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Salmonella Typhimurium encoded cold shock protein E is essential for motility and biofilm formation

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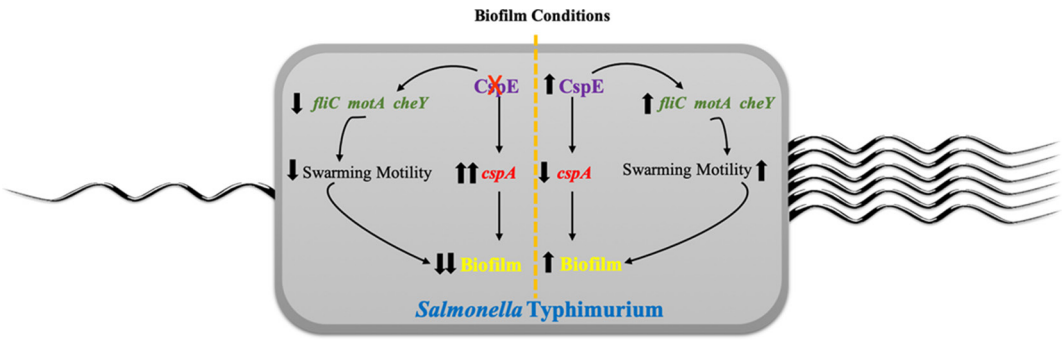

Graphical abstract

CspE regulates biofilm formation through the down-regulation of another cold shock protein, CspA. However, its regulation of motility is independent of CspA.

ABSTRACT

The ability of bacteria to form biofilms increases their survival under adverse environmental conditions. Biofilms have enormous medical and environmental impact; consequently, the factors that influence biofilm formation are an important area of study. In this investigation, the roles of two cold shock proteins (CSP) during biofilm formation were investigated in *Salmonella* Typhimurium, which is a major foodborne pathogen. Among all CSP transcripts studied, the expression of *cspE* (STM14_0732) was higher during biofilm growth. The *cspE* deletion strain (Δ*cspE*) did not form biofilms on a cholesterol coated glass surface; however, complementation with WT *cspE*, but not the F30V mutant, was able to rescue this phenotype. Transcript levels of other CSPs demonstrated up-regulation of *cspA* (STM14_4399) in Δ*cspE*. The *cspA* deletion strain (Δ*cspA*) did not affect biofilm formation; however, Δ*cspE*Δ*cspA* exhibited higher biofilm formation compared to Δ*cspE*. Most likely, the higher *cspA* amounts in Δ*cspE* reduced biofilm formation, which was corroborated using *cspA* over-expression studies. Further functional studies revealed that Δ*cspE* and Δ*cspE*Δ*cspA* exhibited slow swimming but no swarming motility. Although *cspA* over-expression did not affect motility, *cspE* complementation restored the swarming motility of Δ*cspE*. The transcript levels of the major genes involved in motility in Δ*cspE* demonstrated lower expression of the class III (*fliC*, *motA*, *cheY*), but not class I (*flhD*) or class II (*fliA*, *fliL*), flagellar regulon genes. Overall, this study has identified the interplay of two CSPs in regulating two biological processes: CspE is essential for motility in a CspA-independent manner whereas biofilm formation is CspA-dependent.

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Abbreviations: c-di-GMP, cyclic di guanylyl monophosphate; CSP, cold shock protein; DGC, di guanylyl cyclase; EPS, extra-polymeric substance; h, hours; qPCR, quantitative real time Polymerase Chain Reaction; 5'-UTR, 5' untranslated region.

Supplementary material is available with the online version of this article.

INTRODUCTION

Microbes harbour specialised stress defense mechanisms and one such mechanism is the response to a sudden temperature drop, or the cold shock response [1]. One of the major consequences of the temperature change is the stabilization of RNA secondary structure [2], thereby halting transcription and translation. Bacteria respond to this stress by synthesizing a family of 67–73 amino acid containing proteins known as Cold Shock Proteins (CSP) [3]. The major CSP in *E. coli* are the RNA chaperones CspA, CspB, CspG and CspI, RNA helicase CsdA, ribosome binding factor RbfA, PNPase, transcription factor NusA, translation initiation factors IF1 and IF2 etc. [4].

The major CSP in *E. coli* is CspA which is a RNA chaperone that melts the nucleic acid secondary structure thereby facilitating transcription and translation during cold shock [5, 6]. However, this temperature downshift doesn't affect the mRNAs of genes encoding the cold shock proteins [4] due to the presence of structural elements that promote translation at low temperature. The *cspA* mRNA undergoes a temperature-dependent structural reorganisation at low temperature, resulting from stabilization of an otherwise thermodynamically unstable folding intermediate. The 'low temperature' structure is more efficiently translated and less susceptible to degradation than the 37 °C structure. Thus, the *cspA* mRNA acts as a thermometer, sensing temperature downshifts by adopting a functionally distinct structure. *E. coli* encodes nine CSPs of which only four (CspA, CspB, CspG and CspI) are cold-inducible [7]. Several members of the CSP family are non-cold inducible and their expression is induced during different stresses [8]. A variety of functions have been attributed to the CspA homologs such as adaptation to stationary phase and nutrient deprivation [9], antibiotic synthesis [10], UV sensitivity [11], regulation of expression of several stress response proteins [12], camphor resistance and chromosome condensation [13], resistance to toluene [14] and bile [15].

