Comparison of *Salmonella enterica* serovars Typhi and Typhimurium reveals typhoidal-specific responses to bile

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Running title: *Salmonella* Typhi bile responses

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ABSTRACT

Salmonella enterica serovars Typhi and Typhimurium cause typhoid fever and gastroenteritis respectively. A unique feature of typhoid infection is asymptomatic carriage within the gallbladder, which is linked with *S.* Typhi transmission. Despite this, *S.* Typhi responses to bile have been poorly studied. RNA-Seq of *S.* Typhi Ty2 and a clinical *S.* Typhi isolate belonging to the globally dominant H58 lineage (129-0238), as well as *S.* Typhimurium 14028, revealed that 249, 389 and 453 genes respectively were differentially expressed in the presence of 3% bile compared to control cultures lacking bile. *fad* genes, the *actP-acs* operon, and putative sialic acid uptake and metabolism genes (t1787-t1790) were upregulated in all strains following bile exposure, which may represent adaptation to the small intestine environment. Genes within the *Salmonella* pathogenicity island 1 (SPI-1), encoding a type IIII secretion system (T3SS), and motility genes were significantly upregulated in both *S.* Typhi strains in bile, but downregulated in *S.* Typhimurium. Western blots of the SPI-1 proteins SipC, SipD, SopB and SopE validated the gene expression data. Consistent with this, bile significantly increased *S.* Typhi HeLa cell invasion whilst *S.* Typhimurium invasion was significantly repressed. Protein stability assays demonstrated that in *S.* Typhi the half-life of HilD, the dominant regulator of SPI-1, is three times longer in the presence of bile; this increase in stability was independent of the acetyltransferase Pat. Overall, we found that *S.* Typhi exhibits a specific response to bile, especially with regards to virulence gene expression, which could impact pathogenesis and transmission.

INTRODUCTION

In humans, the outcome of infection with *Salmonella enterica* primarily depends on the infecting serovar; whilst non-typhoidal, broad host range serovars such as *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) cause self-limiting gastroenteritis, infection with human-restricted typhoidal serovars, such as *Salmonella enterica* serovar Typhi (*S.* Typhi) result in typhoid fever (1). The virulence of both serovars depends on the activity of two type III secretion systems (T3SS) carried on *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2), which secrete a pool of over 40 effectors to subvert host cell processes resulting in invasion, immune evasion, and intracellular growth (2). The SPI-1 T3SS is active when *Salmonella* are extracellular, and its activity permits *Salmonella* invasion of non-phagocytic cells and also promotes early adaptation to the intracellular environment (2). Expression of the SPI-1 T3SS and its associated genes (several of which are encoded outside of the SPI-1 pathogenicity island) is controlled by a hierarchy of regulators (HilD, HilA, HilC, RtsA, InvF). These regulators are controlled by a variety of factors including two-component systems, RNA binding proteins, and global regulators, which respond to a range of environmental stimuli (3, 4).

Typhoid is an acute illness characterized by high fever, malaise and abdominal pain (5). *S.* Typhi causes systemic infection during which the pathogen colonises the intestine and mesenteric lymph nodes, the liver, spleen, bone marrow and gallbladder (5). It is estimated that there are more than 20 million typhoid fever cases per year, resulting in more than 200,000 deaths (6). Although with adequate treatment most patients recover from the acute phase of *S.* Typhi infection, *S.* Typhi can persist asymptomatically within the gallbladder following clinical recovery (7). Overall, 10% of those infected will carry *S.* Typhi within their gallbladder for up to three months, whilst 1-3% will continue to harbour *S.* Typhi for longer than one year (5, 8). Given the host-restriction of *S.* Typhi, chronic gallbladder

63 carriage represents a key environmental reservoir of *S.* Typhi bacteria, enabling typhoid
64 transmission (7, 9).

65 Although the exact mechanism(s) by which *S.* Typhi persists within the gallbladder are
66 debated (7), it certainty encounters high bile concentrations during carriage, as the
67 gallbladder is where bile is stored and concentrated prior to secretion into the small intestine,
68 where it plays a role in the emulsification and absorption of fats (10). In part due to its
69 detergent activity, bile is also a potent antimicrobial agent (10, 11). However enteric
70 pathogens – including *Salmonella* – are intrinsically resistant to bile (12), and instead often
71 utilise bile as a means to regulate gene expression and virulence (10, 13). In *S.* Typhimurium,
72 expression of the SPI-1 and motility genes are repressed by bile exposure, resulting in a
73 significant repression of epithelial cell invasion (14, 15).

74 Despite the importance of asymptomatic carriage, the behaviour of *S.* Typhi within bile
75 remains poorly understood (7). As the transcriptomic responses of *S.* Typhimurium to bile
76 under various conditions have been well characterised (15–18), the behaviour of *S.*
77 Typhimurium has become an accepted model as to how *Salmonella* in general behaves in bile
78 (11, 19). However a study comparing changes in protein expression by 2D gel electrophoresis
79 within *S.* Typhimurium and *S.* Typhi following exposure to 3% bile found there was "little
80 overlap apparent between proteins affected by bile in *S.* Typhi and in *S.* Typhimurium" (12),
81 suggesting that the response to bile between these serovars differs. Furthermore, a study
82 comparing the genomes of *S.* Typhimurium LT2 to *S.* Typhi CT18 revealed that less than
83 90% of genes are shared between the two strains, with over 600 genes present in CT18 not
84 found in LT2 (20); therefore *S.* Typhimurium cannot be used to model regulation of *S.* Typhi
85 specific genes, which include key virulence factors such as the Vi antigen, and the CdtB and
86 HlyE/ClyA toxins (20).

87 The need to better understand *S.* Typhi infection has been intensified by the recent spread of
88 haplotype 58 (H58), also known as 4.3.1 (21, 22). Following its emergence around 30 years
89 ago, *S.* Typhi strains belonging to haplotype H58 have clonally expanded worldwide to
90 become the dominant cause of multi-drug resistant (MDR) typhoid within endemic regions
91 (21). As yet, the reasons underlying the relative success of H58 strains remain unknown.

92 The aim of this study was to compare global bile responses between *S.* Typhi and *S.*
93 Typhimurium isolates, which in turn might explain differences in pathogenesis and reveal
94 processes important for the carrier state.

