Interplay of cold shock protein E with an uncharacterized protein, YciF, lowers porin expression and enhances bile resistance in *Salmonella Typhimurium*

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Running Title: *Salmonella Typhimurium* CspE regulates bile resistance

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ABSTRACT

Bacterial cold shock proteins (CSPs) function as RNA chaperones. To assess CSP’s roles in the intracellular human pathogen, *Salmonella Typhimurium*, we analyzed their expression in varied stress conditions. We found that cold shock protein E (cspE or STM14_0732) is up-regulated during bile salt-induced stress and that an *S. Typhimurium* strain lacking cspE (ΔcspE) displays dose-dependent sensitivity to bile salts, specifically to deoxycholate. We also found that an uncharacterized gene, yciF (STM14_2092) is up-regulated in response to bile stress in WT, but not in the ΔcspE strain. Complementation with WT CspE, but not with a F30V CspE variant, abrogated the bile sensitivity of ΔcspE as did multicopy over expression of yciF. Northern-blotting experiments with rifampicin disclosed that the regulation of yciF expression is most likely due to the RNA stabilizing activity of CspE. Importantly, electrophoretic mobility shift assay indicated that purified CspE, but not the F30V variant, directly binds yciF mRNA. We also observed that the extra-cytoplasmic stress response (ESR) pathway is augmented in the bile treated ΔcspE strain, as judged by induction of RpoE regulon genes (rpoE, degP and rynB) and downstream ESR genes (hfg, rne and PNPase). Moreover, the transcript levels of the porin genes -ompD, ompF and ompC- were higher in bile salts-stressed ΔcspE and correlated with higher intracellular accumulation of the fluorescent DNA stain bisBenzimide H 33258, indicating greater cell permeability. In conclusion, our study has identified YciF a CspE target involved in the regulation of porins and in countering bile stress in *S. Typhimurium*.

Microbes face myriad stresses due to changes in the environment. Consequently, gene regulation plays important roles during their adaptation to different environmental milieu. For example, most bacteria react to a sudden decrease in temperature, *i.e.* a shift from 30°C to 10°C, by a cold shock response (1). This temperature alteration causes major physiological changes in the cell, including changes in composition and organisation of lipids, leading to alternations in membrane fluidity, increase in superhelicity and compaction of DNA by the introduction of negative supercoils, and overall decreased metabolic rate (2). A family of genes involved in this response are the Cold Shock Proteins (CSPs) consisting of 67-73 amino acids that are evolutionarily conserved across all three domains of life and are implicated to function as RNA-chaperones (3).

The Cold Shock Domain (CSD) containing proteins are characterised structurally and functionally in several organisms, *e.g.* *E. coli* (4), *Bacillus subtilis* (5). Various binding preferences have been reported for CSPs, namely, for poly(dT) and poly(dC) ssDNA (6), for the cold shock primer sequence ATTTG [(7),(8)], or for AT rich regions (9); however *in vivo* evidence for substrate specificity is lacking. Numerous amino acid residues have been identified to be important for nucleotide binding, which largely centre...
around two motifs termed ribonucleoprotein site (RNP)1 (KGFGF) and RNP2 (VFVH) (10). Not all CSPs are cold-inducible and some are reported to fulfil non-cold stress related functions. *E. coli* harbours nine CspA paralogs (CspA-I) of which CspA, CsbB, CspG and CspI are cold inducible (11). *cspA* is also constitutively expressed at ‘normal’ growth temperatures (37°C), as are *cspC* and *cspE* (12), whereas *cspD* is induced during nutrient starvation (13). The major CSP from *Escherichia coli*, CspA has been described as a multifunctional nucleic acid binding protein and essential for mRNA stabilization after temperature downshift (14). In *Listeria monocytogenes*, CspA enables hemolysis by regulating the production of the pore-forming cytolysin listeriolysin (15). A cold shock induced RNA binding protein CspR plays a post-transcriptional function in the Gram-positive opportunistic human pathogen, *E. faecalis* and has a role in the virulence, organ colonization and its survival in macrophages (16).

The three-dimensional structure of CSPs is fairly similar, e.g. the *E. coli* encoded CspE superimposes appropriately with the *S. Typhimurium* CspE. It comprises of five antiparallel β strands forming a classic OB fold with the characteristic RNP1 and RNP2 motifs conserved on the nucleic acid interacting surfaces (8). CspE is characterised to function in varied conditions. The nucleic acid melting ability and transcription anti-terminator activity of CspE is critical for growth at low temperature (17). It functions as a 'housekeeping RNA chaperone' under general stress conditions (18), enhances translation of several mRNAs (9) and is important in imparting camphor resistance (19). It interferes with bacteriophage lambda Q-mediated transcription anti-termination (20), regulation of the poly(A)-mediated 3’-5’ exonuclease activity of polynucleotide phosphorylase and cleavage and poly(A) tail removal by RNase E (21).

The intracellular pathogen, *Salmonella Typhimurium* (*S. Typhimurium*) causes typhoid-like infection in mice and is a well-established model to study the roles of proteins during stress and infection. In high-income countries, non-typhoidal salmonellae mainly cause a self-limiting enterocolitis in immunocompetent individuals (22). Up to 5% of patients develop secondary bacteremia, but attributable mortality is low (1–5%) (23). In sub-Saharan Africa, bacteraemia is commonly presented by invasive non-typhoidal salmonellae, in both children and adults, especially in regions of HIV and malaria prevalence. Despite anti-microbial treatments, fatality rates are 22–47% in African adults and children (24). Another troubling observation is the rise of multi-drug resistant strains in *S. Typhimurium*. In fact, DT104, is one of the leading causes of animal and human salmonellosis (25). DT104 is resistant to the commonly used antibiotics e.g. ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (26). These aspects reinforce the notion that it is important to study all aspects of investigations with respect to the biology of *Salmonella*.

In *S. Typhimurium*, six CSPs have been identified: CspA–E and CspH (8). Of these, cold inducibility of CspA, CspB and CspH has been reported [(3), (27–29)], although their functions have not yet been elucidated. A recent study (30) in *S. Typhimurium* SL1344 identified a plethora of downstream targets and physiological functions that CspE and CspC may regulate. In the present study, we focus on the role of a Cold Shock Protein E (STM14_0732) from *S. Typhimurium* 14028s, in imparting bile resistance. Bile is synthesized by the liver, stored in the gall bladder and plays a major role in dissolution and absorption of fats. In addition, bile demonstrates antimicrobial activity by solubilizing membrane lipids, disrupting membranes, damaging proteins, DNA and RNA and leading to the death of bacteria [(31), (32)]. Commensal bacteria are also able to modify bile salts both in the small and large intestine (31). This balance between the bile salt abundance and modification is beneficial to both the host and microbiome, especially in the cases of invading microbes which can be competed out by the commensals. There is an inverse correlation between bile concentration and bacterial colonisation: areas of high bile concentration (gall bladder) have low bacterial communities. A striking feature of chronic residence of *Salmonella* is the host distal ileum, an area of low bile concentration (33), and biliary tract or gall bladder, areas of high bile concentration (34). To escape the high concentrations of bile, *Salmonella* either form biofilms on the gallstone surface (35) or invades the gallbladder epithelium (36). Importantly, the colonization of the gall bladder by *Salmonella* is
thought to be essential for the chronic asymptomatic carrier state, which is observed in 3-5% of infected individuals (34). These aspects underscore the importance of bile in the biology of Salmonella. Here we show the mechanistic regulation of bile resistance controlled by S. Typhimurium encoded CspE. Furthermore, the identification of YciF (STM14_2092) as a CspE-regulated protein, and its role in regulating the outer membrane porins and cell permeability during bile stress are the highlights of this study.

**Results**

**Transcript levels of S. Typhimurium encoded cspE increases in the presence of bile salts.**

Salmonella faces a plethora of stresses within the host: acidic pH in the stomach, detergent-like stress from bile, osmotic stress in the intestine. We attempted to replicate these stresses in vitro and study the involvement of CSPs in these stresses. The WT strain was grown in a rich media (LB) and subjected to several stress-inducing agents: bile stress [3% bile salts (w/v)], osmotic stress [5% NaCl (w/v)] and acidic pH stress (LB at pH 4.8). Transcript levels of four cold shock genes cspA, cspB, cspC and cspE were analyzed at the 3rd and 8th hour of growth. cspA and cspB did not show any quantifiable changes. cspC transcripts were not detected (S1A Fig). As a control, we showed that the primers used were able to amplify the genomic copy of cspC (S1B Fig). However, only cspE seemed consistently and significantly up-regulated at the 3rd hour and 8th hour of growth, under the bile salt stress (Fig 1A). The observations indicated that bile stress induced the expression of cspE.

