.0.025841-0

12. Cespedes C, Saïd-Salim B, Miller M, Lo S-H, Kreiswirth BN, Gordon RJ, et al. The Clonality of Staphylococcus aureus Nasal Carriage. J Infect Dis. 2005;191: 444–452. doi:10.1086/427240

13. Katayama Y, Ito T, Hiramatsu K. A New Class of Genetic Element, Staphylococcus Cassette Chromosome mec, Encodes Methicillin Resistance in Staphylococcus aureus. Antimicrob Agents Chemother. 2000;44: 1549–1555.

14. Hanssen A-M, Ericson Sollid JU. SCCmec in staphylococci: genes on the move. FEMS Immunol Med Microbiol. 2006;46: 8–20. doi:10.1111/j.1574-695X.2005.00009.x

15. Maree M, Thi Nguyen LT, Ohniwa RL, Higashide M, Msadek T, Morikawa K. Natural transformation allows transfer of SCCmec-mediated methicillin resistance in Staphylococcus aureus biofilms. Nat Commun. 2022;13: 2477. doi:10.1038/s41467-022-29877-2

16. Scharn CR, Tenover FC, Goering RV. Transduction of Staphylococcal Cassette Chromosome mec Elements between Strains of Staphylococcus aureus. Antimicrob Agents Chemother. 2013;57: 5233–5238. doi:10.1128/AAC.01058-13

17. Chambers HF, DeLeo FR. Waves of Resistance: Staphylococcus aureus in the Antibiotic Era. Nat Rev Microbiol. 2009;7: 629–641. doi:10.1038/nrmicro2200

18. Ray GT, Suaya JA, Baxter R. Trends and Characteristics of Culture-Confirmed Staphylococcus aureus Infections in a Large U.S. Integrated Health Care Organization. J Clin Microbiol. 2012;50: 1950–1957. doi:10.1128/JCM.00134-12

19. Graffunder EM, Venezia RA. Risk factors associated with nosocomial methicillin-resistant Staphylococcus aureus (MRSA) infection including previous use of antimicrobials. J Antimicrob Chemother. 2002;49: 999–1005. doi:10.1093/jac/dkf009

20. Dantas G, Sommer MO. Context matters — the complex interplay between resistome genotypes and resistance phenotypes. Curr Opin Microbiol. 2012;15: 577–582. doi:10.1016/j.mib.2012.07.004

21. Gordon NC, Price JR, Cole K, Everitt R, Morgan M, Finney J, et al. Prediction of Staphylococcus aureus Antimicrobial Resistance by Whole-Genome Sequencing. J Clin Microbiol. 2014;52: 1182–1191. doi:10.1128/JCM.03117-13

22. Bruinsma N, Kristinsson KG, Bronzwaer S, Schrijnemakers P, Degener J, Tiemersma E, et al. Trends of penicillin and erythromycin resistance among invasive Streptococcus pneumoniae in Europe. J Antimicrob Chemother. 2004;54: 1045–1050. doi:10.1093/jac/dkh458

23. Collignon P, Beggs JJ, Walsh TR, Gandra S, Laxminarayan R. Anthropological and socioeconomic factors contributing to global antimicrobial resistance: a univariate and multivariable analysis. Lancet Planet Health. 2018;2: e398–e405. doi:10.1016/S2542-5196(18)30186-4

24. Ward and admissions information. In: GOSH Hospital site [Internet]. [cited 16 May 2022]. Available: https://www.gosh.nhs.uk/wards-and-departments/ward-and-admissions-information/

25. Annual reports. In: GOSH Hospital site [Internet]. [cited 19 May 2022]. Available: https://www.gosh.nhs.uk/about-us/our-corporate-information/publications-and-reports/annual-reports/

26. Leclerc QJ, Naylor NR, Aiken AM, Coll F, Knight GM. Feasibility of informing syndrome-level empiric antibiotic recommendations using publicly available antibiotic resistance datasets. Wellcome Open Res. 2020;4: 140. doi:10.12688/wellcomeopenres.15477.2

27. Public Health England. SMI Q 5: inoculation of culture media for bacteriology. 2017 Sep.

28. Lipsitch M, Samore MH. Antimicrobial Use and Antimicrobial Resistance: A Population Perspective - Volume 8, Number 4—April 2002 - Emerging Infectious Diseases journal - CDC. Emerg Infect Dis. 2002 [cited 26 May 2022]. doi:10.3201/eid0804.010312

29. Cooper BS, Medley GF, Stone SP, Kibbler CC, Cookson BD, Roberts JA, et al. Methicillin-resistant *Staphylococcus aureus* in hospitals and the community: Stealth dynamics and control catastrophes. Proc Natl Acad Sci. 2004;101: 10223–10228. doi:10.1073/pnas.0401324101

30. McBryde ES, Pettitt AN, McElwain DLS. A stochastic mathematical model of methicillin resistant Staphylococcus aureus transmission in an intensive care unit: Predicting the impact of interventions. J Theor Biol. 2007;245: 470–481. doi:10.1016/j.jtbi.2006.11.008

31. Di Ruscio F, Guzzetta G, Bjørnholt JV, Leegaard TM, Moen AEF, Merler S, et al. Quantifying the transmission dynamics of MRSA in the community and healthcare settings in a low-prevalence country. Proc Natl Acad Sci. 2019;116: 14599–14605. doi:10.1073/pnas.1900959116

32. Leclerc QJ, Wildfire J, Gupta A, Lindsay JA, Knight GM. Growth-Dependent Predation and Generalized Transduction of Antimicrobial Resistance by Bacteriophage. mSystems. 2022;0: e00135-22. doi:10.1128/msystems.00135-22

33. Leclerc QJ, Lindsay JA, Knight GM. Modelling the synergistic effect of bacteriophage and antibiotics on bacteria: killers and drivers of resistance evolution. bioRxiv. 2022 [cited 29 Mar 2022]. doi:10.1101/2022.03.02.480504

34. Stanczak-Mrozek KI, Laing KG, Lindsay JA. Resistance gene transfer: induction of transducing phage by sub-inhibitory concentrations of antimicrobials is not

correlated to induction of lytic phage. J Antimicrob Chemother. 2017;72: 1624–1631. doi:10.1093/jac/dkx056

35. McCarthy AJ, Loeffler A, Witney AA, Gould KA, Lloyd DH, Lindsay JA. Extensive Horizontal Gene Transfer during Staphylococcus aureus Co-colonization In Vivo. Genome Biol Evol. 2014;6: 2697–2708. doi:10.1093/gbe/evu214

36. Million-Weaver S, Camps M. Mechanisms of plasmid segregation: have multicopy plasmids been overlooked? Plasmid. 2014;0: 27–36. doi:10.1016/j.plasmid.2014.07.002

37. Melnyk AH, Wong A, Kassen R. The fitness costs of antibiotic resistance mutations. Evol Appl. 2015;8: 273–283. doi:10.1111/eva.12196

38. Foster TJ. Antibiotic resistance in Staphylococcus aureus. Current status and future prospects. FEMS Microbiol Rev. 2017;41: 430–449. doi:10.1093/femsre/fux007

39. R Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2020. Available: https://www.R-project.org/

40. Public Health England. SMI B 29: investigation of specimens for screening for MRSA. 2020 May.

41. Wishart DS, Feunang YD, Guo AC, Lo EJ, Marcu A, Grant JR, et al. DrugBank 5.0: a major update to the DrugBank database for 2018. Nucleic Acids Res. 2018;46: D1074–D1082. doi:10.1093/nar/gkx1037

42. Sollid JUE, Furberg AS, Hanssen AM, Johannessen M. Staphylococcus aureus: Determinants of human carriage. Infect Genet Evol. 2014;21: 531–541. doi:10.1016/j.meegid.2013.03.020

43. Pei S, Morone F, Liljeros F, Makse H, Shaman JL. Inference and control of the nosocomial transmission of methicillin-resistant Staphylococcus aureus. Cooper B, Jha P, editors. eLife. 2018;7: e40977. doi:10.7554/eLife.40977

44. Dingle KE, Didelot X, Quan TP, Eyre DW, Stoesser N, Golubchik T, et al. Effects of control interventions on Clostridium difficile infection in England: an observational study. Lancet Infect Dis. 2017;17: 411–421. doi:10.1016/S1473-3099(16)30514-X

45. Public Health England. Clostridioides difficile infection: how to deal with the problem. 2008 Dec. Available: https://www.gov.uk/government/publications/clostridium-difficile-infection-how-to-deal-with-the-problem

46. Khosravi AD, Jenabi A, Montazeri EA. Distribution of genes encoding resistance to aminoglycoside modifying enzymes in methicillin-resistant Staphylococcus aureus (MRSA) strains. Kaohsiung J Med Sci. 2017;33: 587–593. doi:10.1016/j.kjms.2017.08.001

47. Rahimi F. Characterization of Resistance to Aminoglycosides in Methicillin-Resistant Staphylococcus aureus Strains Isolated From a Tertiary Care Hospital in Tehran, Iran. Jundishapur J Microbiol. 2016;9: e29237. doi:10.5812/jjm.29237

48. Evans DR, Griffith MP, Sundermann AJ, Shutt KA, Saul MI, Mustapha MM, et al. Systematic detection of horizontal gene transfer across genera among multidrug-resistant bacteria in a single hospital. Lipsitch M, Ferguson NM, Lipsitch M, editors. eLife. 2020;9: e53886. doi:10.7554/eLife.53886

49. Wichelhaus TA, Böddinghaus B, Besier S, Schäfer V, Brade V, Ludwig A. Biological Cost of Rifampin Resistance from the Perspective of Staphylococcus aureus. Antimicrob Agents Chemother. 2002;46: 3381–3385. doi:10.1128/AAC.46.11.3381-3385.2002

50. Munro APS, Blyth CC, Campbell AJ, Bowen AC. Infection characteristics and treatment of Staphylococcus aureus bacteraemia at a tertiary children's hospital. BMC Infect Dis. 2018;18: 387. doi:10.1186/s12879-018-3312-5

51. Bogaert D, van Belkum A, Sluijter M, Luijendijk A, de Groot R, Rümke H, et al. Colonisation by Streptococcus pneumoniae and Staphylococcus aureus in healthy children. The Lancet. 2004;363: 1871–1872. doi:10.1016/S0140-6736(04)16357-5

52. Suryati B, Watson M. Staphylococcus aureus bacteraemia in children: A 5-year retrospective review. J Paediatr Child Health. 2002;38: 290–294. doi:10.1046/j.1440-1754.2002.00787.x

53. Hill TCJ, Walsh KA, Harris JA, Moffett BF. Using ecological diversity measures with bacterial communities. FEMS Microbiol Ecol. 2003;43: 1–11. doi:10.1111/j.1574-6941.2003.tb01040.x

54. Grundmann H, Aanensen DM, Wijngaard CC van den, Spratt BG, Harmsen D, Friedrich AW, et al. Geographic Distribution of Staphylococcus aureus Causing Invasive Infections in Europe: A Molecular-Epidemiological Analysis. PLOS Med. 2010;7: e1000215. doi:10.1371/journal.pmed.1000215

55. Murray CJ, Ikuta KS, Sharara F, Swetschinski L, Aguilar GR, Gray A, et al. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. The Lancet. 2022;399: 629–655. doi:10.1016/S0140-6736(21)02724-0

56. McCarthy AJ, Lindsay JA. The distribution of plasmids that carry virulence and resistance genes in Staphylococcus aureus is lineage associated. BMC microbiology. 2012;12: 1-8. doi: 10.1186/1471-2180-12-104

57. Worby CJ, Lipsitch M, Hanage WP. Within-Host Bacterial Diversity Hinders Accurate Reconstruction of Transmission Networks from Genomic Distance Data. PLOS Comput Biol. 2014;10: e1003549. doi:10.1371/journal.pcbi.1003549

58. Tonkin-Hill G, Ling C, Chaguza C, Salter SJ, Hinfonthong P, Nikolaou E, et al. Pneumococcal within-host diversity during colonisation, transmission and treatment. bioRxiv. 2022. p. 2022.02.20.480002. doi:10.1101/2022.02.20.480002

59. Feliziani S, Marvig RL, Luján AM, Moyano AJ, Rienzo JAD, Johansen HK, et al. Coexistence and Within-Host Evolution of Diversified Lineages of Hypermutable Pseudomonas aeruginosa in Long-term Cystic Fibrosis Infections. PLOS Genet. 2014;10: e1004651. doi:10.1371/journal.pgen.1004651

60. Cheng S, Fleres G, Chen L, Liu G, Hao B, Newbrough A, et al. Within-host genotypic and phenotypic diversity of contemporaneous carbapenem-resistant *Klebsiella pneumoniae* from blood cultures of patients with bacteremia. bioRxiv. 2022 [cited 31 May 2022]. doi:10.1101/2022.05.26.493675

6 General Discussion

6.1 Summary of findings

Understanding the dynamics of HGT of AMR is essential to design appropriate interventions reducing the health burden of AMR. In Chapter 2, I identified a research gap in our understanding of the dynamics of transduction of AMR, and how to best represent them mathematically (Leclerc, Lindsay and Knight, 2019). Before this thesis, three studies attempted to model transduction of AMR, yet only made theoretical conclusions regarding this process, and were not robustly parameterised as they relied on parameter values from multiple sources and did not generate their own data to inform their model parameters (Volkova *et al.*, 2014; Fillol-Salom *et al.*, 2019; Arya *et al.*, 2020). This lack of fundamental understanding of the dynamics of transduction of AMR is particularly worrying for the nosocomial pathogen *S. aureus*, in which antibiotic-resistance is a major threat and transduction is the main driver of HGT of resistance genes. I suggested that this research gap is notably due to limited availability of appropriate experimental data to inform the design of mathematical models. The optimal solution to overcome this was therefore to create a framework to study transduction of AMR that encompassed both laboratory and modelling work, to design a validated mathematical model of transduction. This model is essential to substantially drive our understanding of this process forward, building upon *in vitro* data to explore phage-bacteria dynamics under many different conditions, and generating hypotheses to guide further lab work. This knowledge can then be used to identify conditions where phage spread AMR genes in a bacterial population via transduction, and inform the design of potential interventions targeting transducing phage to prevent this.

In Chapter 3, by successfully generating *in vitro* data on phage predation and generalised transduction of AMR in *S. aureus*, I was able to represent these dynamics in a mathematical model (Leclerc *et al.*, 2022). I estimated using the model that 1 transducing phage carrying an AMR gene was generated for 10^8 lytic phage. While this was consistent with previous attempts to estimate this (Jiang and Paul, 1998; Mašlaňová *et al.*, 2016), I showed here for the first time that this proportion is sufficient

for multidrug-resistant bacteria to be generated consistently via generalised transduction in *S. aureus*, even in the absence of antibiotics to act as a selection pressure. I also identified surprising equilibria in phage and bacterial numbers, which varied depending on the starting phage concentration. These *in vitro* findings could only be replicated by representing phage predation as a saturated process, and modelling phage burst size as dependent on bacterial growth. To the best of my knowledge, these two elements had been previously described separately (Schrag and Mittler, 1996; Weld, Butts and Heinemann, 2004; Roach *et al.*, 2017), but never before successfully combined in a single mathematical model. My model has therefore significant improved our understanding of phage predation and transduction dynamics, and how to best represent them.

I then extended this analysis of phage-bacteria interactions in Chapter 4, to examine the joint dynamics of antibiotics, phage, and *S. aureus*, as these likely often coexist in various environments (Leclerc, Lindsay and Knight, 2022). I extended my model to represent the effect of antibiotics on bacteria, and generated further *in vitro* data to parameterise this. Antibiotics can act alongside phage both as a selection pressure for double antibiotic-resistant bacteria generated by transduction, and as killers of susceptible bacteria. In this work I reconciled the conflicted existing literature on the joint effect of phage and antibiotics, showing that they although they act in synergy to kill bacteria, by inhibiting bacterial growth the antibiotics also inhibit phage growth. I generated new insights into these complex interactions by introducing the importance of transduction of AMR. I showed that if phage are stuck at a low concentration due to the effect of the antibiotics inhibiting their replication, and are hence unable to exert a strong killing pressure on bacteria, they may instead mostly affect the bacterial population by generating multidrug-resistant bacteria via transduction, which can then replicate to high concentrations as they are given a selective advantage by the antibiotics. Overall, the joint effect of the phage and antibiotics on the bacterial population varied depending on their starting concentration and presence timing, with a trade-off between a higher risk of generating multidrug-resistant bacteria (if phage are introduced before antibiotics), and a slower bacterial killing rate (if antibiotics are introduced before phage). This forms an important hypothesis to guide future *in vitro* experiments and clinical trials of phage therapy, which currently aim to combine phage and antibiotics to treat bacterial infections.

Finally, in Chapter 5 I explored how these experimental and modelling findings may translate to *in vivo* settings by quantifying within-host *S. aureus* AMR diversity and discussing how this may be shaped by multiple factors, including transduction. While previous studies on within-host *S. aureus* diversity have been specifically designed for this purpose and relied on genomic data, but only sampled individuals at one time point (Cespedes *et al.*, 2005; Mongkolrattanothai *et al.*, 2011; Knight *et al.*, 2012; Stanczak-Mrozek *et al.*, 2015; Muenks *et al.*, 2016), here I showed for the first time that within-host AMR diversity in *S. aureus* and changes in that diversity over time could be detected using routinely collected hospital data only. Importantly, this diversity was detected using antibiograms only, and therefore corresponds to phenotypic AMR diversity, which is likely to have clinical implications and affect the treatment of bacterial infections. Of the 906 events where I detected a change in phenotypic resistances in within-host *S. aureus* populations, 74.83% could not be explained by either concurrent antibiotic use selecting for resistant subpopulations, or between-patient transmission of bacteria. In addition, the range of individual resistances which changed suggests that the mechanism driving this evolution can affect all resistances, although not at equal rates. I therefore suggest that generalised transduction may be responsible for this, as it can lead to the transfer of any bacterial gene, with variable rates depending on the gene (Stanczak-Mrozek, Laing and Lindsay, 2017), and has previously been shown to drive substantial within-host evolution in *S. aureus* (McCarthy *et al.*, 2014). More generally, my work in Chapter 5 highlighted the importance of maintaining ongoing discussions with clinicians when attempting to use routinely collected hospital data to support epidemiological analyses, as some trends in AMR may be attributable to changes in testing strategies at the hospital level.

6.2 Strengths and implications

6.2.1 Importance of transduction as a mechanism of AMR spread

My results highlight the necessity to better understand the role of transduction in AMR spread and evolution, and not assume by default that it is too rare to be relevant

compared to other mechanisms of HGT such as conjugation and transformation, as previous work suggested (Volkova *et al.*, 2014). My estimate of 1 transducing phage carrying an AMR gene per 10^8 lytic phage is coherent with previous attempts to quantify this (Jiang and Paul, 1998; Mašlaňová *et al.*, 2016), but the added value of my modelling approach in Chapter 3 is that I have shown this value is sufficient to consistently lead to multidrug-resistant *S. aureus* appearing *in vitro* in less than 8h in the absence of antibiotics, hence without the need for a selection pressure. I further highlighted the importance of transduction in Chapter 4, where I found that multidrug resistance evolution remained possible even at a lower hypothetical transduction probability of 1 transducing phage per 10^9 lytic phage. Finally, I suggested that the absence of a substantial link between antibiotic usage and changes in detected AMR diversity within-host (Chapter 5) implies that this diversity must be shaped by other factors *in vivo*, including frequent generalised transduction.

6.2.2 Improved understanding of phage predation dynamics

The model I developed combines two aspects of phage predation that have only been previously considered separately: a saturated interaction between phage and bacteria, and a link between phage burst size and bacterial growth. This type of saturated interaction was originally suggested in (Roach *et al.*, 2017), and appears to better represent the biology of phage predation over a wide range of bacteria and phage concentrations than the traditionally used linear interaction, since over one time step multiple phage may bind to the same bacteria (Abedon and Katsaounis, 2018). The link between phage predation and bacterial growth has been previously seen in several settings, but to the best of my knowledge never successfully represented using the same logistic function as the one used to represent bacterial growth, reducing burst size as the bacterial population approaches carrying capacity. This is an interesting insight since, in the environment, including in persistent infections, bacteria may spend most of their time in stationary phase (Gefen *et al.*, 2014). These modelling results offer a biological explanation as to how bacteria and phage can coexist for prolonged periods of time across a broad range of environments, without the phage systematically eradicating the bacteria.

6.2.3 Reconciling evidence on the joint effect of phage and antibiotics

I reconciled existing conflicted evidence on the joint effect of phage and antibiotics on bacteria, showing that faster killing of bacteria by phage and antibiotics together is not incompatible with an inhibition of phage replication by antibiotics (Leclerc, Lindsay and Knight, 2022). If the initial concentration of phage is already sufficiently high, this can generate a killing pressure on bacteria to complement the action of antibiotics, without the need for the phage to replicate. This observation is consistent with previous work which described these concentration thresholds governing the interactions between phage and bacteria (Payne and Jansen, 2001). However, this inhibition becomes a problem if the phage concentration is too low and is unable to increase because antibiotics prevent replication, and if we consider the impact of transduction. In that scenario, the multidrug-resistant bacteria initially generated by transduction will replicate to high concentrations, as they will not be affected by antibiotics, and the phage will be at a concentration too low to kill the bacteria faster than they replicate. This creates an opportunity for multidrug-resistant bacteria to persist for potentially long periods of time, during which they may further evolve to gain resistance to phage predation for example, or spread to other individuals or environments without phage, where they may freely replicate. My results are in agreement with experimental work in *S. aureus* exploring the joint effect of phage and antibiotics, which suggested that (Berryhill *et al.*, 2021). However, the novelty of my approach here is that I explored a wide range of possible timings and concentrations, instead of only 30 minutes and fixed concentrations in (Berryhill *et al.*, 2021), and included transduction in these dynamics. My conclusions form an interesting hypothesis regarding the joint role of phage and antibiotics to drive AMR evolution, which is currently being investigated through novel *in vitro* experiments by other members of the research group.

6.2.4 Implications for phage therapy

The results highlighted above on phage predation and the joint effect of antibiotics and phage on bacteria also have important implications for phage therapy. Firstly, as for antibiotics, correct pharmacodynamics representation of the action of phage on

bacteria is important, as this understanding is required to design efficient phage therapies. Consequently, ensuring that phage predation is appropriately represented is essential. For example, failing to account for potential reductions in phage predation that may occur when bacteria are at stationary phase could lead to unsuccessful treatment, if the initial dose of phage is too low to clear the bacterial population. Additionally, since phage and antibiotics are generally given together to patients during phage therapy (Brives and Pourraz, 2020), understanding their joint effect on bacteria is essential. My results can inform the design of phage therapy, by helping to identify optimal timings, concentrations and pairing with antibiotics. These elements should be considered in future clinical trials of phage therapy, as robust data to directly translate these findings from *in vitro* to *in vivo* is not currently available. These trials could include multiple treatment arms, with antibiotics only, antibiotics and phage simultaneously, antibiotics followed by phage, and phage followed by antibiotics, measuring the clearance rate of bacteria.