Salmonella Typhimurium is a foodborne pathogen that causes self-limiting gastroenteritis in humans [16], which may develop into a severe systemic disease in immunocompromised individuals [17]. *S.* Typhimurium is a host generalist which has a wide variety of hosts and, hence its successful propagation is dependent on its ability to adjust to multiple lifestyles relative to the environment. One of the major concerns of *Salmonella* infection is the rise in invasive non-typhoidal salmonellae (iNTS) in under-developed nations [18]. In Sub-Saharan Africa, iNTS disease is caused by two distinct lineages of *S.* Typhimurium ST313, both carrying MDR-encoding Tn21 elements on plasmid pSLT and the prophages, BTP1 and BTP5 [19]. *S.* Typhimurium ST313 was recently found to cause 2.7% of the *S.* Typhimurium-associated gastroenteritis in the UK. A key feature that distinguishes UK-ST313 isolates from SSA ST313 is the absence of the BTP1 and BTP5 prophages [20]. These aspects reinforce the notion that it is important to study all aspects of the biology of *Salmonella*.

S. Typhimurium encodes six CspA homologs: CspA–E and CspH [21] and only CspA, CspB and CspH are cold inducible, [22–25]. The roles of CSPs in regulating biofilm formation, cellular aggregation and virulence have been investigated in a variety of bacteria. In *E.coli,* the CSP member, CspD, is essential for adaptation to nutrient starvation and stationary growth phase; in addition, it also acts as the essential RNase for the toxin-antitoxin system MqsRA that regulates biofilm formation [26]. In the *S.* Typhimurium SL1344 strain, CspC and CspE, combine to regulate biofilm formation [27]. The *msaB* of *S.* aureus which is homologous to CspA of *E. coli* and belongs to the *msaABCR* operon, has been shown to regulate biofilm development and virulence [28]. The analysis of a *V. cholerae* mutant lacking the cold shock gene *cspV* revealed that it regulates genes involved in biofilm formation and type VI secretion, specifically the infectious life cycle of the pathogen [29]. In *Listeria monocytogenes,* CSPs have been reported to be involved in regulation of virulence, cellular aggregation, and flagella-based motility [30]. These studies led us to investigate the roles of *csps* during biofilm formation in the intracellular pathogen, *S.* Typhimurium.

The ability of strains to form biofilms affects their increased persistence and survival [31]. Up to 40% of human and livestock diseases are biofilm-related and have enormous medical and economic impacts [32, 33]. The cells within a biofilm behave differently to their planktonic state, with respect to their gene regulation [34]. Most naturally occurring biofilms are polymicrobial in nature [35] wherein anti-microbial resistance of one bacterial strain may enable the survival of others in the biofilm. The formation and structure of a biofilm is affected by numerous factors, including bacterial species [36] e.g. *Salmonella, Vibrio, E.coli* etc., available surface area [37], nutrients [38, 39], and other environmental conditions [40]. The major gene regulation of biofilm formation occurs through CsgD, a transcriptional regulator, which feeds a multitude of pathways. Several environmental cues are sensed which either individually or combinatorically activate CsgD, thereby activating the downstream di-guanylyl cyclase (DGC), AdrA [41]. This activation leads to increased production of c-di-GMP [41], which regulates a wide array of bacterial responses, including the transition from motility to sessility [42], virulence [43], chemotaxis [44], transcriptional regulation of flagellar genes [45], non-flagella mediated motility [46] and quorum sensing process [47].

Salmonella associated biofilms have been implicated in chronic infections associated with the serovar Typhi [48]. In *Salmonella,* the best characterised biofilm phenotype is the red, dry and rough (rdar) morphotype on Congo Red agar plates, indicative of curli fimbriae and cellulose production [31]. These components form the major bulk of the extracellular matrix scaffold in *Salmonella* [49]. The proteinaceous curli fimbriae enable proximal cell-cell interactions while cellulose enables long-range interactions over the wide expanse of the biofilm. The large extracellular protein, BapA is an integral component of the extracellular matrix in the air-water interface of biofilms [50]. Several other additive factors add to the final outcome of a sturdy biofilm, namely, flagella [51],

O-antigen capsule [52], type I fimbriae [53] and other uncharacterised polysaccharides [54]. In an earlier study [15], we had observed that CspE lacking *S.* Typhimurium 14028 s was sensitive to bile stress. In order to implicate an *in vivo* scenario of attenuated host colonisation, we investigated the possible regulation of biofilm by CspE. In the present study, we focus on the role of a Cold Shock Protein E (STM14_0732) from *S.* Typhimurium 14028 s, in regulating biofilm and motility. We have also identified CspA to be a functional mediator in the CspE-mediated pathway for biofilm formation.

METHODS

Chemicals, bacterial strains and growth conditions

Cholesterol (Sigma Aldrich) was used at concentration of 5 mg ml^{-1} for coating glass coverslip (BlueStar, India). The bacterial strains and plasmids used in this study are listed in Table S1. All strains were grown in Luria–Bertani (LB) medium consisting of 10 g l^{-1} tryptone (HiMedia Laboratories, Mumbai, India), 10 g l^{-1} NaCl (Merck, Darmstadt, Germany) and 5 g l^{-1} yeast extract (HiMedia Laboratories) at 37 °C, except for strains containing pKD46 which were grown at 30 °C, with constant shaking at 160 r.p.m. Single-colony cultures grown for 8 h served as pre-inoculum cultures for all experiments. Antibiotics were used at the following concentrations: Ampicillin-100µg ml^{-1} (HiMedia Laboratories), Chloramphenicol- 30 µg ml^{-1} (HiMedia Laboratories), and Kanamycin- 50 µg ml^{-1} (Sigma Aldrich, Missouri, USA). Arabinose (HiMedia Laboratories) was used at 40 mM for induction of Red recombinase by pKD46 [15].

