



21 **ABSTRACT** *Escherichia coli* K1 strains are major causative agents of invasive disease of the  
22 new born. The age dependency of infection can be reproduced in the neonatal rat.  
23 Colonization of the small intestine following oral administration of K1 bacteria leads rapidly  
24 to invasion of the blood circulation; bacteria that avoid capture by the mesenteric lymphatic  
25 system and evade antibacterial mechanisms in the blood may disseminate to cause organ-  
26 specific infections such as meningitis. Some *E. coli* K1 surface constituents, in particular the  
27 polysialic acid capsule, are known to contribute to invasive potential but a comprehensive  
28 picture of the factors that determine the fully virulent phenotype has not so far emerged.  
29 We constructed a library and constituent sub-libraries of ~775,000 Tn5 transposon mutants  
30 of *E. coli* K1 strain A192PP and employed transposon-directed insertion site sequencing  
31 (TraDIS) to identify genes required for fitness for infection in the two-day-old rat.  
32 Transposon insertions were lacking in 357 genes following recovery on selective agar; these  
33 genes were considered essential for growth in nutrient replete medium. Colonization of the  
34 mid-section of the small intestine was facilitated by 167 *E. coli* K1 gene products. Restricted  
35 bacterial translocation across epithelial barriers precluded TraDIS analysis of gut-to-blood  
36 and blood-to-brain transits; 97 genes were required for survival in human serum. The study  
37 revealed that a large number of bacterial genes, many not previously associated with  
38 systemic *E. coli* K1 infection, are required to realise full invasive potential.

39 **IMPORTANCE** *Escherichia coli* K1 strains cause life-threatening infections in newborn  
40 infants. They are acquired from the mother at birth and colonize the small intestine, from  
41 where they invade the blood and central nervous system. It is difficult to obtain information  
42 from acutely ill patients that shed light on physiological and bacterial factors determining  
43 invasive disease. Key aspects of naturally occurring age-dependent human infection can be

44 reproduced in neonatal rats. Here, we employ transposon-directed insertion site sequencing  
45 to identify genes essential for *in vitro* growth of *E. coli* K1 and genes that contribute to  
46 colonization of susceptible rats. The presence of bottlenecks to invasion of the blood and  
47 cerebrospinal compartments precluded insertion sequencing analysis but we identified  
48 genes for survival in serum.

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63 **INTRODUCTION**

64 Early-onset sepsis and associated septicemia and meningitis are major causes of morbidity  
65 and mortality in the first weeks of life. In the developed world, encapsulated *Escherichia coli*  
66 and Group B streptococci are responsible for the large majority of these infections (1-3).  
67 Over 80% of *E. coli* blood and cerebrospinal fluid isolates from infected neonates express  
68 the  $\alpha$ ,2-8-linked polysialic acid (polySia) capsular K1 polysaccharide (4, 5), a polymer  
69 facilitating evasion of neonatal immune defenses due to its structural similarity to the  
70 polySia modulator of neuronal plasticity in the developing human embryo (6). Infections  
71 arise due to colonization of the neonatal gastrointestinal (GI) tract by maternally derived *E.*  
72 *coli* K1 at or soon after birth, from where the bacteria invade the systemic circulation to gain  
73 entry into the central nervous system (CNS) (7, 8).

74       Essential features of the human infection can be reproduced in the neonatal rat,  
75 enabling investigation of the pathogenesis of *E. coli* K1 neonatal invasive infections (9-11). In  
76 susceptible two-day-old (P2) rat pups the protective mucus layer in the small intestine is  
77 poorly developed but matures to full thickness over the P2-P9 period, coincident with the  
78 development of resistance to invasive infection from GI-colonizing *E. coli* K1 (12). Thus, oral  
79 administration of *E. coli* K1 initiates stable colonization of the small intestine in both P2 and  
80 P9 pups but elicits lethal systemic infection only in younger animals (13). In the absence of  
81 an effective mucus barrier at P2, the colonizing bacteria make contact with the apical  
82 surface of enterocytes in the mid-region of the small intestine before translocation to the  
83 submucosa by an incompletely defined transcellular pathway (12). They subsequently gain  
84 access to the blood compartment by evading mesenteric lymphatic capture (10, 14). *E. coli*  
85 K1 cells strongly express polySia in blood (15) and the capsule may protect the bacteria from  
86 complement attack during this phase of the infection by facilitating binding of complement

87 regulatory factor H to surface-bound C3b to prevent activation of the alternative pathway  
88 (16, 17). Following hematogenous spread, the bacteria enter the CNS *via* the blood-  
89 cerebrospinal barrier at the choroid plexus epithelium to colonize the meninges (15). Some  
90 microorganisms that invade the CNS enter across the cerebral microvascular endothelium of  
91 the arachnoid membrane (18), although the restricted distribution of *E. coli* K1 within the  
92 CNS suggests this is not a primary route of entry for this pathogen.

93         Only a limited number of pathogenic bacteria have the capacity to invade the CNS  
94 from a remote colonizing site and the large majority elaborate a protective capsule that  
95 facilitates avoidance of host defenses during transit to the site of infection (19). Although  
96 the polySia capsule is clearly necessary for neonatal pathogenesis of *E. coli* K1 (11), the large  
97 majority of bacterial virulence factors that facilitate transit from GI tract to brain are  
98 unknown. A number of potential virulence factors associated with neonatal bacterial  
99 meningitis have been defined by phylogenetic analysis (20) and there is good evidence that  
100 the genotoxin colibactin and the siderophore yersiniabactin contribute to the pathogenesis  
101 of *E. coli* K1 in the experimental rat (21-23); however, a more detailed understanding of  
102 virulence mechanisms of *E. coli* K1 invasive disease will present opportunities for new  
103 modes of therapy for these devastating infections.

104         Transposon insertion sequencing (24, 25), a combination of traditional transposon  
105 mutagenesis and massively parallel DNA sequencing, is a powerful tool for the genome-wide  
106 enhanced genetic screening of large pools of mutants in a single experiment. It has recently  
107 been used to determine the full complement of genes required for expression of the K1  
108 capsule by an *E. coli* uropathogenic isolate (26). The technique can be used to detect  
109 variations in genetic fitness of individual mutants undergoing selection in colonized and  
110 infected hosts. There are a number of variations of this procedure but all rely on the

111 creation of a pool of insertion mutants in which every locus has been disrupted at multiple  
112 sites; determination of the site of transposon insertion by sequencing of transposon  
113 junctions within chromosomal DNA before and after applying selective pressure will identify  
114 mutants attenuated under the selective condition (27). Thus, genes that confer fitness  
115 during *Klebsiella pneumoniae* (28) and *Acinetobacter baumannii* (29) lung persistence,  
116 systemic and mucosal survival of *Pseudomonas aeruginosa* (30), and spleen colonization in  
117 the mouse of uropathogenic *E. coli* (31) have been identified by this approach. In this study,  
118 we employ transposon-directed insertion site sequencing (TraDIS) (24) to interrogate a  
119 library of ~775,000 Tn5 mutants or constituent sub-libraries of *E. coli* K1 strain A192PP for  
120 genes essential for growth *in vitro* and GI colonization, invasion and systemic survival in  
121 susceptible P2 rat pups. In addition, we identified “bottlenecks” (32) to systemic invasion  
122 that restrict population diversity and limit the potential for transposon insertion site analysis  
123 of infection in the GI-colonized neonatal rat.

## 124 RESULTS

125 **Generation of a Tn5 mutant library and identification of essential genes.** To provide  
126 sufficient saturation density for the identification of *E. coli* K1 genes essential for growth *in*  
127 *vitro* and of those conferring fitness in a range of defined environments, approximately 300  
128 individual pools, each of  $1\text{-}5 \times 10^3$  transposon mutants of *E. coli* A192PP, were constructed  
129 and combined to form a library containing over  $7.75 \times 10^5$  mutants. Linker PCR was  
130 performed on randomly selected mutants to confirm that Tn5 had inserted into random  
131 genomic locations (Fig. S1). TraDIS was performed on pooled but uncultured mutants to  
132 identify Tn5 insertion sites within the 5.52 Mbp genome of A192PP (33). Sequences of  
133 indexed amplicons were determined and  $2 \times 10^6$  sequence reads containing Tn5 were

134 mapped onto the *E. coli* K1 A192PP genome. Reads mapped to 237,860 unique Tn5 insertion  
135 sites, and were distributed along the entire genome (Fig. 1A).

136 As the Tn5 library contained a high transposon insertion density, genes with no or  
137 limited Tn5 insertion sites are likely to be essential for growth in nutrient-replete media  
138 such as Luria-Bertani (LB) broth. We calculated insertion indices for each gene by  
139 normalizing the number of insertions in each gene by gene length. Insertion index values for  
140 two technical replicates were highly correlated (Spearman's  $\rho = 0.9589$ ) (Fig. 1B). A  
141 density plot of insertion indices produced a bimodal distribution, with a narrow peak  
142 representing genes with no or a limited number of Tn5 insertions and a broad peak  
143 containing genes with a large number of Tn5 insertions (Fig. 1C); the former comprised  
144 genes that confer lethality when mutated and the latter genes that can be mutated without  
145 affecting bacterial viability. To identify genes significantly lacking Tn5 insertions and  
146 therefore essential for *in vitro* growth, gamma distributions from the density plot were used  
147 to determine  $\log_2$  likelihood ratios. Examples of essential genes containing no or limited Tn5  
148 insertions are shown in Fig. 1D. A total of 357 genes were predicted to be essential for the *in*  
149 *vitro* growth of *E. coli* K1 A192PP and these are shown in Table S1, together with KEGG  
150 (Kyoto Encyclopedia of Genes and Genomes) descriptors for genes involved in metabolic  
151 pathways.