From a transduction perspective, my results confirm that generalised transduction can consistently lead to the spread of AMR genes, yet to the best of my knowledge there have not been any attempts to evaluate the potential consequences of this process during phage therapy. As highlighted in the Introduction, generalised transduction remains a possibility during phage therapy, as even exclusively lytic phage are capable of this, therefore only excluding temperate phage would not guarantee the absence of transduction, as this would only exclude specialised transduction. Echoing recommendations from previous reviews (Raj and Karunasagar, 2019; Hassan *et al.*, 2021), I suggest that future studies of phage therapy should acknowledge the risk of generalised transduction and evaluate the impact of this on *in vivo* bacterial evolution during therapy, by monitoring the diversity of the bacterial population during treatment to detect potential transduction events. Overall, future studies and clinical trials of phage therapy should investigate varying timings of phage and antibiotics, instead of only investigating their simultaneous application, and consider the risk of transduction during treatment.

6.2.5 Implications for *S. aureus* AMR diversity

The results presented in this thesis highlight the biological dynamics underpinning HGT of AMR by generalised transduction, and suggest that these dynamics may shape *S. aureus* AMR diversity *in vivo*. Understanding this process is essential to understand how new resistances may arise in *S. aureus* populations, and vary in prevalence over time. This in turn will have implications for the incidence of infections by antibiotic-resistant bacteria, and the treatment of these infections. These are questions that span years, if not decades, hence the importance of studying these evolutionary dynamics at that scale, which requires the appropriate data. As part of this thesis, I have shown that routinely collected data can be used to detect phenotypic AMR diversity in *S. aureus* populations within-host. This complements previous studies on this topic, which identified frequent diversity in *S. aureus* populations, but only focused on genotypic diversity (Cespedes *et al.*, 2005; Mongkolrattanothai *et al.*, 2011; Knight *et al.*, 2012; Stanczak-Mrozek *et al.*, 2015; Muenks *et al.*, 2016). The fact that I was able to detect phenotypic diversity on the other hand suggests more clearly that this diversity may have direct clinical implications. This changing diversity highlights the flexible nature of the public health threat of AMR, which requires constant surveillance. Overall, it is encouraging to see that large datasets obtained from routine surveillance can be used to study within-host AMR diversity, as these are more readily available than genotypic data, and can therefore substantially improve our ability to study diversity and its implications for AMR prevalence.

6.3 Limitations and next steps

6.3.1 The impact of antibiotic resistance fitness costs

The results presented in this thesis give rise to many subsequent questions, and could be enriched by further work. Firstly, although here I found that there was no substantial fitness cost to AMR genes carried by my *S. aureus* bacteria, it may still be relevant to explore these, to better understand conditions under which multidrug-resistant bacteria generated by transduction may or may not persist. Previous work has shown that many antibiotic resistance genes in *S. aureus* do not carry a substantial fitness

cost (Knight, Budd and Lindsay, 2013), yet this may vary depending on the environment and the genes (see (Wichelhaus *et al.*, 2002) for an example with rifampicin resistance genes). In addition, fitness costs may be more relevant for other bacteria than *S. aureus* (Melnyk, Wong and Kassen, 2015), which is important to bear in mind when trying to extend the results from this thesis to other species (further discussed below).

6.3.2 Transduction of multiple AMR genes

My experimental and modelling work only focused on tracking two antibiotic resistance genes: *ermB*, granting erythromycin resistance, and *tetK*, granting tetracycline resistance. Although this relative simplicity was necessary to robustly parameterise the model, in reality, *S. aureus* and particularly MRSA carry multiple AMR genes which may be transferred independently, as well as single MGEs carrying multiple resistance genes, such as plasmids (Haaber, Penadés and Ingmer, 2017). The nature of these plasmids should also be considered, as two closely related plasmids will not co-exist stably in a single bacterium (Novick, 1987). This complexity will eventually have to be captured for us to truly understand the importance of transduction in shaping bacterial diversity. Simply extending the models developed as part of this thesis would not be an appropriate solution, as these are compartmental models and so would require an exponential increase in the number of bacteria and phage compartments included for each additional resistance gene represented. In this case, it would rapidly become unfeasible to keep track of all the interactions between the compartments, ensure they are properly included in the equations, and parameterise them all. Instead, agent-based models may allow for greater flexibility in the way the different combinations of resistances are represented. Although an agent-based model would be more computationally expensive, hence simulating a population of 10^9 bacteria would not be feasible, this may still allow us to generate at least an initial description of the rapid shuffling of AMR genes by transduction.

6.3.3 Further experimental work on the joint effect of phage and antibiotics on *S. aureus*

The work conducted in Chapter 4 has given rise to multiple hypotheses regarding the dynamics of antibiotics, phage and bacteria. Further experiments should be conducted to examine how these model hypotheses translate to *in vitro*. This could be done by combining the experimental methods presented in Chapter 3 with those in Chapter 4 by co-culturing the two single-resistant *S. aureus* strains with phage, adding antibiotics at varying concentrations and timepoints during the co-cultures, and counting bacteria and phage over time. These experiments are now being conducted by other members of the research group. Similarities and differences between model-predicted and observed bacteria and phage numbers would allow us to further refine our understanding of the joint effect of phage and antibiotics on bacteria. For example, recent work has found that aminoglycosides directly inhibit phage replication (Kever *et al.*, 2022). This differs from my approach, as I only considered that antibiotics inhibit phage replication indirectly, by inhibiting the bacterial machinery required by phage to replicate. Linked to this, it is important to highlight that I only focused on the effect of two antibiotics: erythromycin and tetracycline, as these were the resistance genes in the bacteria I used in my experiments. Further experiments should also use other antibiotics, as these may interact with phage differently.

6.3.4 The direct impact of antibiotics on transduction

Throughout this thesis, I have only considered that antibiotics interact with bacteria by killing the susceptible and selecting for resistant ones, and with phage by limiting phage growth via the reduction in bacterial growth. However, recent work suggests that antibiotics can directly modify transduction dynamics in *S. aureus*, by inducing a higher proportion of transducing phage relative to lytic phage (Stanczak-Mrozek, Laing and Lindsay, 2017). However, there is still limited data on this interaction, hence I was not able to include it in my model. This effect might vary depending on which antibiotic is added, and the concentration at which it is added. This could be implemented in the model I developed in Chapter 4 by including an additional parameter to increase or decrease the proportion of new phage released upon burst which are transducing

phage carrying an AMR gene, depending on the concentration of antibiotic. However, as for the joint killing effect of phage and antibiotics mentioned in the previous section, new *in vitro* data to parameterise this would still be required.

6.3.5 Decay of bacteria, phage and antibiotics

A notable exclusion in my model is bacteria, phage, and antibiotic decay, as I did not generate data to parameterise these values. However, I included phage and antibiotic decay in the equations for sensitivity testing in Chapters 3 and 4. Bacteria decay could be added in a similar method using a linear decay rate, but would not be easily distinguishable from growth *in vitro* and would hence be difficult to parameterise. Exploring these effects using parameter values obtained from other sources may be acceptable in a first instance, but limits the robustness of the resulting conclusions. These decay rates would likely vary between different *in vitro* and *in vivo* environments. As these would affect phage and antibiotics, they would affect the pressures exerted by these on bacteria, and may therefore affect conditions under which multidrug-resistant bacteria can appear via transduction and be selected for by the antibiotics, as seen in the Supplementary Material of Chapter 4.

6.3.6 The role of the immune system

In addition, I have not included the potential effect of the immune system in my mathematical model. This was because the main focus here was on an *in vitro* setting, where the immune system was absent. Interestingly, previous work has shown that immune cells can target both bacteria and phage (Hodyra-Stefaniak *et al.*, 2015). This may mean that the immune system overall prevents the appearance of new multidrug-resistant bacteria via transduction, by removing them while they are only present at low numbers, but also by neutralising phage and hence preventing transduction. In a therapy setting, it would be necessary to consider the role of the immune system, as previous work has shown that this can be crucial to guarantee bacterial eradication during phage therapy (Roach *et al.*, 2017). My model could be extended to include this, since previous compartmental models have already been designed for this purpose and could hence be integrated here (Banuelos *et al.*, 2021), but the data to

parameterise this would require further, complex microbiological work in consultation with immunologists. Finally, previous work in piglets found that transduction occurred at higher rates *in vivo* than *in vitro* (McCarthy *et al.*, 2014). Although the mechanisms behind these higher rates are currently unknown, the immune system may play a role in this, by acting as a stress mechanism and leading to more frequent release of phage from the bacteria.

6.3.7 Bacterial resistance to phage

In this work, I assumed that all the bacteria in my environment were equally susceptible to phage infection. This is because I have not seen evidence of resistance appearing in my *in vitro* experiments presented in Chapter 3. However, as mentioned in the Introduction of this thesis, it is well-known that phage and bacteria are actively engaged in a constant arms race, each co-evolving to overcome the actions of the other (Koskella and Brockhurst, 2014; Hampton, Watson and Fineran, 2020). Resistance to phage would likely facilitate the appearance of multidrug-resistant bacteria, as these would be able to replicate more freely instead of remaining at low concentrations and being rapidly cleared by phage. This type of further evolution would likely matter over longer periods of time than the 24-48h periods I have considered in my analyses. Mathematical models have been previously used to understand how this co-evolution can lead to bacterial resistance to phage (Cairns *et al.*, 2009), therefore these components could be integrated in my own model.

6.3.8 Stochasticity

All the models I used in this thesis are deterministic. I made this choice as my aim was to capture the fundamental dynamics of generalised transduction and phage predation, and deterministic models can be fitted to data at a lesser computational cost than stochastic models. However, in reality stochasticity may be important to determine if multidrug-resistant bacteria can persist over time, since these are initially present at low numbers only and are therefore at risk of stochastic fade-out (Arya *et al.*, 2020). This variability could be implemented in the model by rewriting the equations to be solved discreetly instead of continuously, and converting rates to probabilities.

However, the parameter values may change, as deterministic models unrealistically allow for fractions of bacteria to be present at any time, hence a small transduction rate can still easily lead to the appearance of multidrug-resistant bacteria over time. On the other hand, once this rate is converted to a probability in a stochastic model, multidrug-resistant bacteria may no longer appear as consistently as seen *in vitro*.

6.3.9 The need for future *in vivo* work

My ability to measure the impact of transduction as a driver of AMR evolution *in vivo* was restricted in this work due to the limited availability of appropriate data. Generating new, detailed data to study evolution of AMR *in vivo* typically requires longitudinal sampling of patients, with substantial costs and resources required. As shown in Chapter 5, I can detect evidence of within-host AMR diversity in routinely collected data from a hospital diagnostics laboratory, but this approach is not powerful enough to fully capture this diversity, understand how it evolves, and identify the drivers of this evolution. In addition, individuals are not routinely screened for phage, hence it is difficult to identify instances where transduction occurred *in vivo*. I think that extending the coverage of routinely collected data to better capture diversity (e.g. by systematically subculturing multiple isolates per patient sample, and screening samples for phage) may represent an interesting opportunity to strengthen this research, whilst still generating data that will be clinically relevant. In the case of antibiotic treatment for example, the natural presence of phage capable of transduction may explain instances of treatment failure, if these generate multidrug-resistant strains which are then selected for by the antibiotics. Naturally, this extension of routine surveillance would require substantial investments, but would also reduce the gap between the research and clinical domains, and may therefore lead to a more efficient and cost-effective process to generate interventions improving the health of patients.

6.3.10 Transduction in other bacteria, phage, and environments

Finally, it is important to note that all the questions answered in this thesis have focused on *S. aureus*. Transduction is known to occur in other bacterial species and with other phage, but it is unclear to what extent the findings here apply to these other organisms. In *E. coli*, transduction of AMR is currently considered as negligible, due to the previous modelling study which suggested that it happens 1000 times less than conjugation (Volkova *et al.*, 2014). However, as pointed out repeatedly in this thesis I believe that this study lacks data to support this conclusion, as it was not robustly parameterised. In any case, even if transduction in *E. coli* really does occur 1000 times less than conjugation, this may still be sufficient to consistently lead to AMR spread under various conditions, and should therefore be studied. Although the general principles of the experimental framework I developed to study transduction may be applicable to other bacteria (co-culture two single-resistant strains with phage, observe double-resistant bacteria generated by transduction, count phage and bacteria over time), the exact experimental methods have been designed for *S. aureus* and 80α phage specifically, and will certainly be different for other organisms. On the other hand, the structure of the model I developed is generalisable to other systems of generalised transducing phage and bacteria, as it captures what I believe to be the generalisable, relevant biological characteristics of phage predation and generalised transduction, and could therefore be adapted to focus on other strains of bacteria and phage showing similar dynamics of lysis and generalised transduction. However, the parameter values I have estimated for bacterial growth and phage predation will most likely vary depending on the phage, bacteria, and environment studied.

Differences in the biological characteristics of the interaction between bacteria and phage may impact the results presented here. For example, repeating my experiments using a phage which frequently undergoes lysogeny, as opposed to 80α for which I could not detect lysogeny in my system, may lead to a greater concentration of multidrug-resistant bacteria, as these may be protected from killing by phage via lysogenic immunity. This has been previously suggested by the observation of auto-transduction (Haaber *et al.*, 2016). On the other hand, a more lytic phage may minimise the importance of transduction, as multidrug-resistant bacteria generated by

transduction may be rapidly killed by phage before they can replicate to reach high concentrations. Changes in phage burst size may impact these dynamics more unpredictably, since a higher burst size would simultaneously lead to more lytic and transducing phage, generating simultaneously a higher killing pressure on bacteria, and a higher incidence of transduction.

Overall, the biology of phage and bacteria can substantially vary depending on the organisms and the environment. Although understanding the dynamics of transduction across species and environments will require further work, I have shown in this thesis that these invisible dynamics can be revealed by combining mathematical modelling and *in vitro* work. My work focusing on *S. aureus* therefore forms an important basis which future studies in other species can build upon.

6.4 Conclusion

In conclusion, the work presented in this thesis further highlighted the importance of generalised transduction as a key mechanism of AMR spread in *S. aureus*, capable of consistently leading to multidrug-resistant bacteria. By combining *in vitro* work and mathematical models, this work has revealed the invisible dynamics of generalised transduction, but also the dynamics of phage predation in *S. aureus*. This extended modelling work has reconciled conflicted literature on the joint effect of phage and antibiotics on bacteria, whilst considering the added complexity of transduction, with implications for future research on this topic and phage therapy. Finally, this work suggests that transduction may contribute to frequent evolution of AMR in *S. aureus* populations within-host, by shaping the substantial diversity I detected using phenotypic data from routine hospital surveillance.

Overall, through interdisciplinary investigations combining mathematical modelling, *in vitro* work and analysis of routinely collected data, I generated novel insights into the contribution of phage predation dynamics and generalised transduction to the evolution of antimicrobial resistance in *Staphylococcus aureus*.

7 References

Abedon, S.T. (2019) ‘Phage-Antibiotic Combination Treatments: Antagonistic Impacts of Antibiotics on the Pharmacodynamics of Phage Therapy?’, *Antibiotics*, 8(4), p. 182. Available at: https://doi.org/10.3390/antibiotics8040182.

Abedon, S.T., Danis-Wlodarczyk, K.M. and Wozniak, D.J. (2021) ‘Phage Cocktail Development for Bacteriophage Therapy: Toward Improving Spectrum of Activity Breadth and Depth’, *Pharmaceuticals*, 14(10), p. 1019. Available at: https://doi.org/10.3390/ph14101019.

Abedon, S.T. and Katsaounis, T.I. (2018) ‘Basic Phage Mathematics’, in M.R.J. Clokie, A.M. Kropinski, and R. Lavigne (eds) *Bacteriophages: Methods and Protocols, Volume 3*. New York, NY: Springer (Methods in Molecular Biology), pp. 3–30. Available at: https://doi.org/10.1007/978-1-4939-7343-9_1.

Adaptive Phage Therapeutics, Inc. (2022) *An Open-Label Multicenter Study to Evaluate the Safety and Efficacy of PhageBank^{TM} Phage Therapy in Conjunction With Debridement, Antibiotics, and Implant Retention (DAIR) for Patients With First Time Culture Proven Chronic Prosthetic Joint Infection*. Clinical trial registration NCT05269121. clinicaltrials.gov. Available at: https://clinicaltrials.gov/ct2/show/NCT05269121 (Accessed: 28 March 2022).

Alibayov, B. *et al.* (2014) ‘Staphylococcus aureus mobile genetic elements’, *Molecular Biology Reports*, 41(8), pp. 5005–5018. Available at: https://doi.org/10.1007/s11033-014-3367-3.

Alonso, A. *et al.* (2004) ‘Overexpression of the multidrug efflux pump SmeDEF impairs Stenotrophomonas maltophilia physiology’, *The Journal of Antimicrobial Chemotherapy*, 53(3), pp. 432–434. Available at: https://doi.org/10.1093/jac/dkh074.

Anderson, R.M. and May, R.M. (2010) *Infectious diseases of humans: dynamics and control*. Reprinted. Oxford: Oxford Univ. Press.

Andersson, D.I. and Hughes, D. (2010) ‘Antibiotic resistance and its cost: is it possible to reverse resistance?’, *Nature Reviews Microbiology*, 8(4), pp. 260–271. Available at: https://doi.org/10.1038/nrmicro2319.

Arya, S. *et al.* (2020) ‘A generalised model for generalised transduction: the importance of co-evolution and stochasticity in phage mediated antimicrobial resistance transfer’, *FEMS Microbiology Ecology*, 96(fiaa100). Available at: https://doi.org/10.1093/femsec/fiaa100.

Bacaër, N. (2011) ‘Lotka, Volterra and the predator–prey system (1920–1926)’, in N. Bacaër (ed.) *A Short History of Mathematical Population Dynamics*. London: Springer, pp. 71–76. Available at: https://doi.org/10.1007/978-0-85729-115-8_13.

Baird, R.M. and Lee, W.H. (1995) ‘Media used in the detection and enumeration of Staphylococcus aureus’, *International Journal of Food Microbiology*, 26(1), pp. 15–24. Available at: https://doi.org/10.1016/0168-1605(93)E0028-P.

Banuelos, S. *et al.* (2021) 'Investigating the Impact of Combination Phage and Antibiotic Therapy: A Modeling Study', in R. Segal, B. Shtylla, and S. Sindi (eds) *Using Mathematics to Understand Biological Complexity: From Cells to Populations*. Cham: Springer International Publishing (Association for Women in Mathematics Series), pp. 111–134. Available at: https://doi.org/10.1007/978-3-030-57129-0_6.

Barber, M.Y.R. (1964) 'Naturally Occurring Methicillin-Resistant Staphylococci', *Microbiology*, 35(2), pp. 183–190. Available at: https://doi.org/10.1099/00221287-35-2-183.

Barrangou, R. *et al.* (2007) 'CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes', *Science*, 315(5819), pp. 1709–1712. Available at: https://doi.org/10.1126/science.1138140.

Beck, W.D., Berger-Bächi, B. and Kayser, F.H. (1986) 'Additional DNA in methicillin-resistant Staphylococcus aureus and molecular cloning of mec-specific DNA.', *Journal of Bacteriology*, 165(2), pp. 373–378.

Berryhill, B.A. *et al.* (2021) 'Evaluating the potential efficacy and limitations of a phage for joint antibiotic and phage therapy of Staphylococcus aureus infections', *Proceedings of the National Academy of Sciences*, 118(10). Available at: https://doi.org/10.1073/pnas.2008007118.

Bierowiec, K., Płoneczka-Janeczko, K. and Rypuła, K. (2016) 'Is the Colonisation of Staphylococcus aureus in Pets Associated with Their Close Contact with Owners?', *PLoS ONE*, 11(5), p. e0156052. Available at: https://doi.org/10.1371/journal.pone.0156052.

Birkegård, A.C. *et al.* (2018) 'Send more data: a systematic review of mathematical models of antimicrobial resistance', *Antimicrobial Resistance & Infection Control*, 7(1), p. 117. Available at: https://doi.org/10.1186/s13756-018-0406-1.

Bondy-Denomy, J. *et al.* (2013) 'Bacteriophage genes that inactivate the CRISPR/Cas bacterial immune system', *Nature*, 493(7432), pp. 429–432. Available at: https://doi.org/10.1038/nature11723.

Brives, C. and Pourraz, J. (2020) 'Phage therapy as a potential solution in the fight against AMR: obstacles and possible futures', *Palgrave Communications*, 6(1), pp. 1–11. Available at: https://doi.org/10.1057/s41599-020-0478-4.

Brockhurst, M.A. and Harrison, E. (2021) 'Ecological and evolutionary solutions to the plasmid paradox', *Trends in Microbiology*, 0(0). Available at: https://doi.org/10.1016/j.tim.2021.11.001.

Brooks, R.J. and Tobias, A.M. (1996) 'Choosing the best model: Level of detail, complexity, and model performance', *Mathematical and Computer Modelling*, 24(4), pp. 1–14. Available at: https://doi.org/10.1016/0895-7177(96)00103-3.

Bruinsma, N. *et al.* (2004) 'Trends of penicillin and erythromycin resistance among invasive Streptococcus pneumoniae in Europe', *Journal of Antimicrobial*

Chemotherapy, 54(6), pp. 1045–1050. Available at: https://doi.org/10.1093/jac/dkh458.

Cairns, B.J. *et al.* (2009) 'Quantitative Models of In Vitro Bacteriophage–Host Dynamics and Their Application to Phage Therapy', *PLOS Pathogens*, 5(1), p. e1000253. Available at: https://doi.org/10.1371/journal.ppat.1000253.

Casjens, S.R. and Gilcrease, E.B. (2009) 'Determining DNA Packaging Strategy by Analysis of the Termini of the Chromosomes in Tailed-Bacteriophage Virions', *Methods in molecular biology (Clifton, N.J.)*, 502, pp. 91–111. Available at: https://doi.org/10.1007/978-1-60327-565-1_7.

Cassini, A. *et al.* (2019) 'Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis', *The Lancet Infectious Diseases*, 19(1), pp. 56–66. Available at: https://doi.org/10.1016/S1473-3099(18)30605-4.

Catalán, P. *et al.* (2022) 'Seeking patterns of antibiotic resistance in ATLAS, an open, raw MIC database with patient metadata', *Nature Communications*, 13(1), p. 2917. Available at: https://doi.org/10.1038/s41467-022-30635-7.

Centre Hospitalier Universitaire de Nīmes (2022) *Comparison of the Efficacy of Standard Treatment Associated With Phage Therapy Versus Standard Treatment Plus Placebo for Diabetic Foot Ulcers Monoinfected by Staphylococcus Aureus: a Randomized, Multi-centre, Controlled, 2-parallel-group, Double-blind, Superiority Trial*. Clinical trial registration NCT02664740. clinicaltrials.gov. Available at: https://clinicaltrials.gov/ct2/show/NCT02664740 (Accessed: 28 March 2022).