**A cspE deletion strain is highly susceptible to bile stress.**

To better understand its functional role, a single gene deletion of cspE was generated (ΔcspE). The WT and ΔcspE strains did not show any growth differences in LB or other stress conditions studied (S2 Fig). However, with increasing concentrations of bile salts, i.e. 3% (w/v) and above, ΔcspE showed significant growth reduction (S3A Fig). Subsequently, the kinetic growth of WT and ΔcspE in the absence (0% bile salts) and presence (3% bile salts), was monitored. The ΔcspE strain showed growth retardation in colony forming units (CFU), compared to the WT, in the presence of 3% bile salts (Fig 1B). The specificity was also attributed to the deoxycholate component of bile salts (S3B Fig). The functional involvement of cspE in imparting bile resistance, was determined by using an expression construct of cspE (pcspE- cspE was under the control of the native promoter in the pRS424 plasmid). As seen in Fig1C, the bile sensitive phenotype of ΔcspE was rescued by the complementation of cspE but not by the vector alone transformed cells. Also, this bile resistance regulation by CspE was not a general CSP-dependent phenotype observed, since multicopy over expression of another CSP, namely CspA did not show any phenotype suppression effect in ΔcspE (S4 Fig). In S. Typhimurium 14028s, amongst the CSPs studied, cspE is essential for bile resistance.

**Deletion of cspC does not affect bile resistance of S. Typhimurium 14028s.**

A previous study using S. Typhimurium SL1344 had shown that both CspC and CspE are responsible for resistance to multiple stress, e.g. bile, polymyxin B sulfate, H₂O₂ (30). However, in the WT strain (S. Typhimurium 14028s) used in this study, we were unable to detect any cspC transcripts with either qRT or semi qRT PCR (S1A Fig). Deletion strains of cspE, cspC and cspEcspC were generated in the background of both 14028s and SL1344 wild type strains. We observed that the ΔcspE in SL1344 background was marginally more resistant to bile stress than that in the 14028s background. However, in our system single deletion of cspC (ΔcspC) did not show any significant effect on bile resistance in either strains (S5A Fig). Accordingly, the double deletion strain ΔcspEΔcspC showed a cspE-dependant effect.

To further confirm the role of cspC, a cloning and over-expression approach similar to that of cspE was utilized. The cspC gene, unlike cspE, occurs in an operon with an uncharacterized upstream gene, yobF. The full-length cspC operon (pcspC++) was cloned from SL1344 strain (30) in pRS424 vector. Functional complementation assays were performed with the cspC construct but no phenotypic rescue was seen with respect to the bile sensitivity of ΔcspE (S5B Fig). Therefore, we
conclude that in the S. Typhimurium strain used in this study CspE is mainly responsible for combating bile stress.

**CspE regulates an uncharacterised protein, YciF, during bile salts stress.**

It was important to address the possible mediators involved in the CspE-mediated rescue of bile stress. An earlier study of a bile modulated proteome of S. Typhimurium had identified several novel proteins. Of these, an uncharacterized protein Ycif was up-regulated, whereas another protein PagC was down-regulated, by bile (37). In the present study, the qRT-PCR data demonstrated a concomitant down-regulation of pagC, in WT and ΔcspE with bile salts stress (S6 Fig). Notably, the bile-dependant up-regulation of yciF was observed in the WT strain with bile treatment, but was absent in the ΔcspE strain (Fig 2A). Consequently, we hypothesized that yciF might be a downstream target of CspE.

**Overexpression of YciF suppresses the bile sensitivity of ΔcspE.**

To delineate the role of YciF, the ΔyciF and ΔcspEΔyciF strains were generated and growth kinetics of WT, ΔcspE, ΔyciF and ΔcspEΔyciF in the absence (0%) or presence (3%) of bile salts, was performed. In the absence of bile salts, the growth kinetics was indistinguishable for all four strains (S7 Fig). In the presence of 3% bile salts, the WT and ΔyciF strains showed similar growth whereas the ΔcspE and ΔcspEΔyciF strains showed severe growth attenuation with an approximate 6hr growth delay. To elaborate on the importance of yciF, we utilized the yciF over-expression system (WT/pycif and ΔcspE/pycif). Upon yciF over-expression, phenotype suppression was observed in bile salts treated ΔcspE (Table 1). These observations indicated two major aspects of YciF: First, the genetic data with the four strains demonstrated that YciF may not be the sole downstream player in the bile regulation pathway of CspE. Second, increase in YciF levels are capable of imparting bile resistance even in the absence of CspE. Most likely, CspE regulates multiple proteins and this study has identified one target, i.e. YciF which is important to counter bile stress.

**CspE increases the stability of yciF mRNA.**

To understand the exact mechanism of YciF regulation by CspE, and distinguish between transcriptional regulation and mRNA stability we utilized a rifampicin based approach (38). Over-expression of yciF (under the trc promoter in the pTrc99A plasmid) was utilized. Initial experiments were performed with qRT-PCR, which revealed a reduced level of yciF mRNA in ΔcspE irrespective of rifampicin (S8 Fig). Further confirmation was obtained from Northern hybridization experiments which demonstrated that in the absence of cspE the cells harboured less amounts of yciF mRNA despite using an over-expression system. Furthermore, in the presence of rifampicin, i.e. in the absence of any active transcription, there was hastened decay of the yciF mRNA. It is unlikely that CspE regulates the transcription of yciF. Despite the over-expression of yciF from a heterologous promoter there is a significantly lesser amounts of yciF transcript in the ΔcspE strain (ΔcspE/pycif), compared to the WT, even in the absence of rifampicin (Fig 3B). Most likely, CspE regulates YciF by mRNA stabilization and preventing its degradation.

**YciF regulation is imparted through the function of Phenylalanine-30 residue in CspE during bile salts stress.**

To better understand the regulation of YciF by CspE, we utilized the cspE complementation system (pcspE) along with previously known data about CSP functional mutants. Mutations in residues in the RNP motifs have been reported to abrogate nucleic acid melting function (39). Mutation studies of the *B. subtilis* CspB had identified the F30A mutation in the RNP2 motif, to prevent ssDNA binding (10). This residue appeared to be conserved in CspE across several species in eubacteria and archaeabacteria (S9A Fig). To identify if the same would play a role in the *S. Typhimurium* encoded CspE function, we generated a F30V mutation (S9B Fig). We verified the effect of this mutation on the protein stability both in vitro and in vivo. In vitro stability of the protein was determined by quantitating thermal stability of the proteins from 25°C-95°C and following the denaturation kinetics at 219 nm wavelength (40). There appeared negligible change in the melting temperature (Tm~53°C) of CspE upon F30V mutation (Fig...
For in vivo stability analysis, Western blotting and quantitation was performed on the steady state levels of flag-tagged CspE and its F30V mutant. The CspE F30V was expressed but in lesser amounts, compared to the WT CspE, in both the WT and ΔcspE strains (Fig 4B). Although there were sufficient amounts of CspE F30V present after 8 hr of growth, it was unable to rescue the bile sensitivity of ΔcspE. This experiment further validates the essential role of the interplay of CspE and YciF in bile resistance (Table 1).

To obtain validation of the physical binding of purified CspE to a nucleic acid substrate, we performed Electrophoretic mobility shift assay (EMSA) using a 60mer non-specific ssDNA substrate. The StCspE binds with a high affinity (kd 836 nM ± 0.0902) to the oligonucleotide while the StCspE F30V showed no binding even at 10µM protein concentration (S9C Fig). qRT-PCR revealed the induction of yciF transcripts upon complementation of WT cspE, but not the F30V mutant of CspE (pcspE-F30V), in the bile salts treated ΔcspE (Fig 2B).

We further validated the direct binding of CspE to yciF mRNA using the in vitro transcribed full-length mRNA as a substrate in EMSA. StCspE showed robust binding to the yciF mRNA with kd of 626 nM ± 0.021. The StCspE F30V however showed no binding even at a concentration of 10µM (Fig 4C & D). This data demonstrated that CspE directly binds to the yciF mRNA through the F30 residue in RNP2 which plays an important role, effectuating its nucleic acid binding property.

**Bile salts stress triggers the Envelope Stress Response (ESR) in S. Typhimurium.**

To gain a better understanding of the mechanisms involved in the bile sensitivity of ΔcspE. The transcript levels of several master regulators such as dps, uspA or rpoS were assayed for (data not shown). Only rpoE was significantly and kinetically induced in a bile salts stress scenario, in both the WT and ΔcspE strains (Fig 5); however, the levels were much greater in ΔcspE. Upon membrane damage the bacterial ESR is triggered, mediated by the alternative sigma factor RpoE (41). Subsequently, the transcript levels of the downstream players in the ESR pathway, namely degP, rybB etc were significantly induced in the bile salts-treated ΔcspE strain, at the 8th hour of growth (Fig 5). Downstream factors that are not classically defined as part of the ESR regulon, namely, hfg, rne and PNPase, but are essential in appropriate outcome of the ESR pathway were also assayed. mRNA levels of these genes also appeared up-regulated much more in the bile salts treated ΔcspE strain. These observations indicated that not only the ESR but downstream players of the pathway were also up-regulated to combat a possible membrane damage occurring in the ΔcspE strain upon bile salts exposure. Overall this indicated a greater insult to membrane integrity of the ΔcspE strain.