Generation of single- and double-gene deletion strains

S. Typhimurium 14028 s (WT) was used as the parent strain for all experiments. The Δ*cspE* strain was reported in an earlier study [15] and the Δ*cspA* strain was generated similarly and confirmed using primers that are listed in Table S2. The double deletion of *cspE* and *cspA* were generated by amplifying the region flanking *cspA* and electroporating the amplicon into Δ*cspE* cells harboring pKD46 plasmid (Table S2). To avoid polar effects, antibiotic resistance cassettes were removed by pCP20 transformation. All gene deletions were confirmed by PCR amplification using primers that to anneal ~100 bp upstream and downstream of the gene [15].

Cloning of genes for complementation and over-expression

cspE and *cspA* cloning was performed as mentioned by Ray *et.al.,* 2019 [15]. The positive clones (pcspE or pcspA) and control vector ptrc99A (VA) were then transformed into *S.* Typhimurium WT and Δ*cspE* by electroporation, to generate the following strains: WT/VA, Δ*cspE*/VA, WT/pcspE, Δ*cspE*/pcspE, WT/pcspA and Δ*cspE*/pcspA respectively.

Development of biofilms

Experiments were performed using overnight grown cultures, normalised to 0.1 O.D. (600 nm). Twelve well tissue culture grade sterile microtitre plates (Tarsons, Korea) was used for the plastic based substrate, biofilm assays. On the other hand, 5 mg ml^{-1} cholesterol coated glass cover slips were placed into the same microtitre plates and used for the cholesterol-based substrate biofilm assay. Then 1.5 ml LB without NaCl was used as the culture medium, and changed every 24 h. Unless otherwise mentioned, the experiments were carried out at 27 °C, for 5 days under stagnant conditions [55].

Quantitation of biofilms

Planktonic cells were removed without disturbing the biofilm formed on the substratum. Three washes with 1× PBS were performed to remove any un-adhered cells. Biofilms were then dried, and heat fixed for 1 h at 60 °C. Then 0.33% crystal violet was used to stain the biofilms for 5 mins at room-temperature. The excess stain was removed by three 1× PBS washes. The bound stain was then extracted with 33% acetic acid and absorbance was measured at 570 nm. Images for crystal violet stained biofilms were acquired at 20× magnification, prior to stain extraction [27].

Atomic force microscopy imaging

Biofilms grown on cholesterol coated coverslips were removed on day 5 post the start of the experiment. Then 3–4 MilliQ water washes were performed to remove any non-biofilm cells. The samples were then air-dried at room temperature in a laminar flow hood for 1 h. The coverslips were then fixed onto a magnetic stub using double-sided carbon tape and transferred to the AFM stage for imaging. All AFM measurements were performed using an NX-10 atomic force microscope (Park Systems). A high-force-constant (~40 Nm−1) silicon AFM probe tip (Acta; Park Systems) was used with a resonating frequency of 300 kHz. The AFM instrument (Bioengineering facility) was operated in the non-contact mode [56].

Scanning electron microscopy imaging

Biofilms were grown on cholesterol coated coverslips for 5 days as previously mentioned. After washing, the biofilms were air-dried for 1–2 h, fixed over-night with 2.5% glutaraldehyde in PBS. The excess fixative was removed by 2–3 washes of MilliQ water. The biofilms were then dehydrated in a gradient of 30–100% ethanol, with 3 mins treatment in each ethanol grade. The films were then dried in a laminar flow, attached to an aluminium stub with carbon tape, 5 nm gold-sputter coated for 19 secs and imaged using the Sirion SEM (AFMM Facility) in high vacuum at 10000×.

Biofilm fitness assay

GFP harbouring WT 14028 s (WT/GFP) and RFP harbouring deletion strains (Δ*cspE*/RFP, Δ*cspA*/RFP and Δ*cspE*Δ*cspA*/RFP) were cultured over-night in the presence of the appropriate antibiotics. Ratios of inoculum were then adjusted according to the O.D. (600 nm), and inoculated in low osmolarity LB (without NaCl) in cholesterol coated cover-slip containing 12 well tissue culture grade polystyrene petri-plate (Tarsons, Korea). Biofilm

formed coverslips were removed on day 5 and processed as mentioned in the confocal microscopy protocol (Supplementary information).

Gene expression using quantitative Real Time-PCR (qPCR)

The methodology followed was similar to that of Ray *et al.* 2019 [15] and primer sequences are listed in Table S3. In the 2 and 5 day old WT alone biofilm assays, absolute amounts of the transcripts in each strain were represented. However, in the 2 and 5 day old WT and Δ*cspE* biofilm assays, the WT biofilm cells at each time-point (2 and 5 days) was normalised to 1 [ΔC_T (target gene- housekeeping gene) for WT was calculated and this was divided by itself. The resultant value was raised as a negative to the power of two to yield the $2^{-\Delta\Delta C_T}$ value for the WT strain, which would be one. For the rest of the strains and conditions, the numerator of ΔC_T (target gene- housekeeping gene) was from the strain itself and the denominator of ΔC_T (target gene- housekeeping gene) was that from the WT strain for the same gene. The resultant value was then raised as a negative to the power of two to obtain the $2^{-\Delta\Delta C_T}$ value or fold difference of the gene expression between the target strain and the WT]. Similarly, for quantification of transcripts in 12 and 18 h grown swarming cells, the WT cells at each time-point (12 and 18 h) was normalised to one, and transcript levels in all other samples were calculated as fold-change to this reference value.

