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

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

Despite the importance of asymptomatic carriage, the behaviour of *S.* Typhi within bile remains poorly understood (7). As the transcriptomic responses of *S.* Typhimurium to bile under various conditions have been well characterised (15–18), the behaviour of *S.* Typhimurium has become an accepted model as to how *Salmonella* in general behaves in bile (11, 19). However a study comparing changes in protein expression by 2D gel electrophoresis within *S.* Typhimurium and *S.* Typhi following exposure to 3% bile found there was “little overlap apparent between proteins affected by bile in *S.* Typhi and in *S.* Typhimurium” (12), suggesting that the response to bile between these serovars differs. Furthermore, a study comparing the genomes of *S.* Typhimurium LT2 to *S.* Typhi CT18 revealed that less than 90% of genes are shared between the two strains, with over 600 genes present in CT18 not found in LT2 (20); therefore *S.* Typhimurium cannot be used to model regulation of *S.* Typhi specific genes, which include key virulence factors such as the Vi antigen, and the CdtB and HlyE/ClyA toxins (20).
The need to better understand S. Typhi infection has been intensified by the recent spread of haplotype 58 (H58), also known as 4.3.1 (21, 22). Following its emergence around 30 years ago, S. Typhi strains belonging to haplotype H58 have clonally expanded worldwide to become the dominant cause of multi-drug resistant (MDR) typhoid within endemic regions (21). As yet, the reasons underlying the relative success of H58 strains remain unknown.

The aim of this study was to compare global bile responses between S. Typhi and S. Typhimurium isolates, which in turn might explain differences in pathogenesis and reveal 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
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
(ΔinvA) 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 ΔhilD 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. Typh 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. Typh Ty2, deletions were constructed and HilD stability determined as previously.
Unfortunately, a Δlon Ty2 strain had severe growth defects and could not be tested. Although
HilD stability was decreased in a Δpat 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, Salmonella 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
presence of gallstones, it is believed that S. Typhi forms biofilms on gallstone surfaces (7, 55). Biofilm formation on gallstones depends on several factors including the presence of flagellar filaments (56), increased flagellar expression may therefore also promote biofilm formation. As such, increases in expression of SPI-1 and motility associated genes in bile may promote S. Typhi colonisation of the gallbladder, and therefore reflect adaptation to this environment.

In terms of understanding how S. Typhi and S. Typhimurium differ with regards to SPI-1 expression in bile, our results, in combination with previous findings (23), demonstrate that HilD is differentially regulated by bile at the level of protein stability (consistent with the idea that HilD is largely controlled at the post-transcriptional level (40)), resulting in significant differences in the expression of downstream genes, including the SPI-1 master regulator, hilA (Figure 7). The factor(s) responsible for mediating changes in HilD stability in response to bile remains to be established, however this response does not appear to rely on Lon (23) or Pat (this study). A recent transposon screen which aimed to identify factors responsible for bile-mediated SPI-1 repression in S. Typhimurium failed to identify any regulatory factor other than HilD (23). There are several reasons why such an approach may have failed, including the involvement of essential genes or redundancy. Unfortunately attempts to further identify regulatory mechanisms in S. Typhi are confounded by the limited characterisation of SPI-1 regulatory processes within S. Typhi. The overall effect of bile on invasion between S. Typhi and S. Typhimurium may also not be entirely regulatory; for example the translocon protein SipD has been reported to interact with bile salts (57), but SipD is one of several T3SS-associated proteins reported to be 'differentially evolved' (as determined by non-synonymous amino acid changes) between typhoidal and non-typhoidal 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 ΔinvA and Δpat *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% CO₂ 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 (OD₆₀₀ ~1.8), when SPI-1 expression is induced (18) (data not shown).
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\textsubscript{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: $(t \times \ln(2)) / (\ln(No/Nf))$, 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\textsubscript{600} was recorded, and 1 ml of culture pelleted and resuspend in 1 ml Z buffer (0.06M Na\textsubscript{2}HPO\textsubscript{4}, 0.04M NaH\textsubscript{2}PO\textsubscript{4}, 0.01M KCl, 0.001M MgSO\textsubscript{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 vortexted 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 Na₂CO₃. The absorbance of the samples was measured at 405 nm and 540 nm using a FLUOSStar 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.
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FIGURE LEGENDS

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

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

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

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

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

| Gene | RNA-Seq 14028 | Ty2 129-0238 | RT-qPCR 14028 | Ty2 129-0238 |
|------|---------------|--------------|---------------|--------------|
| hilD | -4.08         | 1.23         | 3.15          | 1.42         | 2.44         |
|      |               |              | (-3.48 (± 0.71)) | (± 0.25)     | (± 0.73)     |
| hilA | -6.98         | 1.54         | 3.67          | -6.51        | 1.71         | 3.37         |
|      |               |              | (-6.51 (± 0.64)) | (± 0.39)     | (± 0.39)     |
| prgH | -6.36         | 1.57         | 4.02          | -6.00        | 1.68         | 4.00         |
|      |               |              | (-6.00 (± 0.74)) | (± 0.73)     | (± 0.48)     |
| sopB | -6.95         | 1.11         | 4.21          | -3.85        | 1.38         | 4.13         |
|      |               |              | (-3.85 (± 0.44)) | (± 0.59)     | (± 0.27)     |
| flhD | -1.72         | 1.05         | 1.33          | -1.25        | 1.93         | 2.31         |
|      |               |              | (-1.25 (± 0.43)) | (± 0.38)     | (± 1.13)     |
| Name | Locus tag | Product | Log2 fold change |
|------|-----------|---------|-----------------|
| 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 |
| 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 |
| orgA | 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 |

± indicates standard deviation

Table 3. Genes downregulated in *S. Typhimurium* and upregulated in *S. Typhi* in bile
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    | ΔhilA::lacZ Kan^K    | This study |
| 14028             | ICC1764    | ΔhilD::lacZ Kan^K    | This study |
| *S. Typhi*        |            |                      |        |
| Ty2               | ICC1500    | WT                   | G. Dougan   |
| Ty2               | ICC1630    | ΔhilA::lacZ Kan^K    | This study |
| Ty2               | ICC1762    | ΔhilD::lacZ Kan^K    | This study |
| Ty2               | ICC1556    | ΔinvA Kan^K          | (64)   |
| Ty2               | ICC1756    | Δpat Kan^K           | 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\(^K\) | (64) |
| pWSK29-Spec HilD-4HA Ty2 | Spec E.V. Spec HilD-4HA, constitutive promoter | This study |
| pWSK29-Spec HilD-4HA Tm | Spec E.V. Spec HilD-4HA, constitutive promoter | This study |
A

- HilD stability low
- Basal expression of HilD
- Basal expression of downstream genes (e.g. hilA)

B

- HilD stability increased
- HilD expression increased
- Increased expression of downstream genes (e.g. hilA)

C

- HilD stability increased
- HilD expression increased
- Increased expression of downstream genes (e.g. hilA)