**ΔcspE harbors elevated outer membrane protein (OMP)/porin amounts.**

Next, the expression of genes involved in influx and efflux that are likely to be affected during ESR were studied. As, reduced expression of efflux pumps have been reported to lead to bile sensitivity. Consequently, a strain lacking acrAB has been shown to be highly sensitive to bile salts (33). The major efflux pump genes acrA, acrB and tolC transcripts showed a similar up-regulation upon bile salts stress in WT and ΔcspE strains (S11 Fig). This indicates that the efflux mechanism was functional and likely to be similar, in both the strains. The ESR generally culminates in the degradation of the OMP transcripts, leading to reduction of OMP protein amounts and lowering cell permeability (42). However, the bile salts-treated ΔcspE, displayed significantly higher levels of the porin transcripts, namely ompD, ompC and ompF (Fig 6A). Concurrently, the qualitative estimation of porins revealed a similar result (Fig 6B). The major OMPs were identified using trypsin digestion followed by mass spectrometry. Upon cspE complementation and yciF mediated phenotype suppression, there was lesser induction of the ESR pathway components, rpoE and rybB (S12 Fig) and the porin transcripts were significantly lower during bile stress (Fig 6B). These experiments demonstrated a clear link between CspE and porin mRNA amounts during bile stress.
Bile salts treated ΔcspE manifests increased permeability, which is regulated by CspE and YciF.

Higher levels of porins would make the cell more porous, thereby allowing increased entry of damaging agents. This aspect was functionally tested by studying, the intracellular accumulation of DNA-staining dye bisBenzimide H 33258 (43). The accumulation of the dye was greater in the bile salts treated ΔcspE compared to WT and untreated controls (Fig 7A). Correlating with earlier data, cspE complementation and yciF mediated phenotype suppression was able to reduce the permeability significantly, while the CspE\textsuperscript{F30V} mutant was not effective (Fig 7B). These data suggest that CspE and YciF are likely to be involved in the OMP mRNA degradation steps during the ESR pathway.

Discussion

Genetic and biochemical studies have identified several factors that determine bile resistance in enteric bacteria: lipopolysaccharide (44), enterobacterial common antigen (45), efflux pumps (46), regulatory genes such as phoPQ (47), toxR-toxT (48), marAB (49), porins (50). In the present study, we demonstrate the importance of a cold-shock protein, CspE, in imparting bile resistance in S. Typhimurium 14028s. Compared to physiologically relevant stress conditions such as acidic pH and high salt concentration, cspE was moderately up-regulated in a bile salts environment (Fig 1A) and was functionally important for resistance to bile salts (Figs 1B, 1C and S3A), specifically to the bile component, deoxycholate (S3B Fig). Moreover, this regulation is specific to CspE, since an over expression of another cold shock protein (CspA) did not show a functional rescue (S4 Fig). A previous study had shown that both cspC and cspE were required to counter several stresses in the SL1344 strain (30). Several investigations have reported strain specific bile tolerance, that cannot be extended as a generalized behavior of the species (32). Most likely strain differences exist, and this study demonstrates that CspE plays a major role in countering bile stress in S. Typhimurium. In addition, we identified the F30V variant in S. Typhimurium CspE [(11),(41)] to be important for substrate binding and bile resistance (Figs 4, S9B,C & Table 1).

It was important to identify CspE-regulated proteins that play important roles during bile stress. Increased transcript levels of yciF in bile salts supplemented milieu was detected (Fig 3A), in a CspE-dependant manner (Fig 2A). The functional characterisation of YciF in this study was conducted in two ways: genetic deletion of yciF and multi-copy over-expression. The single gene deletion of yciF did not exemplify any effect in bile salts stress, and neither was there an added effect of the yciF deletion in the cspE deletion background (S7 Fig). If yciF was the only downstream effector of CspE, its gene deletion should have presented a phenotype. On the contrary, multicopy over-expression of yciF suppressed the bile sensitivity of ΔcspE (Table 1). Also, this phenotypic suppression by yciF was not due to alteration of plasmid copy number of the yciF harbouring pTrc99A vector in WT and ΔcspE (plasmid copy numbers were same in both strains, ~900 copies). This indicated that there are multiple pathways that CspE regulates one of which might be through the regulation of YciF. Consequently, shifting the equilibrium by over-expressing YciF, facilitates suppression of bile sensitivity in the ΔcspE strain.

YciF is a hypothetical protein (51), belonging to the yciGFE-katN (52) operon and was first identified as a member of the RpoS regulon in Salmonella (53). The functions of yciG (53) and yciF (52) are unascertained, whereas yciE encodes an acid-shock protein and katN codes for a non-haem catalase (52). The structure of E. coli YciF reveals a dimeric organization in solution and is structurally similar to the di-iron binding proteins, ruberythrin and sulerythrin (54). Notably, CspE is widely distributed and present in diverse bacteria, including enterobacteriaceae, archaea, firmicutes (S9A Fig). On the other hand, a global alignment revealed a restricted presence of YciF in only six prokaryotic genera. Of these, four belong to family Enterobacteriaceae (Salmonella, Escherichia, Citrobacter and Klebsiella) and two belong to the non enterobacteriales (Achromobacter and Vibrio). Hence, this regulatory pathway could be very specific to those genera that encounter high amounts of bile salts. The CspE\textsuperscript{F30V} did not show any effect on the regulation of yciF mRNA levels (Fig 2B). Finally, the F30V complemented ΔcspE did not show any phenotypic rescue in bile sensitivity unlike the
WT complemented ΔcspE (Table 1). Overall, it appears that in S. Typhimurium CspE is a nodal regulator of multiple pathways of bile resistance, and YciF is a downstream player in one such pathway. Further studies are likely to identify other targets of CspE that play important roles during various conditions.

CspE is important for mRNA binding and stabilizing a plethora of genes (30). The transcription inhibitor, rifampicin, was used in Northern hybridization experiments to understand the mechanisms by which CspE regulates YciF. In the absence of rifampicin, the ΔcspE strain harbored lesser yciF transcripts compared to the WT. This reduction in transcript levels were exaggerated in the presence of rifampicin (upon transcription inhibition) and the kinetics of this degradation was faster in ΔcspE as compared to the WT (Fig 3B). It is possible that deletion of CspE leads to increased non-specific activity of RNases, leading to major down-regulation of several transcripts. To address this issue, qRT-PCR and Northern blotting experiments showed that acrB was up-regulated with bile in the WT strain. However, no difference was observed in the mRNA stability in the presence or absence of CspE (S10 Fig). This was unlike the results obtained with yciF (Fig 3) and demonstrates that CspE does not regulate global mRNA stability.

Additionally, structured RNA has been reported to be degraded by PNPase and RNase E, the universal degraders of structured RNA in vivo [(55),(41)]. In the present context, it is possible that CspE increases the half-life of the yciF mRNA by binding to it, unwinding its secondary structure and preventing subsequent degradation. Furthermore, it is unlikely that CspE expends the YciF regulatory role through its anti-transcription terminator property: the yciF mRNA does not harbour any rho-independent transcription terminators. The software ARNold (http://rna.igmors.u-psud.fr/toolbox/arnold/) is specific for identification of Rho-independent transcription terminators in mRNA [(56),(57)]. The software was unable to identify any possible Rho-independent transcription terminators either in the 5′ end of the yciF mRNA or even in the upstream region to the transcription start site or anywhere else in the entire length of the mRNA. Using EMSA with the full-length yciF mRNA we were able to show a direct, cooperative and robust binding of CspE to the mRNA mediated by the F30 residue (Fig 4C). Overall, the data indicates that CspE directly binds to and stabilises the mRNA of yciF thereby preventing its degradation in a bile salts stress condition.

Bile exhibits multifaceted deleterious effects on bacteria (32). LPS is known to be important for resistance to bile (44). We isolated LPS as described by Hitchcock and Brown, 1983 (58) and checked for the components on a 15% SDS-PAGE. However, there was no qualitative difference in LPS in the strains lacking cspE (data not shown). We assayed for several other membrane-associated parameters that would physically regulate the bile tolerance and detected transcript level alterations of the extra-cytoplasmic sigma factor RpoE (Fig 5). Any insult to the membrane integrity activates the ESR cascade involving the extra cytoplasmic sigma factor RpoE, which plays fundamental roles in bacterial virulence and survival. RpoE remains in an inactive membrane bound state in cells. The induction of the ESR leads to controlled proteolysis and release of the active RpoE into the cytosol (59). The cytosolic RpoE governs expression of >80 transcription units in E. coli (60) and Salmonella (61). Most genes of the RpoE core regulon act to synthesize and assemble lipopolysaccharides and OMPs, in order to maintain envelope homeostasis. Activation of the ESR pathway results in the rapid down-regulation of major OMP mRNAs involving multiple mechanisms. First, the major periplasmic protease DegP gets activated upon a stress to the membrane (accumulation of misfolded OMPs), this triggers other periplasmic proteases such as RseP and DegS to cleave and release active RpoE from the membrane-sequestered ensemble of RseA-RpoE (62). Active RpoE then enables transcription of rpoE-controlled major small non-coding RNA (sRNA), rybB which binds to the 5′ end of its target mRNA. Second, Hfq binds to the sRNAs and stabilizes their interaction with the target mRNA. Third, RNase E and PNPase, act to degrade the target mRNAs, thereby preventing further translation and accumulation of unfolded OMPs (41). Our results show that in ΔcspE upon bile salts treatment all the conventional RpoE-mediated ESR pathway components (rpoE, degP and rybB) and the downstream non-conventional players (hfq, rme and PNPase) get up-regulated.
acrB, acrA – ehe interactome of CspE, as light on the cspE (42 A).

aphB, DbpA – cspE ile salts – cspE ile salts – cspE ile salts – cspE ile salts.

