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

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Supplementary Methods

Semi-quantitative reverse transcription (RT)-PCR.RNA was isolated from the WT and Δ*cspE* strains grown in the presence or absence of 3% Bile Salts at 3 and 8 hour time points and RNA was extracted using the TRIzol reagent (Invitrogen) as per the manufacturer's instructions. The succeeding protocol followed was as mentioned in the Materials and Methods section. The cDNA was diluted 1 : 10 and fractionated on an agarose gel (1). Expression levels of *rrlC*, *cspC and acrB* were analysed.

qRT-PCR experiments for mRNA stability: RNA extraction and rifampicin addition was performed as mentioned in Materials and Methods section. qRT-PCR was then performed as a read-out for the relative abundance of the target gene transcripts. WT cells grown in LB, without any rifampicin treatment, and collected at 0 mins in accordance with other experimental sets, was normalised to 1 and all other transcript estimates was accordingly calculated.

Electrophoretic Mobility Shift Assays (EMSA). Electrophoretic mobility shift assays were conducted as previously described (2).Reaction mixtures contained 40 mM Tris-HCl (pH 8.6), 100mM NaCl, 12% glycerol, 4mM EDTA, 1nM ^{32}P-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).

Supplementary Tables

Table S1 :: List of strains and plasmids

Strain	Genotype/features	Source or Reference
S. Typhimurium 14028s (WT)	Wild-type, parent of all *Salmonella* strains	(3)
Δ*cspE*	14028s; *cspE*:: FRT	This study
Δ*yciF*	14028s; *yciF*:: FRT	This study
Δ*cspE*Δ*yciF*	14028s; *cspE*:: FRT , *yciF*:: FRT	This study
Δ*cspC*	14028s; *cspC*:: FRT	This study
WT/VA	14028s; pTrc99A; Ap^r	This study
Δ*cspE*/VA	Δ*cspE*; pTrc99A;Ap^r	This study
WT/pcspE	14028s; pcspE; Ap^r	This study
Δ*cspE*/pcspE	Δ*cspE*; pcspE; Ap^r	This study
WT/pyciF	14028s; pyciF; Ap^r	This study
Δ*cspE*/pyciF	Δ*cspE*; pyciF; Ap^r	This study
WT/pcspA	14028s; pcspA; Ap^r	This study
Δ*cspE*/pcspA	Δ*cspE*; pcspA; Ap^r	This study
WT/pcspE-F30V	14028s; pcspE-F30V; Ap^r	This study
Δ*cspE*/pcspE-F30V	Δ*cspE*; pcspE-F30V; Ap^r	This study
WT/pcspC++-SL1344	14028s; pcspC++-SL1344; Ap^r	This study
Δ*cspE*/pcspC++-SL1344	Δ*cspE* ;pcspC++-SL1344; Ap^r	This study
S. Typhimurium SL1344 (WT)	Wild-type, parent of all deletion strains in SL1344	(4)
Δ*cspE*	SL1344; *cspE*::FRT	This study
Δ*cspC*	SL1344; *cspC*::FRT	This study
Δ*cspE*Δ*cspC*	SL1344; *cspE*:: FRT , *cspC*:: FRT	This study
Δ*cspE*Δ*cspC*	SL1344; *cspE*:: FRT , *cspC*:: FRT	This study

Plasmids	**Genotype/features**	**Source or Reference**
pKD46	Temperature sensitive λ-Red recombinase expression plasmid; Ap^r	(5)
pKD4	Kanamycin resistance (*kan*)	(5)
pCP20	Temperature sensitive FLP recombinase expression plasmid; Ap^r	(6)
pRS424	Ampicillin resistance (Ap^r)	(7)
pTrc99A	Ampicillin resistance (Ap^r)	(6)
pcspE	pRS424 derivative with promoter and *cspE* cloned between *Spe*I and *Xho*I sites	This study
pcspE-F30V	pRS424 derivative with promoter and F30V mutant of *cspE* cloned between *Spe*I and *Xho*I sites	This study
pyciF	pTrc99A derivative with *yciF* cloned between *Bam*HI and *Eco*RI sites	This study
pcspA	pTrc99A derivative with *cspA* cloned between *Bam*HI and *Eco*RI sites	This study
pcspC++-SL1344	pRS424 derivative with *cspC* operon with native promoter from *S.* Typhimurium SL1344 cloned between *Spe*I and *Xho*I sites	This study

Table S2 :: List of primers used for gene deletion and confirmation

PrimerName	OligonucleotideSequence 5'-3'
cspEdeletion primers	
cspE-KO-FP	CACAGCATTTGTGTCTATTTTTCATGTAAAGGTAATTTTGGTGTAGGCTGGAGCTGCTTC
cspE-KO-RP	CGAAAGGGCGGGTTTTGAAATCTTGCTGTCGGACGTATGCGGTCCATATGAATATCCTCCTTAG
yciFdeletion primers	
yciF-KO-FP	GGCTCGCAGTATTTTGCCCAGGAGAATTAGATTTGTGTAGGCTGGAGCTGCTTCG
yciF-KO-RP	CGGAATCATGCAAGCTGATAATTAAAAATACATGGTCCATATGAATATCCTCC
cspC deletion primers	
cspC-KO-FP	CACAGCATTTGTGTCTATTTTTCATGTAAAGGTAATTTTGGTGTAGGCTGGAGCTGCTTC
cspC-KO-RP	CGAAAGGGCGGGTTTTGAAATCTTGCTGTCGGACGTATGCGGTCCATATGAATATCCTCCTTAG
cspE deletion confirmation primers	
cspE-CON-FP	GATGGGGAAGGGTATGCTTCAG
cspE-CON-RP	GCGCTGCTGAATGTGCTG
yciFdeletion confirmationprimers	
yciF-CON-FP	CCGCAACGAGCTTCTGAAGCCGG
yciF-CON-RP	CGGCTGGCCATAGATTCAAGCATCG

Table S3 :: Primers used for cloning *cspE, yciF*and *cspA*

Primer name	**Oligonucleotide sequence 5'- 3'**
p-cspE-pRS-FP	GGACTAGTTGGGGAAGGGTATGCTTCAGTTGTG
cspE-pRS-RP	CGCCTCGAGTCACTTGTCATCGTCATCCTTGTAATCCAGAGCAGTTACGTTTGCAGCGG
yciF-pTrc-FP	CCGGAATTCATGAATATCAAAACCGTTGAAGACC
yciF-pTrc-RP	CGCGGATCCTCACTTGTCATCGTCATCCTTGTAATCCTTGTCATCGTCATCCTTGTAATCTTTCGATTTGCGTTCAGCACTTTTATTAA
cspA-pTrc-FP	CCGGAATTCATGTCCGGTAAAATGACTGGTATCG
cspA-pTrc-RP	CGCGGATCCTCACTTGTCATCGTCATCCTTGTAATCCAGGCTGGTTACGTTGCCAGCTGC
p-yobF-cspC-pRS-FP	GGACTAGTATGCTTACCGGGGCGCAAATGGC
cspC-pRS-RP	CGCCTCGAGTCACTTGTCATCGTCATCCTTGTAATCCATCCAGAGACATTTTGAAG