Cespedes, C. *et al.* (2005) 'The Clonality of Staphylococcus aureus Nasal Carriage', *The Journal of Infectious Diseases*, 191(3), pp. 444–452. Available at: https://doi.org/10.1086/427240.

Chae, C. *et al.* (2020) 'Effect of Pediatric Influenza Vaccination on Antibiotic Resistance, England and Wales', *Emerging Infectious Diseases*, 26(1), pp. 138–142. Available at: https://doi.org/10.3201/eid2601.191110.

Chambers, H.F. and DeLeo, F.R. (2009) 'Waves of Resistance: Staphylococcus aureus in the Antibiotic Era', *Nature reviews. Microbiology*, 7(9), pp. 629–641. Available at: https://doi.org/10.1038/nrmicro2200.

Chen, J. *et al.* (2018) 'Genome hypermobility by lateral transduction', *Science*, 362(6411), pp. 207–212. Available at: https://doi.org/10.1126/science.aat5867.

Clinical and Laboratory Standards Institute (2022) *M100 Performance Standards for Antimicrobial Susceptibility Testing*. 32nd edn. Available at: https://www.nih.org.pk/wp-content/uploads/2021/02/CLSI-2020.pdf (Accessed: 26 May 2022).

Clokie, M.R.J. *et al.* (2011) 'Phages in nature', *Bacteriophage*, 1(1), pp. 31–45. Available at: https://doi.org/10.4161/bact.1.1.14942.

Collignon, P. *et al.* (2018) ‘Anthropological and socioeconomic factors contributing to global antimicrobial resistance: a univariate and multivariable analysis’, *The Lancet Planetary Health*, 2(9), pp. e398–e405. Available at: https://doi.org/10.1016/S2542-5196(18)30186-4.

Couderc, C. *et al.* (2014) ‘Fluoroquinolone Use Is a Risk Factor for Methicillin-Resistant Staphylococcus aureus Acquisition in Long-term Care Facilities: A Nested Case-Case-Control Study’, *Clinical Infectious Diseases*, 59(2), pp. 206–215. Available at: https://doi.org/10.1093/cid/ciu236.

Coyte, K.Z. *et al.* (2022) ‘Horizontal gene transfer increases microbiome stability’, *bioRxiv*, p. 2022.02.25.481914. Available at: https://doi.org/10.1101/2022.02.25.481914.

Dale, G.E. *et al.* (1995) ‘Characterization of the gene for the chromosomal dihydrofolate reductase (DHFR) of Staphylococcus epidermidis ATCC 14990: the origin of the trimethoprim-resistant S1 DHFR from Staphylococcus aureus?’, *Journal of Bacteriology*, 177(11), pp. 2965–2970.

Dalen, R. van, Peschel, A. and Sorge, N.M. van (2020) ‘Wall Teichoic Acid in Staphylococcus aureus Host Interaction’, *Trends in Microbiology*, 28(12), pp. 985–998. Available at: https://doi.org/10.1016/j.tim.2020.05.017.

Davies, N.G. *et al.* (2019) ‘Within-host dynamics shape antibiotic resistance in commensal bacteria’, *Nature Ecology & Evolution*, 3(3), pp. 440–449. Available at: https://doi.org/10.1038/s41559-018-0786-x.

Davis, M.F. *et al.* (2012) ‘Household transmission of meticillin-resistant Staphylococcus aureus and other staphylococci’, *The Lancet Infectious Diseases*, 12(9), pp. 703–716. Available at: https://doi.org/10.1016/S1473-3099(12)70156-1.

Dawson, M.H. and Hobby, G.L. (1944) ‘The Clinical Use of Penicillin: Observations in One Hundred Cases’, *Journal of the American Medical Association*, 124(10), pp. 611–622. Available at: https://doi.org/10.1001/jama.1944.02850100001001.

Deghorain, M. and Van Melderen, L. (2012) ‘The Staphylococci Phages Family: An Overview’, *Viruses*, 4(12), pp. 3316–3335. Available at: https://doi.org/10.3390/v4123316.

Diep, B.A. *et al.* (2006) ‘Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant Staphylococcus aureus’, *The Lancet*, 367(9512), pp. 731–739. Available at: https://doi.org/10.1016/S0140-6736(06)68231-7.

Do, N.T.T. *et al.* (2021) ‘Community-based antibiotic access and use in six low-income and middle-income countries: a mixed-method approach’, *The Lancet Global Health*, 9(5), pp. e610–e619. Available at: https://doi.org/10.1016/S2214-109X(21)00024-3.

Dorado-Morales, P. *et al.* (2021) ‘Fitness Cost Evolution of Natural Plasmids of Staphylococcus aureus’, *mBio*, 12(1), pp. e03094-20. Available at: https://doi.org/10.1128/mBio.03094-20.

Dubois, D. *et al.* (2010) 'Identification of a Variety of Staphylococcus Species by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry', *Journal of Clinical Microbiology*, 48(3), pp. 941–945. Available at: https://doi.org/10.1128/JCM.00413-09.

von Eiff, C. *et al.* (2001) 'Nasal Carriage as a Source of Staphylococcus aureus Bacteremia', *New England Journal of Medicine*, 344(1), pp. 11–16. Available at: https://doi.org/10.1056/NEJM200101043440102.

Enright, M.C. *et al.* (2000) 'Multilocus Sequence Typing for Characterization of Methicillin-Resistant and Methicillin-Susceptible Clones of Staphylococcus aureus', *Journal of Clinical Microbiology*, 38(3), pp. 1008–1015.

EUCAST: Clinical breakpoints and dosing of antibiotics (2022). Available at: https://www.eucast.org/clinical_breakpoints/ (Accessed: 26 May 2022).

European Centre for Disease Prevention and Control (2022) *Surveillance Atlas of Infectious Diseases*. Available at: https://atlas.ecdc.europa.eu/public/index.aspx?Dataset=27&HealthTopic=4 (Accessed: 24 March 2022).

Feil, E.J. *et al.* (2003) 'How Clonal Is Staphylococcus aureus?', *Journal of Bacteriology*, 185(11), pp. 3307–3316. Available at: https://doi.org/10.1128/JB.185.11.3307-3316.2003.

Fillol-Salom, A. *et al.* (2019) 'Bacteriophages benefit from generalized transduction', *PLOS Pathogens*, 15(7), p. e1007888. Available at: https://doi.org/10.1371/journal.ppat.1007888.

Fish, R. *et al.* (2016) 'Bacteriophage treatment of intransigent diabetic toe ulcers: a case series', *Journal of Wound Care*, 25(Sup7), pp. S27–S33. Available at: https://doi.org/10.12968/jowc.2016.25.Sup7.S27.

Fleming, A. (1929) 'On the Antibacterial Action of Cultures of a Penicillium, with Special Reference to their Use in the Isolation of B. influenzæ', *British journal of experimental pathology*, 10(3), pp. 226–236.

Foster, T. (1996) 'Staphylococcus', in S. Baron (ed.) *Medical Microbiology*. 4th edn. Galveston (TX): University of Texas Medical Branch at Galveston. Available at: http://www.ncbi.nlm.nih.gov/books/NBK8448/ (Accessed: 26 May 2022).

Foster, T.J. (2017) 'Antibiotic resistance in Staphylococcus aureus. Current status and future prospects', *FEMS Microbiology Reviews*, 41(3), pp. 430–449. Available at: https://doi.org/10.1093/femsre/fux007.

Fournier, B. and Philpott, D.J. (2005) 'Recognition of Staphylococcus aureus by the Innate Immune System', *Clinical Microbiology Reviews*, 18(3), pp. 521–540. Available at: https://doi.org/10.1128/CMR.18.3.521-540.2005.

French, G.L. (1998) 'Enterococci and Vancomycin Resistance', *Clinical Infectious Diseases*, 27(Supplement_1), pp. S75–S83. Available at: https://doi.org/10.1086/514910.

Fritz, S.A. *et al.* (2014) 'Contamination of Environmental Surfaces With Staphylococcus aureus in Households With Children Infected With Methicillin-Resistant S aureus', *JAMA Pediatrics*, 168(11), pp. 1030–1038. Available at: https://doi.org/10.1001/jamapediatrics.2014.1218.

Gefen, O. *et al.* (2014) 'Direct observation of single stationary-phase bacteria reveals a surprisingly long period of constant protein production activity', *Proceedings of the National Academy of Sciences*, 111(1), pp. 556–561. Available at: https://doi.org/10.1073/pnas.1314114111.

Gelman, A. *et al.* (2015) *Bayesian Data Analysis*. 3rd edn. New York: Chapman and Hall/CRC. Available at: https://doi.org/10.1201/b16018.

Gelman, D. *et al.* (2021) 'Clinical Phage Microbiology: a suggested framework and recommendations for the in-vitro matching steps of phage therapy', *The Lancet Microbe*, 2(10), pp. e555–e563. Available at: https://doi.org/10.1016/S2666-5247(21)00127-0.

Gentry, D.R. *et al.* (2008) 'Genetic Characterization of Vga ABC Proteins Conferring Reduced Susceptibility to Pleuromutilins in Staphylococcus aureus', *Antimicrobial Agents and Chemotherapy*, 52(12), pp. 4507–4509. Available at: https://doi.org/10.1128/AAC.00915-08.

Ghasemnejad, A., Doudi, M. and Amirmozafari, N. (2019) 'The role of the blaKPC gene in antimicrobial resistance of Klebsiella pneumoniae', *Iranian Journal of Microbiology*, 11(4), pp. 288–293.

van Griethuysen, A. *et al.* (1999) 'Rapid Slide Latex Agglutination Test for Detection of Methicillin Resistance in Staphylococcus aureus', *Journal of Clinical Microbiology*, 37(9), pp. 2789–2792.

van Griethuysen, A. *et al.* (2001) 'International Multicenter Evaluation of Latex Agglutination Tests for Identification of Staphylococcus aureus', *Journal of Clinical Microbiology*, 39(1), pp. 86–89. Available at: https://doi.org/10.1128/JCM.39.1.86-89.2001.

Griffith, Fred. (1928) 'The Significance of Pneumococcal Types', *The Journal of Hygiene*, 27(2), pp. 113–159.

Griffiths, A.J. *et al.* (2000) 'Transduction', in *An Introduction to Genetic Analysis*.

Grundmann, H. *et al.* (2010) 'Geographic Distribution of Staphylococcus aureus Causing Invasive Infections in Europe: A Molecular-Epidemiological Analysis', *PLOS Medicine*, 7(1), p. e1000215. Available at: https://doi.org/10.1371/journal.pmed.1000215.

Haaber, J. *et al.* (2016) 'Bacterial viruses enable their host to acquire antibiotic resistance genes from neighbouring cells', *Nature Communications*, 7(1), p. 13333. Available at: https://doi.org/10.1038/ncomms13333.

Haaber, J., Penadés, J.R. and Ingmer, H. (2017) 'Transfer of Antibiotic Resistance in Staphylococcus aureus', *Trends in Microbiology*, 25(11), pp. 893–905. Available at: https://doi.org/10.1016/j.tim.2017.05.011.

Hadas, H. *et al.* (1997) 'Bacteriophage T4 Development Depends on the Physiology of its Host Escherichia Coli', *Microbiology*, 143(1), pp. 179–185. Available at: https://doi.org/10.1099/00221287-143-1-179.

Hall, J.P.J., Brockhurst, M.A. and Harrison, E. (2017) 'Sampling the mobile gene pool: innovation via horizontal gene transfer in bacteria', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 372(1735), p. 20160424. Available at: https://doi.org/10.1098/rstb.2016.0424.

Hall, M.D. *et al.* (2019) 'Improved characterisation of MRSA transmission using within-host bacterial sequence diversity', *eLife*, 8, p. e46402. Available at: https://doi.org/10.7554/eLife.46402.

Hampton, H.G., Watson, B.N.J. and Fineran, P.C. (2020) 'The arms race between bacteria and their phage foes', *Nature*, 577(7790), pp. 327–336. Available at: https://doi.org/10.1038/s41586-019-1894-8.

Hanssen, A.-M. and Ericson Sollid, J.U. (2006) 'SCCmec in staphylococci: genes on the move', *FEMS Immunology & Medical Microbiology*, 46(1), pp. 8–20. Available at: https://doi.org/10.1111/j.1574-695X.2005.00009.x.

Harmsen, D. *et al.* (2003) 'Typing of Methicillin-Resistant Staphylococcus aureus in a University Hospital Setting by Using Novel Software for spa Repeat Determination and Database Management', *Journal of Clinical Microbiology*, 41(12), pp. 5442–5448. Available at: https://doi.org/10.1128/JCM.41.12.5442-5448.2003.

Hassan, A.Y. *et al.* (2021) 'The Age of Phage: Friend or Foe in the New Dawn of Therapeutic and Biocontrol Applications?', *Pharmaceuticals*, 14(3), p. 199. Available at: https://doi.org/10.3390/ph14030199.

Heesterbeek, H. *et al.* (2015) 'Modeling infectious disease dynamics in the complex landscape of global health', *Science (New York, N.Y.)*, 347(6227), p. aaa4339. Available at: https://doi.org/10.1126/science.aaa4339.

den Heijer, C.D.J. *et al.* (2013) 'Prevalence and resistance of commensal Staphylococcus aureus, including meticillin-resistant S aureus, in nine European countries: a cross-sectional study', *The Lancet. Infectious Diseases*, 13(5), pp. 409–415. Available at: https://doi.org/10.1016/S1473-3099(13)70036-7.

Herrell, W.E. (1944) 'The Clinical Use of Penicillin: an Antibacterial Agent of Biologic Origin', *Journal of the American Medical Association*, 124(10), pp. 622–627. Available at: https://doi.org/10.1001/jama.1944.02850100012002.

Hodyra-Stefaniak, K. *et al.* (2015) 'Mammalian Host-Versus-Phage immune response determines phage fate in vivo', *Scientific Reports*, 5(1), p. 14802. Available at: https://doi.org/10.1038/srep14802.

Holden, M.T.G. *et al.* (2004) 'Complete genomes of two clinical Staphylococcus aureus strains: Evidence for the rapid evolution of virulence and drug resistance', *Proceedings of the National Academy of Sciences of the United States of America*, 101(26), pp. 9786–9791. Available at: https://doi.org/10.1073/pnas.0402521101.

Howard-Varona, C. *et al.* (2017) 'Lysogeny in nature: mechanisms, impact and ecology of temperate phages', *The ISME Journal*, 11(7), pp. 1511–1520. Available at: https://doi.org/10.1038/ismej.2017.16.

Huang, S.S. and Platt, R. (2003) 'Risk of methicillin-resistant Staphylococcus aureus infection after previous infection or colonization', *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, 36(3), pp. 281–285. Available at: https://doi.org/10.1086/345955.

Huddleston, J.R. (2014) 'Horizontal gene transfer in the human gastrointestinal tract: potential spread of antibiotic resistance genes', *Infection and Drug Resistance*, p. 167. Available at: https://doi.org/10.2147/IDR.S48820.

Hutchings, M.I., Truman, A.W. and Wilkinson, B. (2019) 'Antibiotics: past, present and future', *Current Opinion in Microbiology*, 51, pp. 72–80. Available at: https://doi.org/10.1016/j.mib.2019.10.008.

Hyman, P. and Abedon, S.T. (2012) 'Smaller Fleas: Viruses of Microorganisms', *Scientifica*, 2012, p. e734023. Available at: https://doi.org/10.6064/2012/734023.

International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (2009) 'Classification of Staphylococcal Cassette Chromosome mec (SCCmec): Guidelines for Reporting Novel SCCmec Elements', *Antimicrobial Agents and Chemotherapy*, 53(12), pp. 4961–4967. Available at: https://doi.org/10.1128/AAC.00579-09.

Jacoby, G.A. (2009) 'AmpC β-Lactamases', *Clinical Microbiology Reviews*, 22(1), pp. 161–182. Available at: https://doi.org/10.1128/CMR.00036-08.

Jassim, S.A.A. and Limoges, R.G. (2014) 'Natural solution to antibiotic resistance: bacteriophages "The Living Drugs"', *World Journal of Microbiology and Biotechnology*, 30(8), pp. 2153–2170. Available at: https://doi.org/10.1007/s11274-014-1655-7.

Jiang, S.C. and Paul, J.H. (1998) 'Gene Transfer by Transduction in the Marine Environment', *Applied and Environmental Microbiology*, 64(8), pp. 2780–2787. Available at: https://doi.org/10.1128/AEM.64.8.2780-2787.1998.

Joice, R. and Lipsitch, M. (2013) 'Targeting Imperfect Vaccines against Drug-Resistance Determinants: A Strategy for Countering the Rise of Drug Resistance', *PLOS ONE*, 8(7), p. e68940. Available at: https://doi.org/10.1371/journal.pone.0068940.

Katayama, Y., Ito, T. and Hiramatsu, K. (2000) 'A New Class of Genetic Element, Staphylococcus Cassette Chromosome mec, Encodes Methicillin Resistance in Staphylococcus aureus', *Antimicrobial Agents and Chemotherapy*, 44(6), pp. 1549–1555.

Keeling, M.J. and Rohani, P. (2008) *Modeling Infectious Diseases in Humans and Animals*, *Modeling Infectious Diseases in Humans and Animals*. Princeton University Press. Available at: https://doi.org/10.1515/9781400841035.

Kermack, W.O. and McKendrick, A.G. (1927) 'A contribution to the mathematical theory of epidemics', *Proceedings of the Royal Society of London. Series A*, 115(772), pp. 700–721. Available at: https://doi.org/10.1098/rspa.1927.0118.

Kever, L. *et al.* (2022) 'Aminoglycoside Antibiotics Inhibit Phage Infection by Blocking an Early Step of the Infection Cycle', *mBio*, 0(0), pp. e00783-22. Available at: https://doi.org/10.1128/mbio.00783-22.

Khan, S.A. and Novick, R.P. (1983) 'Complete nucleotide sequence of pT181, a tetracycline-resistance plasmid from Staphylococcus aureus', *Plasmid*, 10(3), pp. 251–259. Available at: https://doi.org/10.1016/0147-619X(83)90039-2.

Kimmig, A. *et al.* (2021) 'Management of Staphylococcus aureus Bloodstream Infections', *Frontiers in Medicine*, 7. Available at: https://www.frontiersin.org/article/10.3389/fmed.2020.616524 (Accessed: 26 April 2022).

King, S. *et al.* (2016) 'Antimicrobial Stewardship', *Rand Health Quarterly*, 5(3), p. 2.

Kirkeby, C. *et al.* (2017) 'Methods for estimating disease transmission rates: Evaluating the precision of Poisson regression and two novel methods', *Scientific Reports*, 7, p. 9496. Available at: https://doi.org/10.1038/s41598-017-09209-x.

van Kleef, E. *et al.* (2013) 'Modelling the transmission of healthcare associated infections: a systematic review', *BMC Infectious Diseases*, 13(1), p. 294. Available at: https://doi.org/10.1186/1471-2334-13-294.

Kluytmans, J., van Belkum, A. and Verbrugh, H. (1997) 'Nasal carriage of Staphylococcus aureus: epidemiology, underlying mechanisms, and associated risks.', *Clinical Microbiology Reviews*, 10(3), pp. 505–520.

Knight, G.M. *et al.* (2012) 'Shift in dominant hospital-associated methicillin-resistant Staphylococcus aureus (HA-MRSA) clones over time', *Journal of Antimicrobial Chemotherapy*, 67(10), pp. 2514–2522. Available at: https://doi.org/10.1093/jac/dks245.

Knight, G.M. *et al.* (2019) 'Mathematical modelling for antibiotic resistance control policy: do we know enough?', *BMC Infectious Diseases*, 19(1), p. 1011. Available at: https://doi.org/10.1186/s12879-019-4630-y.

Knight, G.M., Budd, E.L. and Lindsay, J.A.Y. 2013 (2013) 'Large mobile genetic elements carrying resistance genes that do not confer a fitness burden in healthcare-associated meticillin-resistant Staphylococcus aureus', *Microbiology*, 159(Pt_8), pp. 1661–1672. Available at: https://doi.org/10.1099/mic.0.068551-0.

Kokjohn, T.A. and Sayler, G.S.Y. (1991) 'Attachment and replication of Pseudomonas aeruginosa bacteriophages under conditions simulating aquatic environments',

Microbiology, 137(3), pp. 661–666. Available at: https://doi.org/10.1099/00221287-137-3-661.

Kondo, K., Kawano, M. and Sugai, M. (2021) 'Distribution of Antimicrobial Resistance and Virulence Genes within the Prophage-Associated Regions in Nosocomial Pathogens', *mSphere*, 6(4), pp. e00452-21. Available at: https://doi.org/10.1128/mSphere.00452-21.

Kong, E.F., Johnson, J.K. and Jabra-Rizk, M.A. (2016) 'Community-Associated Methicillin-Resistant Staphylococcus aureus: An Enemy amidst Us', *PLoS Pathogens*, 12(10), p. e1005837. Available at: https://doi.org/10.1371/journal.ppat.1005837.

Koskella, B. and Brockhurst, M.A. (2014) 'Bacteria–phage coevolution as a driver of ecological and evolutionary processes in microbial communities', *FEMS Microbiology Reviews*, 38(5), pp. 916–931. Available at: https://doi.org/10.1111/1574-6976.12072.

Kuhl, S.A., Pattee, P.A. and Baldwin, J.N. (1978) 'Chromosomal map location of the methicillin resistance determinant in Staphylococcus aureus.', *Journal of Bacteriology*, 135(2), pp. 460–465.

Kwan, T. *et al.* (2005) 'The complete genomes and proteomes of 27 Staphylococcus aureus bacteriophages', *Proceedings of the National Academy of Sciences*, 102(14), pp. 5174–5179. Available at: https://doi.org/10.1073/pnas.0501140102.

LaMarre, J. *et al.* (2013) 'The Genetic Environment of the cfr Gene and the Presence of Other Mechanisms Account for the Very High Linezolid Resistance of Staphylococcus epidermidis Isolate 426-3147L', *Antimicrobial Agents and Chemotherapy*, 57(3), p. 1173. Available at: https://doi.org/10.1128/AAC.02047-12.

Larsen, J. *et al.* (2022) 'Emergence of methicillin resistance predates the clinical use of antibiotics', *Nature*, 602(7895), pp. 135–141. Available at: https://doi.org/10.1038/s41586-021-04265-w.

Larsson, D.G.J. (2014) 'Pollution from drug manufacturing: review and perspectives', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 369(1656), p. 20130571. Available at: https://doi.org/10.1098/rstb.2013.0571.

Leclerc, Q.J. *et al.* (2020) 'Feasibility of informing syndrome-level empiric antibiotic recommendations using publicly available antibiotic resistance datasets', *Wellcome Open Research*, 4, p. 140. Available at: https://doi.org/10.12688/wellcomeopenres.15477.2.

Leclerc, Q.J. *et al.* (2021) 'Importance of patient bed pathways and length of stay differences in predicting COVID-19 hospital bed occupancy in England', *BMC Health Services Research*, 21(1), p. 566. Available at: https://doi.org/10.1186/s12913-021-06509-x.