This compromised ESR status was rescued in susceptible to a greater influx of bile components.

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quantitative evaluation revealed that bile salts determined by STRING.

differences between Typhi and the other serovars specific drug

Salmone specific drug

The mRNA levels of ompD, ompC and ompF were higher in the ΔcspE strain, than the WT, upon bile treatment (Fig 6A). In addition, OmpD was majorly detected in ΔcspE with bile stress (Fig 6B). This observation is especially important because OmpD is the major porin of S. Typhimurium and amounts to almost 1% of the total cell proteome and half of the porins (64).

Also, ompD and the surrounding genomic region is absent in S. Typhi and marks one of the major differences between Typhi and the other Salmonella serovars (65), making it a potential serovar specific drug-target. Importantly, rybB is reported to target ompD, ompC and ompF for degradation (41). In fact, the binding of these sRNAs to the 5' end of the target mRNAs leads to their degradation by multiple mechanisms, e.g. recruitment of RNAse E degradosome machinery (41,61). In fact, the interactome of CspE, as determined by STRING (66), revealed that the foremost and confirmed interacting partners of CspE are RNA helicases of which some are involved in the RNA degradation pathway, e.g. DeaD, RhlB, DpbA (S13 Fig). Qualitative and quantitative evaluation revealed that bile salts treated Salmonella had more intracellular levels of bisBenzimide H33258 compared to the untreated controls (Fig 7). Most likely, the higher levels of porins in bile treated ΔcspE, rendered it susceptible to a greater influx of bile components. This compromised ESR status was rescued in terms of increased rpoE and rybB transcripts and consecutive reduced levels of the porins, upon complementation with cspE or multi-copy overexpression of yciF (Figs S12 and 6B). This observation was further confirmed by the rescue in permeability by multi-copy yciF over-expression and WT cspE complementation, but not by the F30V mutant of cspE (Fig 7B). Most likely, CspE and its mediators, e.g. YciF, are involved in the pathway of porin mRNA decay [(42),(61)], resulting in lower OMPs and reducing permeability upon bile stress (Fig 8).

Therefore, the vast excess of bile accumulation occurs due to dysregulated amounts of OMPs, leading to lower survival of cells in the absence of CspE. Further studies need to be performed to address the precise mechanisms that are involved YciF mediated bile resistance.

There is a need for identification of novel targets and mechanisms of combating bile resistance and genes involved in virulence/carryer status in Salmonella. Two points need to be highlighted: First, in a global expression analysis study (SalCom-Salmonella Compendium V1.0), among Csps, only CspE is majorly induced during bile stress (67). Second, several bile mutants, e.g. phoPQ, acrB etc, are highly sensitive to bile at low amounts (e.g. 1%) (33, 47). However, higher amounts of bile (3% & higher) was required for the increased sensitivity of the ΔcspE strain. The major thrust of the study was to identify the interplay of CspE and YciF and their effective role in regulating bile resistance. This study has identified and shed light on the involvement of CspE, which acts as the master regulator in bile resistance in S. Typhimurium. The strength of the study is that it combines genetic and biochemical approaches to establish the downstream players and the physiological mechanisms regulated by CspE. In addition, this is the first report of functional and mechanistic detailing of yciF. As part of the study, we uncovered the possible physiological mechanism that these two proteins may be regulating OMP porin degradation. Further studies are required to identify additional novel targets of CspE and the detailed mechanisms of porin regulation by CspE and YciF in regulating bile resistance.

Experimental Procedures

Bacterial strains and growth conditions-The bacterial strains and plasmids used in this study are listed in Table S1. All cultures were grown in Luria–Bertani (LB) medium consisting of 10g/l tryptone (HiMedia Laboratories, Mumbai, India),

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10 g/l NaCl (Merck, Darmstadt, Germany) and 5 g/l yeast extract (HiMedia Laboratories) at 37°C, except for strains containing pKD46 which were grown at 30°C, with constant shaking at 160 rpm. Single-colony cultures grown for 8 h served as pre-inoculum cultures for all experiments. Antibiotics were used at the following concentrations: Ampicillin-100 µg/ml (HiMedia Laboratories), Chloramphenicol-30 µg/ml (HiMedia Laboratories), and Kanamycin- 50 µg/ml (Sigma Aldrich, Missouri, USA). Arabinose (HiMedia Laboratories) was used at 40 mM for induction of Red recombinase by pKD46.

**Chemicals**—Bile salts (Sigma Aldrich) was used at concentrations of 1-5% (w/v). Sodium Deoxycholate and Cholate were procured from Sigma Aldrich.

**Generation of single- and double-gene deletion strains**—To construct the single gene deletion strains, one step gene disruption strategy was employed (68). *S. Typhimurium* 14028s (WT) was used as the parent strain for all experiments, unless otherwise mentioned. Briefly, for construction of ∆cspE, primers listed in Table S2 (Sigma Aldrich, Bangalore, India) were designed for amplification of the kan from pKD4 having 40 nt flanking regions of cspE. The resulting PCR product was purified and electroporated into WT cells harboring pKD46, which expresses λ Red recombinase. A similar strategy was followed for the other gene deletions. The double gene deletion strain was generated by amplifying the region flanking the gene from its single-knockout strain and electroporating the amplicon into ∆cspE harboring pKD46 (Table S1). All gene deletions were confirmed by PCR amplification using primers designed to anneal ~100 bp upstream and downstream of the gene (Table S2). Notably, the antibiotic resistance cassettes were removed by pCP20 transformation in the mutant strains used.

**Cloning of genes for trans complementation**—The WT 14028s genomic DNA was used as the template for the PCR amplification of cspE, yciF, cspa and cspC, using specific primers (Table S3) and Phusion DNA Polymerase (New England Biolabs, Massachusetts, USA). cspE and its promoter were targeted for cloning between the SpeI and XhoI sites in the pRS424 plasmid, while yciF and cspa were cloned between the EcoRI and BamHI sites in the pTrc99A plasmid. For cspC, the entire operon comprising its native promoter (pcspC++) were cloned from SL1344 strain. Positive clones were confirmed by sequencing (Aggrigenome, India). The positive clones (pcspE, pcyciF, pcspa, ppcspC++-SL1344) and control vector pRS424 (VA) were then transformed into *S. Typhimurium* WT and ΔcspE by electroporation, to generate the following strains: WT/VA, ΔcspE/VA, WT/pcspE, ΔcspE/pcspE, WT/pcyciF, ΔcspE/pyciF, WT/pcspa, ΔcspE/pcspa, WT/pcspC+-SL1344, ΔcspE/pcspC++-SL1344 respectively.

**Construction of F30V mutant of CspE**—For mutagenesis, plasmid pCspE which contains the structural gene of cspE under the control of its original promoter, was used. Single amino acid substitution was carried out by Quik Change site-directed mutagenesis, using high-fidelity Pfu Turbo DNA polymerase (Stratagene). The following pair of oligonucleotides was used for mutagenesis:

FP-5’GGCAGCAAAGACGTGGCTGTACACTTCTCTGC;
RP-5’GGCAGAAGGTACAGCCACGTCTTTGC TGCC. The oligonucleotides were obtained from Sigma Aldrich, Bangalore, India and confirmed by sequencing.

**Stress assays**—Experiments were performed using overnight grown cultures, using ~5×10⁶ CFU/ml for indicated stress conditions. For the dose assays, normalised cultures along with stress-inducing compounds were grown in a 96-well cell culture plate (SPL Life Sciences, Korea) for 8-9 hrs. O.D. at 600 nm was measured using a Microplate Reader (Tecan, Männedorf, Switzerland). For kinetic assays, the Bioscreen-C Automated Growth Curve Analysis System (Bioscreen, Helsinki, Finland) was used over a period of 16 hrs and O.D. at 600 nm was measured at every 1 hr. For CFU experiments, cultures were collected every 3 hrs and appropriate dilutions were plated on LB Agar plates and incubated overnight at 37°C.