152 COG (Clusters of Orthologous Groups) was used to identify the functional category of  
153 each gene essential for growth *in vitro* from the A192PP whole genome sequence (accession  
154 number PRJEB9141). Genes involved in ribosomal structure (11% of total number of  
155 essential genes) and protein biosynthesis (15%) featured prominently and were significantly  
156 enriched in relation to their representation within the whole genome as were genes  
157 encoding proteins for DNA replication (3%), cell wall (peptidoglycan, lipopolysaccharide)

158 biosynthesis (6.25%) and membrane biogenesis (3%) (Fig. 2). Genes for protein secretion  
159 and export as well as ABC transporter genes were also well represented; the remaining  
160 essential genes were involved in a wide variety of cellular catabolic and anabolic functions.  
161 The list features 254 genes that were found by TraDIS (34) to be essential for growth in Luria  
162 broth of an *E. coli* ST131 multi-drug resistant urinary tract isolate (from a total of 315  
163 essential genes). In similar fashion, 253 genes determined as essential for growth of *E. coli*  
164 K12 MG1655 in LB broth were also identified as essential in the current study (Table S1); the  
165 K12 study employed a comprehensive set of precisely defined, in-frame single-gene deletion  
166 mutants (35), not transposon insertion sequencing.

167 **Maintaining Tn5 library diversity.** The polySia capsule is a major determinant of virulence in  
168 *E. coli* K1 and is central to the capacity of K1 clones to cause neonatal systemic infection (11,  
169 36). PolySia biosynthesis imposes a substantial metabolic burden on producer strains (37).  
170 As TraDIS and other transposon insertion sequencing procedures generally employ growth  
171 in liquid medium for recovery and expansion of the output pool (38), we investigated the  
172 impact of batch culture on the expression of the K1 capsule within the Tn5 library. The  
173 complete Tn5 library was inoculated into LB broth, incubated for 8 h at 37°C and the  
174 proportion of encapsulated and non-encapsulated A192PP bacteria determined by  
175 susceptibility to the *E. coli* K1-specific bacteriophage K1E within the population. Non-  
176 encapsulated mutants initially comprised 4.66% of the bacterial population but by the end  
177 of the incubation period this had risen to 98.24% (Fig. 3A). Growth rates in LB broth of *E. coli*  
178 A192PP and a non-encapsulated mutant of A192PP randomly selected from the Tn5 library  
179 did not differ significantly (Fig. S2A).



180 The cultured Tn5 library was avirulent as determined by administration to P2  
181 neonatal rat pups whereas GI colonization with  $2-6 \times 10^6$  CFU *E. coli* A192PP and the  
182 uncultured Tn5 library were lethal. A similar colonizing inoculum of the cultured (8 h; 37°C)  
183 *E. coli* A192PP-Tn5 library had no impact on survival and all pups remained healthy over the  
184 seven-day observation period (Fig. 3B), even though all animals remained heavily colonized  
185 with K1 bacteria throughout the experiment (data not shown). Thus, culture of the library  
186 prior to challenge resulted in loss of phenotypic diversity and virulence. The complete Tn5  
187 library contained  $2.81 \times 10^5$  unique Tn5 insertions, of which 750 (2.66% of the bacterial  
188 population) possessed transposon insertions in genes determining capsule biosynthesis  
189 (data not shown). The probability that cultured sub-libraries of more than  $5 \times 10^3$  mutants  
190 contained a non-encapsulated mutant was calculated to be  $\geq 0.98$  but only 0.55 for sub-  
191 libraries of  $1 \times 10^3$ . Low complexity libraries of  $10^3$  mutants maintained virulence in P2  
192 neonatal rat pups after culture whereas more complex libraries did not (Fig. S3), due to the  
193 absence of mutants lacking the capacity to express the polySia capsule within the inoculum.  
194 To minimise bias, in all subsequent experiments libraries of sufficient complexity to contain  
195 multiple numbers of non-encapsulated mutants were used; for experiments utilizing  
196 neonatal rats the period between colonization initiation and tissue harvesting was kept to a  
197 minimum and tissue homogenates were cultured directly on to selective agar plates with no  
198 intervening liquid culture step.

199 **Genes required for GI colonization.** *E. coli* A192PP colonize the small intestine of neonatal  
200 rats following oral administration of the bacterial bolus, with  $10^7$ - $10^8$  K1 bacteria/g intestinal  
201 tissue persisting for at least one week (12, 13). Translocation of the neonatal pathogen to  
202 the blood compartment *via* the mesenteric lymphatic system occurs predominantly, and in

203 all likelihood exclusively, across the epithelium of the mid-section of the small intestine  
204 (MSI), even though the density of colonizing bacteria in this region of the GI tract is no  
205 greater than that within neighbouring proximal (PSI) or distal (DSI) locations (12).

206 Few attempts have been made to determine the genes or gene products required by  
207 *E. coli* K1 for colonization of the GI tract (39). To prevent loss of diversity of the *E. coli* K1  
208 A192PP-Tn5 library, we minimized the period of colonization before sampling the *E. coli* K1  
209 population of the MSI. The colonizing *E. coli* K1 population in proximal, middle and distal  
210 regions of the small intestine did not expand beyond 4 h after initiation of colonization (Fig.  
211 4A); GI tissues were therefore excised at this time point. To identify mutants with decreased  
212 capacity to colonize the MSI, P2 rats were fed  $1 \times 10^9$  CFU of an *E. coli* K1 A192PP-Tn5 library  
213 containing  $2 \times 10^5$  mutants, the pups sacrificed after 4 h and *E. coli* K1 bacteria in the MSI  
214 enumerated. The bacterial load of rats colonized with the Tn5 library was comparable to  
215 that of rats colonized with the wildtype strain (data not shown). MSI tissues from four rats  
216 were pooled, homogenized and cultured on LB agar containing kanamycin to ensure that  
217 mutant frequency was not overestimated by inclusion of measurements of DNA from dead  
218 bacteria; kan<sup>R</sup> colonies were then pooled, DNA extracted and the fitness of each mutant  
219 determined by TraDIS. Input and output pools each comprised  $2 \times 10^5$  CFU and the ratio of  
220 input:MSI read counts were expressed as log<sub>2</sub> fold change. A wide distribution of fitness  
221 scores (40) were detected (Fig. 4B). The majority of transposon insertions did not have a  
222 strong negative or positive effect on colonisation of the MSI. A total of 387 transposon  
223 insertions, within 167 genes, had significantly decreased in normalized read counts between  
224 input and output pools (negative log<sub>2</sub> fold change and  $P < 0.05$ ; Table S2). Of the 387  
225 insertion sites, 180 were not detectable in the output pool, demonstrating complete loss in  
226 the output pool. Many of these transposon insertion sites occurred within the same gene

227 (Table S2). For example, within the *neuC* gene, 70 unique transposon insertion sites were  
228 identified as lost during colonization. Transposon-interrupted genes were identified as  
229 important for colonization of the MSI and were grouped into seven arbitrary categories: (i)  
230 genes encoding surface structures, including pili, (ii) genes encoding secretory components,  
231 (iii) genes involved in intermediary metabolism, (iv) stress response genes, (v) cytoplasmic  
232 membrane (CM)-located genes, (vi) genes for iron acquisition and (vii) others and  
233 hypothetical genes.

234 A high proportion of mutations associated with decreased MSI colonizing capacity  
235 were located in genes affecting the biosynthesis of surface structures (Table S2). A few  
236 genes were involved in lipopolysaccharide (LPS) biosynthesis (*yrbH*, *yiaH*) and OM proteins  
237 (*ompG*, *ycbS*) but the majority affected the polySia capsule, with genes of the *neu* operon  
238 (41), accounting for 194 of the 387 colonization-attenuated mutants. There is some  
239 evidence that capsular polysaccharides may promote adhesion to biological and non-  
240 biological surfaces during biofilm formation (37) but there has been little or no  
241 consideration of a role for capsules as mediators of GI colonization.

242 A limited number of genes associated with type II and IV secretion were identified as  
243 required for colonisation of the MSI; these multiprotein complexes translocate a wide range  
244 of proteins and protein complexes across host membranes (42, 43) and are implicated in  
245 adherence and intestinal colonization of enterohemorrhagic *E. coli* in farm animals (44).  
246 Genes for assembly of pilus proteins, including some encoded by the *tra* locus, likely to be  
247 located on plasmids, that initiate conjugation, were also linked to colonization; pili are  
248 virulence factors that may mediate attachment to and infection of host cells (45).  
249 Colonization by both commensals and pathogens is dependent on nutrient scavenging,  
250 sensing chemical signals and regulation of gene expression as the bacteria adapt to a new

251 and potentially hostile environment that in the case of *E. coli* K1 appears to rely on stress  
252 response genes such as *yhiM* (encodes a protein aiding survival at low pH) and the heat  
253 shock protein genes *clpB* and *yrfH*, as well as DNA repair genes. A large number encoded  
254 enzymes involved in the metabolism of sugars (e.g., *gcd*, *rpiR*, *glgC*), amino acids (*dadX*,  
255 *metB*, *tdcB*), fatty acids (*yafH*, *fixA*), growth factors (*bisC*, *yigB*, *thiF*) and other secondary  
256 metabolites (*yicP*). Transporters and permeases involved in central intermediary  
257 metabolism also featured prominently: these included permeases of the major facilitator  
258 superfamily (YjiZ), the hexose phosphate transport protein UhpT, the carnitine transporter  
259 CaiT and a range of CM-located sugar transporters. Of note was the impact of mutation of  
260 the *fucR* L-fucose operon activator on colonization; fucose is abundant in the GI tract and  
261 the fucose-sensing system in enterohemorrhagic *E. coli* regulates colonization and controls  
262 expression of virulence and metabolic genes (46). Availability of free iron is severely limited  
263 in the GI tract and ingestion of iron predisposes to infection (47); the importance of iron  
264 acquisition for *E. coli* K1 during GI colonization is reflected in the requirement for a number  
265 of genes related to iron uptake (e.g., *feoB*, *fepA*).

266 **GI colonizing capacity and virulence of single gene mutants.** To investigate the contribution  
267 of the polySia capsule to colonization of the neonatal rat GI tract, we disrupted the *neuC*  
268 gene of *E. coli* A192PP genes using bacteriophage  $\lambda$  Red recombinase to produce a capsule  
269 free mutant as judged by resistance to *E. coli* K1-specific phage K1E. We also produced other  
270 single gene mutants in genes identified by the TraDIS GI screen: *vasL* (encoding a type IV  
271 secretion system protein), *yfeC* (predicted to form part of a toxin/anti-toxin locus) and two  
272 genes with unknown function, *yaeQ* and *A192PP\_3010* (the latter is present in genomes of  
273 other extra-intestinal *E. coli* pathogens, including IHE3034, UT189, RS218, PMV-1 and S88).