RESULTS

Bile exposure alters global gene expression in *Salmonella*

We performed RNA-Seq on *S.* Typhimurium 14028, *S.* Typhi Ty2 and a clinical *S.* Typhi H58 isolate (129-0238) grown in LB to late-exponential phase in the presence or absence of 3% bile. Given the extensive description of *S.* Typhimurium behaviour in bile (14, 15), *S.* Typhimurium 14028 was considered as a control. 3% ox-bile was chosen for these studies as this concentration robustly affects gene expression in *S.* Typhimurium (14, 15, 23), but does not affect growth of the investigated *Salmonella* strains (Figure S1). Overall following growth in bile, 249 and 389 genes were differentially expressed in *S.* Typhi Ty2 (182 upregulated; 67 downregulated) and 129-2038 (223 upregulated; 166 downregulated) (Figure 1) respectively, while 453 genes were differentially regulated in *S.* Typhimurium 14028 (293 upregulated; 179 downregulated) (Figure 1).

GO enrichment and KEGG pathway analysis on the pools of upregulated and downregulated genes revealed broad differences between *S.* Typhi and *S.* Typhimurium (Figure 1). While *S.* Typhimurium upregulated metabolic processes and downregulated processes linked with pathogenicity, including T3SS, flagella and chemotaxis (motility), in line with previous findings (14, 15, 17), both *S.* Typhi Ty2 and 129-0238 upregulated these processes, whilst downregulating various metabolic pathways (Figure 1). KEGG pathway analysis also revealed that fatty acid degradation (represented by the GO term ‘Fatty acid beta-oxidation’) and tyrosine metabolism were upregulated in all isolates, implicating these processes in general *Salmonella* response to bile.

Similarities in the response to bile between *S.* Typhi and *S.* Typhimurium

The overlap in genes either downregulated or upregulated in bile between all strains was small; only one gene (*pagP*), a PhoP-PhoQ regulated gene involved in modifying lipid A

(24), was downregulated in all strains (Figure 2). Twenty genes were upregulated in all isolates in response to bile (Figure 2) (Table 1), representing genes involved in tyrosine metabolism, sialic acid uptake and utilisation (t1787-1790) (25), and in the production of acetyl-CoA from acetate (*actP-acs*) and fatty acids (*fad* genes). Of the upregulated genes, expression of *acs* and *fadE* was validated by RT-qPCR (Table 2). Upregulation of sialic acid and acetate metabolic pathways may reflect adaptation to the small intestine, where these metabolites are abundant (26), whilst upregulation of *fad* genes are consistent with the ability of *Salmonella* to utilise phospholipids present in bile as a carbon/energy source (27). Interestingly the fatty acid transporter *fadL*, was strongly upregulated in *S*. Typhimurium, but was not upregulated in either *S*. Typhi Ty2 or 129-0238, suggesting that *S*. Typhi may possess additional fatty acid transporters.

Genes implicated in stress responses were also upregulated in bile. All isolates upregulated *msrA*, a sulfoxide reductase upregulated in response to oxidative stress, which is required for growth within macrophages and for full virulence of *S*. Typhimurium *in vivo* (28). *S*. Typhimurium 14028 and *S*. Typhi 129-0238 also activated RpoS-mediated stress responses, with upregulation of *otsAB, spoVR, yeaG, katE, sodC, poxB, ecnB,* and *osmY*, in line with previous findings (17, 29, 30). However, upregulation of these stress-linked genes was not observed in *S*. Typhi Ty2, which is likely due to a frameshift mutation within *rpoS* within this strain (31).

Differences in the response to bile between *S*. Typhi and *S*. Typhimurium

Of special interest are genes that are regulated differently in response to bile between *S*. Typhi and *S*. Typhimurium. The identification of such genes was achieved by determining genes downregulated in *S*. Typhimurium in bile, but upregulated in *S*. Typhi and vice versa. Of the 75 genes upregulated in both *S*. Typhi Ty2 and 129-0238 (Figure 2), the majority

(54/75) were significantly downregulated in *S*. Typhimurium (Table 3). As indicated by the GO and KEGG pathway analyses (Figure 1), genes regulated in this manner predominantly encode proteins associated with the SPI-1 T3SS or motility. To validate these findings, expression of the SPI-1 associated genes *hilD*, *hilA*, *prgH*, and *sopB*, in addition to the flagella associated genes *flhD* and *flgA* was confirmed by RT-qPCR (Table 2).

Additional genes upregulated in *S*. Typhi and downregulated in *S*. Typhimurium include *lpxR* (t1208/STM14_1612), a lipid A modifying protein that modulates the ability of lipid A to stimulate TLR4 (32) and promotes *Salmonella* growth inside macrophages (33), and *srfA/srfB*, virulence factors expressed under SPI-1 inducing conditions (34) and reported to modulate inflammatory signalling (35). Additionally, several hypothetical proteins – t0944 (STM14_2352), t1774 (STM14_1312) and t2782 (STM14_3479) – were upregulated in *S*. Typhi but downregulated in *S*. Typhimurium. Given their regulation pattern, these genes may encode uncharacterised virulence factors or be involved in motility in *Salmonella*.

We also analysed the expression profile of *S*. Typhi specific genes. *S*. Typhi Ty2 carries 453 unique genes relative to *S*. Typhimurium (representing Ty2 homologues of the 601 *S*. Typhi specific genes identified in CT18 (36), in addition to 29 Ty2 specific genes (37)). Only two of these genes were significantly regulated by bile exposure in both *S*. Typhi Ty2 and 129-0238. Both genes, which are upregulated in bile, encode hypothetical proteins: t0349 (STY2749) encodes a GIY-YIG domain containing protein, and t1865 (STY1076) encodes a homologue of the NleG family of T3SS effectors (38, 39). Neither *S*. Typhi isolate demonstrated altered expression of genes encoding the Vi antigen or of the typhoid toxin in bile.

Bile influences SPI-1 expression and *Salmonella* invasion

The most marked differences between *S*. Typhi and *S*. Typhimurium in response to bile was in the expression of SPI-1-associated genes. The majority of genes within the SPI-1

pathogenicity island, in addition to the SPI-1 regulators *rtsA* and *rtsB*, and effector genes carried outside SPI-1 (*sopD*), were significantly upregulated in *S.* Typhi Ty2 and 129-0238 but significantly downregulated in *S.* Typhimurium (Table 3; Figure 3A). Noticeably, *S.* Typhi 129-0238 exhibited significantly elevated expression of SPI-1 genes relative to *S.* Typhi Ty2 (Table 3; Figure 3A).