**RNA extraction**—For total RNA preparation, bacterial cultures grown to the mentioned time-points and approximately 2 O.D. (600 nm) cells were mixed with 0.2 volumes of STOP solution (95% ethanol, 5% phenol) and RNA was extracted using the TRIzol reagent (Invitrogen) as per the manufacturer’s instructions.
Northern hybridization-Rifampicin (final concentration 500 μg/ml) was added to bacterial liquid cultures (growth along-with 3% bile salts), to stop transcription, at the 8th hour of growth. Cells were collected at the indicated time points and RNA was extracted as mentioned. 1% formaldehyde-TBE agarose gel was run at 40V to resolve the total extracted RNA. The RNA was then transferred onto nitran membrane at 5V for 3hrs (Bio-Rad) and were fixed by UV cross linking at 120mJ/cm² (CL1000-UV products). The membranes were blocked using pre-hybridization buffer containing yeast total RNA and Denhardt’s solution (1% bovine serum albumin, 1% ficoll, 1% polyvinylpyrrolidone 40). The Northern blot analysis was performed using 5'-32P end labelled DNA oligomers (69). For yciF, three independent oligomers from different regions of the gene were used (Y1-CAGTITTAATGCGCAGATTAG; Y2-ATTCGATTGGCGGTTAGAC; Y3-CATTGGACGTGCTGTGCAAG). acrB was used as the negative control and two oligomers were used as probes (A-AAGTCCCGCTTGGCAATCAACTCGAAGTCC; A2-CTTCAGAAAGGCGTGGTGC)5S rRNA was used as the loading control, and a single oligomer (5S-CTACGGCGTTTCACTTCTGAGTTC) was used as probe. The blots were initially probed for yciF; exposed to phosphor-imager screen (GE Healthcare) and analysed on BioImage Analyzer (FLA5100, Fuji Film). The yciF probes were then stripped and re-blotted for acrB and then 5S rRNA. The successive procedure was the same as mentioned. Bands were measured using ImageJ (version 1.51j8) and plotted using GraphPad Prism (version 5.0). Expression and Purification of StCspE and StCspEF30V- StCspE and StCspEF30V were amplified using the following primers; FP: 5’- CGCGGATCCATGTCTAAGATTAAGGTACG; RP-5’- CGCGGATCCATGTCTAAGATTAAGGTACG. The PCR products were cloned between the BamHI and SalI sites in the pRSFDuet-1 plasmid. The His-tagged proteins were overexpressed in the E. coli BL21(DE3) strain harboring plasmid constructs. E. coli cells were grown at 180 μg/mL kanamycin, at 37 °C to an A600 of 0.5. Subsequently, the culture was incubated for 1h at 4 °C. StCspE and StCspEF30V expression was initiated by the addition of IPTG to a final concentration of 0.1 mM, followed by incubation for an additional 12 h at 18 °C. Cells were collected by centrifugation for 10 min at 6000 rpm and 4 °C and resuspended in buffer A [50 mM Tris-HCl (pH 8), 100mM NaCl, 1mM EDTA, 5 mM β- mercaptoethanol, and 10% glycerol]. Bacteria were then disrupted by sonication, and the cell debris was removed by centrifugation in a Beckman Ti-45 rotor at 3000 rpm for 1 h at 4 °C. The supernatant was applied to 5 mL of a HiTrap column (GE Healthcare) pre-equilibrated with buffer A. The column was washed with 10 bed volumes of buffer B [50 mM Tris-HCl (pH 8.0), 100mM NaCl, 1mM EDTA, 10-20mM Imidazole, 5 mM β-mercaptoethanol, and 10% glycerol]. The bound proteins were eluted with a linear gradient of Imidazole (30 to 200 mM) in buffer A. All the fractions were analyzed on an SDS–polyacrylamide gel. The fractions containing the StCspE and StCspEF30V were pooled and dialyzed against buffer C [50 mM Tris-HCl (pH 8.0), 100mM NaCl, 1mM EDTA, 5mM β-mercaptoethanol, and 10% glycerol]. The proteins were further dialyzed against buffer D[50mM Tris-HCl (pH 8.0), 100mM NaCl, 1mM EDTA, 5mM β-mercaptoethanol and 50% glycerol]. The final purified protein was run on a SDS-PAGE, checked for purity and identity was confirmed by Mass Spectrometry. The purified proteins were then stored at −80 °C.

Thermal stability determination by Circular Dichroism- Purified enzymes (~500 μM protein) in 400 μl of 20 mM phosphate buffer pH 8.0 were used and ellipticity was monitored from 200 to 250 nm in a 0.2-cm path length cuvette with a bandwidth of 1 nm and response time of 2 s at 20 °C. The thermal denaturation temperature (Tm) was calculated as previously reported (70). In vitro transcription of full-length yciF mRNA and radiolabelling of Northern blot and EMSA substrates- yciF was cloned between the BamHI and SalI sites in the pRSFDuet-1 plasmid using primers listed in Table S3. T7 in vitro transcriptions were carried out using the MEGAscript T7 kit (Ambion). In vitro transcribed mRNA was quality checked by loading the sample on a 5 % PAA, 7 M urea gel and 5’ end labelled with γ-32P (γ32P). The probes for Northern blotting
were radiolabelled at the 5′ end by using [γ-32P]ATP and T4 polynucleotide kinase (Thermo Scientific) as per the manufacturer’s protocol. The DNA substrates were purified using the QIAquick Nucleotide Removal Kit (Qiagen). Oligo probes for Northern hybridization were labelled by the same method.

**Electrophoretic Mobility Shift Assays (EMSA).** EMSA were conducted as previously described (71). yciF mRNA binding reaction mixtures contained 1.25mM Tris-HCl (pH 8.0), 0.05mM DTT, 0.05mM KCl, 0.2mg/ml BSA, 2mM VRC, 2mM 32P-labeled mRNA substrate in a volume of 20µl made up with protein storage buffer. 0.2, 1.6, 3, 10 µM of StCspE was used and 10 µM of StCspE F30V. Reaction mixtures were incubated at 37 °C for 5 min and reactions terminated by the addition of a loading dye [0.1% (w/ v) bromophenol blue and xylene cyanol in 20% glycerol]. Samples were resolved on a 6% native PAGE in a 0.25× TAE buffer at 80 V and 4 °C. For ssDNA binding, reaction mixtures contained 40 mM Tris-HCl (pH 8.6), 100mM NaCl, 12% glycerol, 4mM EDTA, 1nM 32P-labeled substrate ssDNA, and 0.2, 1.6, 3, 10 µM of StCspE and StCspE F30V. Reaction mixtures were incubated at 37 °C for 20 min and reactions terminated by the addition of a loading dye [0.1% (w/ v) bromophenol blue and xylene cyanol in 20% glycerol]. Samples were resolved on a 10% native PAGE in a 0.5× TAE buffer at 80 V and 4 °C. Gels were dried, and the bands were visualized using a Fuji FLA-9000 phosphorimager. The band intensities were quantified in a UVItech gel documentation system using UVI-Band Map software (version 97.04) and plotted using GraphPad Prism (version 5.0).

**Gene expression analysis by qRT-PCR.** Total extracted RNA was treated with the Turbo DNA-Free kit (Life Technologies, Massachusetts, USA). The RNA integrity was analysed by electrophoresis on a 1.5% agarose gel and concentration was estimated using NanoDrop (Thermo Fischer Scientific, Massachusetts, USA). 1µg DNase-treated RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad). The primers (Sigma-Aldrich, Bangalore, India) used for qRT-PCR are listed in Table S4. The expression level of each gene of interest was calculated as the average of three independent cDNA samples. Each cDNA sample and each gene, were performed in triplicates. The cycling conditions were as follows: 95°C for 5mins, followed by 40 cycles of 95°C for 20s, 57°C for 10s and 72°C for 20s. At the end of the final cycle, the amplification specificity and absence of primer dimers were calculated by the melt curve acquired by 81cycles of heating the PCR products from 55-95°C for 20s, with a 0.5°C increase per cycle (CFX Connect, Biorad). The relative quantities of transcripts were determined using the standard curve method and normalised against the mean of the reference gene (rrlC-23S ribosomal RNA). The WT cells grown in LB without any treatment at the early time-point (3hr) was normalised to 1, and all other samples were calculated as fold-change to this reference value.

**Western blotting.** Cells were grown for 8hrs without bile. Approximately 2 O.D. (600nm) cells were taken for lysis and subsequently run on a 12% SDS-PAGE. Western blots were prepared by electoblotting SDS–PAGE gels onto polyvinylidene difluoride (PVDF) membranes and probed with 1:5000 mouse anti-flag primary antibody (E-bioscience) and incubated at 4°C, overnight. Prior to antibody addition, the membranes were blocked for 2 h at 25 °C with 5% skim milk in Tris-buffered saline [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween 20]. The membranes were washed with Tris-buffered saline for 1 min and then incubated with the primary antibody for 12 h at 4 °C (Sigma Aldrich). After washing with Tris-buffered saline five times for 15 min each, the membranes were incubated with the 1:10,000 horseradish peroxidase-conjugated anti-mouse secondary antibody (Sigma-Aldrich) for 3 h at 4 °C (70). Finally, the blots were developed using Chemiluminescent substrates for horseradish peroxidase (Millipore) and imaged with ChemiDoc ImageQuant (GE LAS 4000). Bands were quantified using ImageJ (version 1.51j8) and plotted using GraphPad Prism (version 5.0).