Leclerc, Q.J. *et al.* (2022) 'Growth-Dependent Predation and Generalized Transduction of Antimicrobial Resistance by Bacteriophage', *mSystems*, 0(0), pp. e00135-22. Available at: https://doi.org/10.1128/msystems.00135-22.

Leclerc, Q.J., Lindsay, J.A. and Knight, G.M. (2019) ‘Mathematical modelling to study the horizontal transfer of antimicrobial resistance genes in bacteria: current state of the field and recommendations’, *Journal of The Royal Society Interface*, 16(157), p. 20190260. Available at: https://doi.org/10.1098/rsif.2019.0260.

Leclerc, Q.J., Lindsay, J.A. and Knight, G.M. (2022) ‘Modelling the synergistic effect of bacteriophage and antibiotics on bacteria: killers and drivers of resistance evolution’, *bioRxiv* [Preprint]. Available at: https://doi.org/10.1101/2022.03.02.480504.

Lehman, S.M. *et al.* (2019) ‘Design and Preclinical Development of a Phage Product for the Treatment of Antibiotic-Resistant Staphylococcus aureus Infections’, *Viruses*, 11(1), p. 88. Available at: https://doi.org/10.3390/v11010088.

Levin, B.R. *et al.* (1997) ‘The Population Genetics of Antibiotic Resistance’, *Clinical Infectious Diseases*, 24(Supplement_1), pp. S9–S16. Available at: https://doi.org/10.1093/clinids/24.Supplement_1.S9.

Li, B. *et al.* (2020) ‘Colistin Resistance Gene mcr-1 Mediates Cell Permeability and Resistance to Hydrophobic Antibiotics’, *Frontiers in Microbiology*, 10. Available at: https://www.frontiersin.org/article/10.3389/fmicb.2019.03015 (Accessed: 6 June 2022).

Li, X. *et al.* (2021) ‘A combination therapy of Phages and Antibiotics: Two is better than one’, *International Journal of Biological Sciences*, 17(13), pp. 3573–3582. Available at: https://doi.org/10.7150/ijbs.60551.

Lin, D.M., Koskella, B. and Lin, H.C. (2017) ‘Phage therapy: An alternative to antibiotics in the age of multi-drug resistance’, *World Journal of Gastrointestinal Pharmacology and Therapeutics*, 8(3), pp. 162–173. Available at: https://doi.org/10.4292/wjgpt.v8.i3.162.

Lindsay, J.A. *et al.* (2006) ‘Microarrays Reveal that Each of the Ten Dominant Lineages of Staphylococcus aureus Has a Unique Combination of Surface-Associated and Regulatory Genes’, *Journal of Bacteriology*, 188(2), pp. 669–676. Available at: https://doi.org/10.1128/JB.188.2.669-676.2006.

Lindsay, J.A. (2010) ‘Genomic variation and evolution of Staphylococcus aureus’, *International Journal of Medical Microbiology*, 300(2), pp. 98–103. Available at: https://doi.org/10.1016/j.ijmm.2009.08.013.

Lindsay, J.A. (2014) ‘Staphylococcus aureus genomics and the impact of horizontal gene transfer’, *International Journal of Medical Microbiology*, 304(2), pp. 103–109. Available at: https://doi.org/10.1016/j.ijmm.2013.11.010.

Lipsitch, M. and Samore, M.H. (2002) ‘Antimicrobial Use and Antimicrobial Resistance: A Population Perspective - Volume 8, Number 4—April 2002 - Emerging Infectious Diseases journal - CDC’, *Emerging Infectious Diseases* [Preprint]. Available at: https://doi.org/10.3201/eid0804.010312.

Loh, B. and Leptihn, S. (2020) ‘A Call For a Multidisciplinary Future of Phage Therapy to Combat Multi-drug Resistant Bacterial Infections’, *Infectious Microbes & Diseases*, 2(1), pp. 1–2. Available at: https://doi.org/10.1097/IM9.0000000000000018.

Lopes, A., Pereira, C. and Almeida, A. (2018) ‘Sequential Combined Effect of Phages and Antibiotics on the Inactivation of Escherichia coli’, *Microorganisms*, 6(4), p. 125. Available at: https://doi.org/10.3390/microorganisms6040125.

Lowy, F.D. (2003) ‘Antimicrobial resistance: the example of Staphylococcus aureus’, *Journal of Clinical Investigation*, 111(9), pp. 1265–1273. Available at: https://doi.org/10.1172/JCI200318535.

Lwoff, A. (1953) ‘Lysogeny’, *Bacteriological Reviews*, 17(4), pp. 269–337.

MacFadden, D.R. *et al.* (2019) ‘The Relative Impact of Community and Hospital Antibiotic Use on the Selection of Extended-spectrum Beta-lactamase–producing Escherichia coli’, *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, 69(1), pp. 182–188. Available at: https://doi.org/10.1093/cid/ciy978.

Mahase, E. (2020) ‘UK launches subscription style model for antibiotics to encourage new development’, *BMJ*, 369, p. m2468. Available at: https://doi.org/10.1136/bmj.m2468.

Maree, M. *et al.* (2022) ‘Natural transformation allows transfer of SCCmec-mediated methicillin resistance in Staphylococcus aureus biofilms’, *Nature Communications*, 13(1), p. 2477. Available at: https://doi.org/10.1038/s41467-022-29877-2.

Mašlaňová, I. *et al.* (2016) ‘Efficient plasmid transduction to Staphylococcus aureus strains insensitive to the lytic action of transducing phage’, *FEMS microbiology letters*, 363(19), p. fnw211. Available at: https://doi.org/10.1093/femsle/fnw211.

May, R.M. and Anderson, R.M. (1987) ‘Transmission dynamics of HIV infection’, *Nature*, 326(6109), pp. 137–142. Available at: https://doi.org/10.1038/326137a0.

McCallum, H., Barlow, N. and Hone, J. (2001) ‘How should pathogen transmission be modelled?’, *Trends in Ecology & Evolution*, 16(6), pp. 295–300. Available at: https://doi.org/10.1016/S0169-5347(01)02144-9.

McCarthy, A.J. *et al.* (2011) ‘The Distribution of Mobile Genetic Elements (MGEs) in MRSA CC398 Is Associated with Both Host and Country’, *Genome Biology and Evolution*, 3, pp. 1164–1174. Available at: https://doi.org/10.1093/gbe/evr092.

McCarthy, A.J. *et al.* (2014) ‘Extensive Horizontal Gene Transfer during Staphylococcus aureus Co-colonization In Vivo’, *Genome Biology and Evolution*, 6(10), pp. 2697–2708. Available at: https://doi.org/10.1093/gbe/evu214.

McCarthy, A.J. and Lindsay, J.A. (2012) ‘The distribution of plasmids that carry virulence and resistance genes in Staphylococcus aureus is lineage associated’, *BMC Microbiology*, 12(1), p. 104. Available at: https://doi.org/10.1186/1471-2180-12-104.

McCarthy, A.J. and Lindsay, J.A. (2013) ‘Staphylococcus aureus innate immune evasion is lineage-specific: A bioinfomatics study’, *Infection, Genetics and Evolution*, 19, pp. 7–14. Available at: https://doi.org/10.1016/j.meegid.2013.06.012.

McCarthy, A.J., Lindsay, J.A. and Loeffler, A. (2012) ‘Are all meticillin-resistant Staphylococcus aureus (MRSA) equal in all hosts? Epidemiological and genetic comparison between animal and human MRSA’, *Veterinary Dermatology*, 23(4), pp. 267-e54. Available at: https://doi.org/10.1111/j.1365-3164.2012.01072.x.

McCarthy, A.J., Witney, A.A. and Lindsay, Jodi.A. (2012) ‘Staphylococcus aureus Temperate Bacteriophage: Carriage and Horizontal Gene Transfer is Lineage Associated’, *Frontiers in Cellular and Infection Microbiology*, 2. Available at: https://doi.org/10.3389/fcimb.2012.00006.

McGuinness, W.A., Malachowa, N. and DeLeo, F.R. (2017) ‘Vancomycin Resistance in Staphylococcus aureus’, *The Yale Journal of Biology and Medicine*, 90(2), pp. 269–281.

Meka, V.G. *et al.* (2004) ‘Linezolid Resistance in Sequential Staphylococcus aureus Isolates Associated with a T2500A Mutation in the 23S rRNA Gene and Loss of a Single Copy of rRNA’, *The Journal of Infectious Diseases*, 190(2), pp. 311–317. Available at: https://doi.org/10.1086/421471.

Melnyk, A.H., Wong, A. and Kassen, R. (2015) ‘The fitness costs of antibiotic resistance mutations’, *Evolutionary Applications*, 8(3), pp. 273–283. Available at: https://doi.org/10.1111/eva.12196.

Metcalf, C.J.E. *et al.* (2015) ‘Five challenges in evolution and infectious diseases’, *Epidemics*, 10, pp. 40–44. Available at: https://doi.org/10.1016/j.epidem.2014.12.003.

Miller, R.R. *et al.* (2014) ‘Dynamics of acquisition and loss of carriage of Staphylococcus aureus strains in the community: The effect of clonal complex’, *The Journal of Infection*, 68(5), pp. 426–439. Available at: https://doi.org/10.1016/j.jinf.2013.12.013.

Miller, S.I. (2016) ‘Antibiotic Resistance and Regulation of the Gram-Negative Bacterial Outer Membrane Barrier by Host Innate Immune Molecules’, *mBio*, 7(5), pp. e01541-16. Available at: https://doi.org/10.1128/mBio.01541-16.

Missiakas, D.M. and Schneewind, O. (2013) ‘Growth and Laboratory Maintenance of Staphylococcus aureus’, *Current protocols in microbiology*, CHAPTER 9, p. Unit-9C.1. Available at: https://doi.org/10.1002/9780471729259.mc09c01s28.

Molina, F. *et al.* (2021) ‘A New Pipeline for Designing Phage Cocktails Based on Phage-Bacteria Infection Networks’, *Frontiers in Microbiology*, 12. Available at: https://www.frontiersin.org/article/10.3389/fmicb.2021.564532 (Accessed: 5 June 2022).

Mongkolrattanothai, K. *et al.* (2011) ‘Simultaneous carriage of multiple genotypes of Staphylococcus aureus in children’, *Journal of Medical Microbiology*, 60(3), pp. 317–322. Available at: https://doi.org/10.1099/jmm.0.025841-0.

Moodley, A. *et al.* (2012) 'Comparative Host Specificity of Human- and Pig- Associated Staphylococcus aureus Clonal Lineages', *PLOS ONE*, 7(11), p. e49344. Available at: https://doi.org/10.1371/journal.pone.0049344.

Morikawa, K. *et al.* (2012) 'Expression of a Cryptic Secondary Sigma Factor Gene Unveils Natural Competence for DNA Transformation in Staphylococcus aureus', *PLOS Pathogens*, 8(11), p. e1003003. Available at: https://doi.org/10.1371/journal.ppat.1003003.

Mossong, J. *et al.* (2008) 'Social Contacts and Mixing Patterns Relevant to the Spread of Infectious Diseases', *PLOS Medicine*, 5(3), p. e74. Available at: https://doi.org/10.1371/journal.pmed.0050074.

Mueller, M., de la Peña, A. and Derendorf, H. (2004) 'Issues in Pharmacokinetics and Pharmacodynamics of Anti-Infective Agents: Kill Curves versus MIC', *Antimicrobial Agents and Chemotherapy*, 48(2), pp. 369–377. Available at: https://doi.org/10.1128/AAC.48.2.369-377.2004.

Muenks, C.E. *et al.* (2016) 'Diversity of Staphylococcus aureus strains colonizing various niches of the human body', *Journal of Infection*, 72(6), pp. 698–705. Available at: https://doi.org/10.1016/j.jinf.2016.03.015.

Murray, C.J. *et al.* (2022) 'Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis', *The Lancet*, 399(10325), pp. 629–655. Available at: https://doi.org/10.1016/S0140-6736(21)02724-0.

Nepal, R. *et al.* (2021) 'Prophages encoding human immune evasion cluster genes are enriched in Staphylococcus aureus isolated from chronic rhinosinusitis patients with nasal polyps', *Microbial Genomics*, 7(12), p. 000726. Available at: https://doi.org/10.1099/mgen.0.000726.

Nielsen, E.I. and Friberg, L.E. (2013) 'Pharmacokinetic-Pharmacodynamic Modeling of Antibacterial Drugs', *Pharmacological Reviews*. Edited by D. Andersson, 65(3), pp. 1053–1090. Available at: https://doi.org/10.1124/pr.111.005769.

Niewiadomska, A.M. *et al.* (2019) 'Population-level mathematical modeling of antimicrobial resistance: a systematic review', *BMC Medicine*, 17(1), p. 81. Available at: https://doi.org/10.1186/s12916-019-1314-9.

Novick, R.P. (1987) 'Plasmid incompatibility', *Microbiological Reviews*, 51(4), pp. 381–395.

Ochman, H., Lawrence, J.G. and Groisman, E.A. (2000) 'Lateral gene transfer and the nature of bacterial innovation', *Nature*, 405(6784), pp. 299–304. Available at: https://doi.org/10.1038/35012500.

O'Gara, J.P. (2017) 'Into the storm: Chasing the opportunistic pathogen Staphylococcus aureus from skin colonisation to life-threatening infections', *Environmental Microbiology*, 19(10), pp. 3823–3833. Available at: https://doi.org/10.1111/1462-2920.13833.

O'Neill, J. (2016) 'Tackling drug-resistant infections globally: final report and recommendations'. The Review on Antimicrobial Resistance.

Ooi, M.L. *et al.* (2019) 'Safety and Tolerability of Bacteriophage Therapy for Chronic Rhinosinusitis Due to Staphylococcus aureus', *JAMA Otolaryngology–Head & Neck Surgery*, 145(8), pp. 723–729. Available at: https://doi.org/10.1001/jamaoto.2019.1191.

Opatowski, L. *et al.* (2011) 'Contribution of mathematical modeling to the fight against bacterial antibiotic resistance':, *Current Opinion in Infectious Diseases*, 24(3), pp. 279–287. Available at: https://doi.org/10.1097/QCO.0b013e3283462362.

Osei Sekyere, J. (2018) 'Genomic insights into nitrofurantoin resistance mechanisms and epidemiology in clinical Enterobacteriaceae', *Future Science OA*, 4(5), p. FSO293. Available at: https://doi.org/10.4155/fsoa-2017-0156.

Otter, J.A. and French, G.L. (2010) 'Molecular epidemiology of community-associated meticillin-resistant Staphylococcus aureus in Europe', *The Lancet Infectious Diseases*, 10(4), pp. 227–239. Available at: https://doi.org/10.1016/S1473-3099(10)70053-0.

Park, J.T. *et al.* (1974) 'Mutants of Staphylococci with Altered Cell Walls', *Annals of the New York Academy of Sciences*, 236(1), pp. 54–62. Available at: https://doi.org/10.1111/j.1749-6632.1974.tb41481.x.

Patel, S., Preuss, C.V. and Bernice, F. (2022) 'Vancomycin', in *StatPearls*. Treasure Island (FL): StatPearls Publishing. Available at: http://www.ncbi.nlm.nih.gov/books/NBK459263/ (Accessed: 4 June 2022).

Payne, L.J. *et al.* (2021) 'Identification and classification of antiviral defence systems in bacteria and archaea with PADLOC reveals new system types', *Nucleic Acids Research*, 49(19), pp. 10868–10878. Available at: https://doi.org/10.1093/nar/gkab883.

Payne, R.J.H. and Jansen, V.A.A. (2000) 'Phage therapy: The peculiar kinetics of self-replicating pharmaceuticals', *Clinical Pharmacology & Therapeutics*, 68(3), pp. 225–230. Available at: https://doi.org/10.1067/mcp.2000.109520.

Payne, R.J.H. and Jansen, V.A.A. (2001) 'Understanding Bacteriophage Therapy as a Density-dependent Kinetic Process', *Journal of Theoretical Biology*, 208(1), pp. 37–48. Available at: https://doi.org/10.1006/jtbi.2000.2198.

Payne, R.J.H. and Jansen, V.A.A. (2003) 'Pharmacokinetic Principles of Bacteriophage Therapy', *Clinical Pharmacokinetics*, 42(4), pp. 315–325. Available at: https://doi.org/10.2165/00003088-200342040-00002.

Pei, S. *et al.* (2018) 'Inference and control of the nosocomial transmission of methicillin-resistant Staphylococcus aureus', *eLife*. Edited by B. Cooper and P. Jha, 7, p. e40977. Available at: https://doi.org/10.7554/eLife.40977.

Périchon, B. and Courvalin, P. (2009) 'VanA-Type Vancomycin-Resistant Staphylococcus aureus', *Antimicrobial Agents and Chemotherapy*, 53(11), pp. 4580–4587. Available at: https://doi.org/10.1128/AAC.00346-09.

Petrovic Fabijan, A. *et al.* (2020) 'Safety of bacteriophage therapy in severe Staphylococcus aureus infection', *Nature Microbiology*, 5(3), pp. 465–472. Available at: https://doi.org/10.1038/s41564-019-0634-z.

Pinho, M.G., de Lencastre, H. and Tomasz, A. (2001) 'An acquired and a native penicillin-binding protein cooperate in building the cell wall of drug-resistant staphylococci', *Proceedings of the National Academy of Sciences of the United States of America*, 98(19), pp. 10886–10891. Available at: https://doi.org/10.1073/pnas.191260798.

Powers, T. and Noller, H.F. (1991) 'A functional pseudoknot in 16S ribosomal RNA.', *The EMBO Journal*, 10(8), pp. 2203–2214.

Price, L. *et al.* (2018) 'Effectiveness of interventions to improve the public's antimicrobial resistance awareness and behaviours associated with prudent use of antimicrobials: a systematic review', *Journal of Antimicrobial Chemotherapy*, 73(6), pp. 1464–1478. Available at: https://doi.org/10.1093/jac/dky076.

Rahman, M. *et al.* (2011) 'Characterization of induced Staphylococcus aureus bacteriophage SAP-26 and its anti-biofilm activity with rifampicin', *Biofouling*, 27(10), pp. 1087–1093. Available at: https://doi.org/10.1080/08927014.2011.631169.

Raj, J.R.M. and Karunasagar, I. (2019) 'Phages amid antimicrobial resistance', *Critical Reviews in Microbiology*, 45(5–6), pp. 701–711. Available at: https://doi.org/10.1080/1040841X.2019.1691973.

Rice, L.B. (2008) 'Federal Funding for the Study of Antimicrobial Resistance in Nosocomial Pathogens: No ESKAPE', *The Journal of Infectious Diseases*, 197(8), pp. 1079–1081. Available at: https://doi.org/10.1086/533452.

Roach, D.R. *et al.* (2017) 'Synergy between the Host Immune System and Bacteriophage Is Essential for Successful Phage Therapy against an Acute Respiratory Pathogen', *Cell Host & Microbe*, 22(1), pp. 38-47.e4. Available at: https://doi.org/10.1016/j.chom.2017.06.018.

Robinson, D.A. and Enright, M.C. (2004) 'Evolution of Staphylococcus aureus by Large Chromosomal Replacements', *Journal of Bacteriology*, 186(4), pp. 1060–1064. Available at: https://doi.org/10.1128/JB.186.4.1060-1064.2004.

Rodriguez-Gonzalez, R.A. *et al.* (2020) 'Quantitative Models of Phage-Antibiotic Combination Therapy', *mSystems*, 5(1), pp. e00756-19. Available at: https://doi.org/10.1128/mSystems.00756-19.

Rosenbach, A.J.F. (1884) *Mikro-organismen bei den Wund-Infections-Krankheiten des Menschen*. J.F. Bergmann.

Rouch, D.A. *et al.* (1987) 'The aacA-aphD gentamicin and kanamycin resistance determinant of Tn4001 from Staphylococcus aureus: expression and nucleotide sequence analysis', *Journal of General Microbiology*, 133(11), pp. 3039–3052. Available at: https://doi.org/10.1099/00221287-133-11-3039.

Ryu, S. *et al.* (2014) ‘Colonization and Infection of the Skin by S. aureus: Immune System Evasion and the Response to Cationic Antimicrobial Peptides’, *International Journal of Molecular Sciences*, 15(5), pp. 8753–8772. Available at: https://doi.org/10.3390/ijms15058753.

Salton, M.R.J. and Kim, K.-S. (1996) ‘Structure’, in S. Baron (ed.) *Medical Microbiology*. 4th edn. Galveston (TX): University of Texas Medical Branch at Galveston. Available at: http://www.ncbi.nlm.nih.gov/books/NBK8477/ (Accessed: 26 May 2022).

Santos, S.B. *et al.* (2014) ‘Population Dynamics of a Salmonella Lytic Phage and Its Host: Implications of the Host Bacterial Growth Rate in Modelling’, *PLoS ONE*, 9(7), p. e102507. Available at: https://doi.org/10.1371/journal.pone.0102507.

Sauvage, E. *et al.* (2008) ‘The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis’, *FEMS Microbiology Reviews*, 32(2), pp. 234–258. Available at: https://doi.org/10.1111/j.1574-6976.2008.00105.x.

Scharn, C.R., Tenover, F.C. and Goering, R.V. (2013) ‘Transduction of Staphylococcal Cassette Chromosome mec Elements between Strains of Staphylococcus aureus’, *Antimicrobial Agents and Chemotherapy*, 57(11), pp. 5233–5238. Available at: https://doi.org/10.1128/AAC.01058-13.

Schmitz, F.-J. *et al.* (2000) ‘Prevalence of macrolide-resistance genes in Staphylococcus aureus and Enterococcus faecium isolates from 24 European university hospitals’, *Journal of Antimicrobial Chemotherapy*, 45(6), pp. 891–894. Available at: https://doi.org/10.1093/jac/45.6.891.

Schrag, S.J. and Mittler, J.E. (1996) ‘Host-Parasite Coexistence: The Role of Spatial Refuges in Stabilizing Bacteria-Phage Interactions’, *The American Naturalist*, 148(2), pp. 348–377. Available at: https://doi.org/10.1086/285929.

Sender, R., Fuchs, S. and Milo, R. (2016) ‘Revised Estimates for the Number of Human and Bacteria Cells in the Body’, *PLOS Biology*, 14(8), p. e1002533. Available at: https://doi.org/10.1371/journal.pbio.1002533.

Shallcross, L.J. *et al.* (2013) ‘The role of the Panton-Valentine leucocidin toxin in staphylococcal disease: a systematic review and meta-analysis’, *The Lancet Infectious Diseases*, 13(1), pp. 43–54. Available at: https://doi.org/10.1016/S1473-3099(12)70238-4.

Shkoporov, A.N., Turkington, C.J. and Hill, C. (2022) ‘Mutualistic interplay between bacteriophages and bacteria in the human gut’, *Nature Reviews Microbiology*, pp. 1–13. Available at: https://doi.org/10.1038/s41579-022-00755-4.