Motility assays

For the swimming assays, freshly made 0.3% soft agar (0.3 g per 100 ml LB) was kept at 55 °C until the beginning of the assay. Then 20 ml of soft agar were poured in Petri dishes and dried at room temperature under sterile conditions. The soft agar plates were then inoculated with 2 µl of overnight culture normalised to 1 O.D. (600 nm), in the middle of the plate. The plates were incubated at 37 °C and images were acquired at 6 and 12 h. For swarming assays, 20 ml of freshly made 0.5% nutrient agar (0.5 g per 100 ml LB) with 0.5% glucose were poured for Petri dishes. After drying at room temperature, the plates were inoculated with 5 µl 0.1 O.D. (600 nm) of respective overnight culture. The plates were incubated at 28 °C and images were taken after 12 and 18 h, with ImageQuant LAS4000 (GE Healthcare). The motility experiments were performed as reported in [27] and diameters were estimated using Gwyddion (Version 2.51) [57].

Statistical analysis

All data was analysed using the GraphPad Prism (Version 6.0 c). For analysing biofilm growth in terms of crystal violet quantitation and comparison of steady-state qPCR quantification, one-way ANOVA was performed. All crystal violet staining data is represented as mean±SEM. All qPCR data is represented as mean±SEM. All experiments have been performed in biological and technical triplicates.

RESULTS

S. Typhimurium encoded *cspE* is induced during biofilm formation

Salmonella is known to form matrix-encased biofilms on abiotic and biotic surfaces [58, 59]. Human gallstones and cholesterol-coated surfaces enable formation of a robust biofilm and an *in vitro* system of cholesterol coated surface mimics the *in vivo* gallstones [58]. To study the roles of CSPs, we established a system of robust biofilm formation of *S.* Typhimurium 14028 s, using cholesterol coated cover slips. Next, we utilised qPCR to enumerate the absolute levels of CSP transcripts in the biofilm grown cells in a kinetic manner, i.e. on days 2 and 5. *csgD* was used as the positive control to denote appropriate gene level regulation of biofilm formation [60]. *cspA, cspB* and *cspD* transcripts were downregulated by day 5, whereas *cspH* was unaffected and only *cspE* transcripts were induced (Fig. 1).

S. Typhimurium lacking *cspE* is impaired in biofilm formation

To delineate the role of CspE in biofilm formation, a *cspE* deletion strain (Δ*cspE*) was utilized, as previously reported [15]. The WT strain showed robust biofilm formation in a kinetic manner; however, Δ*cspE* demonstrated significantly impaired biofilm formation even on day 5 of growth (Fig. 2a). To confirm the importance of CspE-dependent biofilm formation, a *cspE* complementation approach was utilized (pcspE –*cspE* was expressed in the pRS424 plasmid under the control of its native promoter). As seen in Fig. 2b, WT/pcspE formed more biofilm on day 2, compared to the vector transformed control (WT/VA). The Δ*cspE*/pcspE showed growth rescue as compared to Δ*cspE*/VA at the early time-point itself. The extent of biofilm formation in Δ*cspE*/pcspE on day 5 was similar to that of WT/pcspE indicating a complete suppression of the mutant phenotype.

To gain further insights into the mechanism of CspE, we utilized the F30V mutant of CspE which has been shown to lack substrate nucleic acid binding properties [15]. In the earlier study, we had shown that the F30 residue in RNP2 motif of CspE was essential for its *yciF* mRNA stabilization function, thereby effectuating bile resistance. In the present study, crystal violet quantitation assays revealed that the WT, but not the F30V mutant, was able to rescue the phenotype of Δ*cspE*. In fact the F30V mutant behaved like a dominant negative and partially reduced biofilm formation in the WT (WT/pcspE-F30V) (Fig. 2b). These observations demonstrated that the nucleic acid binding function of CspE was essential for biofilm formation and mediated by the F30 residue.

CspE negatively regulates CspA during biofilm formation

CspD in *E. coli* is involved in biofilm formation and is known to negatively impact growth of the bacteria [61]. To identify the possible involvement of other CSPs in the biofilm pathway of CspE, the transcripts of all CSPs were compared between the biofilms of WT and Δ*cspE*. Only *cspA* transcripts were significantly up-regulated in Δ*cspE,* on both day 2 and 5 of