**bisBenzimide H 33258 accumulation assay.** Briefly, overnight grown cultures were used to inoculate fresh medium with or without 3% bile salts for 5hrs at 37°C. Bacterial cells were collected by centrifugation at 4000 g, washed twice with PBS and resuspended in 1ml PBS. The O.D. at 600nm was normalised to 0.1 and 180µl was transferred to wells (n=8; 1 strain per column) of a 96-well plate (flat-bottomed, black, Greiner
Bio-one, Kremsmünster, Austria). Every plate contained eight technical replicates (i.e. 1 column) of PBS alone and heat inactivated WT (10mins, 90°C). Replicates (i.e. 1 column) was analysed for every strain and growth condition. The plate was transferred to the Microplate Reader, incubated at 37°C and 20μl of 25μM bis-Benzimide H 33258 (Sigma Aldrich, Missouri, United States) was added to each well using a multi-channel pipette, to attain a final concentration of 2.5 μM. Fluorescence was read from the top of the wells using excitation and emission filters of 355 and 460 nm, respectively, with 25 flashes/well; readings were taken for 45 cycles with a 75s delay between cycles, and a 75% gain (43). Raw fluorescence values were analysed using Excel (Microsoft) that included subtraction of appropriate control blanks from each value of the well of the column and each experiment was repeated thrice.

Imaging of bisBenzimide H 33258 stained Salmonella-Samples were taken as replicates from the bisBenzimide H 33258 accumulation assay, and processed for imaging. Briefly, approximately 20μl of the stained samples were collected at the 15, 30 and 45 min time-points, post addition of H 33258. The samples were fixed with 4% para-formaldehyde (Sigma Aldrich) for 30mins at RT, washed twice with PBS and added onto clean cover slips. The samples were allowed to dry at room temperature for 30mins. The cover slips were then mounted on a glass slide with 3-5μl of mounting medium containing 1% DABCO (Sigma Aldrich) in 1X PBS. Images were acquired with the Zeiss LSM880 with Airy Scan (Carl Zeiss, Oberkochen, Germany) at 63X magnification (IISc Confocal Imaging Facility).

Outer membrane protein isolation, purification and quantitation-Outer membrane proteins (OMPs) were isolated from S.Typhimurium strains grown in LB with or without 3% bile salts (72). Briefly, cells were harvested in the late-log phase (12hrs) of growth and washed twice with 1X PBS. Approximately 4 O.D. (600 nm) cells were used for the extraction. OMP concentrations were determined by the Bradford’s assay, using bovine serum albumin as standard. Equal volume of re-suspended solution was loaded analysed by a 12.5% SDS-PAGE and visualised by staining with Coomassie Brilliant Blue (Sigma Aldrich).

Statistical analysis-All data was analysed using the GraphPad Prism (Version 6.0c). For analysing bacterial growth, one-way ANOVA was performed; for comparison of steady-state RT-PCR quantification, one-way ANOVA was performed. The statistical significance of differences in the accumulation of H33258 was determined using Two-way ANOVA, with each strain compared against appropriate controls such as the parent WT grown in LB medium alone.
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Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: SR and DpN designed the experiments, analyzed and interpreted the data and wrote the manuscript. SR, RDC and MD performed the experiments.
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References

1. Graumann, P., and Marahiel, M. A. (1996) Some like it cold: response of microorganisms to cold shock. *Arch Microbiol* **166**, 293-300
2. Phadtare, S., Alsina, J., and Inouye, M. (1999) Cold-shock response and cold-shock proteins. *Curr Opin Microbiol* **2**, 175-180
3. Horton, A. J., Hak, K. M., Steffan, R. J., Foster, J. W., and Bej, A. K. (2000) Adaptive response to cold temperatures and characterization of cspA in Salmonella typhimurium LT2. *Antonie Van Leeuwenhoek* **77**, 13-20
4. Ermolenko, D. N., and Makhatadze, G. I. (2002) Bacterial cold-shock proteins. *Cell Mol Life Sci* **59**, 1902-1913
5. Morgan, H. P., Wear, M. A., McNae, I., Gallagher, M. P., and Walkinshaw, M. D. (2009) Crystallization and X-ray structure of cold-shock protein E from Salmonella typhimurium. *Acta Crystallogr Sect F Struct Biol Cryst Commun* **65**, 1240-1245
6. Yamanaka, K., and Inouye, M. (2001) Growth-phase-dependent expression of cspD, encoding a member of the CspA family in Escherichia coli. *J Bacteriol* **179**, 5126-5130
7. Jiang, W., Hou, Y., and Inouye, M. (1997) CspA, the major cold shock protein of Escherichia coli, is an RNA chaperone. *J Biol Chem* **272**, 196-202
8. Michaux, C., Martini, C., Shioya, K., Ahmed Lecheheb, S., Budin-Verneuil, A., Cosette, P., Sanguinetti, M., Hartke, A., Verneuil, N., and Giard, J. C. (2012) CspR, a cold shock RNA-binding protein involved in the long-term survival and the virulence of Enterococcus faecalis. *J Bacteriol* **194**, 6900-6908
9. Shenhar, Y., Biran, D., and Ron, E. Z. (2012) Resistance to environmental stress requires the RNA chaperones CspC and CspE. *Environ Microbiol Rep* **4**, 532-539
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19. Hu, K. H., Liu, E., Dean, K., Gingras, M., DeGraff, W., and Trun, N. J. (1996) Overproduction of three genes leads to camphor resistance and chromosome condensation in Escherichia coli. Genetics143, 1521-1532
20. Hanna, M. M., and Liu, K. (1998) Nascent RNA in transcription complexes interacts with CspE, a small protein in E. coli implicated in chromatin condensation. J Mol Biol282, 227-239
21. Feng, Y., Huang, H., Liao, J., and Cohen, S. N. (2001) Escherichia coli poly(A)-binding proteins that interact with components of degradosomes or impede RNA decay mediated by polynucleotide phosphorylase and RNase E. J Biol Chem276, 31651-31656
22. Hohmann, E. L. (2001) Nontyphoidal salmonellosis. Clin Infect Dis32, 263-269
23. Feasey, N. A., Dougan, G., Kingsley, R. A., Heyderman, R. S., and Gordon, M. A. (2012) Invasive non-typhoidal salmonella disease: an emerging and neglected tropical disease in Africa. Lancet379, 2489-2499
24. Gordon, M. A., Graham, S. M., Walsh, A. L., Wilson, L., Phiri, A., Molyneux, E., Zijlstra, E. E., Heyderman, R. S., Hart, C. A., and Molyneux, M. E. (2008) Epidemics of invasive Salmonella enterica serovar enteridis and S. enterica Serovar typhimurium infection associated with multidrug resistance among adults and children in Malawi. Clin Infect Dis46, 963-969
25. Tadesse, D. A., Singh, A., Zhao, S., Bartholomew, M., Womack, N., Ayers, S., Fields, P. I., and McDermott, P. F. (2016) Antimicrobial Resistance in Salmonella enterica serovar Typhimurium in the United States from 1948 to 1995. Antimicrob Agents Chemother60, 2567-2571
26. Poppe, C., Smart, N., Khakhria, R., Johnson, W., Spika, J., and Prescott, J. (1998) Salmonella typhimurium DT104: a virulent and drug-resistant pathogen. Can Vet J39, 559-565
27. Craig, J. E., Boyle, D., Francis, K. P., and Gallagher, M. P. (1998) Expression of the cold-shock gene cspB in Salmonella typhimurium occurs below a threshold temperature. Microbiology144 (Pt 3), 697-704
28. Jeffreys, A. G., Hak, K. M., Steffan, R. J., Foster, J. W., and Bej, A. K. (1998) Growth, survival and characterization of cspA in Salmonella enteritidis following cold shock. Curr Microbiol36, 29-35
29. Kim, B. H., Bang, I. S., Lee, S. Y., Hong, S. K., Bang, S. H., Lee, I. S., and Park, Y. K. (2001) Expression of cspH, encoding the cold shock protein in Salmonella enterica serovar Typhimurium UK-1. J Bacteriol183, 5580-5588
30. Michaux, C., Holmqvist, E., Vasicék, E., Sharan, M., Barquist, L., Westermann, A. J., Gunn, J. S., and Vogel, J. (2017) RNA target profiles direct the discovery of virulence functions for the cold-shock proteins CspC and CspE. Proc Natl Acad Sci U S A114, 6824-6829
31. Hay, A. J., and Zhu, J. (2016) In Sickness and in Health: The Relationships Between Bacteria and Bile in the Human Gut. Adv Appl Microbiol96, 43-64
32. Begley, M., Gahan, C. G., and Hill, C. (2005) The interaction between bacteria and bile. FEMS microbiology reviews29, 625-651
33. Prouty, A. M., Brodsky, I. E., Falkow, S., and Gunn, J. S. (2004) Bile-salt-mediated induction of antimicrobial and bile resistance in Salmonella typhimurium. Microbiology150, 775-783
34. Gonzalez-Escobedo, G., and Gunn, J. S. (2013) Gallbladder epithelium as a niche for chronic Salmonella carriage. Infect Immun81, 2920-2930
35. Prouty, A. M., Schwesinger, W. H., and Gunn, J. S. (2002) Biofilm formation and interaction with the surfaces of gallstones by Salmonella spp. Infect Immun70, 2640-2649
36. Menendez, A., Arena, E. T., Guttmann, J. A., Thorson, L., Vallance, B. A., Vogl, W., and Finlay, B. B. (2009) Salmonella infection of gallbladder epithelial cells drives local inflammation and injury in a model of acute typhoid fever. J Infect Dis200, 1703-1713
37. Prouty, A. M., Brodsky, I. E., Manos, J., Belas, R., Falkow, S., and Gunn, J. S. (2004) Transcriptional regulation of Salmonella enterica serovar Typhimurium genes by bile. FEMS Immunol Med Microbiol41, 177-185
38. Smirnov, A., Wang, C., Drewry, L. L., and Vogel, J. (2017) Molecular mechanism of mRNA repression in trans by a ProQ-dependent small RNA. EMBO J36, 1029-1045