Table S4 :: Primers used for qRT-PCR

Primer Name	Oligonucleotide sequence 5'- 3'
rrlC-FP	GAGCGTTCTGTAAGCCTGTG
rrlC-RP	CGCAGTAACACCAAGTACGG
cspA-FP	AACGCTGATAAAGGCTTCGG
cspA-RP	TTACAGGCTGGTTACGTTGC
cspB-FP	GGGCTTTGGTTTCATTACGC
cspB-RP	TTGACCGCTGATGGACCTTT
cspC-FP	ATCCAAAGGTTTTGGCTTCA
cspC-RP	GTTAACAGCAGCCGGACCT
cspE-FP	GGATTCGGTTTCATTACTCCGG
cspE-RP	CGTTTGCAGCGGAAGGGCC
pagC-FP	TCTGTTGAGCCTGAAGGTATTC
pagC-RP	CGACGTTGAAGCCGTTTATTT
yciF-FP	CTTGAAGAAACCCAGGGTCA
yciF-RP	CGCGTACTTCGTTTTTCTCC
acrA-FP	CTTCCGGGTCGTACCGTTGC
acrA-RP	CGTGCGCGAACGAACATTCC
acrB-FP	AAGTGCTGGATGAGGTCACG
acrB-RP	ACTCGAAGTCAAAGCCGGTT
tolC-FP	AACCGTAACCTGTCGCTGTT
tolC-RP	TGCGAGTTAACCATCCCACC
ompF-FP	GTGGACGGTCTCTCTTTCGG
ompF-RP	TCAGCATATACGGCAGCCAG
ompC-FP	GGTGGATGGTCTGGACTTCG
ompC-RP	TCTGAGAATACTGCGCTGCC
ompD-FP	TCGCGAATAAAACCCAGAAC
ompD-RP	CTTTAGCCGCTTTGGTGAAG
hfq-FP	CGGTCAGCCAGATGGTTTAT
hfq-RP	TCAGTCTCTTCGCTGTCCTG
uspA-FP	GTCTACCAACGCTGGCTACC
uspA-RP	GGCACAATCAGCATGTCAAC
rne-FP	CTTCCTTAACGCGCTGAAAC
rne-RP	GCGACGGTTACGACGATTAT
rpoE-FP	TGCGCTACCAGCATAAAGTG
rpoE-RP	GCCGCTTTCAAAGTTTTCTG

degP-FP	CTGGCTCTGAGTTTAGGTTTGG
degP-RP	GTATTCACCGTGGTGCTACCTT
PNPase-FP	GATGGCCGTGAAAAAGACAT
PNPase-RP	ACGGAGTACGGAGGGAAGTT
rybB-FP	GCCACTGCTTTTCTTTGATG
rybB-RP	AAACCCACCAACCTTGAACC

Supplementary References

1. Bhaskarla, C., Das, M., Verma, T., Kumar, A., Mahadevan, S., and Nandi, D. (2016) Roles of Lon protease and its substrate MarA during sodium salicylate-mediated growth reduction and antibiotic resistance in Escherichia coli. *Microbiology***162**, 764-776
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SUPPLEMENTARY FIGURES

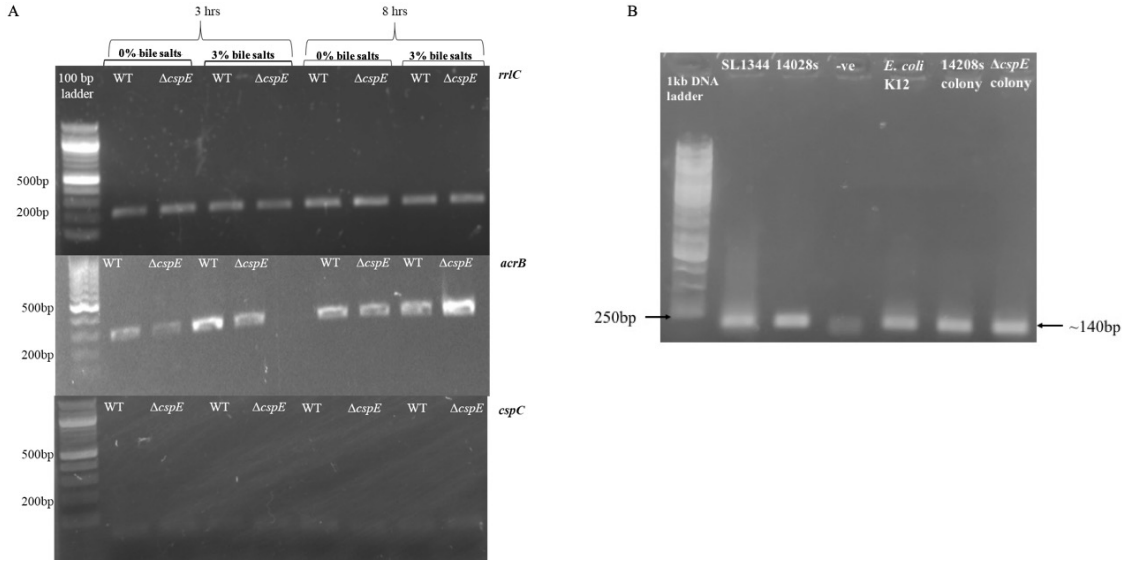


Fig S1. No detectable *cspC* transcript is observed in *S.* Typhimurium 14028s with or without bile salts treatment.