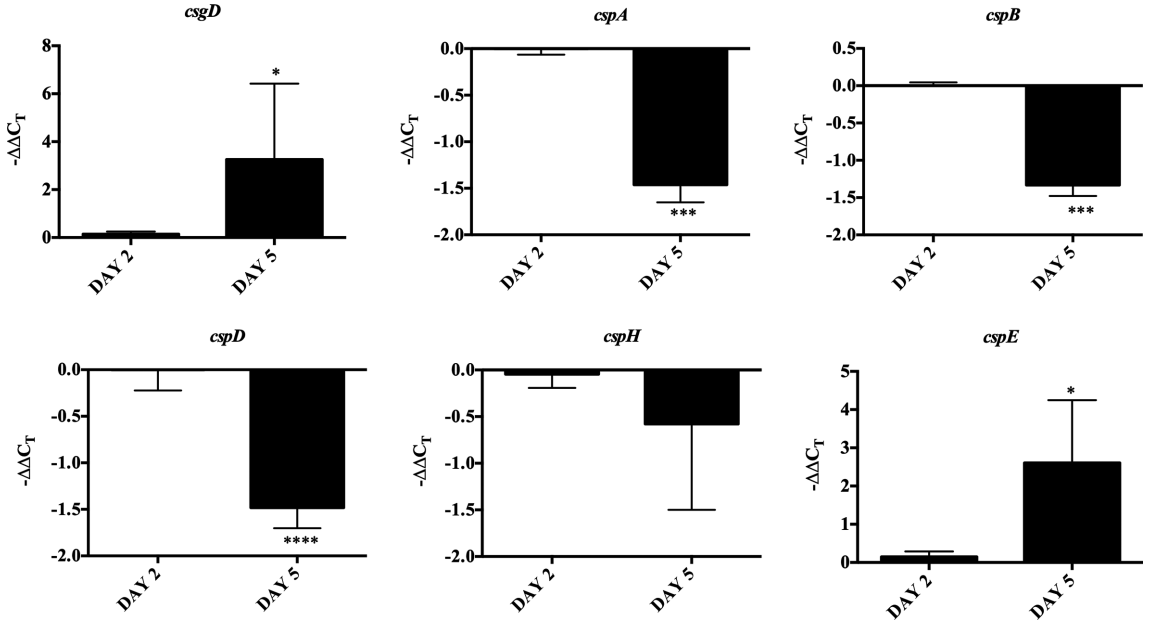


Fig. 1. *cspE* is induced during biofilm formation in *S*. Typhimurium. qPCR estimation of transcript amounts of cold shock proteins from biofilm (days 2 and 5) cells. The amounts of the transcripts has been quantitated by the $-\Delta\Delta C_T$ method and plotted as mean of values from each time-point. Data is representative of three independent experiments and presented as mean±SD. **P*<0.05, ****P*<0.001, *****P*<0.0001

growth (Fig. 3a). Congruent with literature, it appeared that *S*. Typhimurium CspE negatively regulates CspA [62]. To ascertain the roles of *S*. Typhimurium CspA in biofilm formation, a *cspA* single deletion (Δ*cspA*) strain and a double deletion of *cspE* and *cspA* (Δ*cspE*Δ*cspA*) were generated. All four strains did not show any difference in growth under ambient conditions (Fig. 3b). Biofilm formation estimated on day 5 of growth revealed that Δ*cspA* formed a robust biofilm comparable to that of the WT. The Δ*cspE*Δ*cspA* showed a modest biofilm formation, lesser than the WT but greater than that of Δ*cspE,* indicating a partial rescue of the attenuated phenotype of Δ*cspE* (Fig. 4a). Overall, it appeared that high amounts of CspA contributed to the negative effect of CspE deletion during biofilm formation.

cspA over-expression lowers biofilm formation in *S*. Typhimurium

Owing to the partial rescue in biofilm observed upon *cspA* deletion in the *cspE* deleted background, it is possible that in the absence of *cspE, cspA* has a negative effect on biofilm formation. To further ratify this hypothesis, the *cspA* over-expression system [15] was utilized. Although *cspE* complementation demonstrated a positive impact on biofilm formation in both WT and Δ*cspE,* the *cspA* over-expression system displayed a negative effect. The WT/pcspA strain showed markedly reduced biofilm formation, almost to the same level as that of the Δ*cspE*. However, there was no added effect of *cspA* over-expression in the Δ*cspE* strain (Δ*cspE*/pcspA) over and above that of Δ*cspE* (Fig. 4b). It is likely that CspE down-regulates CspA to positively modulate biofilm formation in *S*. Typhimurium.

cspE imparts competitive fitness for biofilm formation to *S*. Typhimurium

Natural biofilms are multi-species, which have distinctly structured and spatially defined communities [63]. One of the most common forms of interactions in multi-species biofilm is competition [64]. Competition majorly implicates the fight for survival, where a more robust strain is likely to outcompete a weaker strain. In the present context, we analysed competition behavior by utilizing the WT (robust strain) and the deletion strains (weaker strains) in a mixed inoculum biofilm formation context. Images acquired on day 5 of growth, indicated that the Δ*cspE* or Δ*cspE*Δ*cspA* did not have any advantage compared to the WT when present in equivalent proportions (Fig. 5). However, when present in equal proportion with the WT, Δ*cspA* was as robust as WT in competing for biofilm formation. Overall, it appears that *cspE* deletion attenuates the ability of *S*. Typhimurium that even excess of it cannot form biofilm by utilizing the structural components of the other species or strain.

Extra-polymeric substances (EPS) are unaffected in Δ*cspE.*

It was important to assess whether EPS components were affected in Δ*cspE* during biofilm formation as the major bulk of organic mass is made up of a meshwork of polymeric extracellular material [65]. The *S*. Typhimurium EPS includes varied proteinaceous components such as adhesive fimbriae (Type I, curli etc.), a large surface protein BapA, flagella, exopolysaccharides such a cellulose, colonic acid