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39. Phadtare, S., and Severinov, K. (2005) Nucleic acid melting by Escherichia coli CspE. *Nucleic Acids Res* **33**, 5583-5590
40. Kelly, S. M., Jess, T. J., and Price, N. C. (2005) How to study proteins by circular dichroism. *Biochim Biophys Acta* **1751**, 119-139
41. Papenfort, K., Pfeiffer, V., Mika, F., Lucchini, S., Hinton, J. C., and Vogel, J. (2006) SigmaE-dependent small RNAs of Salmonella respond to membrane stress by accelerating global omp mRNA decay. *Mol Microbiol* **62**, 1674-1688
42. Vogel, J., and Papenfort, K. (2006) Small non-coding RNAs and the bacterial outer membrane. *Curr Opin Microbio* **9**, 605-611
43. Coldham, N. G., Webber, M., Woodward, M. J., and Piddock, L. J. (2010) A 96-well plate fluorescence assay for assessment of cellular permeability and active efflux in Salmonella enterica serovar Typhimurium and Escherichia coli. *J Antimicrob Chemother* **65**, 1655-1663
44. Picken, R. N., and Beacham, I. R. (1977) Bacteriophage-resistant mutants of Escherichia coli K12 with altered lipopolysaccharide. Studies with concanavalin A. *J Gen Microbio* **102**, 319-326
45. Ramos-Morales, F., Prieto, A. I., Beuzon, C. R., Holden, D. W., and Casadesus, J. (2003) Role for Salmonella enterica enterobacterial common antigen in bile resistance and virulence. *J Bacteriol* **185**, 5328-5332
46. Rosenberg, E. Y., Bertenthal, D., Nilles, M. L., Bertrand, K. P., and Nikaido, H. (2003) Bile salts and fatty acids induce the expression of Escherichia coli AcrAB multidrug efflux pump through their interaction with Rob regulatory protein. *Mol Microb* **48**, 1609-1619
47. van Velkinburgh, J. C., and Gunn, J. S. (1999) PhoP-PhoQ-regulated loci are required for enhanced bile resistance in Salmonella spp. *Infect Immun* **67**, 1614-1622
48. Provenzano, D., and Klose, K. E. (2000) Altered expression of the ToxR-regulated porins OmpU and OmpT diminishes Vibrio cholerae bile resistance, virulence factor expression, and intestinal colonization. *Proc Natl Acad Sci U S A* **97**, 10220-10224
49. Sulavik, M. C., Dazer, M., and Miller, P. F. (1997) The Salmonella typhimurium mar locus: molecular and genetic analyses and assessment of its role in virulence. *J Bacteriol* **179**, 1857-1866
50. Thanassi, D. G., Cheng, L. W., and Nikaido, H. (1997) Active efflux of bile salts by Escherichia coli. *J Bacteriol* **179**, 2512-2518
51. McClelland, M., Sanderson, K. E., Spieth, J., Clifton, S. W., Latreille, P., Courtney, L., Porwollik, S., Ali, J., Dante, M., Du, F., Hou, S., Layman, D., Leonard, S., Nguyen, C., Scott, K., Holmes, A., Grewal, N., Mulvaney, E., Ryan, E., Sun, H., Florea, L., Miller, W., Stoneking, T., Nhan, M., Waterston, R., and Wilson, R. K. (2001) Complete genome sequence of Salmonella enterica serovar Typhimurium LT2. *Nature* **413**, 852-856
52. Robbe-Saule, V., Coynault, C., Ibanez-Ruiz, M., Hermant, D., and Norel, F. (2001) Identification of a non-haem catalase in Salmonella and its regulation by RpoS (sigmaS). *Mol Microbio* **39**, 1533-1545
53. Ibanez-Ruiz, M., Robbe-Saule, V., Hermant, D., Labrude, S., and Norel, F. (2000) Identification of RpoS (sigma(S))-regulated genes in Salmonella enterica serovar typhimurium. *J Bacteriol* **182**, 5749-5756
54. Hindupur, A., Liu, D., Zhao, Y., Bellamy, H. D., White, M. A., and Fox, R. O. (2006) The crystal structure of the E. coli stress protein YciF. *Protein Sci* **15**, 2605-2611
55. Oussenko, I. A., Abe, T., Ujiie, H., Muto, A., and Bechhofer, D. H. (2005) Participation of 3'-to-5' exonuclease activities in the turnover of Bacillus subtilis mRNA. *J Bacteriol* **187**, 2758-2767
56. Gautheret, D., and Lambert, A. (2001) Direct RNA motif definition and identification from multiple sequence alignments using secondary structure profiles. *J Mol Biol* **313**, 1003-1011
57. Macke, T. J., Ecker, D. J., Gutell, R. R., Gautheret, D., Case, D. A., and Sampath, R. (2001) RNAMotif, an RNA secondary structure definition and search algorithm. *Nucleic Acids Res* **29**, 4724-4735
58. Hitchcock, P. J., and Brown, T. M. (1983) Morphological heterogeneity among Salmonella lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J Bacteriol* **154**, 269-277
Salmonella Typhimurium CspE regulates bile resistance

Ruiz, N., Kahne, D., and Silhavy, T. J. (2006) Advances in understanding bacterial outer-membrane biogenesis. *Nat Rev Microbiol* **4**, 57-66

Rhodius, V. A., Suh, W. C., Nonaka, G., West, J., and Gross, C. A. (2006) Conserved and variable functions of the sigmaE stress response in related genomes. *PLoS Biol* **4**, e2

Skovierova, H., Rowley, G., Rezuchova, B., Homerova, D., Lewis, C., Roberts, M., and Kormanec, J. (2006) Identification of the sigmaE regulon of Salmonella enterica serovar Typhimurium. *Microbiology* **152**, 1347-1359

Leiser, O. P., Charlson, E. S., Gerken, H., and Misra, R. (2012) Reversal of the DeltadegP phenotypes by a novel rpoE allele of *Escherichia coli*. *PloS one* **7**, e33979

Nikaido, H. (2003) Molecular basis of bacterial outer membrane permeability revisited. *Microbiol Mol Biol Rev* **67**, 593-656

Santiviago, C. A., Toro, C. S., Hidalgo, A. A., Youderian, P., and Mora, G. C. (2003) Global regulation of the *Salmonella enterica serovar typhimurium* major porin, OmpD. *J Bacteriol* **185**, 5901-5905

Santiviago, C. A., Fuentes, J. A., Bueno, S. M., Trombert, A. N., Hildago, A. A., Socias, L. T., Youderian, P., and Mora, G. C. (2002) The *Salmonella enterica sv. Typhimurium* smvA, yddG and ompD (porin) genes are required for the efficient efflux of methyl viologen. *Mol Microbiol* **46**, 687-698

Szklarczyk, D., Morris, J. H., Cook, H., Kuhn, M., Wyder, S., Simonovic, M., Santos, A., Doncheva, N. T., Roth, A., Bork, P., Jensen, L. J., and von Mering, C. (2017) The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. *Nucleic Acids Res* **45**, D362-D368

Kröger, C., Colgan, A., Srikumar, S., Händler, K., Sivasankaran, S., Hammarlöf, D., Canals, R., Grissom, J., Conway, T., Hokamp, K., and Hinton, J. (2013) An Infection-Relevant Transcriptionic Compendium for *Salmonella enterica Serovar Typhimurium*. *Cell Host & Microbe* **14**, 683-695

Datsenko, K. A., and Wanner, B. L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**, 6640-6645

Shetty, S., and Varshney, U. (2016) An evolutionarily conserved element in initiator tRNAs prompts ultimate steps in ribosome maturation. *Proc Natl Acad Sci U S A* **113**, E6126-E6134

Bhosale, M., Pande, S., Kumar, A., Kairamkonda, S., and Nandi, D. (2010) Characterization of two M17 family members in *Escherichia coli*, Peptidase A and Peptidase B. *Biochem Biophys Res Commun* **395**, 76-81

Thakur, M., Kumar, M. B., and Muniyappa, K. (2016) *Mycobacterium tuberculosis* UvrB Is a Robust DNA-Stimulated ATPase That Also Possesses Structure-Specific ATP-Dependent DNA Helicase Activity. *Biochemistry* **55**, 5865-5883

Villarreal, J. M., Becerra-Lobato, N., Rebollar-Flores, J. E., Medina-Aparicio, L., Carbajal-Gomez, E., Zavala-Garcia, M. L., Vazquez, A., Gutierrez-Rios, R. M., Olvera, L., Encarnacion, S., Martinez-Batallar, A. G., Calva, E., and Hernandez-Lucas, I. (2014) The *Salmonella enterica serovar Typhi* ItrR-ompR-ompC-ompF genes are involved in resistance to the bile salt sodium deoxycholate and in bacterial transformation. *Mol Microbiol* **92**, 1005-1024

Footnotes

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Table 1. Over-expression of yciF suppresses the bile sensitivity of ΔcspE.