To determine if changes in SPI-1 gene expression correlated with changes at the protein level, we compared the intracellular levels of the SPI-1 translocon proteins SipC, SipD, and the SPI-1 effectors SopE (for *S.* Typhi) or SopB (for *S.* Typhi and *S.* Typhimurium) from each strain grown in the absence or presence of bile. Additional *S.* Typhi strains were also included to further expand and validate these findings, namely the $RpoS^{+}$ *S.* Typhi reference strain CT18 (37), and an additional H58 isolate, ERL12148 which belongs to a different sublineage of H58 than 129-0238 (21). All *S.* Typhi strains tested (Ty2, CT18, 129-0238, ERL12148) showed increased levels of SPI-1 proteins, with the H58 strains demonstrating the largest increases in SPI-1 protein expression in bile (Figure 3B, Figure S2). Conversely *S.* Typhimurium 14028 showed decreased levels of SopB, SipD and SipC following growth in bile (Figure 3B, Figure S2); as *S.* Typhimurium 14028 lacks SopE, its lanes (Tm) in the SopE panel are not shown.

Given the significant effect of bile on SPI-1 expression, we investigated the impact of bile on epithelial cell invasion. In line with previous findings (14), *S.* Typhimurium exposed to bile demonstrated significantly reduced invasion, achieving an invasion rate approximately 90% lower than *S.* Typhimurium grown in the absence of bile (Figure 3C). In contrast, all *S.* Typhi strains tested demonstrated significantly increased invasion following bile exposure, with Ty2 and CT18 displaying an approximate 2-fold increase in the number of intracellular bacteria at 2 h post-infection, and both H58 isolates demonstrating even higher increases in invasion (between 4-16 fold) (Figure 3C, Figure S2). A SPI-1 deficient strain of *S.* Typhi Ty2

(Δ*invA*) did not invade HeLa cells in the presence of bile, indicating that the increased invasiveness of *S*. Typhi in bile is SPI-1 dependent (Figure S2).

Transcriptional regulation of SPI-1 regulators in bile

Given the striking difference in SPI-1 expression between *S*. Typhi and *S*. Typhimurium in response to bile, we determined where and how SPI-1 regulation differs between the two serovars. The central regulators governing SPI-1 expression are HilA, often termed the master SPI-1 regulator, and HilD, which is the dominant regulator of HilA (3, 40). The RNA-Seq and RT-qPCR data show that the mRNA levels of these regulators significantly decrease in *S*. Typhimurium in response to bile, but significantly increase in response to bile in the *S*. Typhi strains (Table 2).

In order to determine if these changes are mediated by transcriptional regulation of these genes, we constructed *hilA* and *hilD lacZ* chromosomal transcriptional reporters in *S*. Typhimurium 14028 and *S*. Typhi Ty2 (41). The reporter activity was determined by β-galactosidase assay following growth to late exponential phase in LB with or without 3% bile. In *S*. Typhimurium expression of *hilA* is significantly reduced in the presence of bile, with expression almost 20 fold lower, while expression of *hilD* is unchanged (Figure 4). In contrast, expression of *hilA* in *S*. Typhi significantly increases in bile, with expression over 3 times higher, whilst *hilD* expression is only modestly increased (Figure 4). Taken together, these results indicate that *hilA* is transcriptionally regulated by bile in both *S*. Typhi and *S*. Typhimurium, whilst *hilD* is not subject to transcriptional regulation.

The seeming absence of *hilD* transcriptional regulation in bile (Figure 4) is at odds with the significant changes in mRNA levels observed (Table 2). One explanation is that *hilD:lacZ* reporter strains do not account for HilD-mediated autoregulation, as the chromosomal reporter strains were made in a Δ*hilD* background. HilD autoregulation has previously been

reported in *S*. Typhimurium (42), but has not been characterised in *S*. Typhi. To determine if HilD autoregulation could account for transcriptional changes of *hilD* in bile in *S*. Typhi, the *hilD:lacZ S*. Typhi Ty2 reporter strain was transformed with a plasmid expressing HilD or an empty vector control, and reporter activity assessed by β-galactosidase assay following growth in LB. *hilD* expression from the strain complemented with HilD was significantly higher than *hilD* expression from both the reporter strain alone and the reporter carrying the empty vector (Figure 5), indicating that in *S*. Typhi HilD positively regulates its own transcription, either directly or indirectly.

Bile influences HilD stability

Given that expression of *hilA*, a gene directly regulated by HilD, significantly increases in bile, we investigated if HilD is post-transcriptionally regulated by bile in *S*. Typhi. Previous studies have shown that in *S*. Typhimurium, HilD stability is markedly decreased in the presence of bile, with a reported half-life almost 4 times shorter in LB supplemented with 3% bile, than in LB alone (23). To determine the effect of bile on HilD stability in *S*. Typhi, *S*. Typhi Ty2 was transformed with constitutively expressed HA-tagged HilD (from *S*. Typhi Ty2), subcultured in the presence or absence of bile, and samples taken at regular intervals following the inhibition of protein synthesis. Importantly the HA-tagged HilD used in these studies was functional (Figure 5), indicating that the HA tag used does not disrupt HilD structure or activity. In LB the half-life of HilD was 14 min, while in bile the half-life of HilD increased to 40 min, indicating that HilD is approximately three times more stable in the presence of bile in *S*. Typhi (Figure 6A).

HilD is highly conserved between *S*. Typhi and *S*. Typhimurium (>99% identity; 2 amino acid changes). Since HilD has previously been shown to be less stable in bile in *S*. Typhimurium (23), we next determined if this difference in stability was due to intrinsic

differences between HilD between the serovars, or rather due to differences in factors that act on HilD and influence its stability. To investigate this, we determined the stability of HA-tagged HilD from *S.* Typhimurium 14028 expressed in *S.* Typhi Ty2. As for *S.* Typhi HilD, *S.* Typhimurium HilD was three times more stable in bile, with a recorded half-life increasing from 8 min in LB, to 21 min (Figure 6B).