Shrestha, P. *et al.* (2018) ‘Enumerating the economic cost of antimicrobial resistance per antibiotic consumed to inform the evaluation of interventions affecting their use’, *Antimicrobial Resistance and Infection Control*, 7, p. 98. Available at: https://doi.org/10.1186/s13756-018-0384-3.

Silverman, R.B. and Holladay, M.W. (2014) *The Organic Chemistry of Drug Design and Drug Action*. Academic Press.

Singh-Moodley, A. *et al.* (2020) 'Diversity of SCCmec elements and spa types in South African Staphylococcus aureus mecA-positive blood culture isolates', *BMC Infectious Diseases*, 20(1), p. 816. Available at: https://doi.org/10.1186/s12879-020-05547-w.

Smith, D.R., Temime, L. and Opatowski, L. (2021) 'Microbiome-pathogen interactions drive epidemiological dynamics of antibiotic resistance: A modeling study applied to nosocomial pathogen control', *eLife*, 10, p. e68764. Available at: https://doi.org/10.7554/eLife.68764.

Smith, T.C. (2015) 'Livestock-Associated Staphylococcus aureus: The United States Experience', *PLOS Pathogens*, 11(2), p. e1004564. Available at: https://doi.org/10.1371/journal.ppat.1004564.

Spicknall, I.H. *et al.* (2013) 'A Modeling Framework for the Evolution and Spread of Antibiotic Resistance: Literature Review and Model Categorization', *American Journal of Epidemiology*, 178(4), pp. 508–520. Available at: https://doi.org/10.1093/aje/kwt017.

Spiegelhalter, D.J. *et al.* (2002) 'Bayesian measures of model complexity and fit', *Journal of the Royal Statistical Society: Series B (Statistical Methodology)*, 64(4), pp. 583–639. Available at: https://doi.org/10.1111/1467-9868.00353.

Stanczak-Mrozek, K.I. *et al.* (2015) 'Within-host diversity of MRSA antimicrobial resistances', *Journal of Antimicrobial Chemotherapy*, 70(8), pp. 2191–2198. Available at: https://doi.org/10.1093/jac/dkv119.

Stanczak-Mrozek, K.I., Laing, K.G. and Lindsay, J.A. (2017) 'Resistance gene transfer: induction of transducing phage by sub-inhibitory concentrations of antimicrobials is not correlated to induction of lytic phage', *Journal of Antimicrobial Chemotherapy*, 72(6), pp. 1624–1631. Available at: https://doi.org/10.1093/jac/dkx056.

Tacconelli, E. *et al.* (2018) 'Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis', *The Lancet Infectious Diseases*, 18(3), pp. 318–327. Available at: https://doi.org/10.1016/S1473-3099(17)30753-3.

Tanwar, J. *et al.* (2014) 'Multidrug Resistance: An Emerging Crisis', *Interdisciplinary Perspectives on Infectious Diseases*, 2014, p. 541340. Available at: https://doi.org/10.1155/2014/541340.

Taylor, T.A. and Unakal, C.G. (2022) 'Staphylococcus Aureus', in *StatPearls*. Treasure Island (FL): StatPearls Publishing. Available at: http://www.ncbi.nlm.nih.gov/books/NBK441868/ (Accessed: 26 May 2022).

Tedijanto, C. *et al.* (2018) 'Estimating the proportion of bystander selection for antibiotic resistance among potentially pathogenic bacterial flora', *Proceedings of the*

National Academy of Sciences, 115(51), pp. E11988–E11995. Available at: https://doi.org/10.1073/pnas.1810840115.

Tekle, Y.I. *et al.* (2012) 'Controlling Antimicrobial Resistance through Targeted, Vaccine-Induced Replacement of Strains', *PLOS ONE*, 7(12), p. e50688. Available at: https://doi.org/10.1371/journal.pone.0050688.

The National Institute for Health and Care (2022a) *Daptomycin. BNF: British National Formulary - NICE*. NICE. Available at: https://bnf.nice.org.uk/drug/daptomycin.html (Accessed: 26 April 2022).

The National Institute for Health and Care (2022b) *Vancomycin. BNF: British National Formulary - NICE*. NICE. Available at: https://bnf.nice.org.uk/drug/vancomycin.html (Accessed: 26 April 2022).

Thomas, C.M. and Nielsen, K.M. (2005) 'Mechanisms of, and Barriers to, Horizontal Gene Transfer between Bacteria', *Nature Reviews Microbiology*, 3(9), pp. 711–721. Available at: https://doi.org/10.1038/nrmicro1234.

Tong, S.Y.C. *et al.* (2015) 'Staphylococcus aureus Infections: Epidemiology, Pathophysiology, Clinical Manifestations, and Management', *Clinical Microbiology Reviews*, 28(3), pp. 603–661. Available at: https://doi.org/10.1128/CMR.00134-14.

Tran, T.T., Munita, J.M. and Arias, C.A. (2015) 'Mechanisms of Drug Resistance: Daptomycin Resistance', *Annals of the New York Academy of Sciences*, 1354, pp. 32–53. Available at: https://doi.org/10.1111/nyas.12948.

Trzcinski, K. *et al.* (2000) 'Expression of resistance to tetracyclines in strains of methicillin-resistant Staphylococcus aureus', *The Journal of Antimicrobial Chemotherapy*, 45(6), pp. 763–770. Available at: https://doi.org/10.1093/jac/45.6.763.

Uhlemann, A.-C. *et al.* (2011) 'The Environment as an Unrecognized Reservoir for Community-Associated Methicillin Resistant Staphylococcus aureus USA300: A Case-Control Study', *PLOS ONE*, 6(7), p. e22407. Available at: https://doi.org/10.1371/journal.pone.0022407.

Valério, N. *et al.* (2017) 'Effects of single and combined use of bacteriophages and antibiotics to inactivate Escherichia coli', *Virus Research*, 240, pp. 8–17. Available at: https://doi.org/10.1016/j.virusres.2017.07.015.

Vasala, A., Hytönen, V.P. and Laitinen, O.H. (2020) 'Modern Tools for Rapid Diagnostics of Antimicrobial Resistance', *Frontiers in Cellular and Infection Microbiology*, 10, p. 308. Available at: https://doi.org/10.3389/fcimb.2020.00308.

Ventola, C.L. (2015) 'The Antibiotic Resistance Crisis', *Pharmacy and Therapeutics*, 40(4), pp. 277–283.

Verheust, C. *et al.* (2010) 'Contained use of Bacteriophages: Risk Assessment and Biosafety Recommendations', *Applied Biosafety*, 15(1), pp. 32–44. Available at: https://doi.org/10.1177/153567601001500106.

Versporten, A. *et al.* (2018) ‘Antimicrobial consumption and resistance in adult hospital inpatients in 53 countries: results of an internet-based global point prevalence survey’, *The Lancet Global Health*, 6(6), pp. e619–e629. Available at: https://doi.org/10.1016/S2214-109X(18)30186-4.

Vila, J., Moreno-Morales, J. and Ballesté-Delpierre, C. (2020) ‘Current landscape in the discovery of novel antibacterial agents’, *Clinical Microbiology and Infection*, 26(5), pp. 596–603. Available at: https://doi.org/10.1016/j.cmi.2019.09.015.

Vlazaki, M., Huber, J. and Restif, O. (2019) ‘Integrating mathematical models with experimental data to investigate the within-host dynamics of bacterial infections’, *Pathogens and Disease*, 77(8), p. ftaa001. Available at: https://doi.org/10.1093/femspd/ftaa001.

Volkova, V.V. *et al.* (2014) ‘Modeling the Infection Dynamics of Bacteriophages in Enteric Escherichia coli: Estimating the Contribution of Transduction to Antimicrobial Gene Spread’, *Applied and Environmental Microbiology*, 80(14), pp. 4350–4362. Available at: https://doi.org/10.1128/AEM.00446-14.

Wagenlehner, F. *et al.* (2022) ‘A global perspective on improving patient care in uncomplicated urinary tract infection: expert consensus and practical guidance’, *Journal of Global Antimicrobial Resistance*, 28, pp. 18–29. Available at: https://doi.org/10.1016/j.jgar.2021.11.008.

Waldron, D.E. and Lindsay, J.A. (2006) ‘Sau1: a Novel Lineage-Specific Type I Restriction-Modification System That Blocks Horizontal Gene Transfer into Staphylococcus aureus and between S. aureus Isolates of Different Lineages’, *Journal of Bacteriology*, 188(15), pp. 5578–5585. Available at: https://doi.org/10.1128/JB.00418-06.

Walsh, L. *et al.* (2021) ‘Efficacy of Phage- and Bacteriocin-Based Therapies in Combatting Nosocomial MRSA Infections’, *Frontiers in Molecular Biosciences*, 8. Available at: https://www.frontiersin.org/article/10.3389/fmolb.2021.654038 (Accessed: 5 June 2022).

Wang, R. *et al.* (2018) ‘The global distribution and spread of the mobilized colistin resistance gene mcr-1’, *Nature Communications*, 9, p. 1179. Available at: https://doi.org/10.1038/s41467-018-03205-z.

Waxman, D.J. and Strominger, J.L. (1983) ‘Penicillin-Binding Proteins and the Mechanism of Action of Beta-Lactam Antibiotics’, *Annual Review of Biochemistry*, 52(1), pp. 825–869. Available at: https://doi.org/10.1146/annurev.bi.52.070183.004141.

Weld, R.J., Butts, C. and Heinemann, J.A. (2004) ‘Models of phage growth and their applicability to phage therapy’, *Journal of Theoretical Biology*, 227(1), pp. 1–11.

Wheat, P.F. (2001) ‘History and development of antimicrobial susceptibility testing methodology’, *Journal of Antimicrobial Chemotherapy*, 48(suppl_1), pp. 1–4. Available at: https://doi.org/10.1093/jac/48.suppl_1.1.

Wichelhaus, T.A. *et al.* (2002) 'Biological Cost of Rifampin Resistance from the Perspective of Staphylococcus aureus', *Antimicrobial Agents and Chemotherapy*, 46(11), pp. 3381–3385. Available at: https://doi.org/10.1128/AAC.46.11.3381-3385.2002.

Winstanley, C., O'Brien, S. and Brockhurst, M.A. (2016) 'Pseudomonas aeruginosa Evolutionary Adaptation and Diversification in Cystic Fibrosis Chronic Lung Infections', *Trends in Microbiology*, 24(5), pp. 327–337. Available at: https://doi.org/10.1016/j.tim.2016.01.008.

von Wintersdorff, C.J.H. *et al.* (2016) 'Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer', *Frontiers in Microbiology*, 7. Available at: https://doi.org/10.3389/fmicb.2016.00173.

World Health Organization (2015) *Global action plan on antimicrobial resistance*. Geneva: World Health Organization. Available at: https://apps.who.int/iris/handle/10665/193736 (Accessed: 23 March 2022).

World Health Organization (2021) *Global tuberculosis report 2021*. Geneva: World Health Organization. Available at: https://apps.who.int/iris/handle/10665/346387 (Accessed: 26 May 2022).

Wulf, M. and Voss, A. (2008) 'MRSA in livestock animals—an epidemic waiting to happen?', *Clinical Microbiology and Infection*, 14(6), pp. 519–521. Available at: https://doi.org/10.1111/j.1469-0691.2008.01970.x.

Wyllie, D., Paul, J. and Crook, D. (2011) 'Waves of trouble: MRSA strain dynamics and assessment of the impact of infection control', *Journal of Antimicrobial Chemotherapy*, 66(12), pp. 2685–2688. Available at: https://doi.org/10.1093/jac/dkr392.

Xia, G. and Wolz, C. (2014) 'Phages of Staphylococcus aureus and their impact on host evolution', *Infection, Genetics and Evolution*, 21, pp. 593–601. Available at: https://doi.org/10.1016/j.meegid.2013.04.022.

Yoshida, H. *et al.* (1990) 'Nucleotide sequence and characterization of the Staphylococcus aureus norA gene, which confers resistance to quinolones.', *Journal of Bacteriology*, 172(12), pp. 6942–6949.

Zwanzig, M. *et al.* (2019) 'Mobile Compensatory Mutations Promote Plasmid Survival', *mSystems*, 4(1), pp. e00186-18. Available at: https://doi.org/10.1128/mSystems.00186-18.

8 Appendix

8.1 Supplementary Material for Chapter 2

Supplementary Table 2.1: Summary table of the elements recorded in the 43 studies included in our review. *E. coli*: *Escherichia coli*; *S. marcescens*: *Serratia marcescens*; *A. vinelandii*: *Azotobacter vinelandii*; *K. pneumonia*: *Klebsiella pneumonia*; *B. subtilis*: *Bacillus subtilis*; *E. blattae*: *Escherichia blattae*; *E. fergusonii*: *Escherichia fergusonii*; *E. chrysantemi*: *Erwinia chrysantemi*; *P. fluorescens*: *Pseudomonas fluorescens*; *P. putida*: *Pseudomonas putida*; *P. koreensis*: *Pseudomonas koreensis*; *S. mathophilia*: *Stenotrophomonas mathophilia*; *P. plecoglossicida*: *Pseudomonas plecoglossicida*; *P. veronii*: *Pseudomonas veronii*; *O. tritici*: *Ochrobactrum tritici*; *E. adhaerens*: *Ensifer adhaerens*.

RECORDED ELEMENT	Authors	Year	Transfer mechanism	Bacteria	Aim of the study	Bacterial container	Antibiotic effect considered	Multiple resistances considered	Fitness cost of resistance considered	Source of model parameters	Source of model parameters (continued)	Type of model	Type of parameter values	Sensitivity analysis performed
POSSIBLE VALUES	-	-	**Conjugation OR Transformation OR Transduction**	-	**Evolutionary OR Public health OR Both**	-	**Yes OR No**	**Yes OR No**	**Yes OR No**	**Experimental AND/OR External AND/OR Assume**	**Number of external sources used**	**Deterministic OR Stochastic OR Both**	**Constant OR Sampled**	**Yes OR No**
A high-throughput approach to the culture-based estimation of plasmid transfer rates.	Kneis, David; Hiltunen, Teppo; Hess, Stefanie	2019	Conjugation	*E. coli, S. marcescens*	Evolutionary	Culture	No	No	Yes	Experimental	-	Deterministic	Constant	Yes
A kinetic model for horizontal transfer and bacterial antibiotic resistance	Knopoff, Damian A; Sanchez Sanso, Juan M	2017	Conjugation	None specified	Evolutionary	None specified	Yes	No	No	Assume	-	Deterministic	Constant	No
A kinetic model of gene transfer via natural transformation of Azotobacter vinelandii	Lu, Nanxi; Massoudieh, Arash; Liang, Xiaomeng; Kamai, Tamir; Zilles, Julie L; Nguyen, Thanh H; Ginn, Timothy R	2015	Transformation	*A. vinelandii*	Evolutionary	Culture	No	No	No	Experimental	-	Deterministic	Constant	Yes
A model of antibiotic-resistant bacterial epidemics in hospitals.	Webb, Glenn F; D'Agata, Erika M C; Magal, Pierre; Ruan, Shigui	2005	Conjugation	None specified	Public health	Human	Yes	No	Yes	Assume	-	Deterministic	Sampled	Yes

A network-based approach for resistance transmission in bacterial populations	Gehring, Ronette; Schumm, Phillip; Youssef, Mina; Scoglio, Caterina	2010	Conjugation	None specified	Evolutionary	None specified	Yes	No	Yes	Assume	-	Both	-	Yes
Accounting for mating pair formation in plasmid population dynamics	Zhong, Xue; Krol, Jarosław E; Top, Eva M; Krone, Stephen M	2010	Conjugation	*E. coli*	Evolutionary	Culture	No	No	No	Experimental	-	Deterministic	Constant	Yes
Antibiotics as a selective driver for conjugation dynamics.	Lopatkin, Allison J Huang, Shuqiang Smith, Robert P Srimani, Jaydeep K; Sysoeva, Tatyana A; Bewick, Sharon; Karig, David K; You, Lingchong	2016	Conjugation	*E. coli, K. pneumonia*	Evolutionary	Culture	Yes	No	Yes	Experimental	-	Deterministic	Constant	Yes
Effects of nano-TiO2 on antibiotic resistance transfer mediated by RP4 plasmid	Qiu, Zhigang; Shen, Zhiqiang; Qian, Di; Jin, Min; Yang, Dong; Wang, Jingfeng; Zhang, Bin; Yang, Zhongwei; Chen, Zhaoli; Wang, Xinwei; Ding, Chengshi; Wang,	2015	Conjugation	*E. coli*	Evolutionary	Culture	No	No	No	Experimental	-	Deterministic	Constant	No

	Daning; Li, Jun-Wen													
Episodic selection and the maintenance of competence and natural transformation in Bacillus subtilis	Johnsen, P J; Dubnau, D; Levin, B R	2009	Transformation	*B. subtilis*	Evolutionary	Culture	Yes	No	Yes	Experimental, assume	-	Stochastic	-	No
Estimating the rate of plasmid transfer: an end-point method	Simonsen, L.; Gordon, D. M.; Stewart, F. M.; Levin, B. R.	1990	Conjugation	*E. coli*	Evolutionary	Culture	No	No	Yes	Experimental	-	Deterministic	Constant	Yes
Evaluating targets for control of plasmid-mediated antimicrobial resistance in enteric commensals of beef cattle: a modelling approach	Volkova, V V; Lu, Z; Lanzas, C; Grohn, Y T	2013	Conjugation	*E. coli*	Public health	Beef feedlot's pen	Yes	No	Yes	External, assume	12	Deterministic	Sampled	Yes
Evaluating the effect of horizontal transmission on the stability of plasmids under different selection regimes	Peña-Miller, Rafael; Rodríguez-González, Rogelio; MacLean, R Craig; San Millan, Alvaro	2015	Conjugation	*P. aeruginosa*	Evolutionary	Culture	Yes	No	Yes	External, assume	1	Deterministic	Constant	Yes

Experimental and mathematical models of Escherichia coli plasmid transfer in vitro and in vivo.	Freter, R; Freter, R R; Brickner, H	1983	Conjugation	*E. coli*	Evolutionary	Culture, mice	No	No	Yes	Experimental	-	Deterministic	Constant	No
Fate of sulfadiazine administered to pigs and its quantitative effect on the dynamics of bacterial resistance genes in manure and manured soil	Heuer, Holger; Focks, Andreas; Lamshoeft, Marc; Smalla, Kornelia; Matthies, Michael; Spiteller, Michael	2008	Conjugation	None specified	Evolutionary	Manure	Yes	No	Yes	Experimental	-	Deterministic	Constant	Yes
Genetic Drift Suppresses Bacterial Conjugation in Spatially Structured Populations	Freese, Peter D; Korolev, Kirill S; Jimenez, Jose I; Chen, Irene A	2014	Conjugation	*E. coli*	Evolutionary	Culture	No	No	Yes	Experimental	-	Both	-	Yes
Implications of stress induced genetic variation for minimizing multidrug resistance in bacteria	Obolski, Uri; Hadany, Lilach	2012	Conjugation	None specified	Public health	Human	Yes	Yes	No	Assume	-	Deterministic	Sampled	Yes
Mathematical model for the transport of fluoroquinolone and its resistant bacteria in aquatic environment.	Gothwal, Ritu; Thatikonda, Shashidhar	2018	Conjugation	None specified	Evolutionary	River	Yes	No	Yes	Experimental, external	14	Deterministic	Constant	No

Mathematical model of plasmid-mediated resistance to ceftiofur in commensal enteric Escherichia coli of cattle	Volkova, Victoriya V; Lanzas, Cristina; Lu, Zhao; Gröhn, Yrjö Tapio	2012	Conjugation	*E. coli*	Evolutionary	Cattle	Yes	No	Yes	External, assume	13	Deterministic	Sampled	Yes
Mathematical modeling of bacterial resistance to antibiotics by mutations and plasmids	Ibargueen-Mondragon, Eduardo; Romero-Leiton, Jhoana P; Esteva, Lourdes; Mariela Burbano-Rosero, Edith	2016	Conjugation	*M. tuberculosis*	Both	Human	Yes	No	Yes	External, assume	8	Deterministic	Constant	No
Mathematical modelling of antimicrobial resistance in agricultural waste highlights importance of gene transfer rate	Baker, Michelle; Hobman, Jon L; Dodd, Christine E R; Ramsden, Stephen J; Stekel, Dov J	2016	Conjugation	*E. coli*	Evolutionary	Slurry tank	Yes	No	Yes	External	12	Deterministic	Constant	Yes
Mobile Compensatory Mutations Promote Plasmid Survival	Zwanzig, Martin; Harrison, Ellie; Brockhurst, Michael A; Hall, James P J; Berendonk, Thomas U; Berger, Uta	2019	Conjugation	None specified	Evolutionary	None specified	Yes	No	Yes	External	12	Deterministic	Sampled	Yes
Modeling the Evolutionary Dynamics of	Connelly, Brian D; Zaman, Luis;	2011	Conjugation	None specified	Evolutionary	None specified	Yes	No	Yes	Assume	-	Stochastic	-	Yes

Plasmids in Spatial Populations	McKinley, Philip K; Ofria, Charles													
Modeling the infection dynamics of bacteriophages in enteric Escherichia coli: estimating the contribution of transduction to antimicrobial gene spread	Volkova, Victoriya V; Lu, Zhao; Besser, Thomas; Gröhn, Yrjö T	2014	Transduction	*E. coli*	Evolutionary	Cattle	No	No	Yes	External, assume	13	Deterministic	Sampled	Yes
Modelling dynamics of plasmid-gene mediated antimicrobial resistance in enteric bacteria using stochastic differential equations	Volkova, Victoriya V; Lu, Zhao; Lanzas, Cristina; Scott, H Morgan; Gröhn, Yrjö T	2013	Conjugation	*E. coli*	Evolutionary	Cattle	Yes	No	Yes	External	1	Stochastic	Sampled	Yes
Modelling the spatial dynamics of plasmid transfer and persistence.	Krone, Stephen M; Lu, Ruinan; Fox, Randal; Suzuki, Haruo; Top, Eva M	2007	Conjugation	*E. coli, Ochrobactrum*	Evolutionary	Culture	No	No	Yes	Experimental	-	Stochastic	-	No
Monte Carlo simulations suggest current chlortetracycline drug-residue based withdrawal periods would not control antimicrobial resistance dissemination	Cazer, Casey L.; Ducrot, Lucas; Volkova, Victoriya V.; Gröhn, Yrjö T.	2017	Conjugation	*E. coli*	Evolutionary	Cattle	Yes	No	Yes	External	42	Deterministic	Sampled	Yes