(A) Semi quantitative Reverse Transcriptase PCR was performed using *cspC* internal primers. *rrlC* internal primers was using as the loading control and *acrB* was used as the positive control. (B) Genomic DNA amplification of *cspC* using the internal primers for *cspC.*

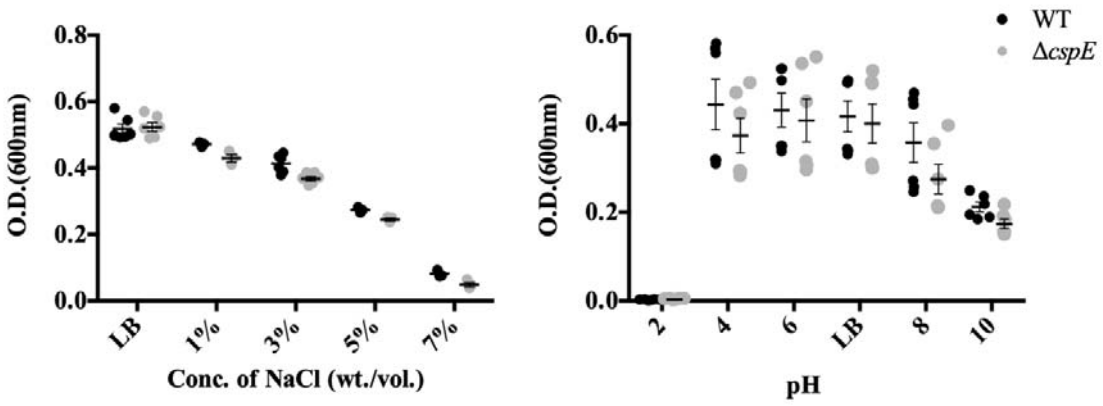


Fig S2.WT and Δ*cspE* doesn't show any growth difference in varying salt (NaCl) concentrations or pH.

(A) Effect of increasing concentrations of NaCl, (B) pH on WT and Δ*cspE* in LB. $5X10^6$ CFU/ml of WT and Δ*cspE* was used to inoculate the respective media conditions and grown at 37℃. O.D. (600nm) was acquired at the 8^{th}hr of growth, in a 96-well polystyrene microtitre plate. Data is presented as mean±SEM and is representative of three independent experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.001$, **** $p<0.0001$.

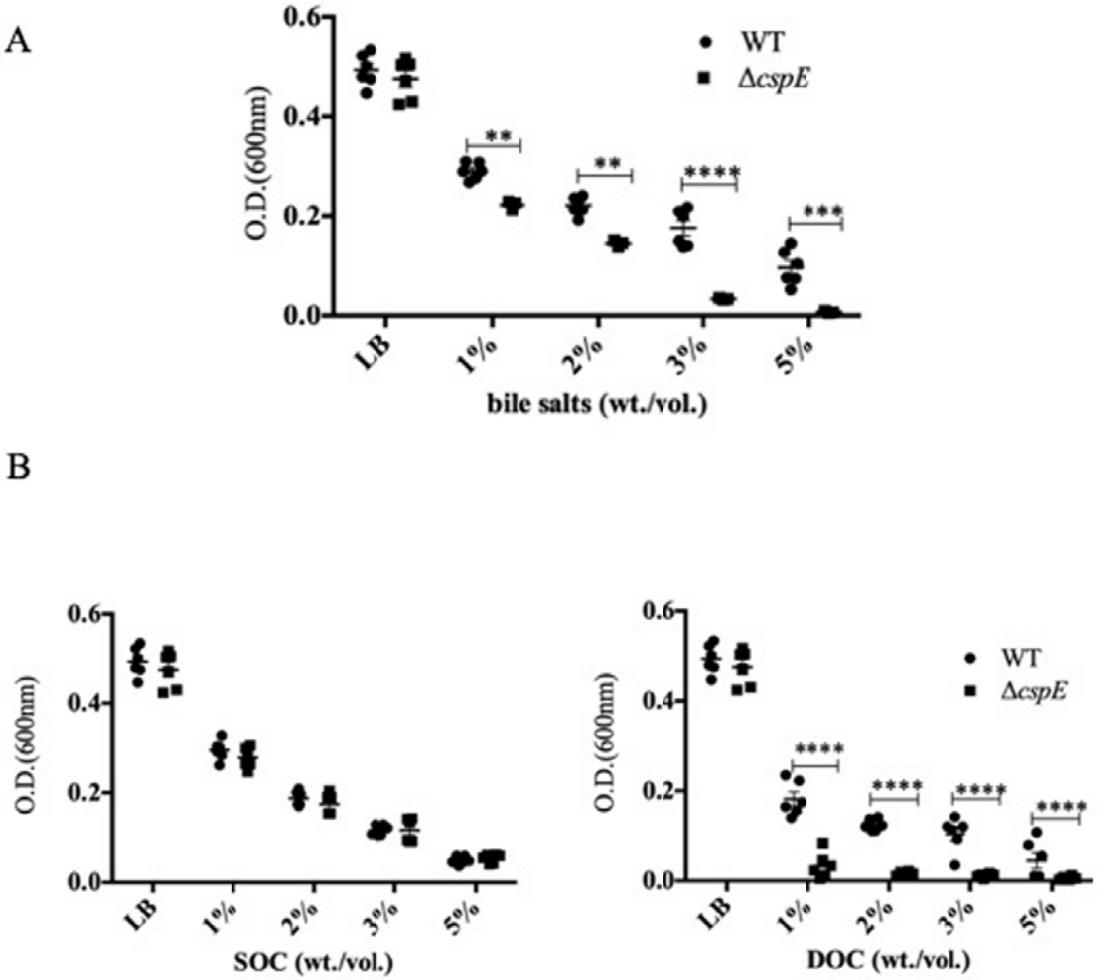


Fig S3. *S.* Typhimurium Δ*cspE* shows a dose dependant sensitivity to increasing concentrations of bile salts, specifically to deoxycholate.

(A) The effect of increasing concentrations of bile salts on WT and Δ*cspE* in LB media. (B) The effect of varying doses of the bile salts components on the growth of WT 14028s and Δ*cspE* namely, Sodium Cholate (SOC), Sodium Deoxycholate (DOC) was studied at the 8th hour time-point. Data is presented as mean±SEM and is representative of three independent experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