Although several factors have been reported to post-transcriptionally regulate HilD (e.g. HilE, CsrA, GreE/GreB, FliZ, Hfq, RNase E (3, 43, 44)), only two have been described to directly influence HilD protein stability: the protease Lon, which degrades HilD (45), and the acetyltransferase Pat, which acetylates HilD to increase stability whilst decreasing DNA binding (46). To determine if these factors were involved in mediating HilD stability in bile in *S.* Typhi Ty2, deletions were constructed and HilD stability determined as previously. Unfortunately, a Δ*lon* Ty2 strain had severe growth defects and could not be tested. Although HilD stability was decreased in a Δ*pat* Ty2 strain, in line with previous findings in *S.* Typhimurium (46, 47), stability of HilD was still increased in the presence of bile, increasing from 4 min in LB to 13 min in the presence of bile (Figure S3), indicating that Pat-mediated acetylation of HilD is not responsible for the increased stability in bile. Overall, our data suggest that factors responsible for governing the stability of HilD in response to bile (other than Pat) differ between *S.* Typhi and *S.* Typhimurium.

DISCUSSION

Transcriptomic analysis of *S.* Typhimurium and *S.* Typhi strains grown in LB or 3% bile permitted the identification of similarities and differences in each serovars' response to bile. Significant differences were observed in the regulation of the invasion-associated SPI-1 T3SS and in motility genes between non-typhoidal and typhoidal serovars. *S.* Typhi strains significantly upregulated these processes, and displayed a significant increase in T3SS-dependent invasion in bile, a response akin to other enteric pathogens (13), including *Vibrio parahaemolyticus* (48), *Vibrio cholera* (49, 50), and *Shigella* (51, 52). All *S.* Typhi strains tested (Ty2, CT18 and two H58 clinical isolates) demonstrated significantly increased invasion in bile, strongly suggesting that this is a common response of *S.* Typhi to bile.

It is interesting to consider why *S.* Typhi and *S.* Typhimurium have such disparate responses to bile. During infection, *Salmonell*a encounters bile within the small intestine, and in the case of *S.* Typhi, within the gallbladder. Following the observation that *S.* Typhimurium invasion was significantly repressed in the presence of bile (14), a model was proposed that *S.* Typhimurium uses bile concentration as a means to sense proximity to the intestinal epithelium; in the lumen where bile concentration is highest, SPI-1 expression would be repressed, as the bacteria get closer to the intestinal cells, bile concentration would decrease, leading to SPI-1 expression and invasion (14). Within the context of this model however, *S.* Typhi would be less invasive when in close contact with the intestinal epithelium, which is consistent with the limited intestinal inflammatory responses induced by *S.* Typhi (1). Moreover, *S.* Typhi has a unique site of infection – the gallbladder (7, 9). One of the mechanisms by which *S.* Typhi has been proposed to persist within the gallbladder is via direct invasion of gallbladder epithelial cells (53, 54); bile-induced increases in SPI-1 expression and invasiveness may therefore promote *S.* Typhi invasion and colonisation of the gallbladder epithelium. Alternatively, as *S.* Typhi carriage is closely associated with the

283 presence of gallstones, it is believed that *S.* Typhi forms biofilms on gallstone surfaces (7,
284 55). Biofilm formation on gallstones depends on several factors including the presence of
285 flagellar filaments (56), increased flagellar expression may therefore also promote biofilm
286 formation. As such, increases in expression of SPI-1 and motility associated genes in bile
287 may promote *S.* Typhi colonisation of the gallbladder, and therefore reflect adaptation to this
288 environment.

289 In terms of understanding how *S.* Typhi and *S.* Typhimurium differ with regards to SPI-1
290 expression in bile, our results, in combination with previous findings (23), demonstrate that
291 HilD is differentially regulated by bile at the level of protein stability (consistent with the
292 idea that HilD is largely controlled at the post-transcriptional level (40)), resulting in
293 significant differences in the expression of downstream genes, including the SPI-1 master
294 regulator, *hilA* (Figure 7). The factor(s) responsible for mediating changes in HilD stability in
295 response to bile remains to be established, however this response does not appear to rely on
296 Lon (23) or Pat (this study). A recent transposon screen which aimed to identify factors
297 responsible for bile-mediated SPI-1 repression in *S.* Typhimurium failed to identify any
298 regulatory factor other than HilD (23). There are several reasons why such an approach may
299 have failed, including the involvement of essential genes or redundancy. Unfortunately
300 attempts to further identify regulatory mechanisms in *S.* Typhi are confounded by the limited
301 characterisation of SPI-1 regulatory processes within *S.* Typhi. The overall effect of bile on
302 invasion between *S.* Typhi and *S.* Typhimurium may also not be entirely regulatory; for
303 example the translocon protein SipD has been reported to interact with bile salts (57), but
304 SipD is one of several T3SS-associated proteins reported to be 'differentially evolved' (as
305 determined by non-synonymous amino acid changes) between typhoidal and non-typhoidal
306 serovars, which results in functional differences (58). Importantly, in *Shigella flexneri*,

interaction of deoxycholate or other bile salts with the SipD homologue, IpaD, promotes the recruitment of the translocator protein, IpaB, 'readying' the T3SS for secretion (59, 60).

Our results also demonstrate that strains belonging to the H58 *S.* Typhi lineage (129-0238 and ERL12148) display significantly increased responses to bile when compared to *S.* Typhi reference strains (Ty2 and CT18). When considering chronic carriage such responses may be advantageous, by increasing the potential of H58 strains to colonise the gallbladder, increasing bacterial burden and subsequently increasing transmission. However, it is currently unknown if this reflects differences between recently isolated clinical strains when compared to more laboratory-adapted reference strains, or is instead due to intrinsic difference in H58 strains compared to other *S.* Typhi haplotypes. H58 isolates have 44 non-synonymous single nucleotide polymorphisms (SNPs) which are not found within the *S.* Typhi reference strain CT18 (21), including several SNPs within the Csr system (*sirA* (L63F), *csrB* (155G>A), *csrD* (A620V)), which is a known regulator of SPI-1 (61). Interestingly, significant phenotypic differences in bile were also observed between the two H58 strains investigated. Further comparisons of H58 strains would be required to determine if the phenotypic differences observed are sublineage-specific or simply reflect diversity within the H58 group.