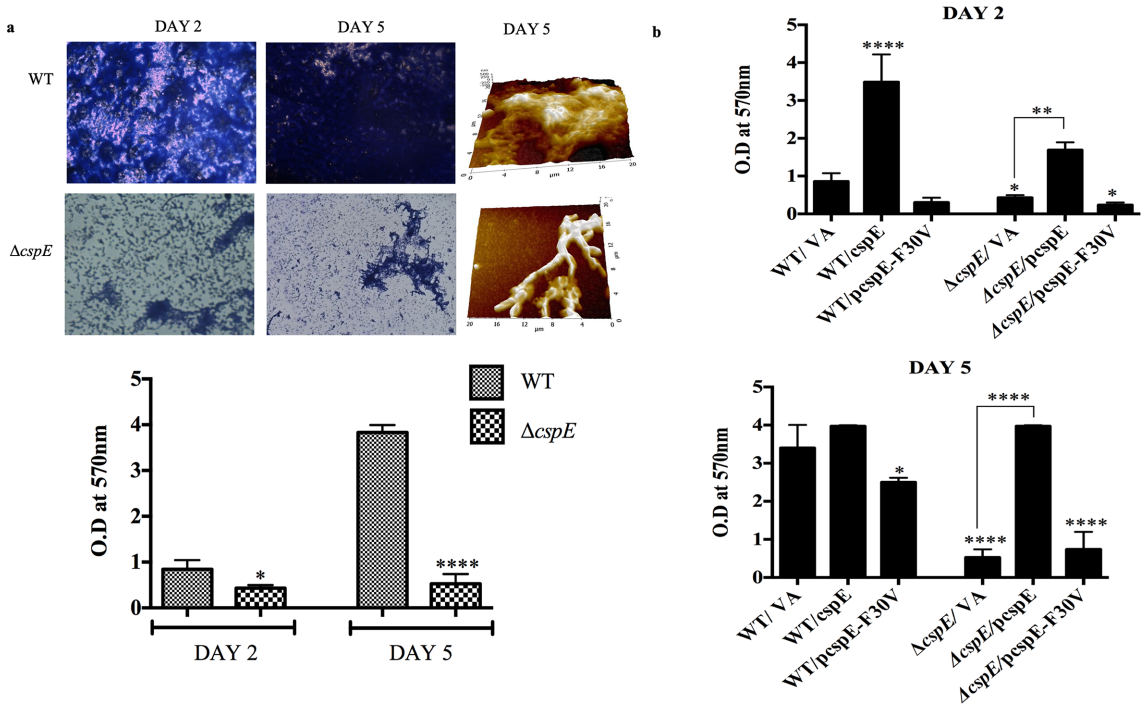


Fig. 2. CspE is essential for biofilm formation in *S.* Typhimurium. (a) Quantitative estimation of crystal violet stained biofilms and representative atomic force microscopy (images) of 5 day old biofilms of WT and Δ*cspE*. (b) Quantitative estimation of crystal violet staining of biofilms of WT/VA, Δ*cspE*/VA, *cspE* complemented strains (WT/pcspE and Δ*cspE*/pcspE) and CspE-F30V complemented strains (WT/pcspE-F30V and Δ*cspE*/pcspE-F30V) on cholesterol coated glass cover-slips. Data is representative of three independent experiments and presented as mean±SEM. **P*<0.05, ***P*<0.01, *****P*<0.0001

and the O-Ag capsule, fatty acids for lipid anchors of the exopolysachharides and more recently extracellular DNA [66]. The most widely used assay of biofilm formation is the rdar morphotype; which mainly detects the important biofilm components, curli and cellulose. In the present study assay with the WT and three deletion strains, showed comparable and equivalent rdar phenotype (Fig S1 a, b). Furthermore, yeast agglutination assay to detect the expression of Type I fimbriae also yielded identical results in all the four strains (Fig. S1 c). These observations denoted that Δ*cspE* did not affect any major EPS components.

cspE deletion affects transcript levels of the genes of the class III operon of the flagellar regulon

Motility has been reported to be essential in the early stages of biofilm formation: cells with the most robust biofilms also showed vigorous motility and lack of motility affected biofilm architecture [67]. Motility is required early, perhaps, to locate a suitable environment or surface on which to form a biofilm. The progressive inhibition of motility is often thought to be an essential event in biofilm formation [65]. Motility inhibition by transcriptional repression of the flagellar operon is slow but may be important for the long-term stability of the biofilm. The complex network of flagellar genes is regulated majorly at the transcriptional level, with them being classified into three major regulons namely class I, II and III. To study the involvement of the flagellar genes, we resorted to qPCR analysis. Representative genes from each of the classes were tested for in motility assays of WT and Δ*cspE*. Of the class I genes, the representative *flhD* appeared to have similar levels of transcripts in both strains. Successively, the Class II representative genes, namely *fliA*, *fliL*, *fliM* also showed comparable levels in both the strains. Strikingly, *fliC*, *yhjH*, *cheY* and *motA*, the representative class III genes showed significantly lesser transcripts in the Δ*cspE* compared to the WT (Fig. 6). Although the lass III flagellar genes are transcriptionally regulated by the sigma factor *fliA* (whose levels were comparable in the two strains), there might be an additional role of CspE since, in its absence, the transcript levels of class II flagellar genes are negatively affected.

Δ*cspE* exhibits slow swimming and no swarming motility in a *cspA*-independent manner

Flagella-mediated motility is of two types swimming (movement in a fluid) and swarming (surface movement). While swimming is essential for the initial migration to the substratum, swarming motility is important for aiding surface movement to enable microcolony formation [65]. Therefore, both motilities were assayed in a kinetic manner. WT and Δ*cspA* exhibited similar swimming and swarming motility. The *cspE* deletion strains (Δ*cspE* and Δ*cspE*Δ*cspA*) showed slower swimming motility (Fig. S2) at the early time-point but were able to draw level with the WT at the later time-point.