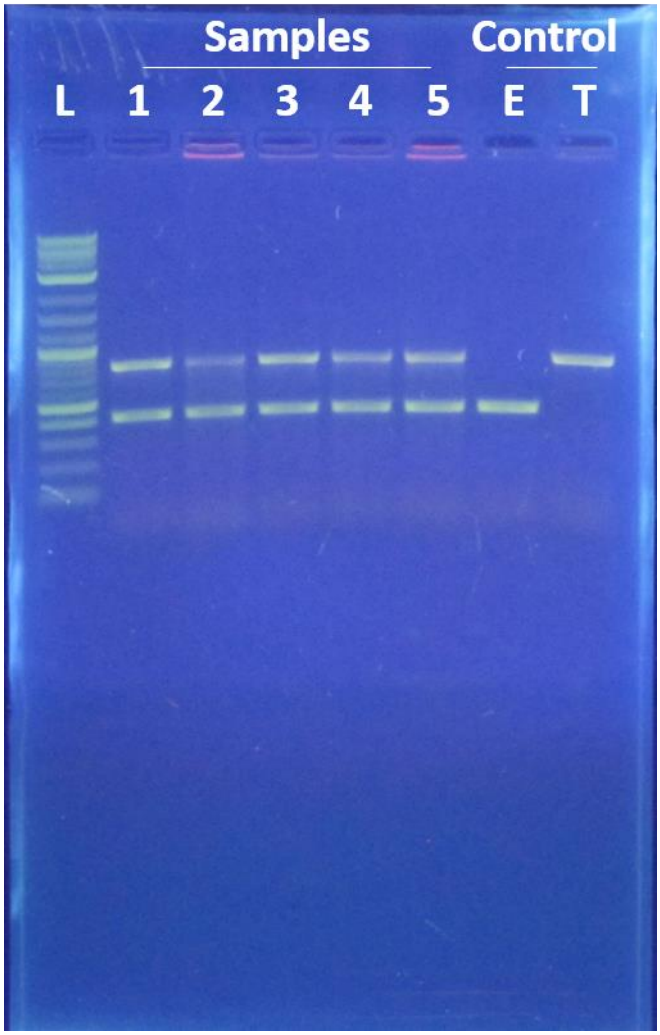
from feedlot to slaughterhouse														
On the meaning and estimation of plasmid transfer rates for surface associated and well-mixed bacterial populations	Zhong, Xue; Droesch, Jason; Fox, Randal; Top, Eva M.; Krone, Stephen M.	2012	Conjugation	*E. coli*	Evolutionary	Culture	No	No	Yes	Experimental, external	3	Stochastic	-	No
Optimal dosing strategies against susceptible and resistant bacteria	Khan, Adnan; Imran, Mudassar	2018	Conjugation	None specified	Public health	Culture	Yes	No	Yes	Assume	-	Deterministic	Constant	No
Plasmids spread very fast in heterogeneous bacterial communities.	Dionisio, Francisco; Matic, Ivan; Radman, Miroslav; Rodrigues, Olivia R; Taddei, François	2002	Conjugation	*E. coli, E. blattae, E. fergusonii, E. chrysantemi*	Evolutionary	Culture	No	No	Yes	Experimental, assume	-	Deterministic	Constant	No
Predictive Modeling of a Batch Filter Mating Process	Malwade, Akshay; Nguyen, Angel; Sadat-Mousavi, Peivand; Ingalls, Brian P	2017	Conjugation	*E. coli*	Evolutionary	Culture	No	No	Yes	Experimental	-	Deterministic	Constant	Yes

Resource competition may lead to effective treatment of antibiotic resistant infections	Gomes, Antonio L C; Galagan, James E; Segrè, Daniel	2013	Conjugation	None specified	Both	Culture, human	Yes	Yes	Yes	External, assume	2	Deterministic	Constant	Yes
Simulating Multilevel Dynamics of Antimicrobial Resistance in a Membrane Computing Model	Campos, Marcelino; Capilla, Rafael; Naya, Fernando; Futami, Ricardo; Coque, Teresa; Moya, Andrés; Fernandez-Lanza, Val; Cantón, Rafael; Sempere, José M.; Llorens, Carlos; Baquero, Fernando	2019	Conjugation	*E.coli, K. pneumonia, E. faecium, P. aeruginosa*	Both	Human	Yes	Yes	Yes	Assume	-	Stochastic	-	No
Source-sink plasmid transfer dynamics maintain gene mobility in soil bacterial communities	Hall, James P J; Wood, A Jamie; Harrison, Ellie; Brockhurst, Michael A	2016	Conjugation	*P. fluorescens, P. putida*	Evolutionary	Culture	No	No	Yes	Experimental, external	2	Deterministic	Constant	Yes
Static recipient cells as reservoirs of antibiotic resistance during antibiotic therapy	Willms, Allan R; Roughan, Paul D; Heinemann, Jack A	2006	Conjugation	None specified	Evolutionary	Culture	Yes	No	Yes	Assume	-	Deterministic	Sampled	No

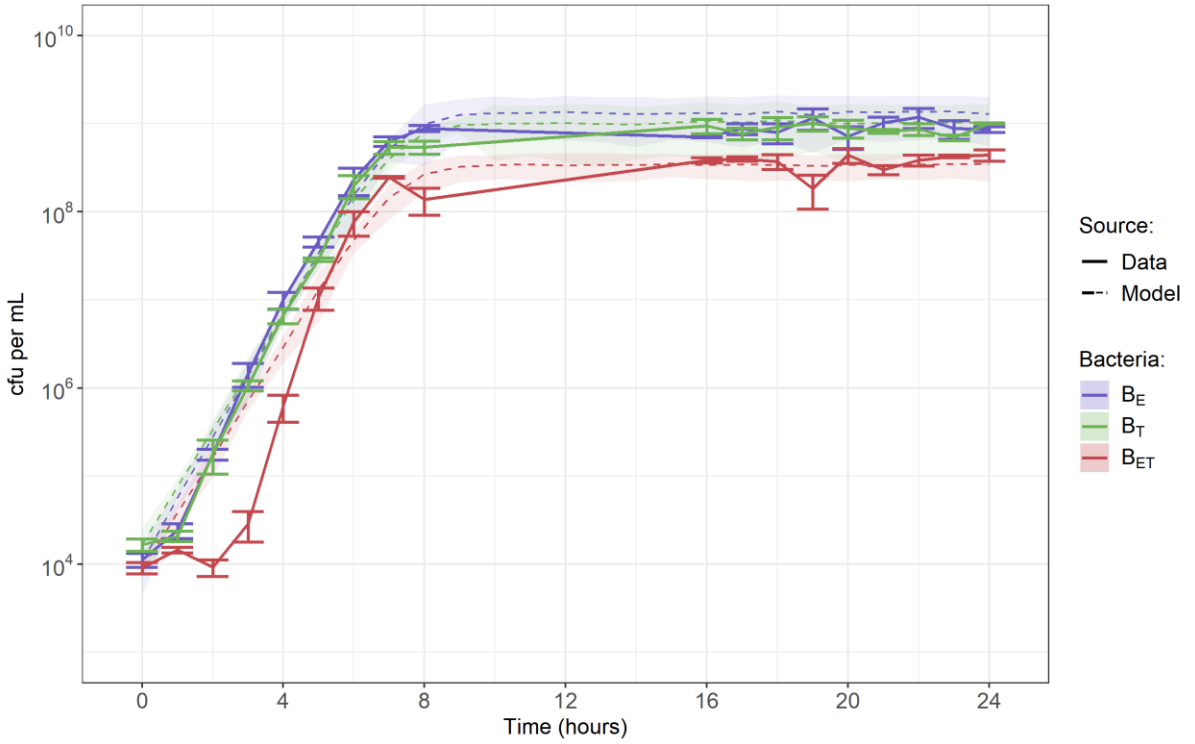
The dominance of bacterial genotypes leads to susceptibility variations under sublethal antibiotic pressure.	Xu, Shilian; Yang, Jiaru; Yin, Chong; Zhao, Xiaohua	2018	Conjugation	None specified	Evolutionary	None specified	Yes	No	Yes	Assume	-	Deterministic	Constant	Yes
The evolution of plasmid-carried antibiotic resistance	Svara, Fabian; Rankin, Daniel J	2011	Conjugation	None specified	Evolutionary	Culture	Yes	No	Yes	Assume	-	Deterministic	Constant	Yes
The Impact of Different Antibiotic Regimens on the Emergence of Antimicrobial-Resistant Bacteria	D'Agata, Erika M. C.; Dupont-Rouzeyrol, Myrielle; Magal, Pierre; Olivier, Damien; Ruan, Shigui	2008	Conjugation	*E. coli*	Evolutionary	Human	Yes	Yes	Yes	External, assume	5	Deterministic	Constant	No
The IncI1 plasmid carrying the blaCTX-M-1 gene persists in in vitro culture of a Escherichia coli strain from broilers	Fischer, Egil AJ; Dierikx, Cindy M; van Essen-Zandbergen, Alieda; van Roermund, Herman JW; Mevius, Dik J; Stegeman, Arjan; Klinkenberg, Don	2014	Conjugation	*E. coli*	Evolutionary	Culture	No	No	Yes	Experimental	-	Deterministic	Constant	No
The influence of horizontal gene transfer on the mean fitness of unicellular populations in static environments.	Raz, Yoav; Tannenbaum, Emmanuel	2010	Conjugation	None specified	Evolutionary	None specified	Yes	No	Yes	Assume	-	Both	-	Yes

The population biology of bacterial plasmids: a hidden Markov model approach.	Ponciano, José M; De Gelder, Leen; Top, Eva M; Joyce, Paul	2007	Conjugation	*P. putida, P. koreensis, S. mathophilia, P. plecoglossicida , P. veronii, O. tritici, E. adhaerens*	Evolutionary	Culture	No	No	Yes	Experimental	-	Both	-	Yes
The Population Biology of Bacterial Plasmids: A PRIORI Conditions for the Existence of Conjugationally Transmitted Factors.	Stewart, F M; Levin, B R	1977	Conjugation	None specified	Evolutionary	None specified	Yes	No	Yes	Assume	-	Deterministic	Constant	Yes
Transitory derepression and the maintenance of conjugative plasmids	Lundquist, Peter D; Levin', Bruce R	1986	Conjugation	*E. coli*	Evolutionary	Culture	No	No	Yes	Experimental, assume	-	Deterministic	Constant	Yes
Within-Host and Population Transmission of bla(OXA-48) in K. pneumoniae and E. coli	Haverkate, Manon R Dautzenberg , Mirjam J D; Ossewaarde, Tjaco J M; van der Zee, Anneke; den Hollander, Jan G; Troelstra, Annet; Bonten, Marc J M; Bootsma, Martin C J	2015	Conjugation	*E. coli, K. pneumonia*	Public health	Human	No	No	No	External, assume	1	Deterministic	Constant	Yes

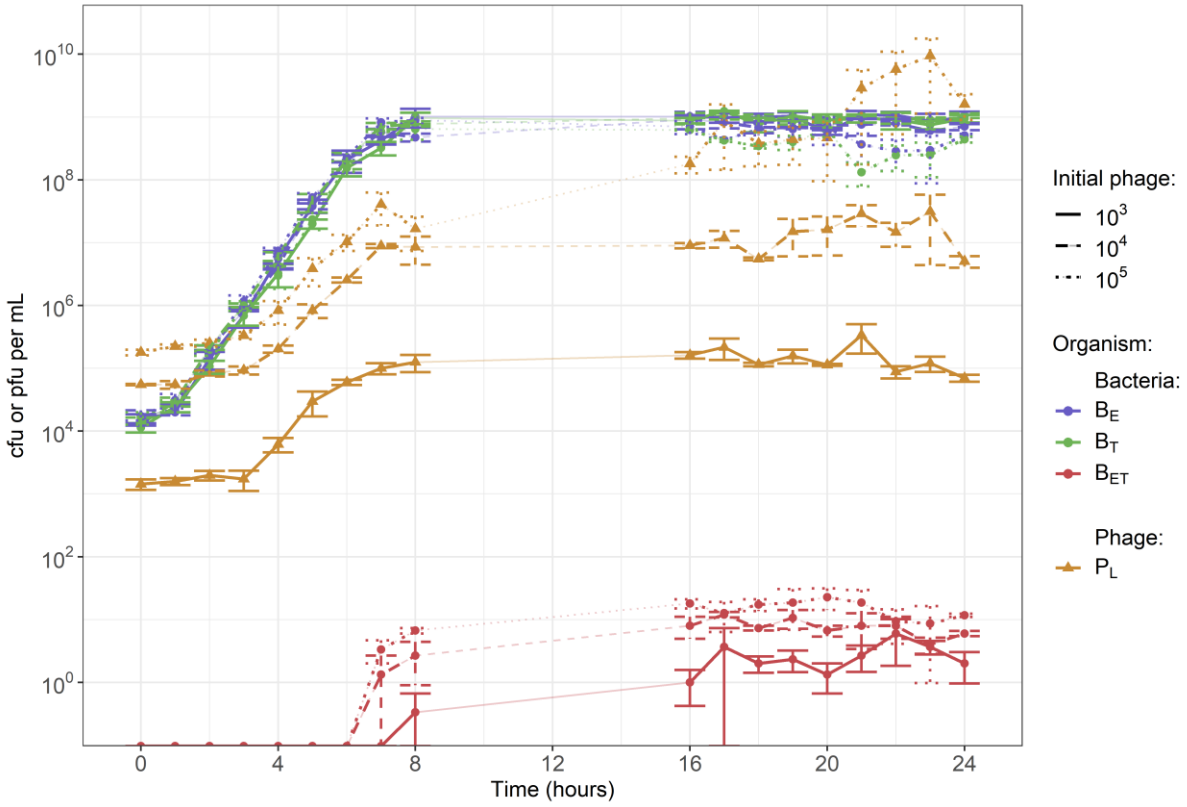
8.2 Supplementary Material for Chapter 3



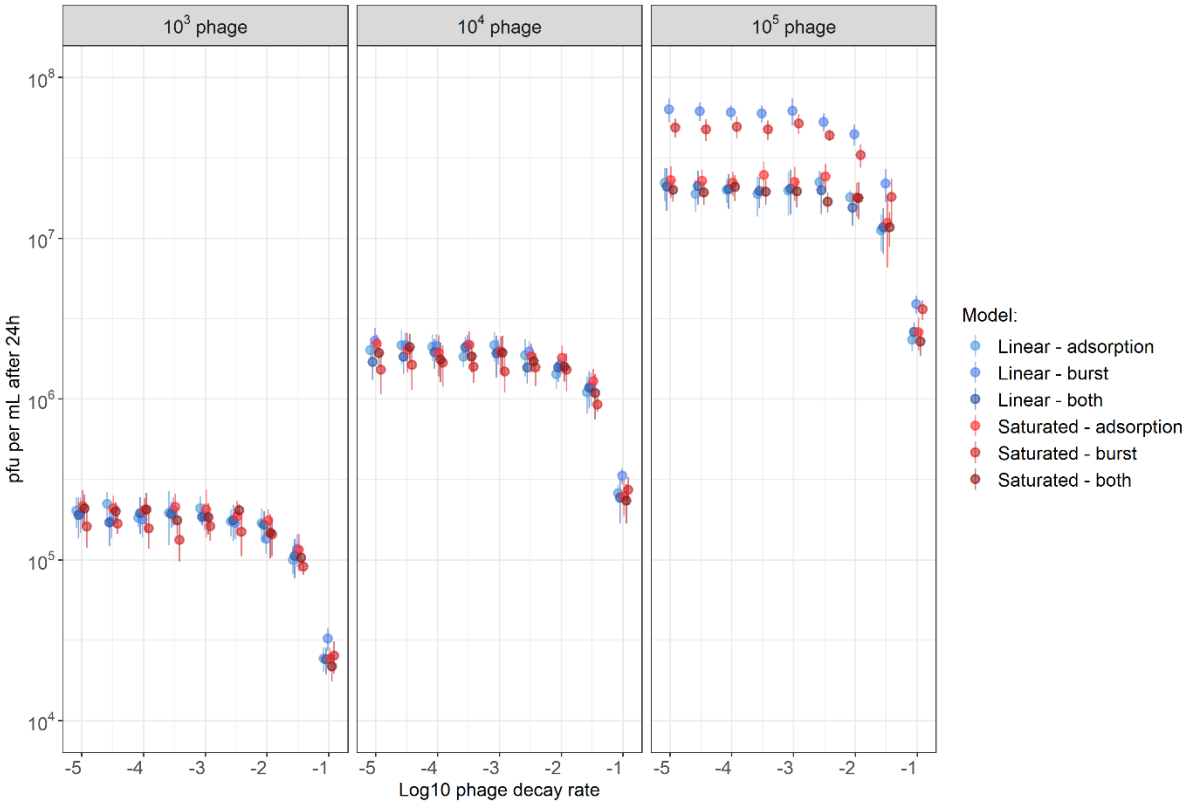
Supplementary Figure 3.1: Confirmation of double-resistant progeny by polymerase-chain reaction. Five single colonies were sampled from a double antibiotic plate (1-5), containing bacteria plated after 24h of co-culture started only with single-resistant parent strains (E and T) and exogenous phage. L: ladder; E: erythromycin resistance gene (*ermB*); T: tetracycline resistance gene (*tetK*).



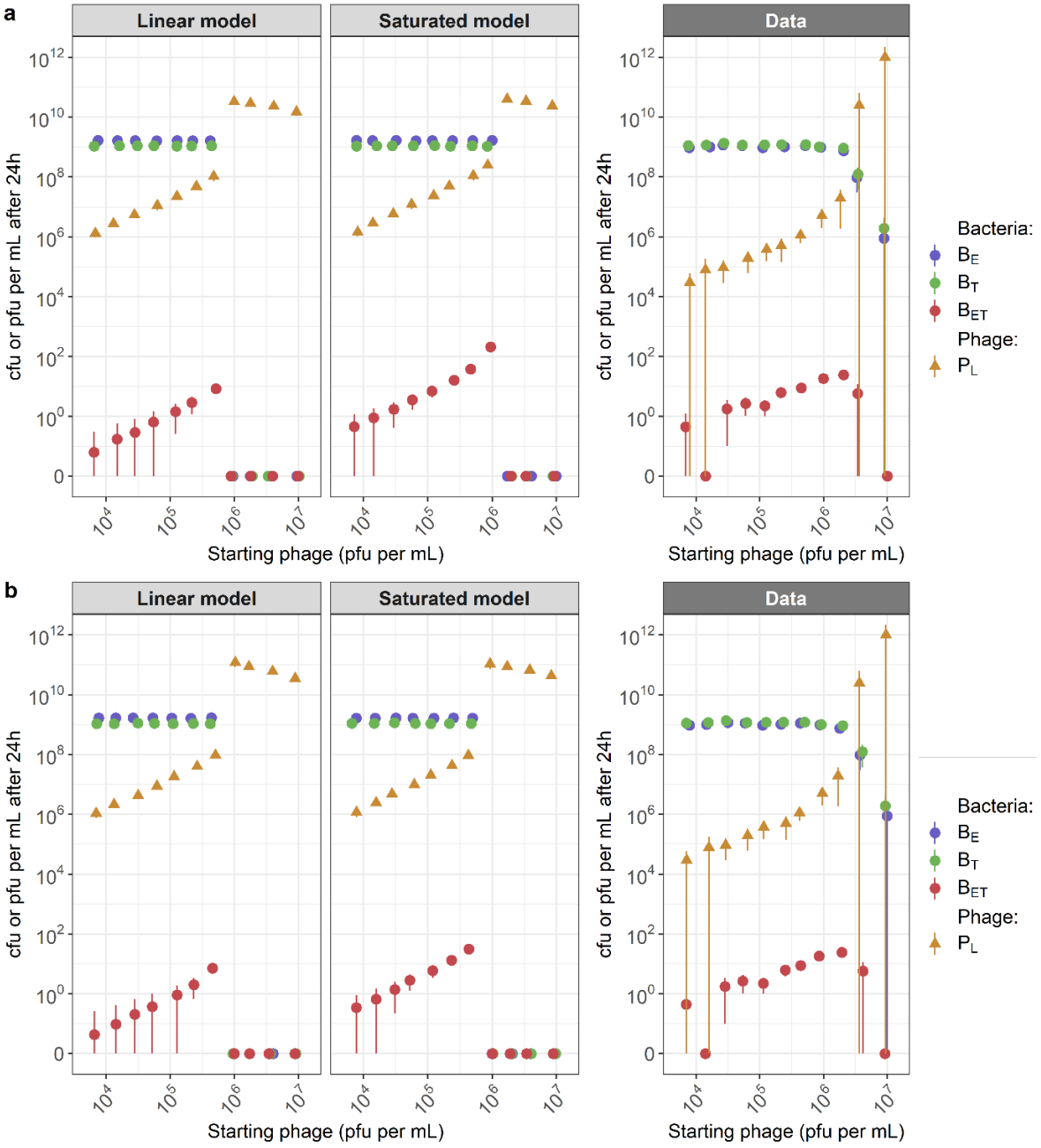
Supplementary Figure 3.2: Growth curves for bacteria in the absence of exogenous phage. B_E: bacteria resistant to erythromycin, B_T: bacteria resistant to tetracycline, B_{ET}: bacteria resistant to both erythromycin and tetracycline. Solid lines correspond to *in vitro* data, and dashed lines to the model output generated using the median values of the parameter distributions obtained by model fitting. Shaded areas indicate error obtained by resampling the model results from a Poisson distribution ten times.