In conclusion, our results confirm that bile is a key regulator of gene expression in *Salmonella*, influencing the expression of almost 10% of the genome, including genes associated with virulence, motility and metabolism. These findings add to the characterisation of *S.* Typhi responses to bile (30, 62), which may ultimately help explain the mechanisms by which *S.* Typhi induces chronic carriage (13).

MATERIALS AND METHODS

Bacterial strains, growth conditions and plasmid construction

The strains and plasmids used in this study are listed in Table 4. *Salmonella* were routinely grown in LB Lennox (Sigma-Aldrich) at 37°C / 200 rpm. Ox bile (3% w/v) (Sigma-Aldrich/Merck-Millipore) was supplemented as indicated.

All oligonucleotides used in this study are listed in Table S1. The Δ*invA* and Δ*pat S*. Typhi Ty2 deletion strains were constructed via lambda red, as previously described (63, 64). Strains with chromosomal integration of the *lacZ* gene were also constructed via lambda red recombination as described (41). Correct integration of introduced cassettes was validated by PCR.

To create HA tagged HilD, pWSK29-Spec-4HA (64) was amplified with a reverse primer containing a PacI digestion site, and HilD was amplified from both *S*. Typhimurium and *S*. Typhi with primers containing NotI and PacI restriction sites. Both products were digested, and HilD cloned into the existing NotI site and the introduced PacI site of pWSK29-Spec-4HA, resulting in constitutively expressed C-terminally tagged HilD-4HA. Plasmid construction was validated by sequencing.

Cell culture and HeLa invasion assays

HeLa cells (ATCC) were maintained in Dulbecco's Modified Eagle medium supplemented with 10% foetal bovine serum (FBS) (Sigma-Aldrich) in a 5% CO_2 at 37°C. The cells were authenticated via short tandem repeat profiling in February 2016 (Microsynth).

Invasiveness of strains was determined by gentamicin protection assays, as previously described (64). Briefly, *Salmonella* strains were cultured overnight at 37°C / 200 rpm in LB or LB supplemented with 3% bile before subculturing 1:33 in LB or LB 3% bile until late exponential phase (OD_{600} ~1.8), when SPI-1 expression is induced (18) (data not shown). To

prevent bile-mediated cell lysis, bacteria were washed twice in LB before addition to cells at an MOI 100:1. As *S.* Typhi is less invasive than *S.* Typhimurium (65), *S.* Typhi infections were performed for 1 h, and *S.* Typhimurium for 15 min, prior to the addition of gentamicin, unless otherwise indicated. At indicated time points, cells were lysed, serially diluted, and plated to enumerate intracellular CFU.

RNA extraction

Salmonella were cultured overnight in LB or LB supplemented with 3% bile (w/v) before subculturing 1:33 until late exponential phase (OD_{600} ~1.8). 6 x 10^8 bacteria were incubated in RNAprotect (Qiagen) at room temperature (RT) for 5 min. Bacteria were digested with lysozyme (15 mg / ml) and proteinase K for 20 min at RT, and RNA extracted using the RNeasy Mini Kit (Qiagen) as per manufacturer's instructions. RNA extractions for RNA-Seq were performed in duplicate then pooled, over three biological repeats. RNA extractions for quantitative reverse transcription PCR (RT-qPCR) were performed in triplicate over three biological repeats. RNA samples for RNA-Seq and RT-qPCR were extracted independently of each other.

RNA sequencing and data analysis

For RNA sequencing, mRNA libraries were multiplexed and prepared by utilisation of the Illumina TruSeq protocol followed by sequencing via paired-end methodology on the Illumina HiSeq version 4 platform. Each lane of Illumina sequence was assessed for quality on the basis of adapter contamination, average base read quality and any unusual G-C bias using FastQC. The median Phred score for all samples was >34. To permit comparison between strains, sequenced reads for each strain were mapped to the Ty2 genome (NC_004631) using the Rockhopper tool (66) with default parameters (Data S1-3). The read alignment coverage for each sample can be found in Table S2. The threshold for

differentially expressed genes was gated as those displaying >2 fold change in expression in 3% bile compared to LB alone, and with an adjusted p value (q value) < 0.05.

GO term enrichment for differentially regulated genes was performed with Panther (67) using the *S.* Typhimurium GO annotation, whilst KEGG pathway analysis was performed with the GAGE R package (68) (R 3.3.1), using the *S.* Typhi (stt) KEGG annotation. The VennDiagram (69) and gplots R packages were used for data visualisation.

Quantitative reverse transcription PCR (RT-qPCR)

2 µg of RNA was treated with DNase (Promega) prior to reverse transcription with M-MLV reverse transcriptase (Promega) according to manufacturer's recommendations. Fast SYBR Green Master Mix (Applied Biosystems) was used for qPCR reactions alongside the Applied Biosystems StepOnePlus system. 20 ng of cDNA was used per reaction, and forward and reverse primers (Table S1) used at final concentration of 0.2 µM. Samples without reverse transcription were included as negative controls. The housekeeping gene, *ftsZ*, was used as the reference gene as it was determined to be least variable gene between strains and between LB with and without 3% bile. qPCR reactions were performed in duplicate on triplicate samples over three biological replicates.

SPI-1 protein expression and stability assays

To determine expression of SPI-1 proteins, *Salmonella* were subcultured in the absence or presence of 3% ox-bile to late exponential phase. 1 mL of culture was pelleted and re-suspended in 2X SDS loading buffer (1M Tris pH 6.8, 2% SDS, 20% glycerol, 5% β-mercaptoethanol, bromophenol blue) in proportion to OD_{600}. To determine HilD stability, *Salmonella* strains previously transformed with 4HA-tagged constructs were subcultured in 10 ml LB with or without the addition of 3% ox-bile until late exponential phase. The OD_{600} was recorded, and chloramphenicol (30 µg/ml) added to inhibit protein synthesis. 1 ml

bacteria were pelleted and re-suspended in 2X SDS loading buffer in proportion to OD_{600}. The cultures were incubated at 37°C / 200 rpm, and 1 ml samples were taken at required time points. Samples were heated at 95°C for 10 min. Whole cell samples were subject to Western blotting, using an anti-HA antibody to detect the protein of interest, and DnaK as a loading control. Following imaging, band density was quantified using ImageJ, and half-life (in minutes) calculated using the equation: (t x ln(2)) / (ln(No/Nf)), where t equals time elapsed between measurements (in minutes), N_0 equals the initial amount, and N_f equals the final amount (23). To determine changes in SPI-1 proteins in bile, band density was quantified using ImageJ, levels of SPI-1 proteins were normalised to the corresponding DnaK value, and fold change in bile relative to LB calculated.