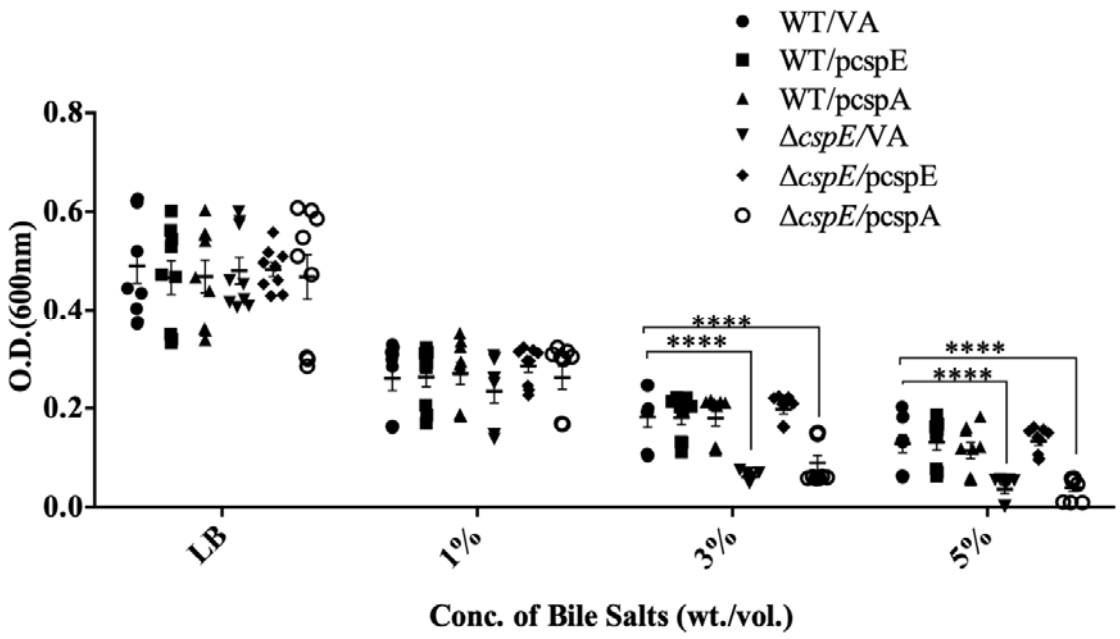


Fig S4. Bile sensitivity of *S.* Typhimurium Δ*cspE* is rescued by complementation of *cspE* but not *cspA*.

The effect of increasing concentrations of bile salts on WT/VA, Δ*cspE*/VA, *cspE* complemented (WT/pcspE and Δ*cspE*/pcspE) and *cspA* complemented strains (WT/pcspA and Δ*cspE*/pcspA) in LB media was studied at the 8th hour time-point. Data is presented as mean±SEM and is representative of three independent experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

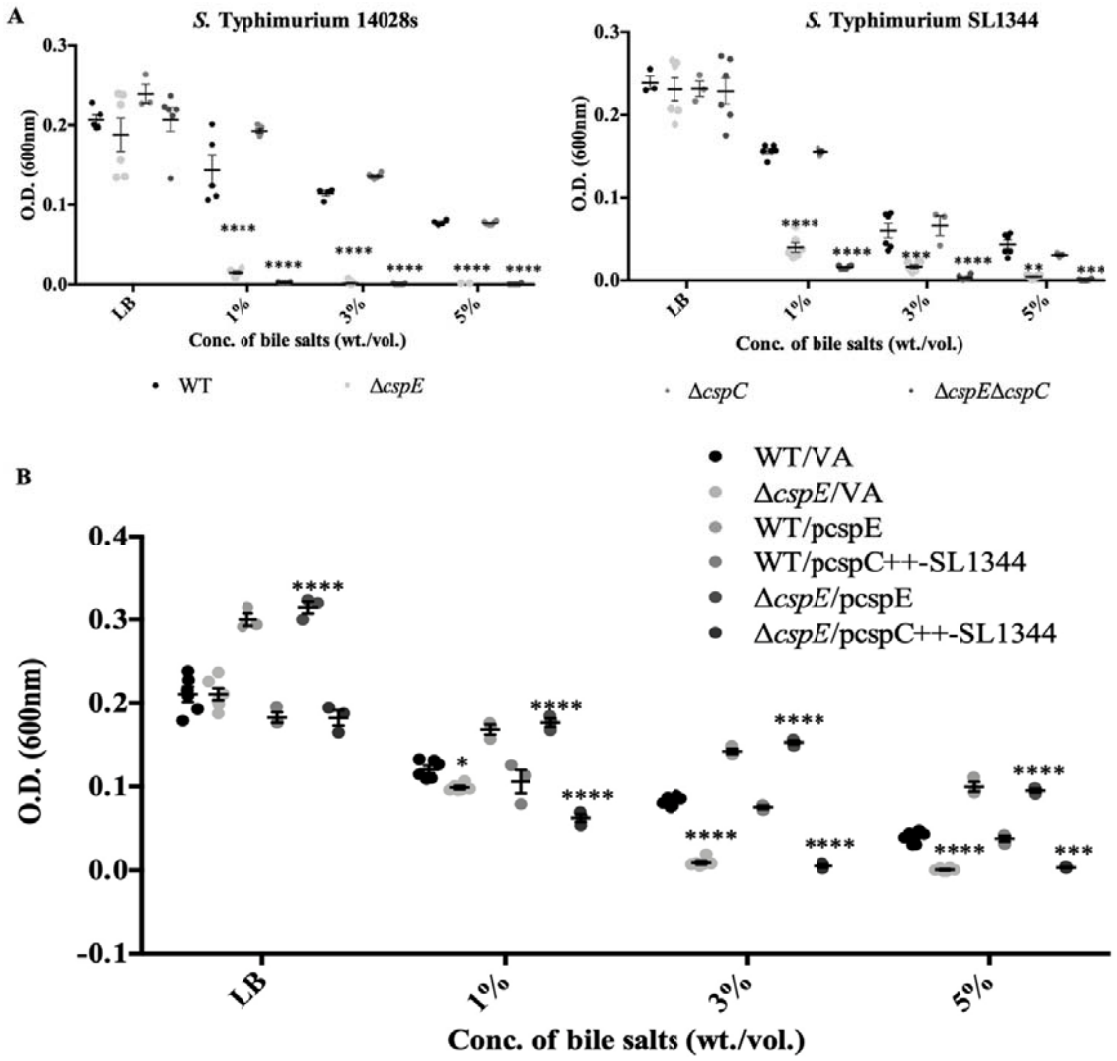


Fig S5. *S.* Typhimurium 14028s encoded *cspC* does not play a major role in bile resistance.

(A) The effect of increasing concentrations of bile salts on *wild type* and genetic deletion strains of *S.* Typhimurium 14028s and SL1344. The strains used were WT, Δ*cspE*, Δ*cspC* and Δ*cspE*Δ*cspC*. (B) The effect of increasing concentrations of bile salts on WT/VA, Δ*cspE*/VA, *cspE* complemented from 14028s (WT/pcspE and Δ*cspE*/pcspE), *cspC* complemented with the entire operon of *cspC* from SL1344 (WT/pcspC++-SL1344 and Δ*cspE*/pcspC++-SL1344) in LB media was studied at the 8th hour time-point. Data is presented as mean±SEM and is representative of three independent experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