274 Growth rates of these mutants, in particular the capsule-negative *neuC* mutant (Fig. S2B),  
275 were indistinguishable from that of the *E. coli* A192PP parent in LB medium. All were  
276 examined for their capacity to colonize the GI tract and cause lethal infection in P2 rat pups  
277 (Fig. 4C and 4D).

278 The *E. coli* A192PP parent strain or single gene mutants ( $2-6 \times 10^6$ ) were administered  
279 orally to P2 rats; all members of a litter of 12 pups received the same strain. Pups were  
280 sacrificed 24 h after initiation of colonization and *E. coli* K1 bacteria in the small intestine  
281 (PSI, MSI and DSI) and colon enumerated. The capacity of all mutants to transit the upper  
282 portion of the alimentary canal, pass through the stomach and colonize the small intestine  
283 was markedly inferior to the wildtype strain (Fig. 4C). Reductions in colonization of the PSI,  
284 MSI and DSI by the mutants, including *E. coli* A192PP $\Delta$ *neuC::kan*, were significant, the only  
285 exception being colonization of the DSI by A192PP $\Delta$ *yfeC::kan*, with no significant difference  
286 between parent and mutant. Interestingly, no increases in the numbers of viable  
287 A192PP $\Delta$ *neuC::kan*, A192PP $\Delta$ *vasL::kan*, A192PP $\Delta$ 3010::*kan* and A192PP $\Delta$ *yaeQ::kan*  
288 recovered from the colon were noted to compensate for reductions in colonization of the  
289 small intestine. There was a significant increase in the colonic burden of viable  
290 A192PP $\Delta$ *yfeC::kan* bacteria compared to the parent strain. We have established (12) that *E.*  
291 *coli* A192PP transits to the blood circulation *via* the mesenteric lymphatic system by  
292 exploiting a vesicular pathway through the GI epithelium only at the MSI. As mutant  
293 numbers colonizing this region of the small intestine were much reduced compared to the  
294 parent strain, we determined the capacity of the single gene mutants to elicit lethal  
295 systemic infection following GI colonization by oral administration of  $2-6 \times 10^6$  bacteria at P2  
296 (Fig. 4D). Four of the five mutants (A192PP $\Delta$ *neuC::kan*, A192PP $\Delta$ *vasL::kan*,  
297 A192PP $\Delta$ 3010::*kan* and A192PP $\Delta$ *yfeC::kan*) displayed significantly reduced lethal potential

298 compared to the A192PP parent. Loss of capsule (A192PP $\Delta$ *neuC::kan*) resulted in complete  
299 loss of lethality over the seven day observation period. Administration of A192PP $\Delta$ *vasL::kan*  
300 elicited a lethal response in 41.6% of pups; 33.3% and 25% survived after receiving,  
301 respectively, A192PP $\Delta$ *3010::kan* and A192PP $\Delta$ *yfeC::kan* at P2. For A192PP $\Delta$ *yaeQ::kan*, 75%  
302 of pups succumbed to lethal infection but did not reach levels of significance when  
303 compared to the 100% lethality engendered by the A192PP parent ( $P > 0.05$ ). Overall, these  
304 data indicate that the TraDIS screen efficiently identified genes important for MSI  
305 colonization that impact on pathogenic potential.

306 **A bottleneck to infection in the neonatal rat prevents identification of genes for**  
307 **translocation across the gastrointestinal epithelium.** Our initial intention was to exploit the  
308 high degree of susceptibility of the P2 neonatal rat to systemic infection, sepsis and  
309 meningitis following oral administration of an effective dose of *E. coli* A192PP in order to  
310 determine all genes required to enable the neonatal pathogen to overcome previously  
311 defined (12-15) physical and immunological barriers to invasion of the blood circulation and  
312 dissemination to the meninges. However, earlier studies indicate that relatively few *E. coli*  
313 K1 bacteria migrate from colonized sites within the GI tract to the blood (10), constraining  
314 the genetic diversity of the translocated bacterial population and eliminating genotypes  
315 from the translocated gene pool in a stochastic manner that does not reflect the fitness of  
316 individual genes to contribute to genotypes with invasive potential (32). We therefore  
317 determined if bottlenecks existed which would compromise the identification of mutants  
318 with attenuated capacity to translocate from the GI tract to the blood compartment; if any  
319 experimental bottlenecks are narrower than the complexity of the *E. coli* A192PP Tn5  
320 library, many relevant transposon insertion mutants will be lost entirely by chance (38).

321 Further, the existence of a restrictive bottleneck would limit the complexity of the library  
322 that could be used for TraDIS evaluation of populations colonizing the MSI (input pool) and  
323 reaching the blood (output pool).

324 We constructed an *E. coli* A192PP $\Delta$ *lacZ* mutant by bacteriophage  $\lambda$  Red  
325 recombineering and confirmed that there was no significant difference in lethal potential  
326 between *E. coli* A192PP and the *lacZ* mutant (Fig. 5A). We then used mixtures of parent and  
327 mutant to investigate the existence of bottlenecks that restrict translocation to the blood  
328 compartment. A 1:1 mixture (total  $2-4 \times 10^6$  CFU) of *E. coli* A192PP and A192PP $\Delta$ *lacZ* was  
329 administered orally to P2 rat pups, the animals sacrificed after 24 h and GI tissue  
330 homogenates plated for quantification of each strain. The competitive index (CI), the ratio of  
331 input A192PP: A192PP $\Delta$ *lacZ* to output A192PP: A192PP $\Delta$ *lacZ*, was calculated for excised PSI,  
332 MSI, DSI, colon and mesenteric lymphatic tissue and for blood. CI values in the PSI, MSI, DSI  
333 and colon were not significantly different from 1 (one-sample *t*-test), indicating that the  
334 composition of the colonizing inoculum was maintained in each rat pup (Fig. 5B). However,  
335 there was more heterogeneity in CI values of bacterial populations from the blood and in  
336 five pups only one strain could be recovered from the blood (four animals parent strain  
337 only, one animal A192PP $\Delta$ *lacZ* only). The highly restrictive bottleneck between GI epithelial  
338 transport and entry into the blood circulation supports the argument that reduced virulence  
339 of the complete, cultured library in comparison to less complex sub-libraries (Fig. S3) is at  
340 least in part due to a reduced likelihood that a fully virulent mutant would randomly escape  
341 capture by the mesenteric lymphatic system. The presence of significant bottlenecks  
342 between the GI tract, blood circulation and brain was confirmed by determination of the  
343 complexity of recovered Tn-5 library populations from these sources (Fig. 5C).

344 **Identification of *E. coli* K1 A192PP genes required for survival in human serum.** Systemic  
345 infection in the neonatal rat is likely to be maintained only if *E. coli* A192PP bacteria survive  
346 in the blood circulation. Due to limited exposure to antigens *in utero* coupled with deficits in  
347 adaptive immunity, neonates depend on innate immunity for protection against infection.  
348 The complement system provides front line innate defense against Gram-negative bacterial  
349 infection and the polySia capsule in turn enables *E. coli* K1 to avoid successful complement-  
350 mediated attack by host immune mechanisms. To obtain insights into *E. coli* K1  
351 pathogenesis during the invasive phase of the infection, and in light of restrictions placed on  
352 the neonatal rat model with regard to the use of TraDIS by the gut-to-blood bottleneck, we  
353 used the *E. coli* A192PP Tn5 library to investigate genes essential for A192PP fitness in  
354 pooled normal human serum, a reliable and plentiful source of all soluble components of  
355 the three complement pathways (48).

356 *E. coli* A192PP is resistant to the bactericidal action of human serum (Fig. 6A). A  
357 portion of the A192PP-Tn5 library containing  $2 \times 10^4$  mutants ( $1 \times 10^9$  CFU) was incubated in  
358 either 30% human serum or 30% heat-inactivated serum (final volume 375  $\mu$ l) at 37°C for 3  
359 h. Kan<sup>R</sup> bacteria in the input and output pools (each  $2 \times 10^5$ ) were collected, DNA extracted  
360 from each pool and transposon insertion sites sequenced. A wide distribution of fitness  
361 scores were detected (Fig. 6B). Mutation of 97 genes (negative log<sub>2</sub>-fold change and  $P < 0.05$ )  
362 resulted in decreased survival in normal serum, but not in heat-inactivated serum (Fig. 6C &  
363 Table S3).

364 A high proportion of genes identified in the TraDIS screen as contributing to  
365 resistance encoded cell surface constituents. It is well established that the polySia capsule  
366 protects *E. coli* K1 from complement attack (16, 17) and three mutations in the *kps* capsule  
367 gene cluster compromised serum survival. The central region of the cluster contains the *neu*



368 genes that direct the biosynthesis, activation and polymerization of the *N*-acetylneuraminic  
369 acid building block of polySia. *neuC* encodes the UDP *N*-acetylglucosamine 2-epimerase that  
370 catalyzes the formation of *N*-acetylmannosamine (49) and the *O*-acetyltransferase *neuD*  
371 acetylates monomeric neuraminic acid at carbon position 7 or 9 (50). *KpsM* is a component  
372 of the multimeric ATP-binding cassette transporter involved in the translocation of the  
373 polySia capsule through a transmembrane corridor to the cell surface (41, 51). Disruption of  
374 the genes encoding these proteins will prevent polySia expression (41); interruption of *rfaH*,  
375 identified in the TraDIS screen, will also prevent capsule expression but its loss will have a  
376 more profound effect on the surface topography of *E. coli* A192PP, as this transcriptional  
377 anti-terminator is required for the expression of operons that direct the synthesis, assembly  
378 and export of LPS core components, pili and toxins in addition to the capsule (52, 53).  
379 Indeed, survival in serum is dependent on anti-termination control by RfaH (54). Another  
380 gene identified that impacts on capsule formation was *bipA*; BipA is a tyrosine-  
381 phosphorylated GTPase that regulates through the ribosome a variety of cell processes,  
382 including some associated with virulence (55, 56). Other genes involved in LPS biosynthesis  
383 and pilus formation were also identified: *waaW* is a UDP-galactose:(galactosyl) LPS alpha1,2-  
384 galactosyltransferase involved in the synthesis of the R1 and R4 LPS core oligosaccharides  
385 (57) and *wzzE* encodes a polysaccharide copolymerase that catalyzes the polymerization of  
386 LPS O-antigen oligosaccharide repeat units into a mature polymer within the periplasmic  
387 space in readiness for export to the cell surface (58). Both mutations will prevent  
388 attachment of LPS O-side chains to the core oligosaccharide of LPS. The 16 genes that  
389 specify pilus synthesis that were identified in the screen included the majority of genes of  
390 the *tra* locus.