SDS-PAGE and Western blotting

Proteins were separated on 12% acrylamide gels followed by semi-dry transfer on to PVDF membrane (GE Healthcare). Membranes were blocked in 5% milk in PBS + 0.05% Tween-20 (Sigma-Aldrich), and probed with either anti-DnaK 8E2/2 (1:10000) (Enzo Life Sciences #ADI-SPA-880), anti-HA HA-7 (1:1000) (Sigma #H3663), anti-SipC, anti-SipD, anti-SopB, or anti-SopE (1:5000) (V. Koronakis, University of Cambridge) primary antibodies, followed by HRP-conjugated secondary antibody (1:10000) (Jackson ImmunoResearch). Chemiluminescence following the addition of EZ-ECL reagent (Geneflow) was detected using the LAS-3000 imager (Fuji).

β-galactosidase assays

β-galactosidase assays were performed as previously described (70). *Salmonella* strains were grown in SPI-1 inducing conditions with or without the addition of 3% ox bile. The OD_{600} was recorded, and 1 ml of culture pelleted and resuspend in 1 ml Z buffer (0.06M Na_2HPO_4, 0.04M NaH_2PO_4, 0.01M KCl, 0.001M $MgSO_4$ and 0.05M β-mercaptoethanol, pH 7). WT

strains were used as negative controls. Samples were permeabilised with the addition of 0.1% SDS and chloroform, and vortexed for 2 min. 20 µl of prepared sample was added to 180 µl Z buffer in a 96 well microplate, and 2-Nitrophenyl β-D-galactopyranoside (ONPG) substrate (4 mg/ml in Z buffer) added. Plates were incubated at RT, then the reaction stopped with the addition of 1M Na_2CO_3. The absorbance of the samples was measured at 405 nm and 540 nm using a FLUOStar Omega plate reader (BMG Labtech).

Statistical analysis

Statistical tests were performed using GraphPad Prism (Version 7.00) for Windows (GraphPad Software, San Diego, California, USA). All data are expressed as mean ± SD. Significance ($p < 0.05$) was determined by unpaired t-test or ANOVA, with correction for multiple comparisons when required.

ACKNOWLEDGEMENTS

We are grateful to Gordon Dougan (Sanger Institute) for providing the *S.* Typhi strains used in this study, to Michael Hensel for providing the p3138 template plasmid for construction of reporter strains via lambda red, and to Vassilis Koronakis (University of Cambridge) for providing the anti-SipC, anti-SipD, anti-SopB and anti-SopE antibodies. RJ is supported by an MRC Centre for Molecular Bacteriology and Infection Grant, ref: MR/J006874/1. GF is supported by a Wellcome Trust Investigator grant.

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671

672 **FIGURE LEGENDS**

673 **Figure 1. Comparison of pathways differentially regulated by bile between *S.* Typhi and**
674 ***S.* Typhimurium.** Overrepresented Gene Ontology (GO) terms within upregulated and
675 downregulated genes following growth in 3% bile for each strain.

676

677 **Figure 2. Gene expression in response to bile differs between *Salmonella* strains.**
678 Comparison of genes upregulated and downregulated in response to bile in *S.* Typhimurium
679 (Tm), *S.* Typhi Ty2 (Ty2) and *S.* Typhi 129-0238 (H58).

680

681 **Figure 3. The effect of bile on SPI-1 expression and activity. (A)** Heatmap showing log2
682 fold change in gene expression for *S.* Typhimurium (Tm), *S.* Typhi Ty2 (Ty2) and *S.* Typhi
683 129-0238 (H58) across the SPI-1 pathogenicity island and for non-SPI-1 carried effectors.
684 Asterisks (*) indicate genes significantly affected by bile across all three strains. **(B)** Western
685 blots of SipC, SipD and SopE of *S.* Typhimurium 14028 (Tm), *S.* Typhi Ty2 (Ty2), and two
686 H58 clinical isolates (ERL12148 and 129-0238) grown in LB with or without 3% bile; SopE
687 panels are not shown for *S.* Typhimurium 14028, as this strain lacks SopE. DnaK was used as
688 a loading control. A representative blot of two independent repeats is shown. Numbers below
689 panels indicate fold change in density when compared to LB; all bands were normalised to
690 their respective DnaK control prior to comparison. **(C)** Strains grown in LB or 3% bile to late
691 exponential phase were added to HeLa cells at an MOI 100 for 30 min. The percentage of
692 intracellular bacteria at 2 h post-infection relative to the inoculum added is shown. n=3, error
693 bars show SD. Invasion rates of strains were compared by *t*-test *(*** = $P < 0.01$, **** = $P <$
694 0.001).

Figure 4. Effect of bile on *hilA* and *hilD* transcription in *Salmonella*. The reporter activity (β-galactosidase units) of *hilA:lacZ* and *hilD:lacZ* in *S.* Typhimurium 14028 **(A & B)** and *S.* Typhi Ty2 **(C & D)** following growth to late exponential phase in LB in the presence or absence of bile. n=3, error bars show SD. Reporter activity between strains was compared by t-test (* = $P < 0.05$, *** = $P < 0.001$).

Figure 5. HilD autoregulation in *S.* Typhi. The reporter activity of a *S.* Typhi Ty2 *hilD:lacZ* chromosomal transcriptional reporter strain complemented with HilD (pWSK29-Spec HilD-4HA (HilD)) or an empty vector control (pWSK29-Spec (EV)), was determined by β-galactosidase assay following growth in LB. n=3, error bars show SD. Reporter activity between strains was compared by one way ANOVA (*** = $P < 0.001$).

Figure 6. Bile promotes HilD stability in *S.* Typhi. WT *S.* Typhi Ty2 constitutively expressing C-terminally 4HA-tagged HilD from **(A)** *S.* Typhi Ty2 or **(B)** *S.* Typhimurium 14028 was grown in LB with or without bile. 30 µg/ml chloramphenicol was added to stop protein synthesis, and samples were collected every 10 min. HilD levels were determined via Western blotting using an anti-HA antibody, and DnaK used as a loading control. A representative blot of three independent repeats is shown. Half-life measurements are averaged from three independent repeats, and standard deviation is shown.