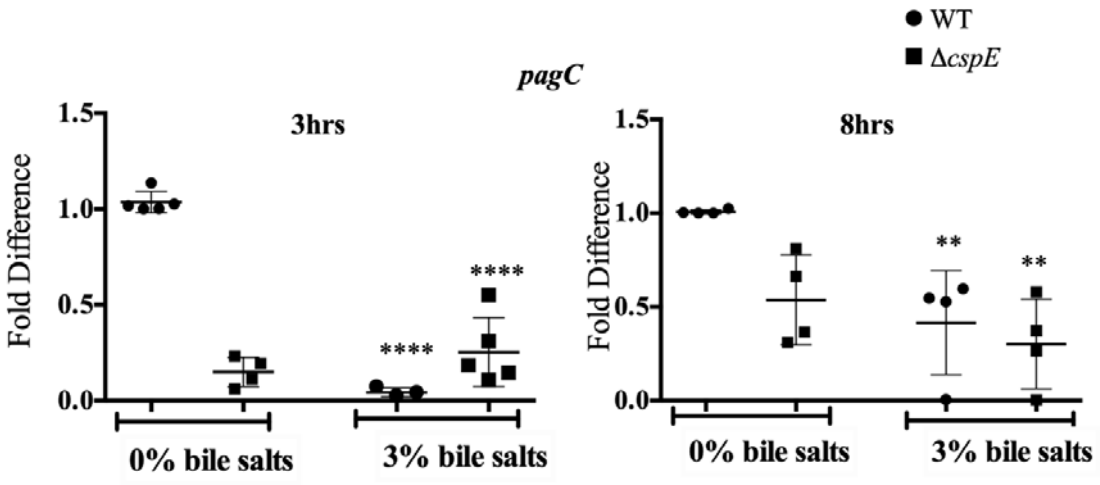


Fig S6. *pagC* is down-regulated in both WT and Δ*cspE*, upon bile salts treatment.

The transcript levels of *pagC* was determined using qRT-PCR for the WT and Δ*cspE* 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. Data is presented as mean±SEM and is representative of three independent experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

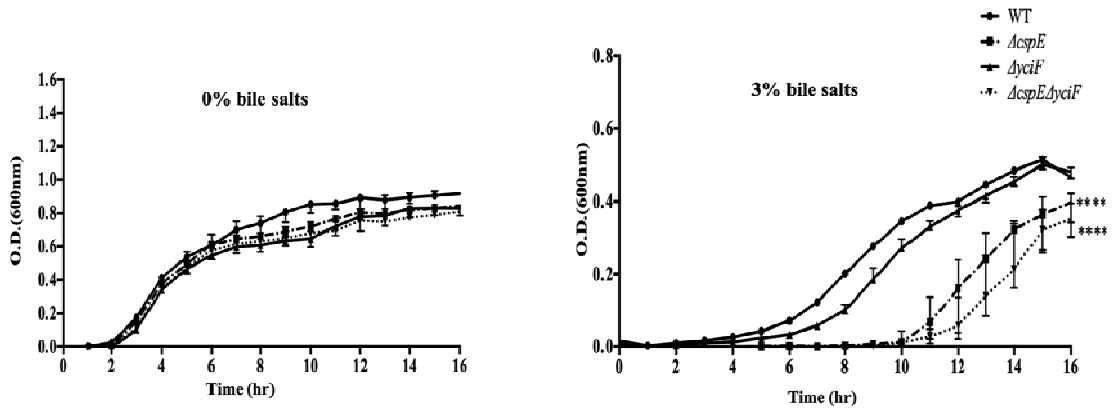


Fig S7. Deletion of *yciF* does not have any effect on the bile resistance of *S.* Typhimurium 14028s.

Kinetic growth analysis in terms of O.D. (600nm) of WT, Δ*cspE*, Δ*yciF* and Δ*cspE*Δ*yciF* strains in the absence (0%) and presence (3%) of bile salts. Data is presented as mean±SEM and representative of three independent experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

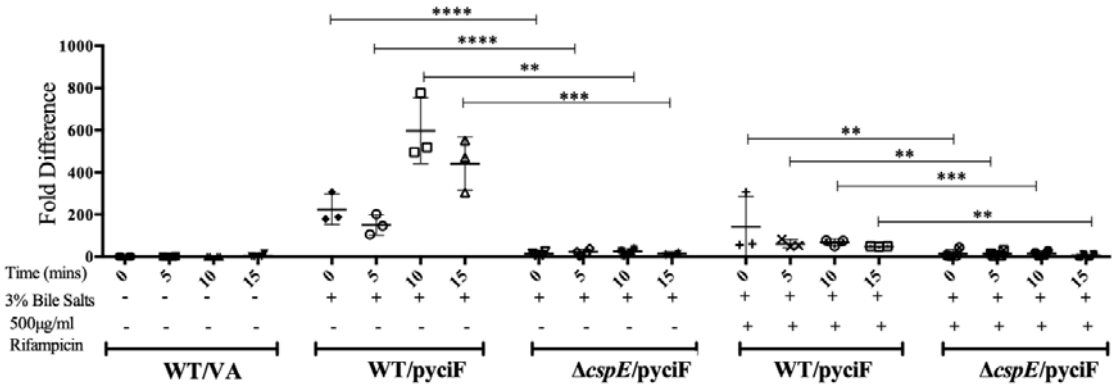


Fig S8. Quantitation of mRNA transcripts by qRT-PCR confirms the mRNA stabilisation property of CspE.

The *yciF* mRNA stability was determined in terms of the relative amounts of its transcript levels, by qRT-PCR, in the *yciF* over-expressing strains (WT/pyciF and Δ*cspE*/pyciF), using 500µg/ml Rifampicin, added at the end of the 8th of growth. mRNA levels in the untreated WT/VA collected at 0 mins, was normalised to 1. All other samples were calculated as fold-change to this reference value. Data is presented as mean±SEM and is representative of three independent experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