391 The TraDIS screen identified a range of proteins that are embedded in the OM (Fig.  
392 6C), none of which had been previously implicated in complement resistance, and which  
393 could influence the topography of the bacterial surface. Of the remaining genes with  
394 assigned function, the majority were involved with cell metabolism and the stress response;  
395 it is well established that metabolic processes are intimately associated with the  
396 complement-mediated bacterial killing process (59, 60). To verify the screen, we  
397 constructed four single gene mutants of *E. coli* A192PP by bacteriophage  $\lambda$  Red  
398 recombineering. Genes with roles in LPS synthesis (*rfaH* and *waaW*), capsule synthesis  
399 (*neuC*) and pilus assembly (*traL*) were mutated; none showed any reduction in growth rate  
400 in LB broth. All displayed significant reductions in complement resistance following  
401 incubation in pooled human serum (Fig. 6D). *E. coli* A192PP $\Delta$ *rfaH* was exquisitely susceptible  
402 with no colonies detected after 30 min. The viability of A192PP $\Delta$ *neuC* was also  
403 compromised with a threefold log reduction in viability over the 3 h incubation period.  
404 Killing of A192PP $\Delta$ *traL* and A192PP $\Delta$ *waaW* was less marked but these mutations  
405 significantly reduced viability. Complementation of the mutants with the functional gene  
406 introduced on a pUC19 vector completely restored resistance in all cases (Fig. 6D). These  
407 genes also contributed to lethality in the P2 neonatal rat (Fig. 6E). The lethal capacity of  
408 A192PP $\Delta$ *neuC*, A192PP $\Delta$ *rfaH* and A192PP $\Delta$ *waaW* was completely attenuated in comparison  
409 to *E. coli* A192PP; 42% of pups administered A192PP $\Delta$ *traL* succumbed to systemic infection  
410 (all  $P < 0.01$ ).

#### 411 **DISCUSSION**

412 Systemic infection with meningeal involvement arises spontaneously after GI colonization of  
413 neonatal rats with a high proportion of *E. coli* K1 isolates and the pathway to infection

414 mirrors to a large extent that of natural infections in the human host. In contrast to models  
415 of bacterial infection that create an artificial pathogenesis bypassing some or all of the  
416 barriers to infection by injection of a bacterial bolus directly into the blood circulation, the  
417 neonatal rat model provides an opportunity to investigate in stepwise fashion the progress  
418 of the pathogen as it transits from gut to blood to brain. TraDIS and other transposon  
419 sequencing methods enable simultaneous and rapid determination of the fitness  
420 contribution of every gene for a given condition and therefore have the potential to enable  
421 the identification of genes that are essential for, or significantly contribute to, each step of  
422 the infection process. However, stochastic loss will become evident if each mutant in the  
423 input pool does not have an equal chance to overcome the physical, physiological and  
424 immunological barriers presented by the host (61). This was clearly the case with epithelial  
425 transit of *E. coli* A192PP, with evidence that on occasion systemic infection arose due to only  
426 one viable bacterial cell entering the blood circulation (Fig. 5B), and complements other  
427 studies showing single or low-cell-number bottlenecks in models of severe infection (62-64).  
428 As translocation from MSI colonizing sites to the blood was not amenable to analysis by  
429 TraDIS we determined genes essential for survival in the presence of complement, a major  
430 component of the innate immune system that protects against extracellular systemic  
431 pathogens (17).

432         The high density of transposon insertion into random genomic positions along the  
433 entire *E. coli* A192PP chromosome, with minimal insertional bias (Fig. 1A), enabled the  
434 identification of genes essential for growth in nutrient replete LB medium. Of the 357 *E. coli*  
435 A192PP genes considered essential, orthologues of 254 (from 315) had been previously  
436 identified using TraDIS in a multi-drug-resistant uropathogenic strain of *E. coli* ST131 grown  
437 in LB (34) and 253 in an *E. coli* K12 strain (35), confirming the existence of a core set of

438 essential genes in *E. coli*. As anticipated, a high proportion of these genes encoded enzymes  
439 involved in a range of key metabolic functions such as carbohydrate, protein and nucleobase  
440 metabolism, and the remainder were associated with essential functions such as transport,  
441 cell organisation and biogenesis.

442         During characterization of the *E. coli* A192PP mutant library we examined the impact  
443 of culture in liquid medium on the expression of the polySia capsule, which places large  
444 demands on cell energy expenditure, as lengthy incubation times before marker selection  
445 may lower library complexity (38). Unexpectedly, we found that prolonged culture of the  
446 library enriched the proportion of non-encapsulated mutants (Fig. 3A). We anticipated that  
447 loss of capsule would enable the non-encapsulated mutants to grow at a faster rate than  
448 capsule-replete mutants and wildtype, and out-compete capsule-bearing library members.  
449 However, growth of a non-encapsulated mutant selected at random from the library was  
450 virtually identical to, and not significantly different from, the *E. coli* A192PP parent strain  
451 (Fig. S2A). There was also no difference in the climax populations of the strains at the end of  
452 the logarithmic phase of growth. In similar fashion, the growth curve for a *neuC* single gene  
453 mutant was identical to *E. coli* A192PP (Fig. S2B). *neuC* is involved in the synthesis of the *N*-  
454 acetylneuraminic acid monomeric unit of polySia, and as a consequence is unable to  
455 elaborate the capsule. It is clearly impractical to evaluate the growth kinetics of every  
456 distinct non-encapsulated mutant in the Tn5 library but it currently appears that differences  
457 in growth rate of individual library members cannot explain the highly reproducible  
458 enrichment that we observed. Indeed, use of transposon insertion libraries is predicated on  
459 the assumption that there are no significant differences in the growth rate of individual  
460 mutants. At present, the basis of the loss of mutants expressing capsule in TraDIS library  
461 cultures cannot be readily explained.

462 A sub-library of  $2 \times 10^5$  mutants was used to establish genes involved in GI  
463 colonization. To minimize bias due to any outgrowth of non-encapsulated mutants on the GI  
464 epithelium we harvested *E. coli* K1 from the MSI after 4 h, by which time maximal CFU had  
465 been achieved; bacteria were plated directly on to solid medium to further avoid outgrowth.  
466 Bias due to this restricted timeline is likely to be low as the majority of genes involved in  
467 adhesion and complement resistance are expressed constitutively. TraDIS identified the  
468 polySia capsule as a major determinant of GI colonization associated with *E. coli* K1. There is  
469 little or no evidence from the literature that capsules of Gram-negative bacteria enhance GI  
470 colonization; indeed, it has been reported that they interfere with adhesive interactions by  
471 obstructing binding of underlying surface molecules to mucosal surfaces (65, 66). The single  
472 gene mutant *E. coli* A192PP $\Delta$ *neuC::kan* displayed a reduced capacity to colonize the MSI  
473 (Fig. 4E), although it should be borne in mind that passage through the upper alimentary  
474 canal and stomach may impact on the number of mutant bacteria gaining access to the  
475 small intestine. In this context it should be noted that capsular exopolysaccharide protects  
476 *E. coli* from the environmental stress of stomach acid (67).

477 Other cell surface structures that are likely to have an impact on adhesion and  
478 colonization of the mucosal layer associated with the MSI were identified by TraDIS. Pili are  
479 established mediators of adhesion of *E. coli* to the host epithelium, although a large  
480 proportion of the evidence comes from enterotoxigenic and enteropathogenic strains (68,  
481 69). LPS and OM protein encoding genes were also implicated, as were genes involved in the  
482 stress response, reflecting ongoing adaptation to a new and hostile environment. The  
483 involvement of genes encoding metabolic enzymes, including some for anaerobic  
484 respiration, equates to increases in bacterial cell numbers in the anaerobic environment of  
485 the small intestine and for iron acquisition genes this reflects the low availability of

486 intestinal luminal iron (47, 70). Genes encoding some components of type II and type IV  
487 secretion systems were found with decreased frequency in the output pool. Members of  
488 these gene categories were also identified by Martindale et al (39) as necessary for GI  
489 colonization of *E. coli* K1 faecal isolate RS228 using signature-tagged mutagenesis; no genes  
490 found in this study were identified in the current study, in spite of the close genetic  
491 relatedness of the strains employed.

492         The intestinal lumen represents a potentially important portal of entry for pathogens  
493 into the host through adhesion, invasion or disruption of the epithelial barrier (71). In  
494 neonatal rats, *E. coli* K1 induces no detectable disruption of barrier integrity but exploits an  
495 intracellular pathway to access the submucosa (12). Only small numbers of bacteria breach  
496 the mesenteric lymphatic barrier in apparently random fashion (Fig. 5) and this precludes  
497 analysis by TraDIS. To accumulate data on genes and gene products facilitating invasion and  
498 survival/replication in the blood circulation, we examined essentiality for avoiding  
499 complement-mediated bactericidal effects. Although not all *E. coli* K1 isolates from cases of  
500 systemic infection are resistant to complement, resistance amongst K1 and K5 capsular  
501 types is more frequently encountered than for other K types (72); *E. coli* O18:K1 strains (such  
502 as A192) are in turn more often resistant than other O:K serotype combinations (73) due to  
503 the capacity of the polySia capsule to prevent complement activation. It is assumed, but not  
504 established, that the polySia capsule surrounding susceptible strains does not completely  
505 mask either OM-located activators of complement or lipid domains on the outer surface of  
506 the cell that are targets for OM intercalation of the C5b-9 membrane attack complex, the  
507 entity responsible for bacterial killing (59). In addition, long and numerous LPS O-side chains  
508 are necessary but not sufficient to enable the target cell to avoid complement killing (74)  
509 and they are able to bind C1 inhibitor to arrest classical or lectin pathway activation at the

510 early C1 stage (75). The importance of these structures for the complement resistance of *E.*  
511 *coli* K1 is supported by the decreased frequency of key LPS and capsule genes in the output  
512 pool along with a large number of OM-embedded proteins.