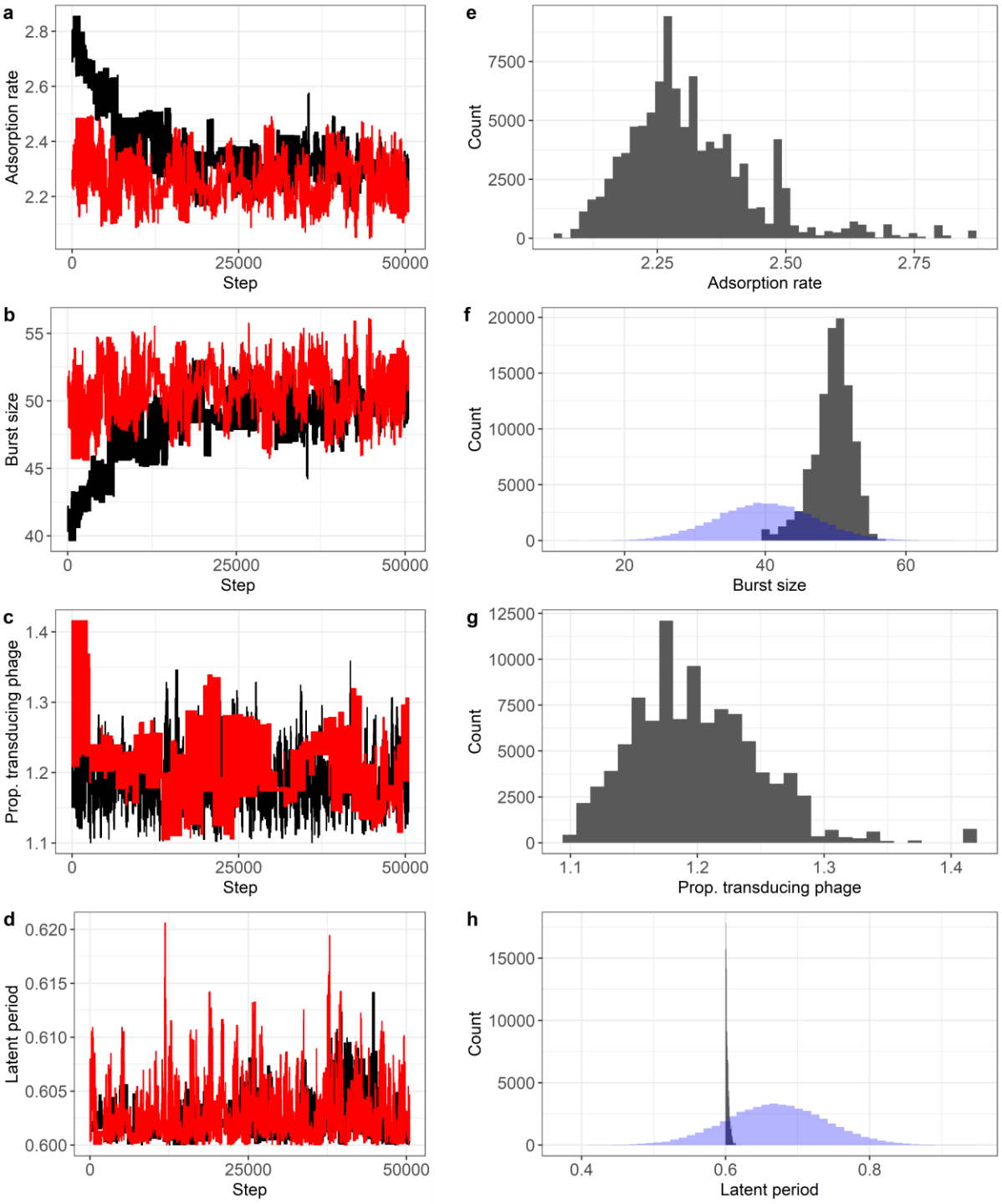
Supplementary Figure 3.3: Transduction co-culture datasets overlaid. The starting concentration of both single-resistant *S. aureus* parent strains (B_E to erythromycin & B_T to tetracycline) is 10^4 colony-forming units (cfu) per mL. The starting concentration of exogenous phage 80α (P_L) is either 10^3 (solid lines), 10^4 (dashed) or 10^5 (dotted) plaque-forming units (pfu) per mL. Error bars indicate mean +/- standard error, from 3 experimental replicates. There is no data for the time period 9h-15h.



Supplementary Figure 3.4: Model results are not affected by phage decay rate over a wide range of values. Previous estimates of phage decay rate per hour are between 10^{-3} *in vitro* and up to 10^{-1} *in vivo* (see reference 44 from Chapter 3). Phage predation in the models is either linear or saturated, with either or both the phage adsorption rate and burst size linked to bacterial growth.

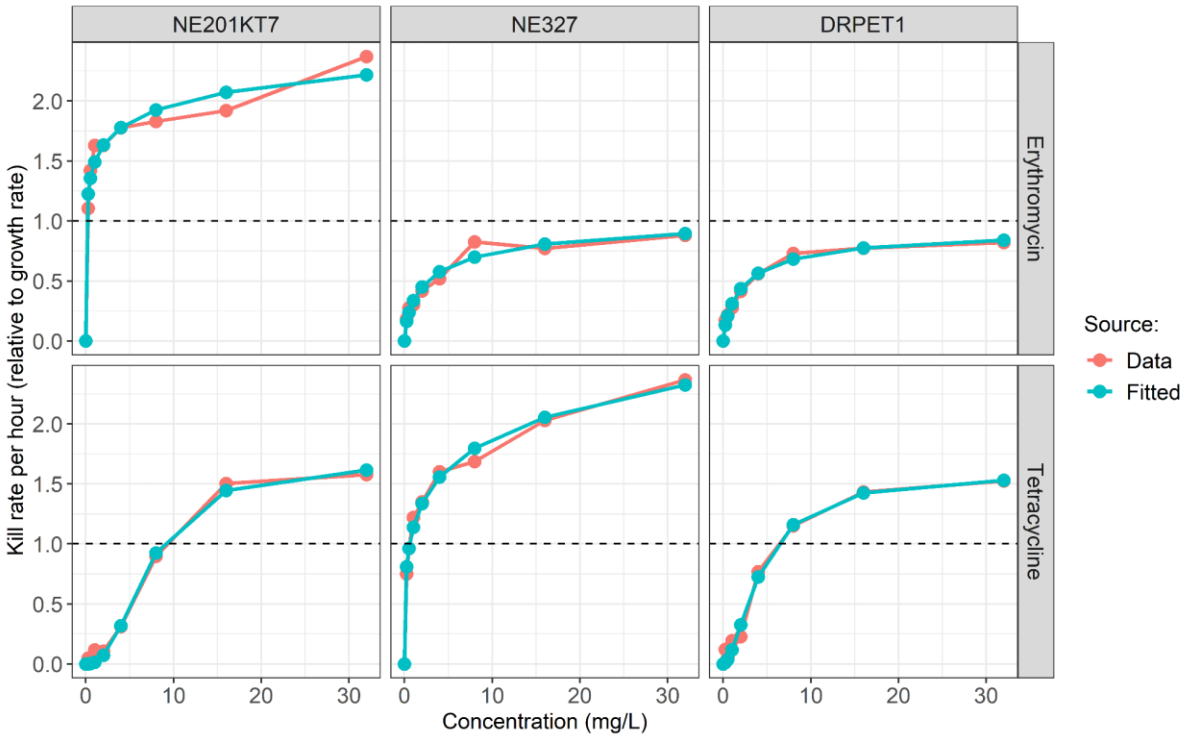


Supplementary Figure 3.5: Model performance in reproducing the 24h data values for different starting concentrations of phage with different links between phage predation and bacterial growth rate: A) Phage adsorption rate decreases as bacterial growth rate decreases. B) Phage burst size and adsorption rate decrease as bacterial growth rate decreases. Phage predation is either linear or saturated in the models. Model parameters are those estimated for the corresponding model as shown in Table 3.1. In the co-culture used to generate the data, each single-resistant parent strain (B_E and B_T) is added at a starting concentration of 10^6 cfu/mL, and no double-resistant progeny (B_{ET}) are initially present. The starting concentration of lytic phage (P_L) varies (x axis).

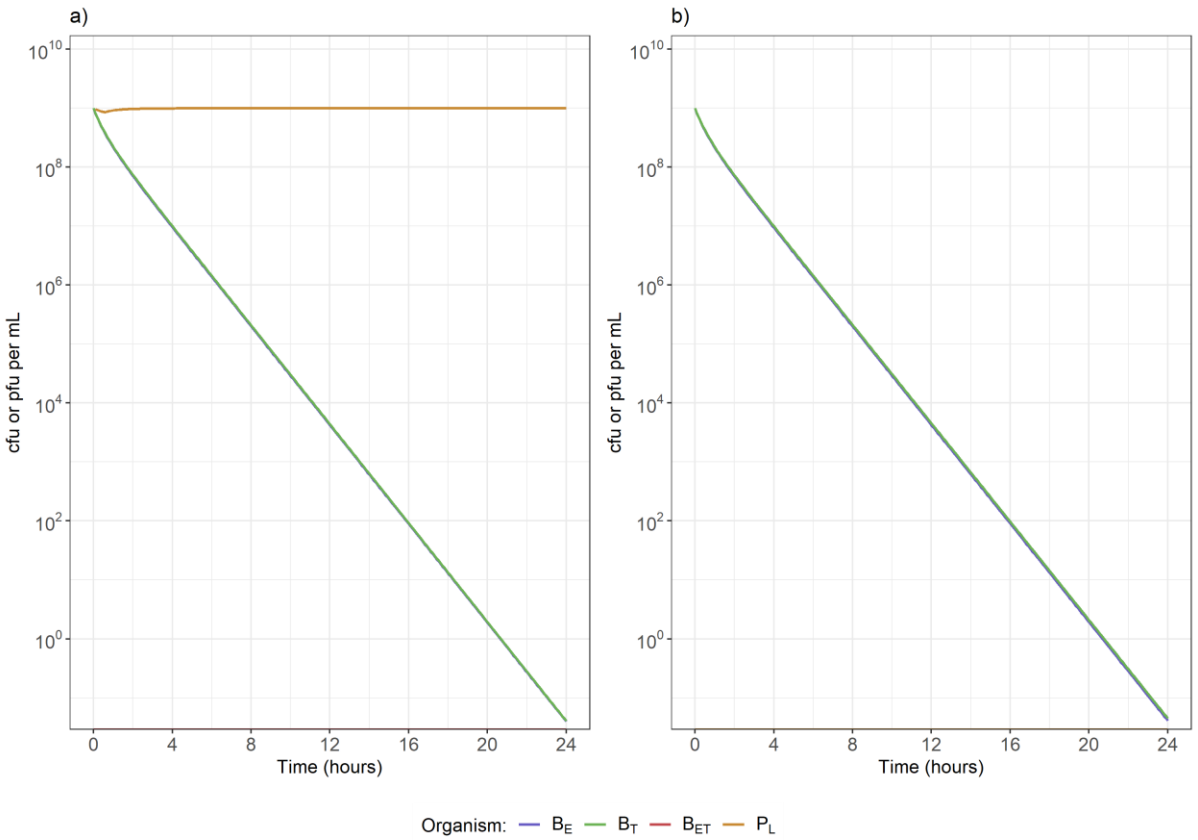


Supplementary Figure 3.6: Convergence and posterior distributions for the best-fitting model (with a saturated interaction and a link between phage burst size and bacterial growth). (a-d) Parameter convergence plots. Fitting was performed using two chains (black and red). **(e-h) Posterior distributions.** The prior distributions for the burst size and latent period are shown in blue. The prior distributions for other parameters were uninformative and not shown.

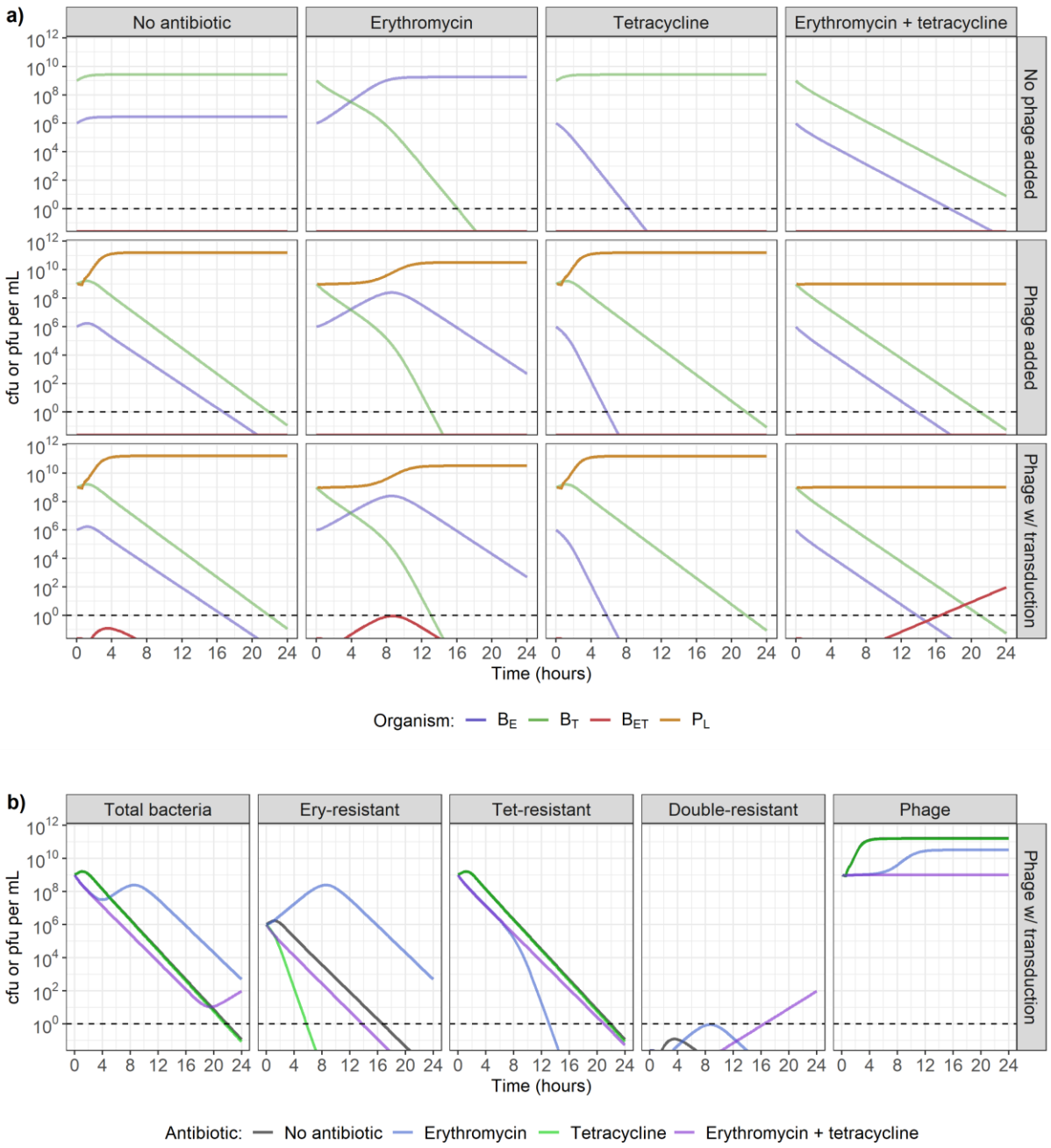
8.3 Supplementary Material for Chapter 4



Supplementary Figure 4.1: Antibacterial effect of erythromycin and tetracycline measured *in vitro* (pink) and obtained after fitting Hill equations (blue). Effect is relative to bacterial growth, such that a value greater than 1 indicates killing (net negative growth), while a value between 0 and 1 indicates only a decrease in growth rate. NE201KT7 contains a tetracycline-resistance gene (*tetK*), NE327 contains an erythromycin-resistance gene (*ermB*) and DRPET1 contains both resistance genes. The Hill equation is shown in Equation 4.3.

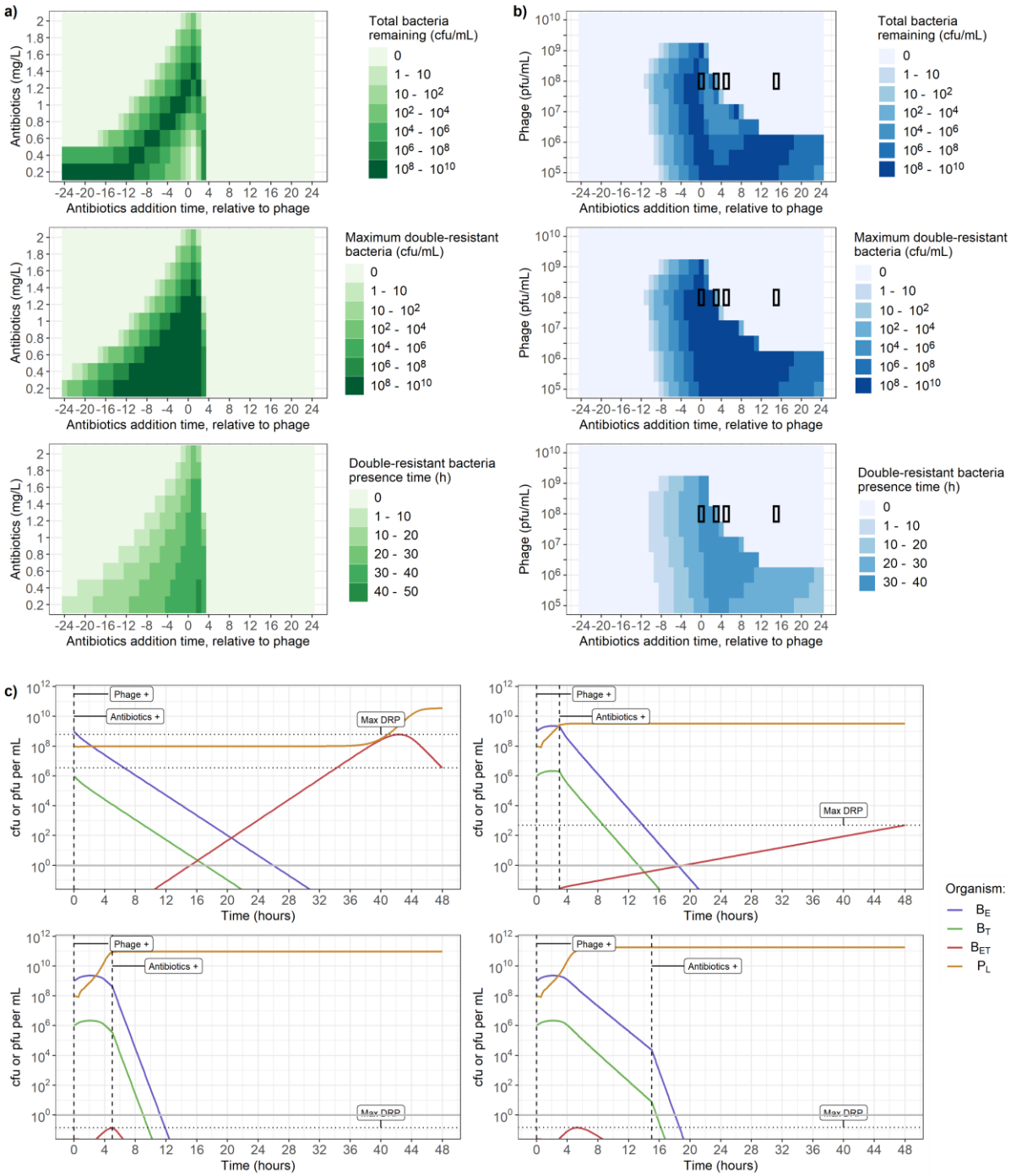


Supplementary Figure 4.2: a) The antibacterial effect of 1 mg/L of both erythromycin and tetracycline alongside 10^9 pfu/mL of phage is equivalent to b) the effect of 4.58 mg/L of erythromycin and 1.14 mg/L of tetracycline in the absence of phage. This was estimated by setting the concentration of phage to 0 in b) and fitting the concentrations of erythromycin and tetracycline to reproduce the decrease in bacteria numbers seen in a). cfu: colony-forming units; pfu: plaque-forming units.



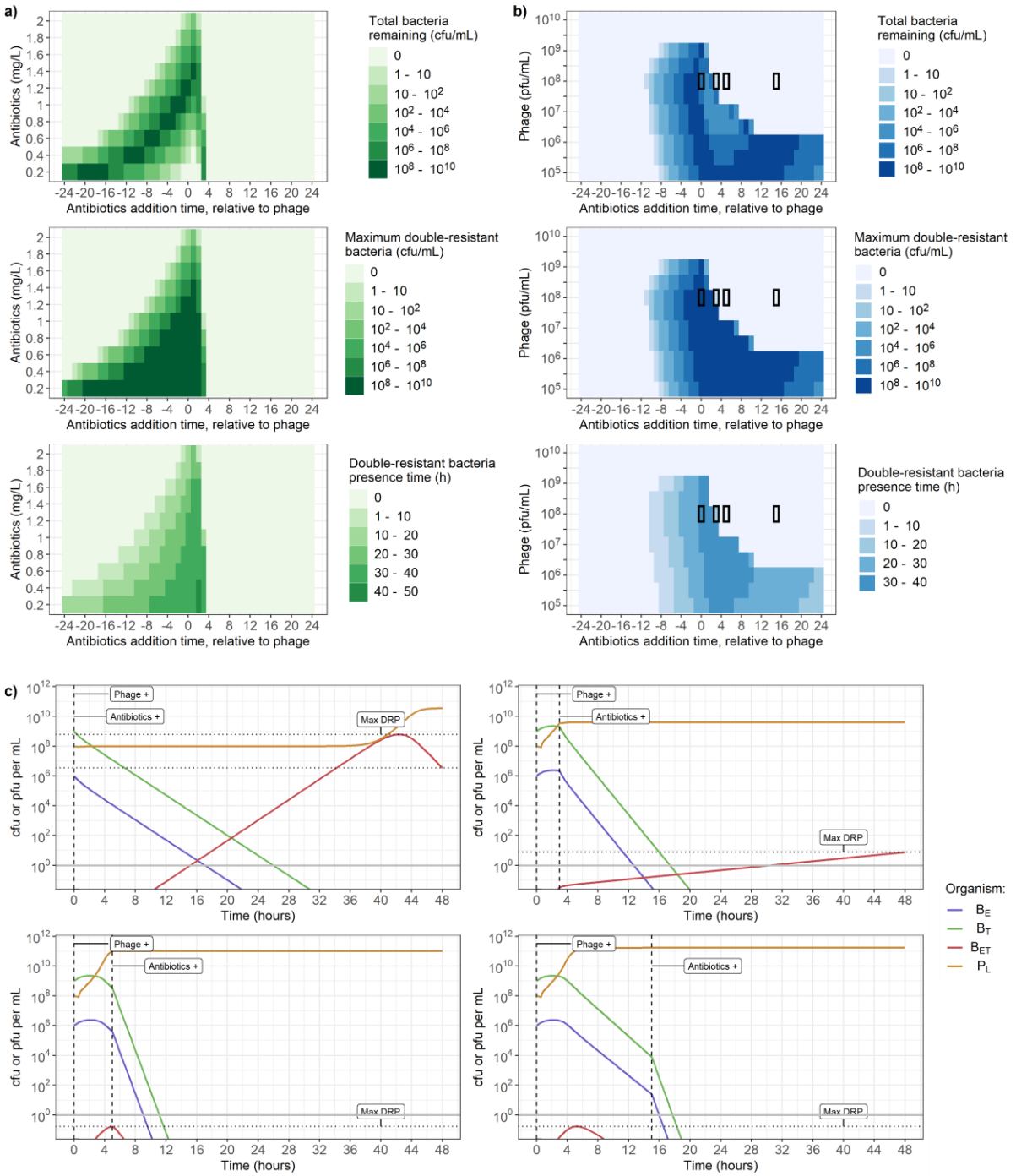
Supplementary Figure 4.3: a) Model-predicted bacterial dynamics in the presence of no antibiotics (1st column), erythromycin only (2nd column), tetracycline only (3rd column), or both erythromycin and tetracycline (4th column), combined with either no phage (top row), phage incapable of transduction (middle row), or phage capable of generalised transduction (bottom row). Tetracycline-resistant bacteria (B_T) are initially present at a concentration of 10^9 colony-forming units (cfu)/mL, and erythromycin-resistant bacteria (B_E) at 10^6 cfu/mL. Antibiotics and/or phage (P_L) are present at the start of the simulation, at concentrations of 1 mg/L and 10^9 plaque-forming units (pfu)/mL

respectively. Double-resistant bacteria (B_{ET}) can be generated by generalised transduction only. Dashed line indicates the detection threshold of 1 cfu or pfu/mL. **b) Change in bacteria (single-resistant to erythromycin, single-resistant to tetracycline, or double-resistant) and phage numbers depending on the antibiotic exposure, in the presence of phage capable of generalised transduction.**



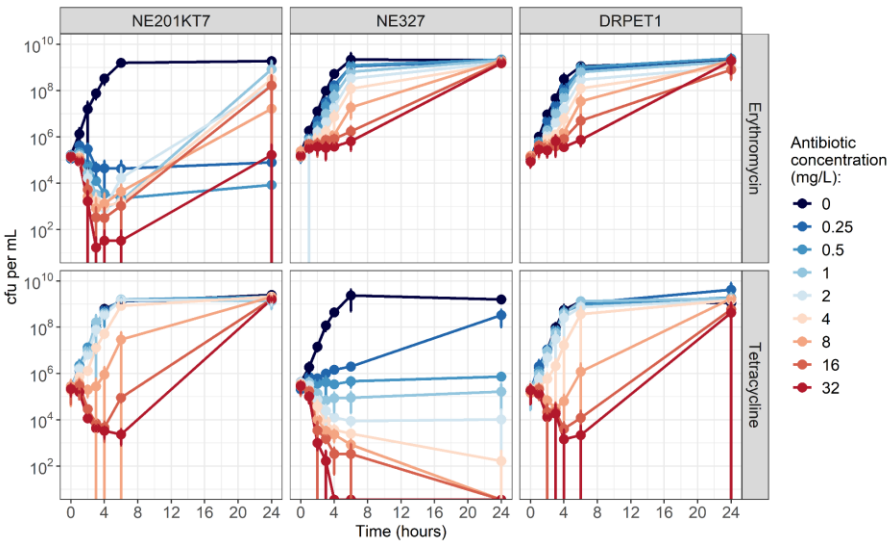
Supplementary Figure 4.4: Effect of varying antibiotic and phage timing and concentration when the tetracycline-resistant bacterial strain (B_T) is in minority (10^6 cfu/mL). a-b) Varying timing and dose of antibiotic and phage affects total bacterial count after 48h (top), maximum concentration of double-resistant bacteria (B_{ET}) (middle), and time when the concentration of B_{ET} is greater than 1 colony-forming unit (cfu) per mL (bottom). a) Adding 10^9 plaque-forming units (pfu) per mL of phage, and between 0.2 and 2.2 mg/L of both erythromycin and tetracycline. b) Adding 1 mg/L of both erythromycin and tetracycline, and between 10^5 and 10^{10}

pfu/mL of phage. The x-axis indicates the time when antibiotics were added, relative to when phage were added. For example, the value “4” indicates that phage were present at the start of the simulation, and antibiotics were introduced 4h later. The segments with black borders correspond to the dynamics shown in c). **c) Phage and bacteria dynamics over 48h for 4 conditions taken from panel b.** In all 4 conditions, phage are initially present at a concentration of 10^8 pfu/mL, while erythromycin and tetracycline are both introduced at concentrations of 1 mg/L after either 0h, 3h, 5h or 15h, stated on the plots, with the timing indicated by the vertical dashed lines. Solid line indicates the detection threshold of 1 cfu or pfu/mL. The concentrations of single-resistant bacteria (B_E, blue, and B_T, green) overlap and cannot be distinguished.