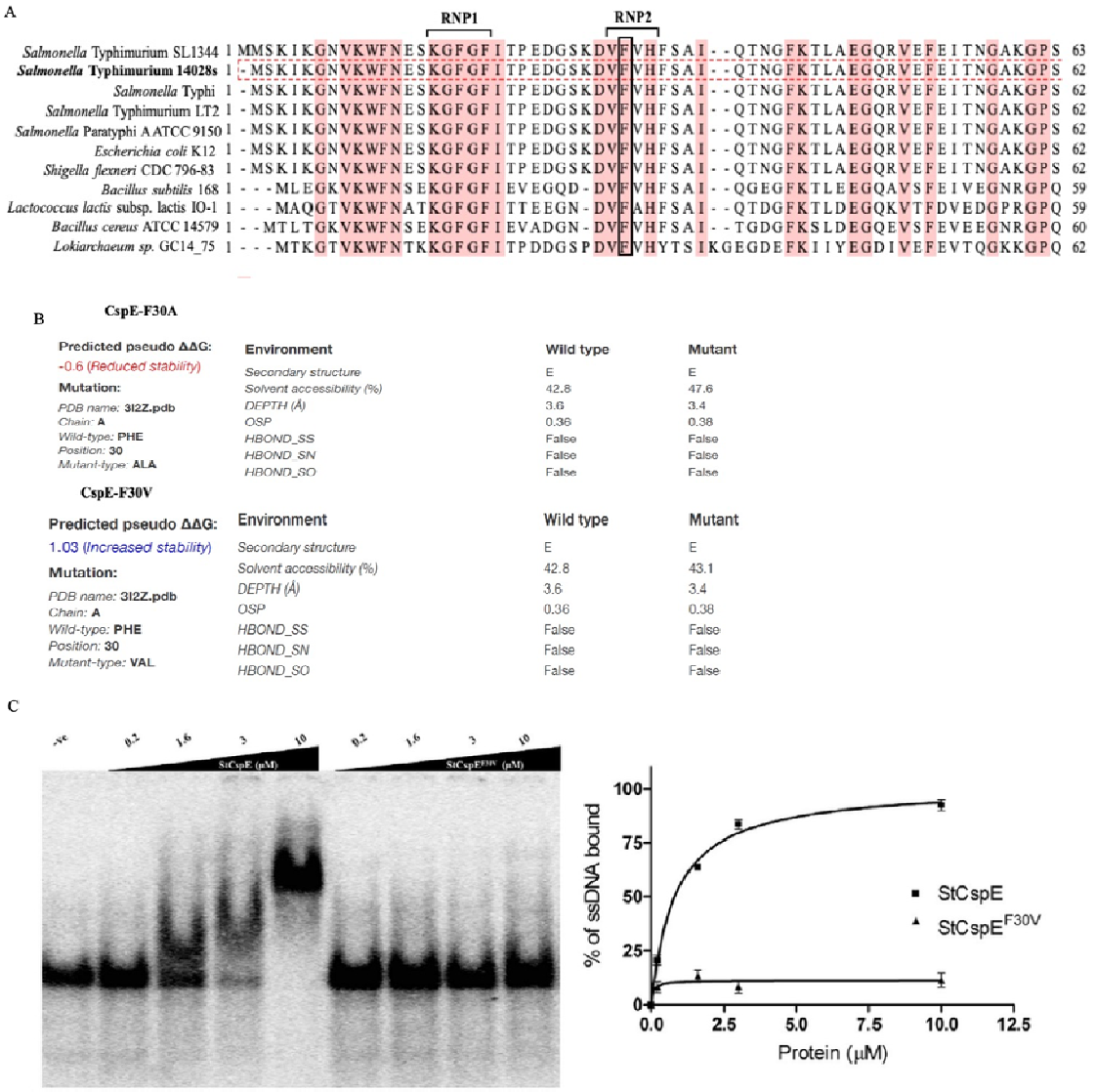
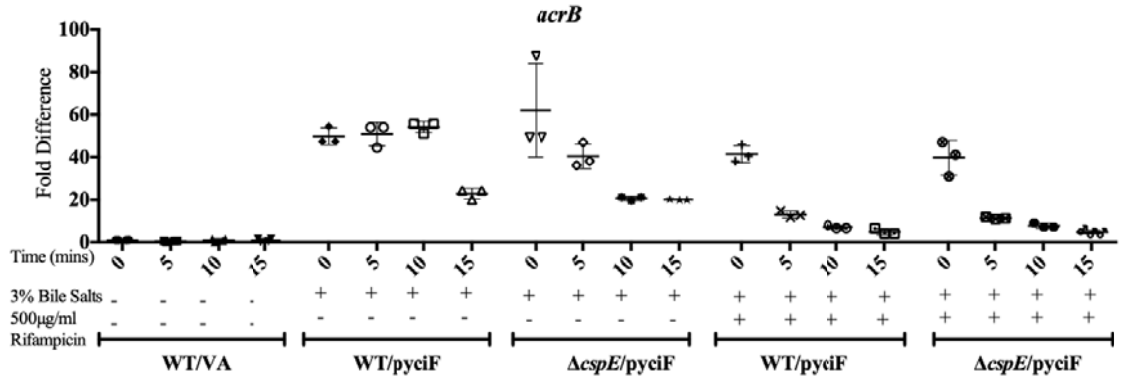


Fig S9. F30 in RNP2 of CspE is essential for ssDNA binding function of CspE.

(A) ClustalW was used to align the CspE sequences from several species within the eubacterial domain. Accession Nos for sequences in MSA: *S.* Typhimurium SL1344 (A0A0H3N9E2_SALTS); *S.* Typhimurium 14028s (A0A0F6AYC2_SALT1); *S.* Typhi (Q8XGU4_SALTI); *S.* Typhimurium LT2 (Q7CQZ5_SALTY); *S.* Paratyphi A ATCC 9150 (A0A0H2WQN8_SALPA); *E. coli* K12 (CSPE_ECOLI); *Shigella flexneri* CDC 796-8 (E7TCU3_SHIFL); *Bacillus subtilis* 168 (P32081|CSPB_BACSU); *Lactococcus lactis* subsp. lactis IO-1 (H5SWC0_LACLL); *Bacillus cereus* ATCC 14579 (CSPE_BACCR); *Lokiarchaeum sp.* GC14_75 (A0A0F8VY40_9ARCH). (B) The software Site Directed Mutator was used for the prediction of the F30V mutation, that indicates a stabilizing effect on the secondary structure (http://marid.bioc.cam.ac.uk/sdm2/prediction). (C)EMSA was performed with ^{32}P labeled substrate ssDNA at 1 nM in the absence or presence of 0.2, 1.6, 3 and 10μM of StCspE and StCspE^{F30V}. Binding of StCSpE and StCspE^{F30V} to nucleic acid substrate containing a single CCAT site. The filled triangle represents increasing concentrations of purified proteins. Graphical representation of complex formation by StCspE and StCspE^{F30V}, as a function of protein concentration. Data is presented as mean±SEM and representative of three independent

experiments. The best fit curve was obtained by subjecting the data to non-linear regression analysis in GraphPad Prism (version 5), using the equation for one site specific binding with a Hill slope.