513         A small number of OM proteins, such as TraT and Iss, have been implicated as  
514 determinants of complement resistance (74) but they have been introduced into low-  
515 resistance backgrounds in high copy number; their role in the intrinsic resistance of clinical  
516 isolates is unclear and no mechanisms have been invoked to account for increases in  
517 resistance. The insertion of large numbers of protein molecules into the OM may  
518 fortuitously alter the biophysical properties of the bilayer, reducing the surface area and  
519 fluidity of lipid patches that are essential for binding and assembly of the C5b-9 membrane  
520 attack complex. The identification by TraDIS of a range of OM proteins as putative  
521 complement resistance determinants creates an opportunity to systematically investigate  
522 their precise function through generation of single gene mutants and we intend to pursue  
523 this line of investigation. We suggest that the architecture of the external surface of the OM,  
524 together with other more external macromolecular structures such as polysaccharide  
525 capsules, influences the capacity of the pore-generating C5b-9 complex to perturb the  
526 integrity of the OM. Thus, the surface of susceptible strains contains a sufficient number of  
527 exposed lipid domains to facilitate C5b-9 generation and penetration whereas the spatial  
528 and temporal organization of the OM of resistant bacteria is dominated by supramolecular  
529 protein assemblages to a degree where insufficient hydrophobic domains are available to  
530 act as C5b-9 assembly and binding sites, and this state persists throughout the growth cycle.  
531 The data we have generated in this study is compatible with this hypothesis. An array of  
532 metabolic genes emerged as essential for maintenance of the complement resistant  
533 phenotype (Fig. 6D) and may be indicative of repair processes invoked due to complement

534 attack. Exposure of resistant *E. coli* to complement results in minor perturbation of  
535 membrane integrity and metabolic homeostasis (76, 77) and C5b-9 intercalation into the  
536 OM has profound effects on cellular metabolic parameters (60).

537 TraDIS has also been employed by Schembri and coworkers to define the serum  
538 resistome of a globally disseminated, multidrug resistant clone of *E. coli* ST131 (34). They  
539 identified, and in most cases validated, 56 genes that contributed to the high level of  
540 complement resistance displayed by this pathogen. In similar fashion to our study, genes  
541 involved in the synthesis and expression of cell surface components were prominent. A  
542 number of genes contributing to LPS biosynthesis such as those of the *waa* operon, the *wzz*  
543 locus and *rfaH*, were common to both studies, as was the gene encoding the  
544 intermembrane protein AcrA. Genes of the plasmid-encoded *tra* locus, which we  
545 determined to be components of the *E. coli* A192PP serum resistome, were not present in *E.*  
546 *coli* ST131 (34) but other OM-located proteins may fulfill a similar role in reducing the fluidic  
547 properties of the bilayer. In contrast to the well-established role of the *E. coli* K1 polysialyl  
548 polymer in prevention of complement activation, no capsule genes were identified as  
549 components of the serum resistome of *E. coli* ST131, but different ST131 isolates express  
550 different capsule types due to extensive mosaicism at the capsule locus (78) and these  
551 uronic acid-containing polymers are unlikely to prevent complement activation (75). Thus,  
552 the different strategies employed by the two strains to prevent successful complement  
553 attack, together with differences in the bacterial surface composition and topography,  
554 probably explain variations in the serum resistomes of these related pathogens.

555 In summary, we identified *E. coli* K1 genes required for growth in standard  
556 laboratory liquid medium and for colonization of the GI tract of P2 neonatal rat pups. Both  
557 data sets provide insights into the biology of K1 neuropathogens and could provide the basis



558 for drug discovery programs for identification of selective antibacterial or colonization-  
559 inhibiting agents. In our rodent model, the stochastic nature of invasion of blood and  
560 probably brain prevented TraDIS analysis of gene essentiality for crossing gut epithelial and  
561 choroid plexus borders but some indication of genes necessary for survival in blood were  
562 obtained from output pool analyses after incubation of *E. coli* A192PP in human serum, a  
563 potent source of complement.

#### 564 **MATERIALS AND METHODS**

565 **Ethics statement.** Animal experiments were approved by the Ethical Committee of the UCL  
566 School of Pharmacy and the United Kingdom Home Office and were conducted in  
567 accordance with national legislation.

568 **Bacteria and culture conditions.** *E. coli* strain A192PP was obtained by serial passage in P2  
569 neonatal rats of *E. coli* A192 (serotype O18:K1) isolated from a patient with septicemia (79),  
570 as described earlier (11). Carriage of the polysialyl K1 capsule was determined with phage  
571 K1E (80): colonies were streaked onto MH agar, 10  $\mu$ l of  $\sim 10^9$  PFU/ml phage suspension  
572 dropped on each streak, the plates incubated overnight at 37°C and the proportion of  
573 encapsulated bacteria within cultures quantified by comparing the ratio of phage-  
574 susceptible and phage-resistant colonies. *E. coli* A192PP single gene mutants (Table 1) were  
575 constructed using bacteriophage  $\lambda$  Red recombination (81); the oligonucleotides employed  
576 for construction of targeted mutants, for confirmation of targeted mutants and for  
577 construction of complemented mutants are shown in Tables S3-S5. All were cultured in  
578 Luria-Bertani (LB) and on LB agar at 37°C; media were supplemented with either 100  $\mu$ g/ml  
579 ampicillin or 50  $\mu$ g/ml kanamycin as required.

580 **Tn5 library construction.** The EZ-Tn5 <KAN-2> Tnp transposome (Epicentre Biotechnologies)  
581 was introduced into *E. coli* A192PP by electroporation. Transformants were selected by  
582 overnight growth on LB plates containing 50 µg/ml kanamycin. Pools of 1-5 x 10<sup>3</sup> colonies  
583 were collected and frozen at -80°C in PBS containing 20% glycerol. Aliquots of individual  
584 pools were combined to create larger populations of mutants of up to 7.75 x 10<sup>5</sup>. Genomic  
585 DNA was extracted from 1 ml cultures using the PurElute Bacterial Genomic Kit (Edge  
586 Biosystems) following standard protocol.

587 **Linker PCR of Tn5 insertion sites.** Linker PCR was used to test individual transformant  
588 colonies and to confirm individual random-insertion events. DNA (2.5 µg) was digested with  
589 AluI restriction enzyme (Promega) and purified using MinElute PCR purification kit (QIAGEN).  
590 A linker, formed by annealing of oligonucleotides 254 (5'CGACTGGACCTGGA<sup>3'</sup>) and 256  
591 (5'GATAAGCAGGGATCGGAACCTCCAGGTCCAGTCG<sup>3'</sup>), was ligated to purified fragments (50  
592 ng) with Quick Ligation kit (NEB). Linker PCR was performed with linker- and transposon-  
593 specific oligonucleotides (258 5'GATAAGCAGGGATCGGAACC<sup>3'</sup> and  
594 5'GCAATGTAACATCAGAGATTTTGAG<sup>3'</sup> respectively) using HotStart Taq Mastermix kit  
595 (QIAGEN) and thermocycling conditions of 95°C for 5 min, 35 cycles of 94°C for 45 s, 56°C for  
596 1 min and 72°C for 1 min, and 72°C for 10 min. Resulting amplicons were separated on 1.5%  
597 agarose gels at 100 V for 60 min.

598 **Illumina sequencing.** For sequencing of Tn5 insertion sites, approximately 2 µg of genomic  
599 DNA was degraded to ~ 500 bp fragments by ultrasonication using a Covaris instrument.  
600 Fragments were end-repaired and A-tailed using the NEBNext DNA library preparation  
601 reagent kit for Illumina sequencing (NEB). Adapters Ind\_Ad\_T  
602 (ACACTCTTCCCTACACGACGCTCTCCGATC\*T; where \* indicates phosphorothionate) and

603 Ind\_Ad\_B (pGATCGGAAGAGCGGTTTCAGCAGGAATGCCGAGACCGATCTC) were annealed and  
604 ligated to DNA fragments. PCR was performed with transposon- and adapter- specific  
605 primers Tn-FO  
606 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCGGGGATCCTCTAGAGTCGACCTGC<sup>3'</sup> and  
607 Adapt-RO  
608 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTCTTCCCTACACGACGCTCTTCCGATC<sup>3</sup>  
609 '. Tn-FO and Adapt-RO contain a forward overhang and reverse overhang for indexing of  
610 amplicons by Nextera index primers (Illumina). PCR was performed using HotStart Taq  
611 Mastermix kit (QIAGEN) and thermocycling conditions of 95°C for 5 min, 22 cycles of 94°C for  
612 45 s, 56°C for 1 min and 72°C for 1 min, and 72°C for 10 min. Resulting amplicons were  
613 separated on 1.5% agarose gels at 70 V for 90 min, and those between 150 and 700 bp  
614 selected and purified using QIAquick Gel Extraction kit (QIAGEN). Samples were indexed with  
615 oligonucleotides from Nextera XT Index Kit (Illumina) using HotStart ReadyMix (Kapa  
616 Biosystems) and thermocycling conditions of 95°C for 3 min, 8 cycles of 95°C for 30 s, 55°C  
617 for 30 s and 72°C for 30 s, and 72°C for 5 min applied. Indexed amplicons were purified  
618 using the AMPure XP system (Agencourt). The final concentration of samples was confirmed  
619 using Qubit dsDNA BR assays (ThermoFisher Scientific). Indexed amplicons were sequenced  
620 on an Illumina Mi-Seq platform as 151-bp paired-end reads following manufacturer's  
621 protocol (Illumina).