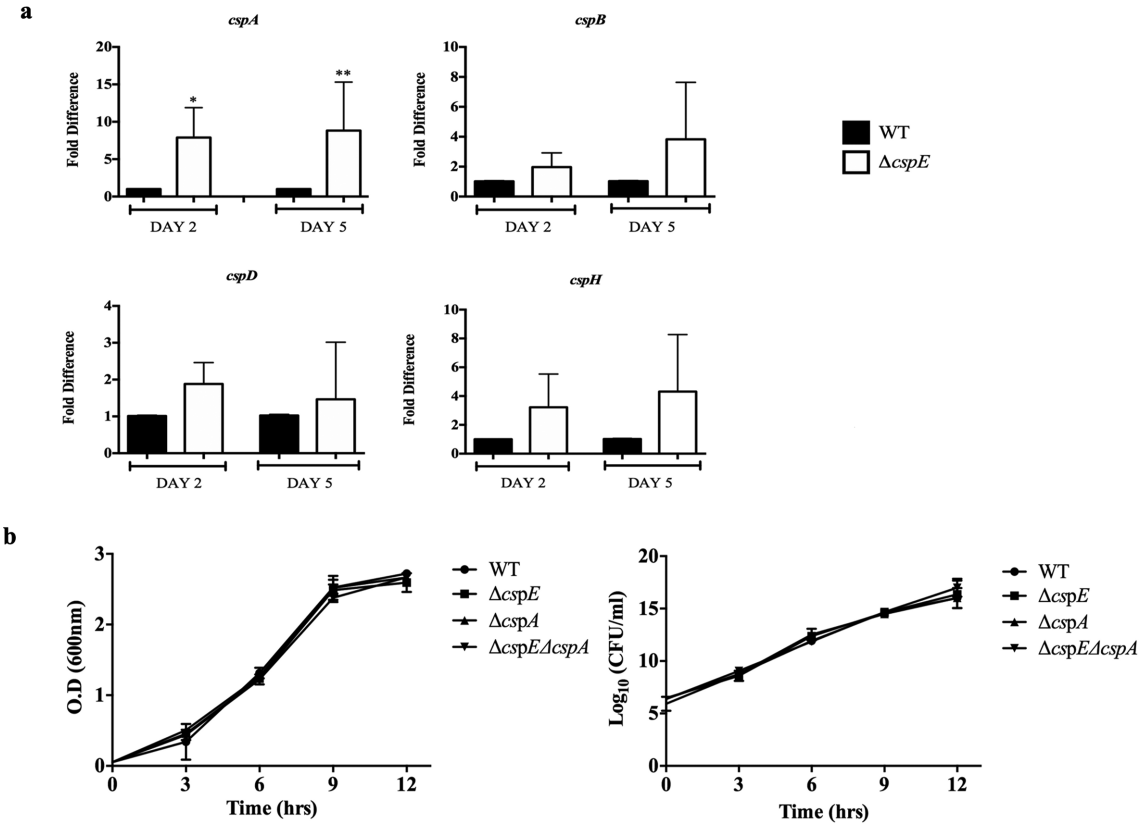


Fig. 3. CspE negatively regulates *cspA* expression during biofilm formation. (a) qRT-PCR estimation of transcripts of CSPs from biofilms (days 2 and 5) of WT and Δ*cspE*. In the panels, values for WT at the respective time-points is normalised to one and fold difference is compared to these values. Data is representative of three independent experiments and presented as mean±SEM. **P*<0.05, ***P*<0.01. (b) Kinetic growth analysis of WT, Δ*cspE*, Δ*cspA* and Δ*cspE*Δ*cspA* was conducted in terms of O.D. (600 nm) and Log_{10} c.f.u. ml^{-1} over a period of 12 h of growth.

However, they exhibited no swarming motility at both time-points (Fig. 7a).

To understand whether the CspE mediated biofilm regulatory pathway overlapped with the regulation of swarming motility, the *cspE* complementation (pcspE) and *cspA* over-expression system (pcspA) was used. The *cspE* complementation showed a positive effect on swarming motility, rescuing the abrogated motility in Δ*cspE*. However, *cspA* over-expression did not show any effect on the motilities of WT or Δ*cspE* (Fig. 7b). These observations suggested a probable hypothesis that CspE might be a central regulator of multiple pathways. Its biofilm regulator pathway may involve the down-regulation of CspA to aid biofilm formation, while regulation of swarming motility was independent of CspA (Fig. 8).

DISCUSSION

In response to nutrient limitation and stressful conditions, many microorganisms including *Salmonella* form biofilms. Multiple proteins and pathways have been reported to regulate biofilm formation. In the present study, the roles of *S.* Typhimurium encoded CspE and CspA during biofilm formation was addressed. *S.* Typhimurium 14028 s formed better and kinetically faster biofilms on a cholesterol coated surface, which is implicated in mimicking the *in vivo* situation of gallstones [55, 58]. Transcript analysis of the CSPs from biofilm cells, demonstrated that only *cspE* was induced whereas *cspA*, *cspB* and *cspD* were down-regulated (Fig. 1). Further confirmation of CspE in biofilm formation was obtained using Δ*cspE* which showed diminished biofilm growth (Fig. 2a). In *Salmonella* enterica SL1344 strain, both CspC and CspE are required for resistance to stress, biofilm formation etc. [27]; however, this is not the case with the 14028 s strain [15]. Importantly, complementation with WT CspE, but not the F30V mutant, recued biofilm formation by Δ*cspE* (Fig. 2b). In the bile resistance regulatory pathway of CspE, we had identified the indispensable role of the F30 residue for the nucleic acid substrate binding of CspE. In this study, the F30V mutant of CspE also showed a compromised phenotype in the biofilm regulatory pathway of CspE; most likely, the RNA chaperoning activity of CspE is mediated by substrate nucleic acid binding [15].