Figure 7. Proposed model of how bile influences SPI-1 expression in *S*. Typhi. (A) HilD is at the top of the SPI-1 regulatory hierarchy, where it regulates its own expression and the expression of HilA. HilD also regulates expression of the additional regulators HilC and RtsA, which also control HilA expression. **(B)** In the absence of bile the turnover of HilD is high, the expression of *hilD* is at a basal level and as a result the expression of *hilA* is low **(C)** In the presence of bile HilD is more stable, leading to enhanced expression of *hilD*, *hilA* and thus SPI-1.

723 **TABLES**

724 **Table 1. Genes upregulated by bile in all strains**

			Log2 fold change		
Name	**Locus tag**	**Product**	**Tm**	**Ty2**	**H58**
fadI	t0475	3-ketoacyl-CoA thiolase	4.12	2.55	2.52
fadJ	t0476	multifunctional fatty acid oxidation complex subunit alpha	3.32	2.06	2.17
fadE	t2541	acyl-CoA dehydrogenase	7.44	4.70	4.18
fadB	t3315	multifunctional fatty acid oxidation complex subunit alpha	7.52	2.92	1.57
fadA	t3316	3-ketoacyl-CoA thiolase	7.66	2.88	1.57
actP	t4179	acetate permease	3.41	1.58	1.27
-	t4180	hypothetical protein	3.44	1.72	1.32
acs	t4181	acetyl-CoA synthetase	3.91	2.11	1.31
acnA	t1625	aconitate hydratase	3.18	1.81	1.59
argT	t0509	lysine-arginine-ornithine-binding periplasmic protein	3.36	2.29	1.33
argD	t1182	bifunctional succinylornithine transaminase/acetylornithine transaminase	5.61	2.72	1.20
-	t0677	gentisate 1,2-dioxygenase	2.51	3.91	3.38
-	t0678	FAA-hydrolase-family protein	2.09	3.21	2.87
-	t0679	glutathione-S-transferase-family protein	2.09	2.89	2.49
-	t0680	salicylate hydroxylase	1.27	2.09	2.03
-	t1787	oxidoreductase	3.62	3.53	1.32
-	t1789	hypothetical protein	3.17	4.04	1.44
-	t1790	N-acetylneuraminic acid mutarotase	2.78	4.07	1.32
gabT	t2687	4-aminobutyrate aminotransferase	5.18	2.93	1.74
msrA	t4462	methionine sulfoxide reductase A	1.68	1.67	1.30

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727 **Table 2. Log2 fold change in gene expression determined by RNA-Seq and RT-qPCR**

	RNA-Seq			RT-qPCR		
Gene	**14028**	**Ty2**	**129-0238**	**14028**	**Ty2**	**129-0238**
hilD	-4.08	1.23	3.15	-3.48 (± 0.71)	1.42 (± 0.25)	2.44 (± 0.73)
hilA	-6.98	1.54	3.67	-6.51 (± 0.64)	1.71 (± 0.39)	3.37 (± 0.39)
prgH	-6.36	1.57	4.02	-6.00 (± 0.74)	1.68 (± 0.73)	4.00 (± 0.48)
sopB	-6.95	1.11	4.21	-3.85 (± 0.44)	1.38 (± 0.59)	4.13 (± 0.27)
flhD	-1.72	1.05	1.33	-1.25 (± 0.43)	1.93 (± 0.38)	2.31 (± 1.13)

flgA	-1.29	1.37	1.70	-0.98 (± 0.27)	1.99 (± 0.44)	1.37 (± 0.84)
fadE	7.44	4.70	4.18	3.55 (± 2.13)	3.75 (± 0.16)	4.75 (± 0.09)
acs	3.91	2.11	1.31	2.03 (± 1.77)	0.87 (± 0.67)	2.37 (± 0.59)

± indicates standard deviation

Table 3. Genes downregulated in *S.* Typhimurium and upregulated in *S.* Typhi in bile

			Log2 fold change		
Name	**Locus tag**	**Product**	**Tm**	**Ty2**	**H58**
fliO	t0899	flagellar biosynthesis protein FliO	-1.87	1.57	1.35
fliN	t0900	flagellar motor switch protein FliN	-1.55	1.44	1.62
fliM	t0901	flagellar motor switch protein FliM	-1.71	1.40	1.71
fliL	t0902	flagellar basal body protein FliL	-1.74	1.41	1.78
fliK	t0903	flagellar hook-length control protein	-1.67	1.33	2.08
fliJ	t0904	flagellar biosynthesis chaperone	-1.37	1.43	2.25
fliI	t0905	flagellum-specific ATP synthase	-1.43	1.25	1.69
fliH	t0906	flagellar assembly protein H	-1.45	1.41	1.57
fliG	t0907	flagellar motor switch protein G	-1.44	1.34	1.53
fliF	t0908	flagellar MS-ring protein	-1.89	1.32	1.41
fliE	t0909	flagellar hook-basal body protein FliE	-2.49	1.76	2.01
flhD	t0952	transcriptional activator FlhD	-1.72	1.05	1.33
flgJ	t1738	flagellar rod assembly protein/muramidase FlgJ	-1.56	1.30	1.38
flgI	t1739	flagellar basal body P-ring biosynthesis protein FlgA	-1.69	1.41	1.39
flgH	t1740	flagellar basal body L-ring protein	-1.71	1.42	1.68
flgC	t1745	flagellar basal body rod protein FlgC	-1.86	1.39	1.79
flgB	t1746	flagellar basal-body rod protein FlgB	-2.05	1.40	1.73
flgA	t1747	flagellar basal body P-ring biosynthesis protein FlgA	-1.29	1.37	1.70
sprB	t2768	AraC family transcriptional regulator	-3.76	1.97	4.11
sprA	t2769	AraC family transcriptional regulator	-3.29	1.97	3.29
-	t2770	hypothetical protein	-3.69	1.22	2.11
orgA	t2771	oxygen-regulated invasion protein	-3.90	1.34	1.79
orgA	t2772	oxygen-regulated invasion protein	-5.65	1.62	3.50
prgJ	t2774	pathogenicity island 1 effector protein	-6.05	1.43	3.83
prgI	t2775	pathogenicity island 1 effector protein	-6.15	1.41	3.89
prgH	t2776	pathogenicity island 1 effector protein	-6.36	1.57	4.02
hilA	t2778	invasion protein regulator	-6.98	1.54	3.67
iagB	t2779	cell invasion protein	-6.64	1.35	3.83
sicP	t2781	chaperone	-3.06	1.40	3.19
-	t2782	hypothetical protein	-3.10	1.56	2.98