622 **Bioinformatic and statistical analysis.** Raw sequence reads that passed Trimmomatic quality  
623 control filters (82) and contained the Tn5 transposon were mapped to the *E. coli* K1 A192PP  
624 reference genome (14) using Bowtie (83), permitting zero mismatches and excluding reads  
625 that did not map to a single site. The reference genome assembly contains ORFs located on

626 contigs that were mapped to the IHE3034 chromosome and ORFs located on other contigs  
627 that are likely to map to plasmids and other mobile genetic elements. An in-house pipeline  
628 based on the SAMtools (<http://samtools.sourceforge.net>) and BCFtools toolkits was utilised  
629 on the alignment files to determine insertion sites and coverage. To identify essential and  
630 non-essential genes, the insertion index was calculated for each gene by dividing the  
631 number of unique insertions in the gene by gene length. Observed insertion index values  
632 were fitted to a bimodal distribution with a gamma distribution (or an exponential  
633 distribution for genes with no observed insertion sites) corresponding to essential and non-  
634 essential genes. A  $\log_2$  likelihood, and corresponding  $P$  values, of each gene belonging to  
635 essential or non-essential sets was calculated using R software. To compare the fitness of  
636 individual mutants in input and output populations, reads were normalised and tested for  
637 differential base means by calculating  $\log_2$ -fold changes and corresponding  $P$  values at a  
638 false discovery rate of 0.1 using DESeq with R software. Raw read data for all transposon  
639 insertions have been deposited in the European Nucleotide Archive (ENA); accession  
640 numbers are as follows: ERR2235345 and ERR2235346 for identification of essential genes  
641 for replicates 1 and 2; ERR2235567 for input population; ERR2235568 for output population  
642 of rat MSI genes; ERR2235569 for output population of serum-exposed *E. coli* A192PP;  
643 ERR2235570 for output population of bacteria exposed to heat-inactivated serum.

644 **Colonisation and infection of neonatal rats.** Timed-birth Wistar rat pup litters (usually  $n =$   
645 12) were purchased from Harlan UK, delivered at P2 and colonized on the same day. Pups  
646 were retained throughout each experiment with the natural mothers in a single dedicated  
647 cage under optimal conditions (19-21°C, 45-55% humidity, 15-20 changes of air/h, 12 h  
648 light/dark cycle) and were returned to the mother immediately after colonization. Mothers

649 had unrestricted access to standard rat chow and water. The procedure has been described  
650 in detail (84). In brief, all members of P2 rat pup litters were fed 20 $\mu$ l of mid-logarithmic-  
651 phase *E. coli* (2-6 x 10<sup>6</sup> CFU unless otherwise stated) from an Eppendorf micropipette. GI  
652 colonization was confirmed by culture of perianal swabs on MacConkey agar and  
653 bacteremia detected by MacConkey agar culture of blood taken *post mortem*. Disease  
654 progression was monitored by daily evaluation of symptoms of systemic infection and  
655 neonates culled by decapitation and recorded as dead once a threshold had been reached:  
656 pups were regularly examined for skin color, agility, agitation after abdominal pressure,  
657 presence of a milk line, temperature, weight and behaviour in relation to the mother.  
658 Neonates were culled immediately when abnormalities for three of these criteria were  
659 evident. After sacrifice, GI tissues were excised aseptically without washing, colon  
660 separated and the SI segmented into 2 cm portions representing proximal, middle and distal  
661 small intestinal tissue. Tissues were then transferred to ice-cold phosphate-buffered saline,  
662 and homogenized. Bacteria were quantified by serial dilution culture on MacConkey agar  
663 supplemented with 25  $\mu$ g/ml kanamycin as appropriate. The presence of *E. coli* K1 was  
664 confirmed with phage K1E: 20 lactose-fermenting colonies were streaked onto MH agar, 10  
665  $\mu$ l of  $\sim$ 10<sup>9</sup> PFU/ml phage suspension dropped on each streak and the plates incubated  
666 overnight. *E. coli* K1 bacteria were quantified by multiplying total CFU by the proportion of  
667 K1E susceptible colonies. In all cases at least 19 colonies were susceptible to the K1 phage;  
668 *E. coli* K1 was never found in samples from non-colonized pups.

669 **Susceptibility to human serum.** Serum was obtained from healthy volunteers and used  
670 immediately. Bacteria were grown to late logarithmic phase in LB broth in an orbital  
671 incubator (minimum 200 orbits/min), 500  $\mu$ l culture removed, washed twice with gelatin-

672 Veronal buffered saline plus magnesium and calcium ions (pH 7.35) (GVB<sup>++</sup>), and suspended  
673 in an equal volume of GVB<sup>++</sup>. Fresh human serum was diluted 1:3 in GVB<sup>++</sup> and pre-warmed  
674 to 37°C. Bacterial suspensions and serum solutions were mixed 1:2 to give a final  
675 concentration of  $\sim 10^7$  CFU/ml and incubated at 37°C for 3 h in a total volume of 125  $\mu$ l  
676 containing 22% serum. Surviving *E. coli* were quantified by serial dilution and overnight  
677 incubation on LB agar. Pre-warmed, heat-inactivated (56°C, 30 min) serum served as  
678 control.

679

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928 **LEGENDS**

929 **FIG 1.** A high-density transposon library for identification of genes essential for *in vitro*  
930 growth of *E. coli* K1 A192PP. (A) Distribution of Tn5 insertions along the *E. coli* K1 A192PP  
931 genome. The number of sequence reads mapped to each single genomic location are  
932 plotted to show representation of the entire genome. (B) Insertion index values for two  
933 biological replicates are strongly correlated. (C) Density plot showing the frequency of  
934 insertion index values for all genes. A bimodal distribution is evident, with the left peak  
935 representing “essential” genes in which Tn5 insertion is lethal for growth on selective Luria-  
936 Bertani agar; the left peak represents “non-essential” genes into which Tn5 inserted without  
937 induction of lethality. Green and red lines indicate gamma distributions used to estimate  
938 likelihood ratios and *P* values. (D) Tn5 insertion site reads plotted to a 9 Kb region of the *E.*  
939 *coli* A192PP genome. The height of each line on the y axis indicates the number of reads at  
940 each Tn5 insertion site. The genes *lytB* and *dapB* possess no insertion sites, indicating they  
941 are putative essential genes.

942 **FIG 2.** Essential *E. coli* A192PP genes in each selected KEGG (Kyoto Encyclopedia of Genes  
943 and Genomes) functional orthologs (KO). Gene frequencies (light grey; expressed as % of  
944 essential coding DNA sequences [CDSs] for each category) are compared to their frequency  
945 within the whole genome (dark grey). KO (x/y) where y is the number of CDSs in the whole  
946 genome and x is the number of identified essential genes.

947 **FIG 3.** Culture of the *E. coli* K1 A192PP-Tn5 library results in loss of population diversity and  
948 enrichment of non-encapsulated mutants. (A) Changes in the proportion of *E. coli*  
949 encapsulated and non-capsulated A192PP bacteria during culture of the *E. coli* A192PP-Tn5  
950 library in LB media at 37°C (200 orbits/min) (*n* = 3; ±1SD; Student’s *t*, *p* < 0.01). CFU of

951 encapsulated and non-encapsulated bacteria were determined from the proportion of  
952 bacteria susceptible to the K1E bacteriophage. (B) Survival of P2 rats colonized with *E.*  
953 *coli* K1 A192PP, the uncultured *E. coli* A192PP-Tn5 library and the cultured (LB broth; 8 h;  
954 37°C) *E. coli* A192PP-Tn5 library. Pups ( $n = 12$  for each group) were colonized with  $2-4 \times 10^6$   
955 CFU by the oral route. Log-rank [Mantel-Cox] to compare survival of the cultured library  
956 with wildtype strain and the uncultured library: ns, non-significant, \*  $P < 0.05$ , \*\*  $P < 0.01$ .

957 **FIG 4.** Identification using a high-density transposon library of genes promoting GI  
958 colonization of *E. coli* A192PP in the neonatal rat. (A) Colonization of PSI, MSI, DSI and colon  
959 after oral administration of  $2-6 \times 10^6$  CFU *E. coli* K1 A192PP to P2 pups. (B) Log<sub>2</sub>-fold change  
960 and average Tn5 insertion site read abundance of each gene after MSI colonization of P2 rat  
961 pups ( $n = 4$ ) over a 4 h stabilization period expressed as MA-plot. An inoculum containing  $2 \times$   
962  $10^4$  unique *E. coli* K1 A192PP-Tn5 mutants was prepared and  $1 \times 10^9$  CFU administered  
963 orally. *E. coli* colonies ( $2 \times 10^5$ ) were recovered from the inoculum (input pool) and from MSI  
964 homogenates (output pool) by culture on to LB agar containing 50 µg/ml kanamycin. Red  
965 data points represent Tn5 insertion sites determined as differentially expressed in the  
966 output pool compared to the input pool using a negative binomial test with a false discovery  
967 rate of 0.1. (C) Mutations in 167 genes significantly decreased fitness for colonization of the  
968 MSI and encoded proteins with a range of functions. (D) Colonization of P2 rat intestine by  
969 *E. coli* K1 A192PP and single gene mutants. Bacteria ( $2-4 \times 10^6$ ) were administered orally to  
970 P2 rats ( $n = 12$ /group). Pups were sacrificed and the *E. coli* K1 burden in intestinal sections  
971 enumerated 24 h after initiation of colonization. Parent and mutant strain CFU values were  
972 compared using Student's *t*-test: \*  $P < 0.05$ , \*\*  $P < 0.01$ . (E) Survival of P2 rats colonized with  
973 *E. coli* K1 A192PP and single gene mutants. Bacteria ( $2-4 \times 10^6$ ) were administered orally to

974 P2 rats ( $n = 12$ /group). Log-rank [Mantel-Cox] test: ns, non-significant, \*  $P < 0.05$ , \*\*  $P <$   
975 0.01.