A



B

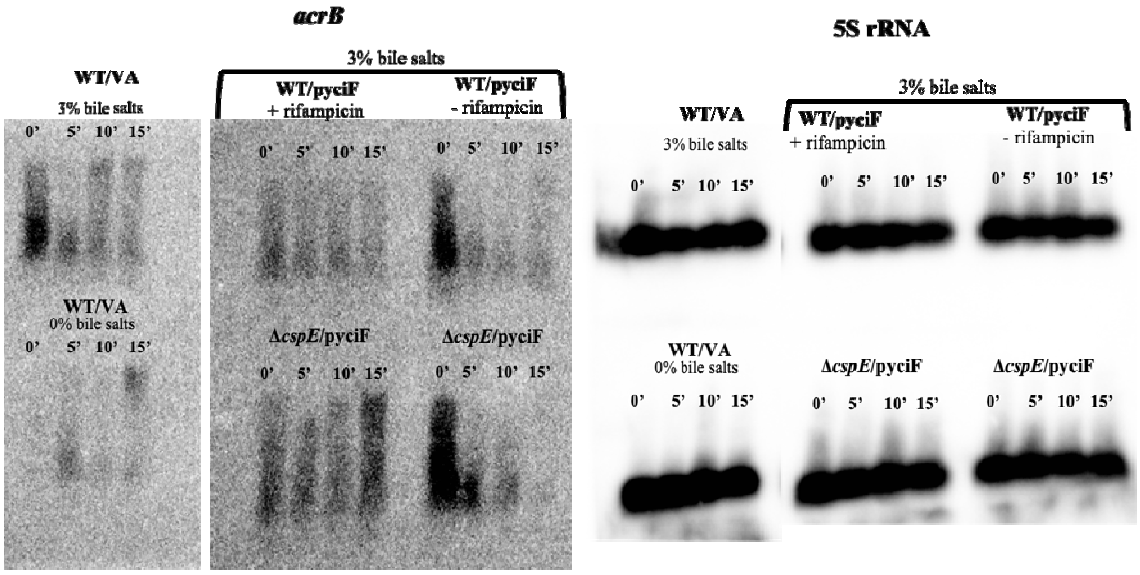


Fig S10. *acrB* is induced by bile but is not regulated by CspE.

(A) qRT-PCR quantitation of *acrB* mRNA. *rrlC* was used as the house-keeping normalization control. (B) The *acrB* mRNA stability was determined in terms of the relative amounts of its transcript levels. The relative mRNA levels of *acrB* were determined upon bile treatment in the presence of absence of rifampicin, in WT/pyciF and Δ*cspE*/pyciF. 5S rRNA was used as the loading control.

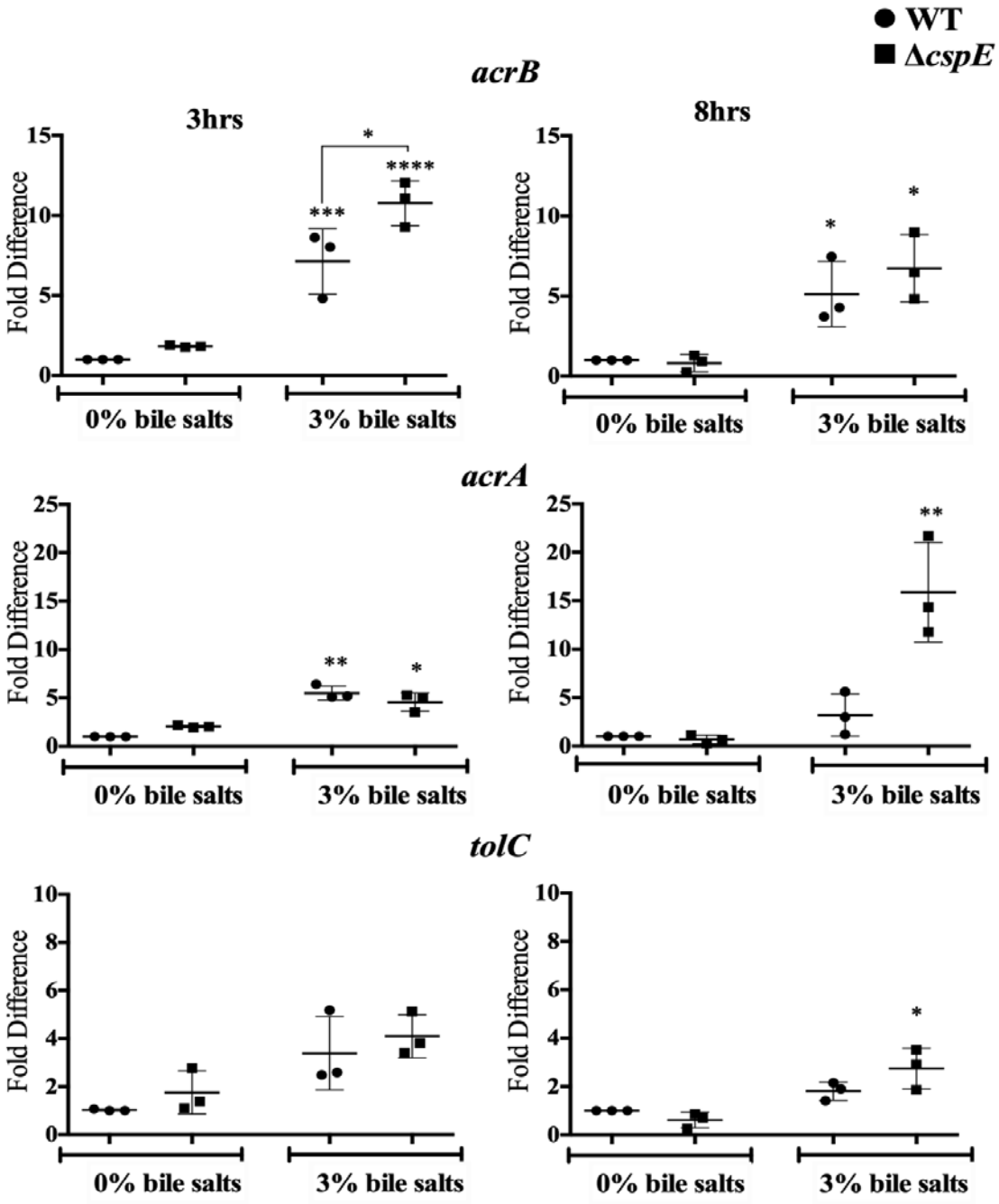


Fig S11. The major efflux pump AcrAB-TolC components do not show any differential expression between bile salts-treated WT and Δ*cspE*.