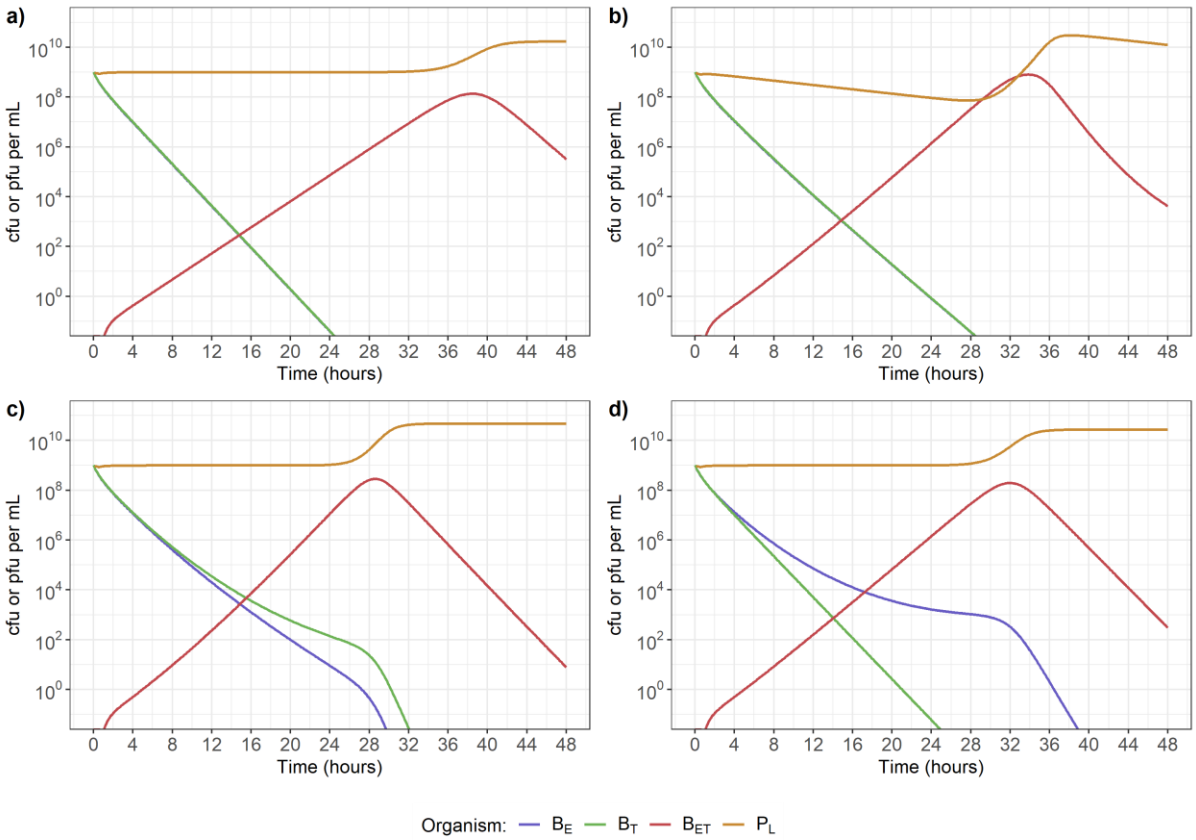


Supplementary Figure 4.5: Effect of varying antibiotic and phage timing and concentration when the erythromycin-resistant bacterial strain (B_E) is in minority (10^6 cfu/mL). a-b) Varying timing and dose of antibiotic and phage affects total bacterial count after 48h (top), maximum concentration of double-resistant bacteria (B_{ET}) (middle), and time when the concentration of B_{ET} is greater than 1 colony-forming unit (cfu) per mL (bottom). a) Adding 10^9 plaque-forming units (pfu) per mL of phage, and between 0.2 and 2.2 mg/L of both erythromycin and tetracycline. b) Adding 1 mg/L of both erythromycin and tetracycline,

and between 10^5 and 10^{10} pfu/mL of phage. The x-axis indicates the time when antibiotics were added, relative to when phage were added. For example, the value "4" indicates that phage were present at the start of the simulation, and antibiotics were introduced 4h later. The segments with black borders correspond to the dynamics shown in c). **c) Phage and bacteria dynamics over 48h for 4 conditions taken from panel b.** In all 4 conditions, phage are initially present at a concentration of 10^8 pfu/mL, while erythromycin and tetracycline are both introduced at concentrations of 1 mg/L after either 0h, 3h, 5h or 15h, stated on the plots, with the timing indicated by the vertical dashed lines. Solid line indicates the detection threshold of 1 cfu or pfu/mL. The concentrations of single-resistant bacteria (B_E, blue, and B_T, green) overlap and cannot be distinguished.



Supplementary Figure 4.6: Growth curves of NE201KT7 (tetracycline-resistant, left), NE327 (erythromycin-resistant, middle) and DRPET1 (double-resistant, right), exposed to varying concentrations of erythromycin (top) or tetracycline (bottom). The minimum inhibitory concentration values for bacteria at 24h were identical to the ones for stock bacteria, suggesting that antibiotic decay rather than acquired resistance is responsible for the increase in bacteria numbers after 24h. Error error bars indicate mean +/- standard deviation, from 3 replicates. cfu: colony-forming units. Note that cfu per mL are shown on a log-scale.



Supplementary Figure 4.7: Impact of phage and antibiotic decay on phage and bacteria dynamics over 48h. The conditions shown are: no decay (a), phage decay (b), erythromycin decay (c), and tetracycline decay (d). In all 4 conditions, phage and antibiotics (erythromycin and tetracycline) are initially present at concentrations of 10^9 pfu/mL and 1 mg/L respectively. Rates of decay are set to either 0 or 0.1 per hour.

8.4 Supplementary Material for Chapter 5

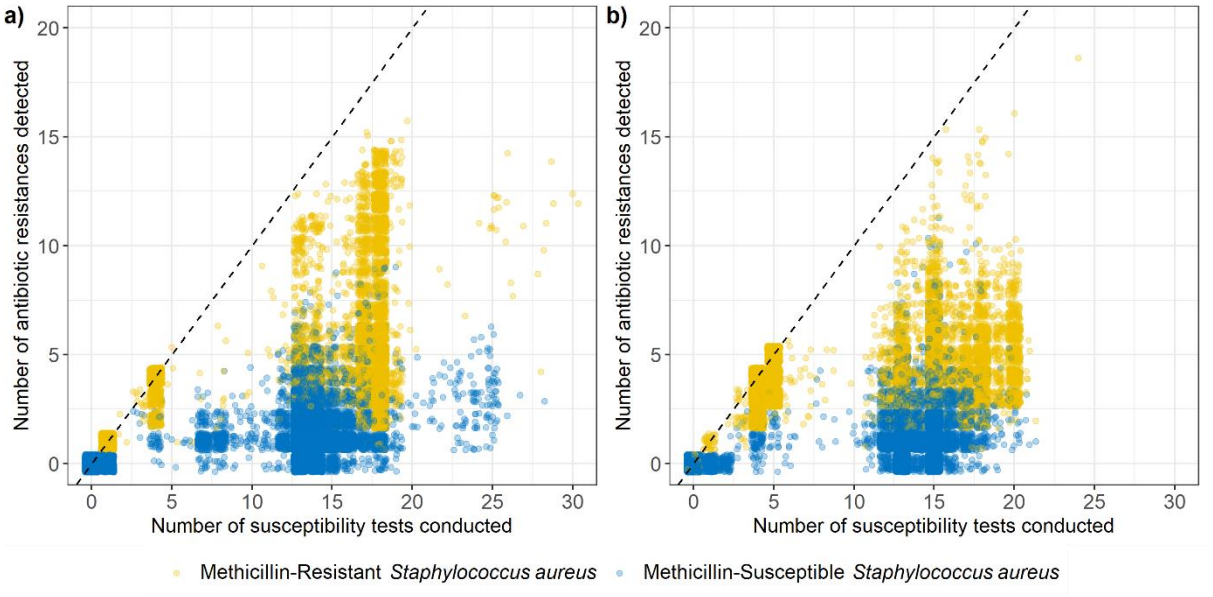
Supplementary Table 5.1: Matching information for antibiotics and antibiotic classes.

Antibiotic	Class	Antibiotic	Class
Amikacin	Aminoglycoside	Daptomycin	Lipopeptide
Gentamicin	Aminoglycoside	Azithromycin	Macrolide
Tobramycin	Aminoglycoside	Clarithromycin	Macrolide
Rifaximin	Ansamycin	Erythromycin	Macrolide
Bedaquiline	Bedaquiline	Metronidazole	Metronidazole
Ertapenem	Carbapenem	Mitomycin	Mitomycin
Imipenem	Carbapenem	Aztreonam	Monobactam
Meropenem	Carbapenem	Mupirocin	Mupirocin
Cefalexin	Cephalosporin	Nitrofurantoin	Nitrofuran
Cefiderocol	Cephalosporin	Linezolid	Oxazolidinone
Cefixime	Cephalosporin	Tedizolid	Oxazolidinone
Cefotaxime	Cephalosporin	Amoxicillin	Penicillin
Cefoxitin	Cephalosporin	Ampicillin	Penicillin
Ceftazidime	Cephalosporin	Benzylpenicillin	Penicillin
Ceftolozane	Cephalosporin	Co-Amoxiclav	Penicillin
Ceftriaxone	Cephalosporin	Flucloxacillin	Penicillin
Cefuroxime	Cephalosporin	Phenoxymethylpenicillin	Penicillin
Chloramphenicol	Cephalosporin	Piperacillin	Penicillin
Chlorhexidine	Chlorhexidine	Pivmecillinam	Penicillin
Clofazimine	Clofazimine	Temocillin	Penicillin
Dapsone	Dapsone	Colistin	Polypeptide
Ethambutol	Ethambutol	Protionamide	Protionamide

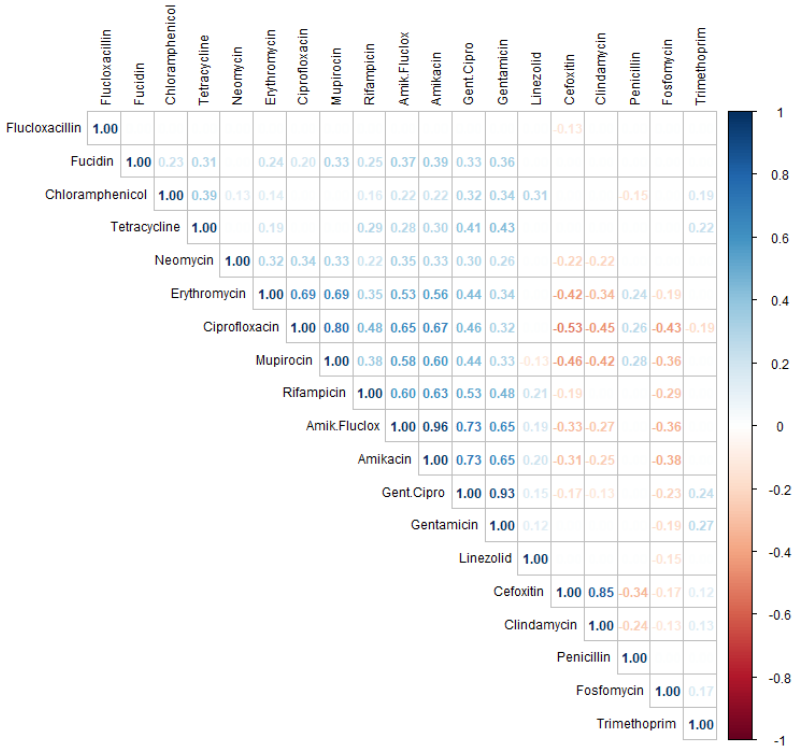
Ciprofloxacin	Fluoroquinolone
Levofloxacin	Fluoroquinolone
Moxifloxacin	Fluoroquinolone
Ofloxacin	Fluoroquinolone
Fosfomycin	Fosfomycin
Fucidin	Fucidin
Teicoplanin	Glycopeptide
Vancomycin	Glycopeptide
Isoniazid	Isoniazid
Clindamycin	Lincosamide

Pyrazinamide	Pyrazinamide
Rifampicin	Rifamycin
Co-Trimoxazole	Sulfonamide
Sulfadiazine	Sulfonamide
Trimethoprim	Sulfonamide
Doxycycline	Tetracycline
Minocycline	Tetracycline
Tetracycline	Tetracycline
Tigecycline	Tetracycline
Thalidomide	Thalidomide

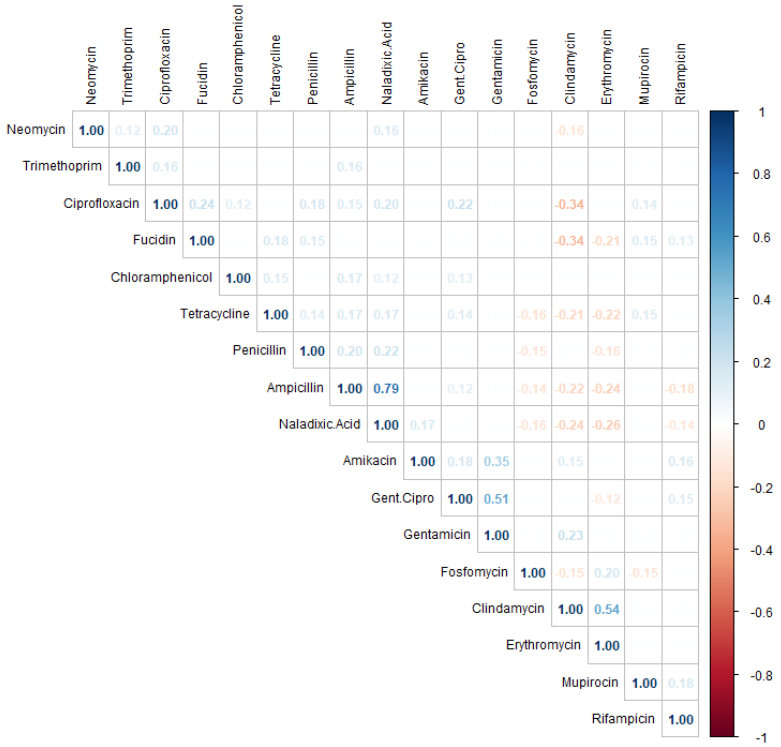
Supplementary Figure 5.1: Proportion of all *S. aureus* isolates which are methicillin-resistant (a) and ethnicity of patients in our dataset (b). Vertical green dashed line shows the date when lockdown began in the UK during the first wave of the COVID-19 pandemic (29/03/20). Patients with no recorded ethnicity are excluded from the analysis.



Supplementary Figure 5.2: Number of susceptibility tests conducted versus antibiotic resistances detected for each *S. aureus* isolate in our dataset. Each point corresponds to an isolate in the dataset, with the position of the points jittered on the figure to prevent the overlap of isolates with the same number of susceptibility tests and antibiotic resistances detected. The dashed line shows the maximum number of resistance that can be detected (equal to the number of susceptibility tests conducted). **a)** Isolates collected between 2000 and 2010. **b)** Isolates collected between 2011 and 2021.



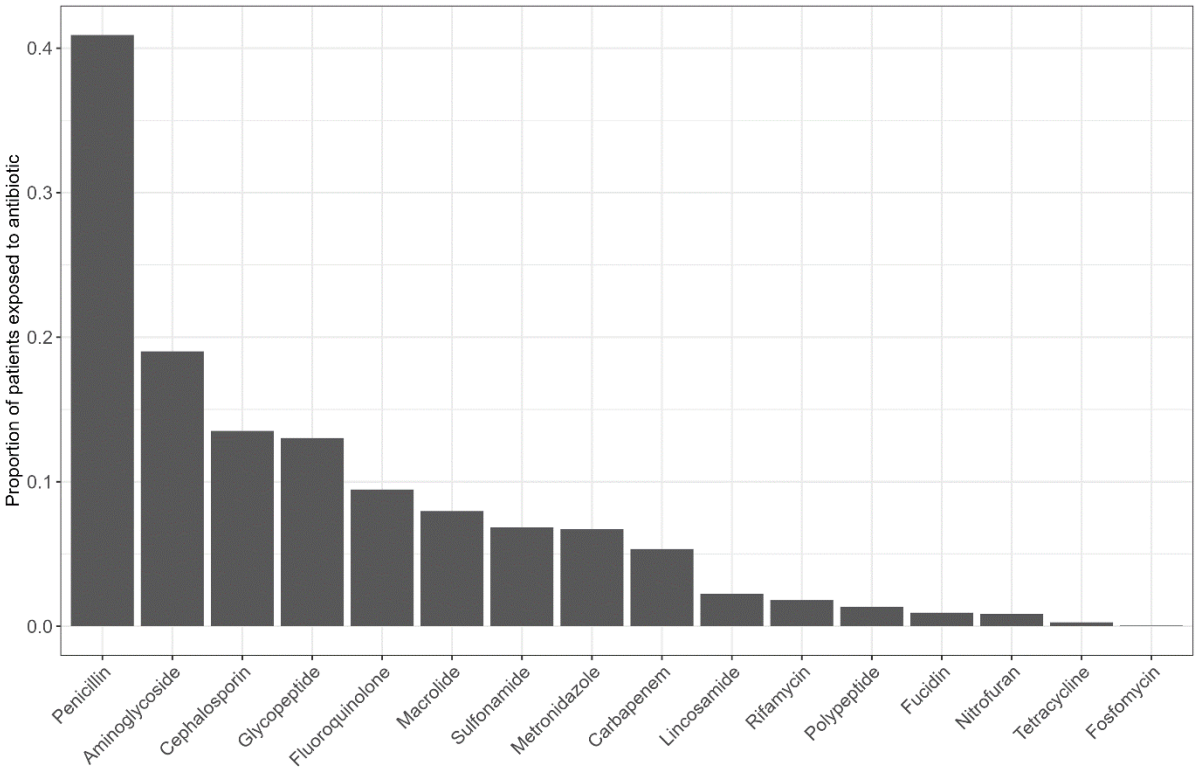
Supplementary Figure 5.3: Spearman's correlation coefficients between proportions of methicillin-resistant *S. aureus* isolates resistant to different antibiotics over time. Antibiotics included are those with at least 50 susceptibility tests conducted over the entire time period (2000-2021). Only significant coefficients are shown (p value < 0.05).



Supplementary Figure 5.4: Spearman's correlation coefficients between proportions of methicillin-susceptible *S. aureus* isolates resistant to different antibiotics over time. Antibiotics included are those with at least 50 susceptibility tests conducted over the entire time period (2000-2021). Only significant coefficients are shown (p value < 0.05).

Supplementary Figure 5.5: Change in proportion of *S. aureus* isolates resistant to antibiotics over time, out of those tested for resistance to the corresponding antibiotic. Amik.Fluclox: joint amikacin and flucloxacillin resistance, Gent.Ceftaz: joint gentamicin and ceftazidime resistance, Gent.Cipro: joint gentamicin and ciprofloxacin resistance, Gent.Pip.Taz: joint gentamicin and piperacillin-tazobactam resistance, Pip.Taz.Amik: joint piperacillin-tazobactam and amikacin resistance, Pip.Taz.Cipro: joint piperacillin-tazobactam and ciprofloxacin resistance.

Supplementary 5.6: Change in proportion of *S. aureus* isolates tested for different antibiotic susceptibilities over time. Amik.Fluclox: joint amikacin and flucloxacillin resistance, Gent.Ceftaz: joint gentamicin and ceftazidime resistance, Gent.Cipro: joint gentamicin and ciprofloxacin resistance, Gent.Pip.Taz: joint gentamicin and piperacillin-tazobactam resistance, Pip.Taz.Amik: joint piperacillin-tazobactam and amikacin resistance, Pip.Taz.Cipro: joint piperacillin-tazobactam and ciprofloxacin resistance.



Supplementary Figure 5.7: Proportion of all patients with a positive *S. aureus* swab exposed to antibiotics of different classes. Patients may have been exposed to more than one antibiotic.