sipF/iacP	t2783	acyl carrier protein	-5.62	1.46	3.43
sipA	t2784	pathogenicity island 1 effector protein	-5.84	1.55	3.60
sipD	t2785	pathogenicity island 1 effector protein	-6.24	1.48	3.87
spaS	t2789	surface presentation of antigens protein SpaS	-5.70	1.24	3.29
spaQ	t2791	virulence-associated secretory protein	-7.26	1.40	3.00
spaP	t2792	surface presentation of antigens protein SpaP	-6.87	1.43	3.25
spaO	t2793	surface presentation of antigens protein SpaO	-6.72	1.60	3.66
spaN	t2794	antigen presentation protein SpaN	-6.66	1.58	3.91
spaM	t2795	virulence-associated secretory protein	-6.91	1.76	3.83
spaL/invC	t2796	ATP synthase SpaL	-6.61	1.53	3.43
Spak/invB	t2797	virulence-associated secretory protein	-6.04	1.91	4.01
invA	t2798	virulence-associated secretory protein	-6.50	1.40	3.34
invE	t2799	cell invasion protein	-6.86	1.35	3.59
invG	t2800	virulence-associated secretory protein	-7.12	1.37	3.60
invF	t2801	AraC family transcriptional regulator	-6.97	1.27	3.84
invH	t2802	cell adherance/invasion protein	-4.54	1.57	2.97
sopD	t2846	hypothetical protein	-3.76	1.05	4.33
rtsB	t4220	GerE family regulatory protein	-7.59	1.99	3.58
rtsA	t4221	AraC family transcriptional regulator	-7.33	1.80	3.83
-	t0944	lipoprotein	-2.25	1.20	2.22
-	t1774	hypothetical protein	-2.09	1.46	2.60
lpxR	t1208	hypothetical protein	-7.02	1.19	3.44
srfA	t1503	virulence effector protein	-1.75	1.64	1.81
srfB	t1504	virulence effector protein	-1.48	1.58	1.88

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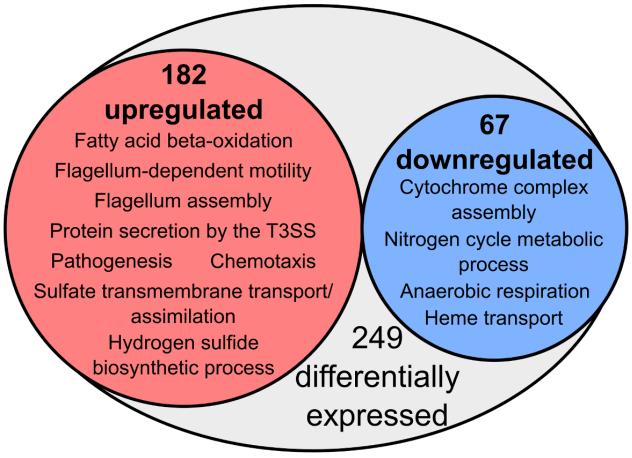
732 **Table 4. Strains and plasmids used in this study**

Strain or plasmid	**Identifier**	**Genotype or comments**	**Source**
Strains			
***S*. Typhimurium**			
14028	ICC797	WT	(64)
14028	ICC1765	Δ*hilA*:*lacZ* Kan^R	This study
14028	ICC1764	Δ*hilD*:*lacZ* Kan^R	This study
***S*. Typhi**			
Ty2	ICC1500	WT	G. Dougan
Ty2	ICC1630	Δ*hilA*:*lacZ* Kan^R	This study
Ty2	ICC1762	Δ*hilD*:*lacZ* Kan^R	This study
Ty2	ICC1556	Δ*invA* Kan^R	(64)
Ty2	ICC1756	Δ*pat* Kan^R	This study

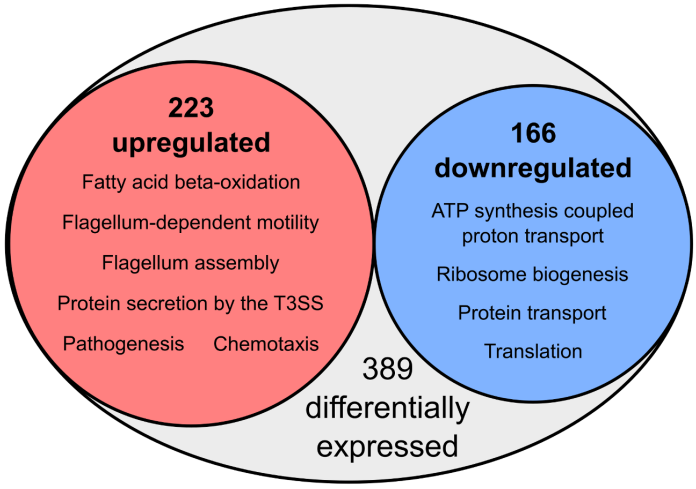
CT18	ICC1502	WT	G. Dougan
129-0238	ICC1503	WT, H58 isolate	G. Dougan (21)
ERL12148	ICC1504	WT, H58 isolate	G. Dougan (21)
Plasmids			
pKD4	pICC893	Kanamycin cassette template plasmid	(63)
p3138	pICC2515	LacZ and kanamycin cassette template plasmid	(41)
pKD46	pICC1298	Lambda red recombinase plasmid	(63)
pWSK29-Spec E.V.	pICC2489	Empty vector, spectinomycin^{R}	(64)
pWSK29-Spec HilD-4HA Ty2		*S.* Typhi Ty2 HilD-4HA, constitutive promoter	This study
pWSK29-Spec HilD 4HA Tm		*S.* Typhimurium 14028 HilD-4HA, constitutive promoter	This study

733

S. Typhi Ty2 (Ty2)



S. Typhi 129-0238 (H58)



S. Typhimurium 14028 (Tm)