976 **FIG 5.** Bottleneck to systemic infection in the neonatal rat. (A) Survival of rats colonized at  
977 P2 by oral administration of *E. coli* K1 A192PP or A192PP $\Delta$ *lacZ::kan*.  $n = 12$  pups for both  
978 groups. Log-rank [Mantel-Cox] test: ns, non-significant, \*  $P < 0.05$ , \*\*  $P < 0.01$ . (B)  
979 Competitive indices of intestinal colonization and gut-to-blood transit of *E. coli* K1 A192PP  
980 and A192PP $\Delta$ *lacZ::kan*. A 1:1 mixture of *E. coli* K1 A192PP and A192PP $\Delta$ *lacZ::kan* (total 2-4  
981  $\times 10^6$  CFU) was administered orally to P2 pups. After 24 h, ratios of A192PP and  
982 A192PP $\Delta$ *lacZ::kan* were enumerated in segmented GI tissues and in the blood as indicated  
983 using selective media. Animals in which only *E. coli* K1 A192PP or A192PP $\Delta$ *lacZ::kan* were  
984 detected in the blood are coloured red and blue respectively, indicating the existence of a  
985 bottleneck to infection. (C) Loss of diversity of *E. coli* K1 A192PP-Tn5 populations recovered  
986 from the blood (red) and brain (blue) following translocation from the GI tract (black).

987 **FIG 6.** Identification using a high-density transposon library of genes contributing to the  
988 complement resistance of *E. coli* A192PP. (A) Survival of *E. coli* A192PP and *E. coli* K12 strain  
989 MG1655 in 22% pooled human serum. The latter was used a serum susceptible control;  $n =$   
990 3, error bars represent range of values. (B) Log<sub>2</sub>-fold change and average Tn5 insertion site  
991 read abundance of each gene after incubation of  $1 \times 10^6$  CFU containing  $2 \times 10^4$  unique *E.*  
992 *coli* K1 A192PP-Tn5 mutants in 22% pooled human serum for 3 h at 37°C. Colonies ( $2 \times 10^5$ )  
993 were obtained by culture of diluted aliquots on LB agar containing 50  $\mu$ g/ml kanamycin. The  
994 inoculum served as the input pool. Red data points represent Tn5 insertion sites determined  
995 as differentially expressed in the output pool compared to the input pool using a negative  
996 binomial test with a false discovery rate of 0.1. (C) Survival of  $1 \times 10^6$  *E. coli* K1 A192PP and

997 single gene mutants in 22% normal human and heat-inactivated (56°C; 30 min) serum. Final  
998 volume of the reaction mixture was 1.5 ml;  $n = 3$ , error bars represent range of values.  
999 Complementation with the functional gene restored resistance in all cases. (D) Survival of  
1000 P2 rats colonized with *E. coli* K1 A192PP and single gene mutants. Bacteria ( $2-4 \times 10^6$ ) were  
1001 administered orally to P2 rats ( $n = 12/\text{group}$ ). Log-rank [Mantel-Cox] test: ns, non-significant,  
1002 \*  $P < 0.05$ , \*\*  $P < 0.01$ .

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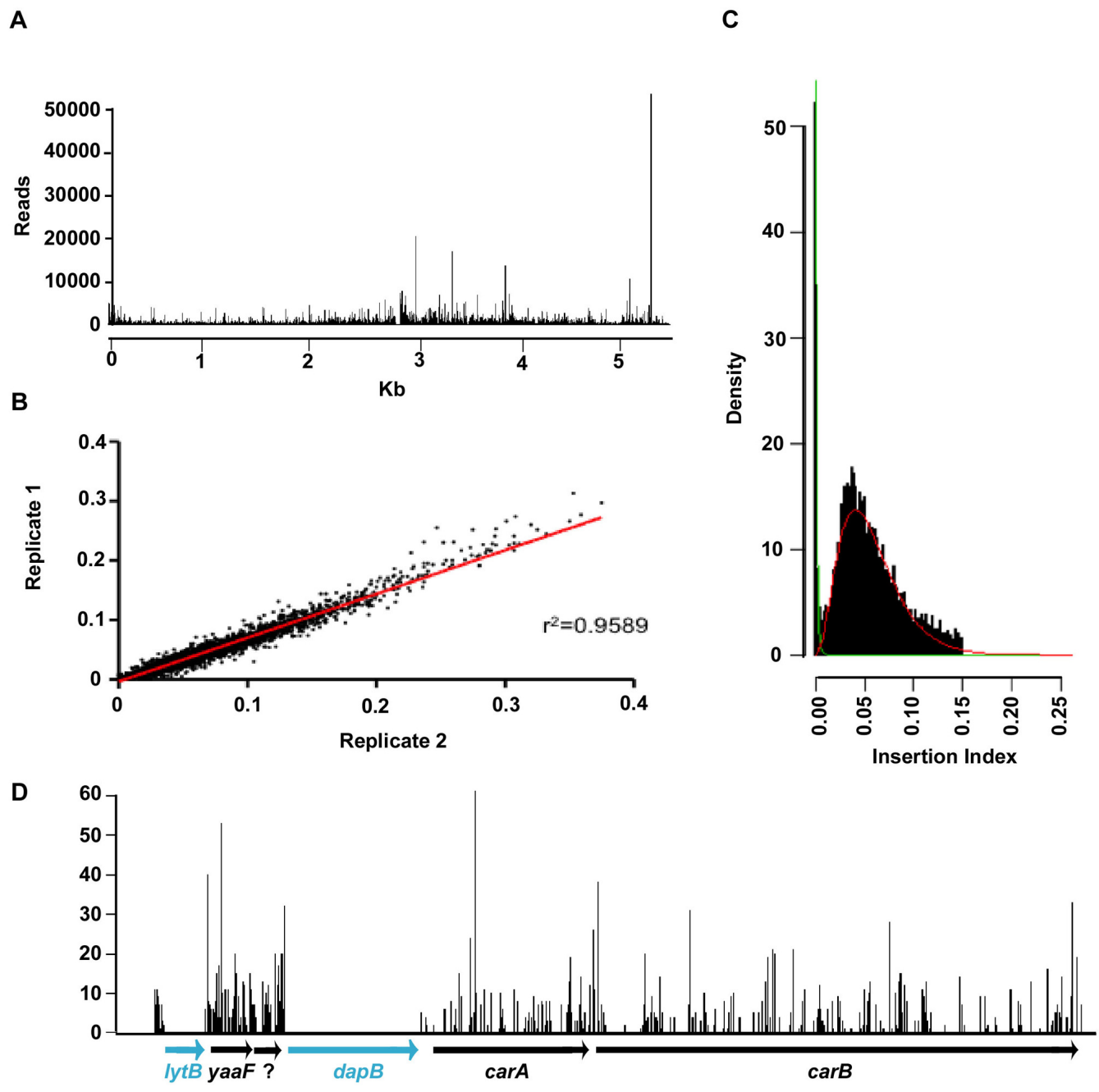
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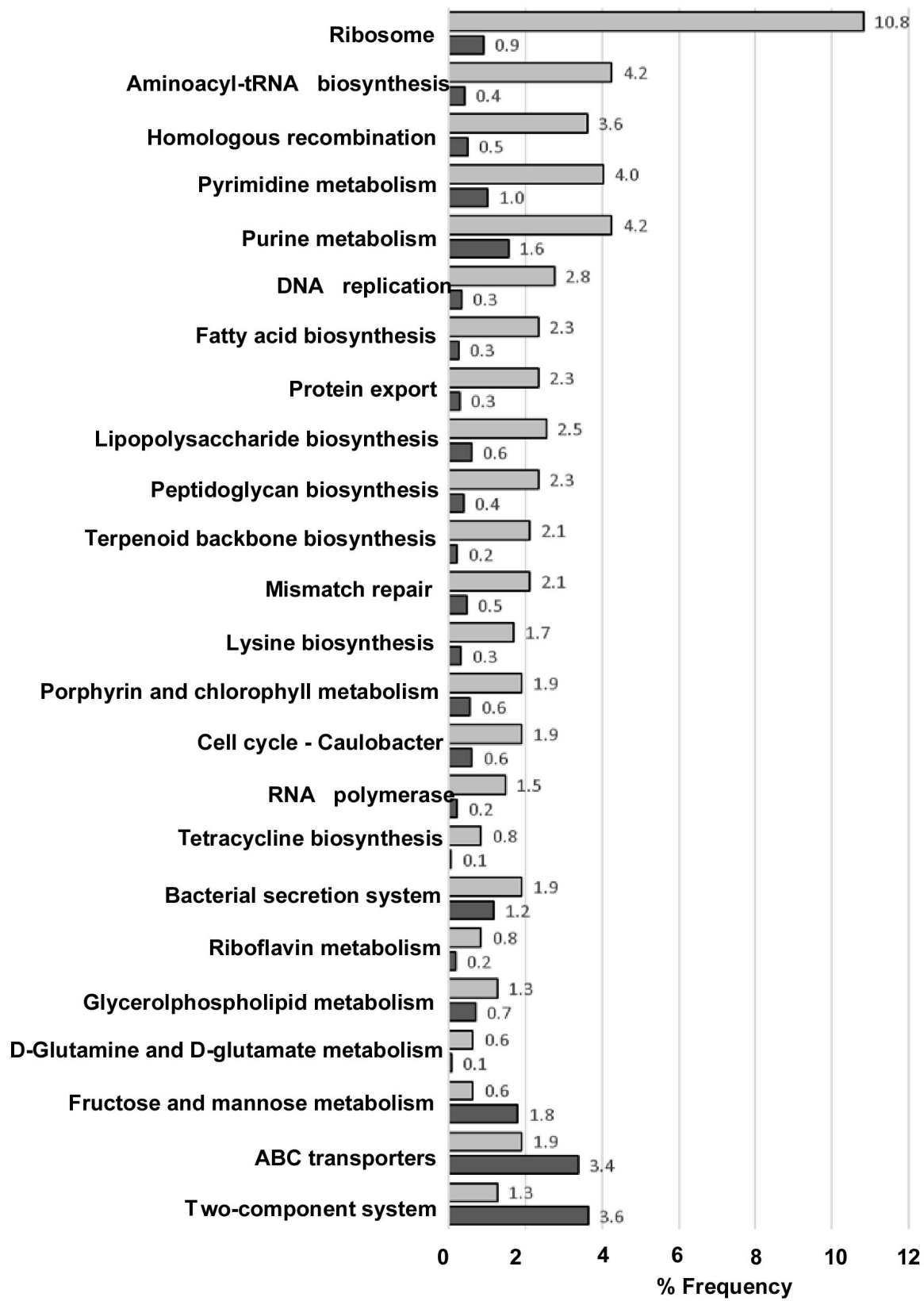
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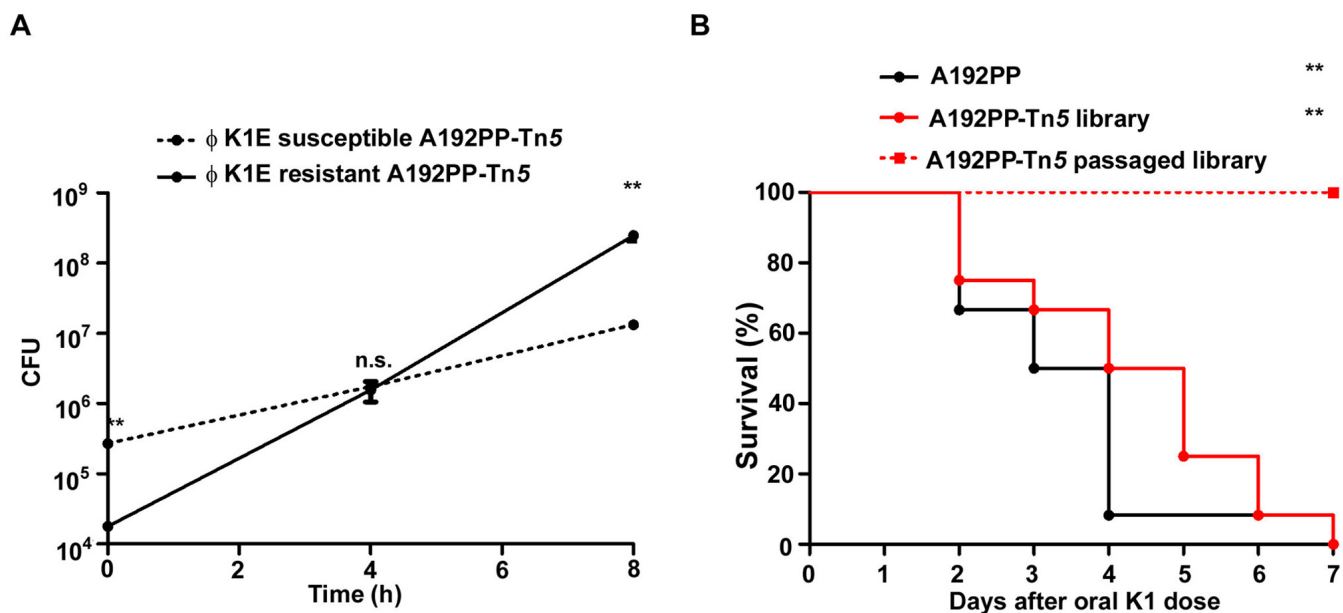
1020 **TABLE 1.** Strains used in this study