Transcript levels of *acrB*, *acrA* and *tolC* were using qRT-PCR for the WT and Δ*cspE* 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. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

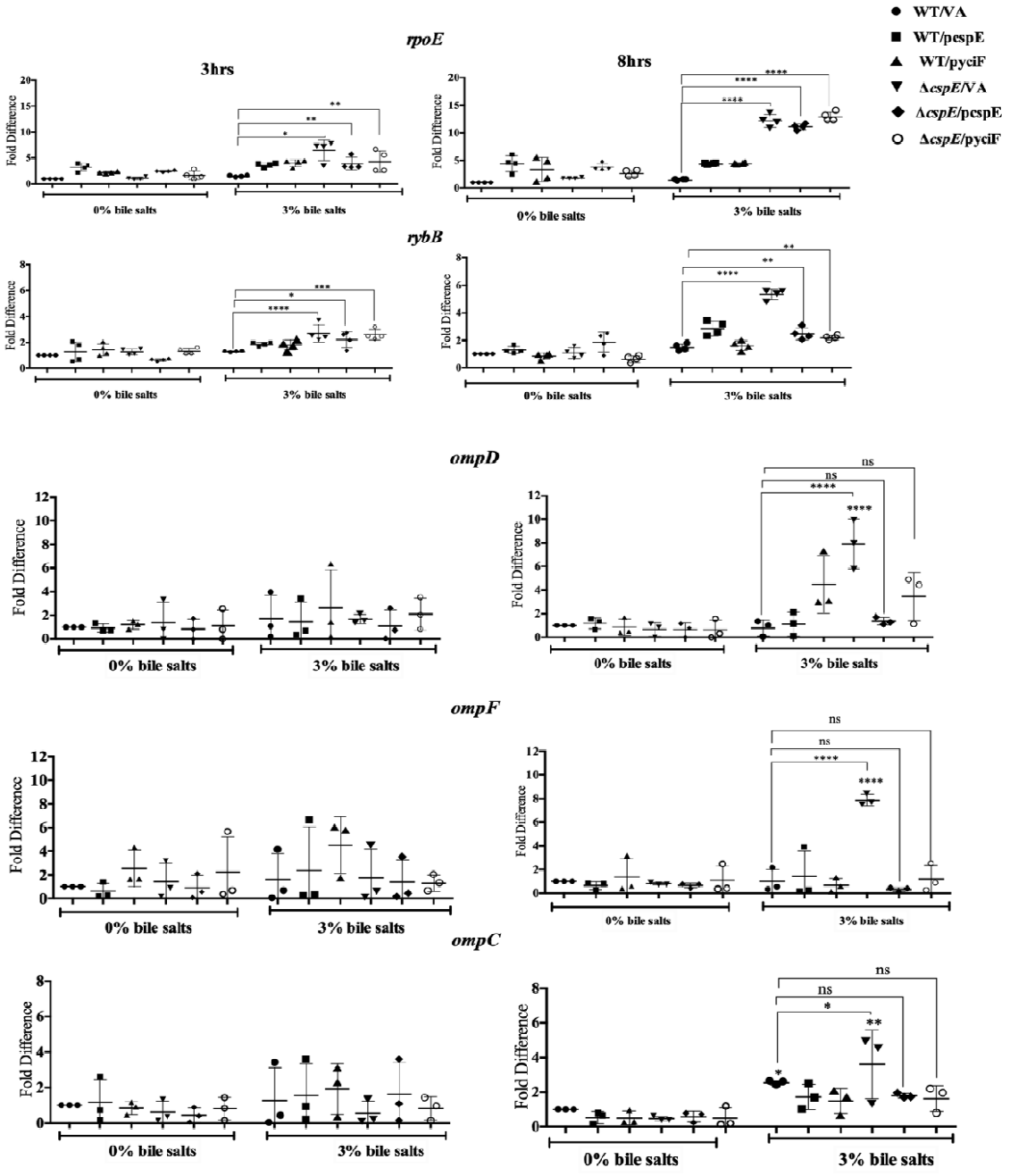
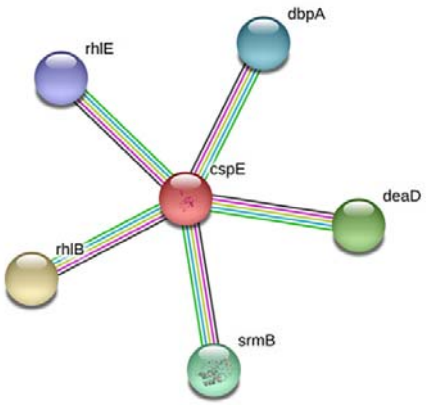
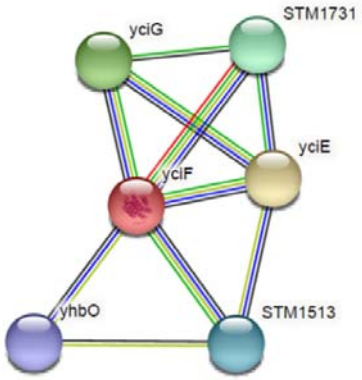


Fig S12. Complementation of *cspE* and over-expression of *yciF* rescues perturbed transcript levels of ESR components and porins, in bile salts-treated Δ*cspE*.

Transcript levels of *rpoE, rybB* and OMPs (*ompD*, *ompF*, *ompC*) were determined using qRT-PCR for the WT/VA, Δ*cspE*/VA, *cspE* complemented strains (WT/pcspE and Δ*cspE*/pcspE),and *yciF* over-expression (WT/pyciF and Δ*cspE*/pyciF) strains, with (3%) or without (0%) pre-treatment 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. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.



node	annotation
srmB	• ATP-dependent RNA helicase SrmB; • DEAD-box RNA helicase involved in the assembly of the 50S ribosomal subunit at low temperature. • Exhibits RNA-stimulated ATP hydrolysis and RNA unwinding activity
dbpA	• ATP-dependent RNA helicase DbpA; • DEAD-box RNA helicase involved in the assembly of the 50S ribosomal subunit. • Has an RNA-dependent ATPase activity, which is specific for 23S rRNA, and a 3' to 5' RNA helicase activity that uses the energy of ATP hydrolysis to destabilize and unwind short rRNA duplexes
rhlE	• ATP-dependent RNA helicase RhlE; • DEAD-box RNA helicase involved in ribosome assembly. • Has RNA-dependent ATPase activity and unwinds double-stranded RNA
rhlB	• ATP-dependent RNA helicase RhlB; • DEAD-box RNA helicase involved in RNA degradation. • Has RNA-dependent ATPase activity and unwinds double-stranded RNA
deaD	• ATP-dependent RNA helicase DeaD; • DEAD-box RNA helicase involved in various cellular processes at low temperature, including ribosome biogenesis, mRNA degradation and translation initiation



yciE	*Cytoplasmic protein (168 aa)*
yciG	*Cytoplasmic protein (60 aa)*
STM1731	*Catalase (292 aa)*
STM1513	*Hypothetical protein (60 aa)*
yhbO	*Intracellular proteinase (172 aa)*

Fig S13. Many RNA helicases and degradation related protein constitute the CspE interaction network.

S. Typhimurium LT2 was used as the query organism to generate a protein-protein interaction network of CspE and YciF, using the available software STRING. Interactions were established using data available from KEGG Pathways, PFAM Protein Domains and INTERPRO Protein Domains and Features databases.