Quantification of kinetic growth determined in terms of log_{10} (CFU/ml) values of WT and ΔcspE with the following vectors- VA, pcespF, pcespF-F30V and pyciF grown with and without 3% bile salts. Values are represented as mean±SEM. Statistical analysis was performed using two-way ANOVA and the different comparisons have been represented using the following symbols: # :: p<0.001- Comparison of each strain treated with 3% bile salts with respect to WT/VA grown in 3% bile salts; a :: p<0.001, b :: p<0.001, c :: p<0.01, d :: p<0.05- Comparison of each strain grown in 3% bile salts with respect to ΔcspE/VA grown in 3% bile salts.

| Time (hrs) | WT/VA | WT/pcsP | WT/pcsP-F30V | WT/ pyciF | ΔcspE/ VA | ΔcspE/ pcsP | ΔcspE/ pcsP-F30V | ΔcspE/ pyciF |
|------------|-------|---------|---------------|-----------|-----------|------------|-----------------|-------------|
| 0          | 5.72±.59 | 5.52±.48 | 6.25±.07 | 5.51±.53 | 5.83±.28 | 5.54±.11 | 6.35±.07 | 5.33±.29 |
| 6          | 9.35±.18 | 9.48±.17 | 8.68±.38 | 9.42±.13 | 9.65±.11 | 9.57±.08 | 8.63±.22 | 9.51±.06 |
| 12         | 11.14±.44 | 11.48±.15 | 10.55±.13 | 10.72±.15 | 10.58±.02 | 11.36±.42 | 10.47±.16 | 10.81±.42 |

| Time (hrs) | WT/VA | WT/pcsP | WT/pcsP-F30V | WT/ pyciF | ΔcspE/ VA | ΔcspE/ pcsP | ΔcspE/ pcsP-F30V | ΔcspE/ pyciF |
|------------|-------|---------|---------------|-----------|-----------|------------|-----------------|-------------|
| 0          | 5.69±.64 | 4.59±.15 | 6.47±.12 | 5.1±.52 | 4.06±.82 | 5.15±.44 | 4.01±.33 | 5.73±.23 |
| 6          | 8.52±.23 | 8.80±.26 | 8.37±.18 | 7.79±.51 | 4.59±.73 # | 8.50±.414 * | 5.1±.34 # | 7.61±.2 |
| 12         | 10.55±.24 | 11±.21 | 10.56±.31 | 10.04±.5 | 5.71±.34 # | 10.71±.72 a | 5.68±.47 # | 11.31±.26 a |
Salmonella Typhimurium CspE regulates bile resistance

Fig 1. *Salmonella* Typhimurium encoded *cspE* is up-regulated during bile stress and is essential for resistance

(A) Transcript levels of *cspA*, *cspB*, and *cspE* were determined using qRT-PCR for the WT strain, under the indicated stress conditions, at the 3rd and 8th hour of growth. The relative quantities of transcripts were calculated against the mean of the reference gene (*rrlC*). Transcript levels of target gene in control set (WT cells grown in LB), at the 3rd and 8th hour time-point was normalised to 1, and all other samples were calculated as fold-change to this reference value. (B) Kinetic growth analysis of WT and Δ*cspE* strains were analysed in terms of CFU/ml over a period of 12 hrs of growth, in the absence (0%) or presence (3%) of bile salts. (C) Kinetic growth analysis, calculated in terms of CFU/ml of WT/VA, Δ*cspE*/VA and *cspE* complemented (WT/pcspE and Δ*cspE*/pcspE) was studied for 12 hrs in the absence (0%) and presence (3%) of bile salts. Data is presented as mean±SEM and representative of three independent experiments. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.
Fig 2. *S.* Typhimurium encoded cspE positively regulates yciF.

(A) The transcript levels of yciF was determined using qRT-PCR for the WT and ΔcspE strains, in the absence (0%) and presence (3%) of bile salts, at the 3rd and 8th hour of growth. In all panels, values are normalized by those obtained for the WT strain grown in 0% bile salts, at the indicated time point. (B) The transcript levels of endogenous yciF in the WT/VA, ΔcspE/VA, cspE complemented (WT/pcspE and ΔcspE/pcspE) and cspE-F30V complemented (WT/pcspE-F30V and ΔcspE/pcspE-F30V) strains was determined using qRT-PCR, in the absence (0%) and presence (3%) of bile salts, at the 8th hour of growth. In all panels, values are normalized by those obtained for the WT strain grown in 0% bile salts, at the indicated time point. Data is presented as mean±SEM and representative of three independent experiments. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.
Fig 3. S. Typhimurium encoded CspE increases the stability of yciF mRNA.

The yciF mRNA stability was determined in terms of the relative amounts of its transcript levels. (A) The relative mRNA levels of yciF was determined upon bile treatment in the WT/VA strain, by Northern blotting and quantitation. (B) The mRNA levels of yciF was determined with or without transcription inhibition by rifampicin. The mRNA levels were quantitated relative to the amounts at 0 mins post addition of rifampicin, in the yciF over-expressing strains (WT/pyciF and ΔcspE/pyciF), using 500µg/ml rifampicin, added at the end of the 8th of growth. Data is presented as mean±SEM and is representative of three independent experiments. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.
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Fig 4. S. Typhimurium encoded CspE binds to yciF mRNA and the F30 residue is essential for this role.

(A) Thermal stability curve of purified StCspE and StCspE<sup>F30V</sup> proteins obtained using CD spectroscopy at 219 nm wavelength. (B) Qualitative and quantitative analysis of in vivo stability of 2X flag-tagged StCspE and StCspE<sup>F30V</sup> obtained using Western blotting with antibody against the flag tag. (C) Binding of StCspE and StCspE<sup>F30V</sup> to full length yciF mRNA. Lane1- mRNA alone; lanes 2-6 increasing concentration of StCspE, lane 7- StCspE<sup>F30V</sup>. The filled triangle represents increasing concentrations of purified protein. (D) Graphical representation of complex formation by StCspE, as a function of protein concentration. Data is presented as mean±SEM and representative of three independent experiments.
Fig 5. The extra-cytoplasmic stress response (ESR) pathway is greatly induced in the ΔcspE strain, upon bile stress.

Transcript levels of the ESR components, degP, rpoE, rybB, hfq, rne and PNPase were determined using qRT-PCR for the WT and ΔcspE strains in the absence (0%) or presence (3%) of bile salts, at the 3rd and 8th hour of growth. In all panels, values are normalized by those obtained for the WT strain grown in 0% bile salts, at the indicated time point. Data is presented as mean±SEM and representative of three independent experiments. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.
Salmonella Typhimurium CspE regulates bile resistance

Fig 6. Bile salts treated ΔcspE exhibits higher OMP amounts, which is lowered upon cspE complementation or yciF over-expression.

(A) Transcript levels of Outer Membrane Proteins (ompD, ompF, ompC). (B) 12.5% SDS-PAGE analysis of isolated OMPs. Data is presented as mean±SEM and representative of three independent experiments. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.
Salmonella Typhimurium CspE regulates bile resistance

Fig 7. ΔcspE exhibits increased permeability upon bile salts stress, which is rescued upon cspE complementation, but not by the F30V mutant of CspE.

(A) Representative fluorescence image 45 minutes after addition of bisBenzimide H 33258, WT and ΔcspE, without (0%) or with (3%) pre-treatment with bile salts for 5 hrs. (B) Quantification of bisBenzimide H 33258 accumulation in WT/VA, ΔcspE/VA, cspE complemented (WT/pcsE and ΔcspE/pcsE), cspE-F30V complemented strains (WT/pcsE-F30V and ΔcspE/pcsE-F30V) and yciF over-expressing strains (WT/pyciF and ΔcspE/pyciF), without (0%) or with (3%) pre-treatment with bile salts for 5 hrs. Data is presented as mean±SEM and representative of three independent experiments. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.
Fig 8. A graphical representation of the CspE and YciF mediated down-regulation of porins to impart bile resistance in *S. Typhimurium*.

Bile stress induces the upregulation of *cspE* and the ESR pathway. CspE through multiple pathways regulates bile resistance, one of which involves YciF. CspE increases *yciF* mRNA stability, thereby enabling translation and further functions of YciF. Complementation of *cspE* or overexpression of *yciF* suppresses the bile sensitivity of the Δ*cspE* strain by establishing the appropriate culmination of the ESR pathway, with respect to porin degradation.
Interplay of cold shock protein E with an uncharacterized protein, YciF, lowers porin expression and enhances bile resistance in Salmonella Typhimurium
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