Strain	Description
<i>E. coli</i> K1	
A192PP	018:K1:H7; virulent in neonatal rat model of infection
A192PP $\Delta$ <i>lacZ::kan</i>	<i>lacZ</i> mutant of A192PP; Kan <sup>r</sup>
A192PP $\Delta$ <i>neuC::kan</i>	<i>neuC</i> mutant of A192PP; Kan <sup>r</sup>
A192PP $\Delta$ <i>neuC::kan</i> + pUC19. <i>neuC</i>	Complemented A192PP $\Delta$ <i>neuC::kan</i> ; Kan <sup>r</sup> Amp <sup>r</sup>
A192PP $\Delta$ <i>rfaH::kan</i>	<i>rfaH</i> mutant of A192PP; Kan <sup>r</sup>
A192PP $\Delta$ <i>rfaH::kan</i> + pUC19. <i>rfaH</i>	Complemented A192PP $\Delta$ <i>rfaH::kan</i> ; Kan <sup>r</sup> Amp <sup>r</sup>
A192PP $\Delta$ <i>traL::kan</i>	<i>traL</i> mutant of A192PP; Kan <sup>r</sup>
A192PP $\Delta$ <i>traL::kan</i> + pUC19. <i>traL</i>	Complemented A192PP $\Delta$ <i>traL::kan</i> ; Kan <sup>r</sup> Amp <sup>r</sup>
A192PP $\Delta$ <i>vasL::kan</i>	<i>vasL</i> mutant of A192PP; Kan <sup>r</sup>
A192PP $\Delta$ <i>waaW::kan</i>	<i>waaW</i> mutant of A192PP; Kan <sup>r</sup>
A192PP $\Delta$ <i>waaW::kan</i> + pUC19. <i>waaW</i>	Complemented A192PP $\Delta$ <i>waaW::kan</i> ; Kan <sup>r</sup> Amp <sup>r</sup>
A192PP $\Delta$ <i>yaeQ::kan</i>	<i>yaeQ</i> mutant of A192PP; Kan <sup>r</sup>
A192PP $\Delta$ 0678:: <i>kan</i>	0678 mutant of A192PP; Kan <sup>r</sup>
A192PP $\Delta$ 3010:: <i>kan</i>	3010 mutant of A192PP; Kan <sup>r</sup>
<i>E. coli</i> K12	
MG1655	F- lambda- <i>ilvG- rfb-50 rph-1</i>
MG1655 $\Delta$ <i>lacZ::kan</i>	<i>lacZ</i> mutant of MG1655; Kan <sup>r</sup>

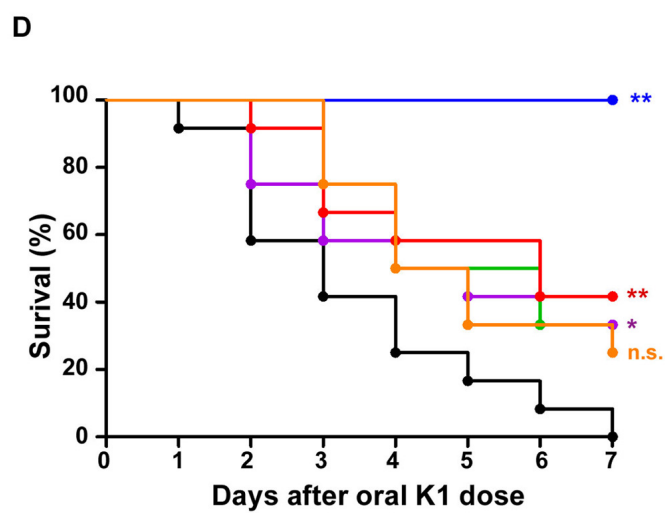
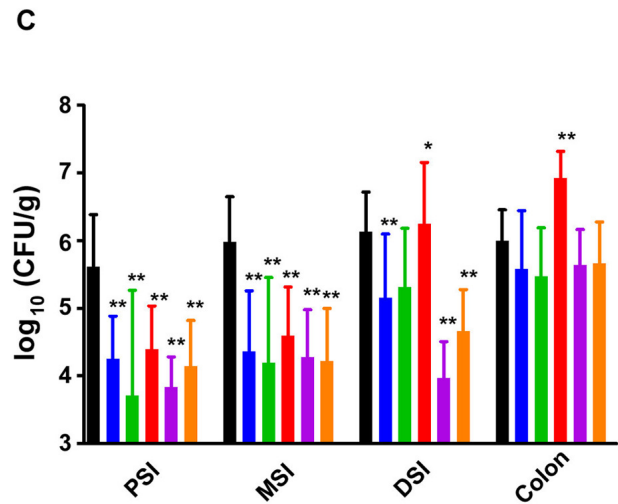
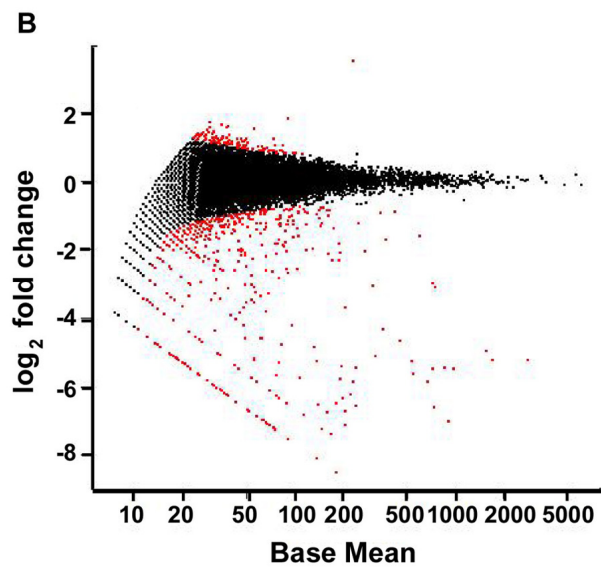
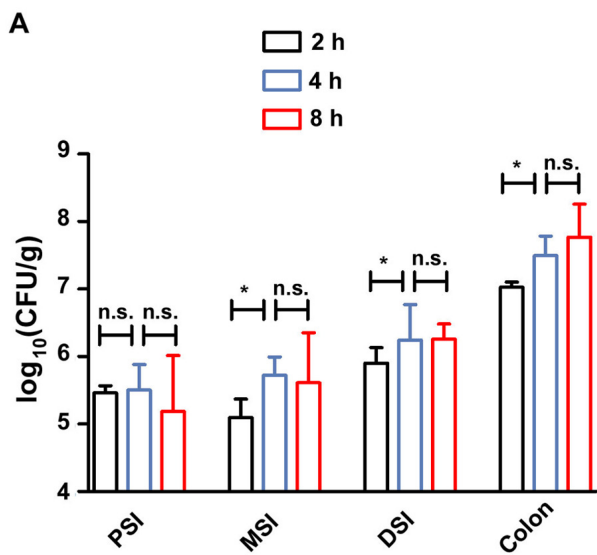
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A192PP  $\Delta vasL$   $\Delta 3010$   
 $\Delta neuC$   $\Delta 0678$   $\Delta yaeQ$