Naturally occurring biofilms are usually multi-species [64] and entail production and sharing of products. In such multi-species biofilms, cheater populations or EPS non-producers

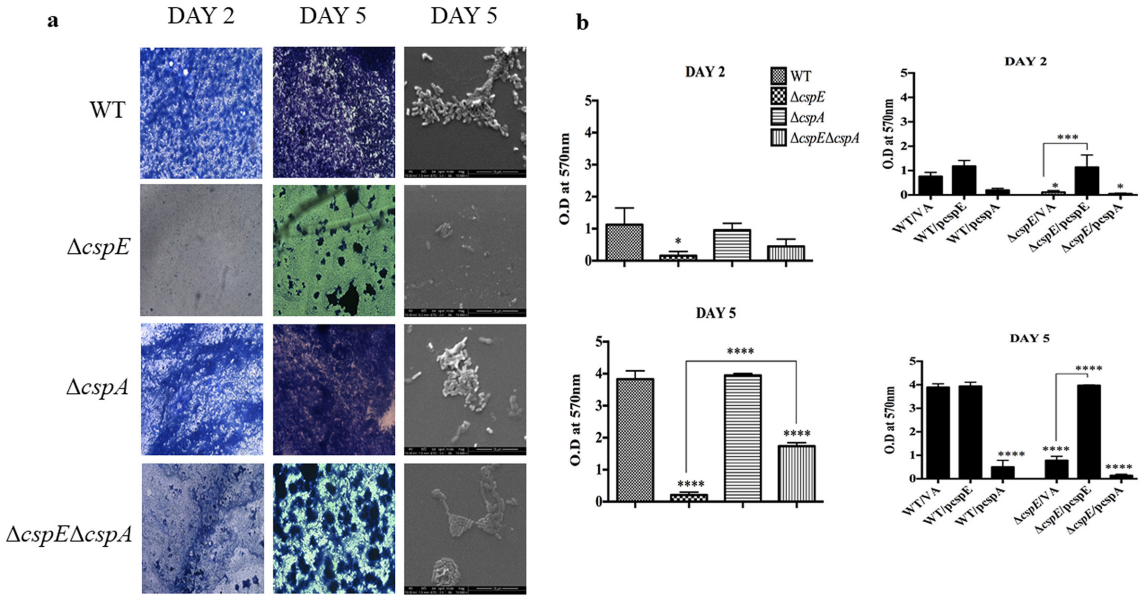


Fig. 4. Higher expression of CspA lowers biofilm formation in *S.* Typhimurium. (a) Representative Scanning Electron Microscopy (day 5) and quantitative estimation of crystal violet staining of biofilms of WT, Δ*cspE*, Δ*cspA* and Δ*cspE*Δ*cspA* grown on cholesterol coated cover slips in 24 well tissue culture grade plates. Quantitative estimation of crystal violet stained (days 2 and 5) images of biofilms at 28 °C of WT/VA and Δ*cspE*/VA with *cspE* complementation (WT/pcspE and Δ*cspE*/pcspE) and cspA over-expression (WT/pcspA and Δ*cspE*/pcspA). The data is representative of three independent experiments and presented as mean±SEM. **P*<0.05, ****P*<0.001, *****P*<0.0001

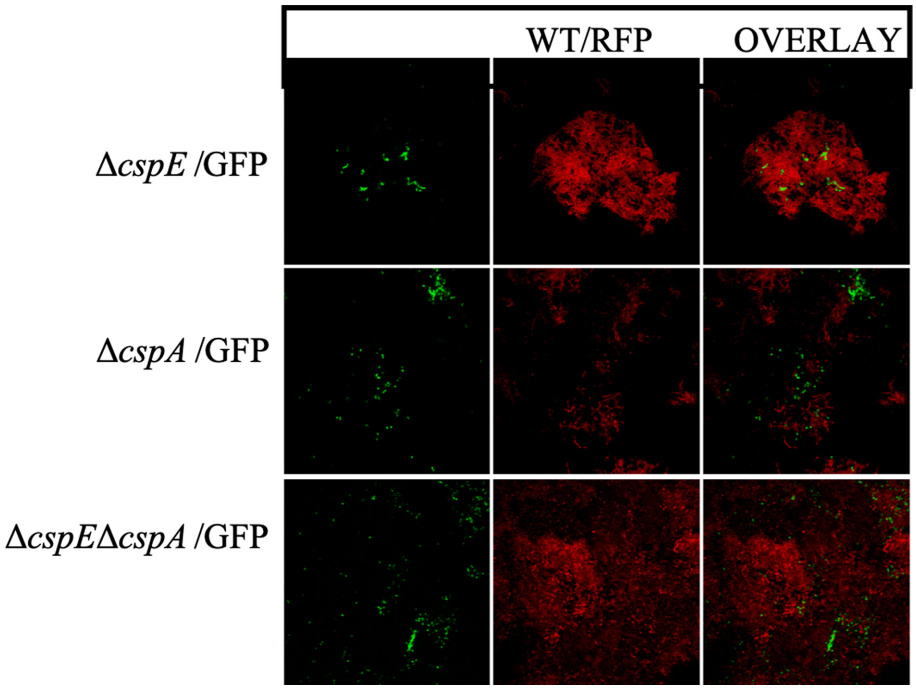


Fig. 5. CspE increases the *in vitro* biofilm fitness of *S.* Typhimurium. Representative Confocal images (100×,Zeiss LSM Meta) from a 5 day old biofilm, grown on cholesterol coated coverslip. WT harboured RFP expressing plasmid (WT::RFP), while Δ*cspE*, Δ*cspA* and Δ*cspE*Δ*cspA* expressed plasmid encoded GFP (Δ*cspE*::GFP, Δ*cspA*::GFP, Δ*cspE*Δ*cspA*::GFP). Strains were grown in the indicated ratios, where one indicates a c.f.u. equivalent of 0.1 O.D. Data is representative of three independent experiments.

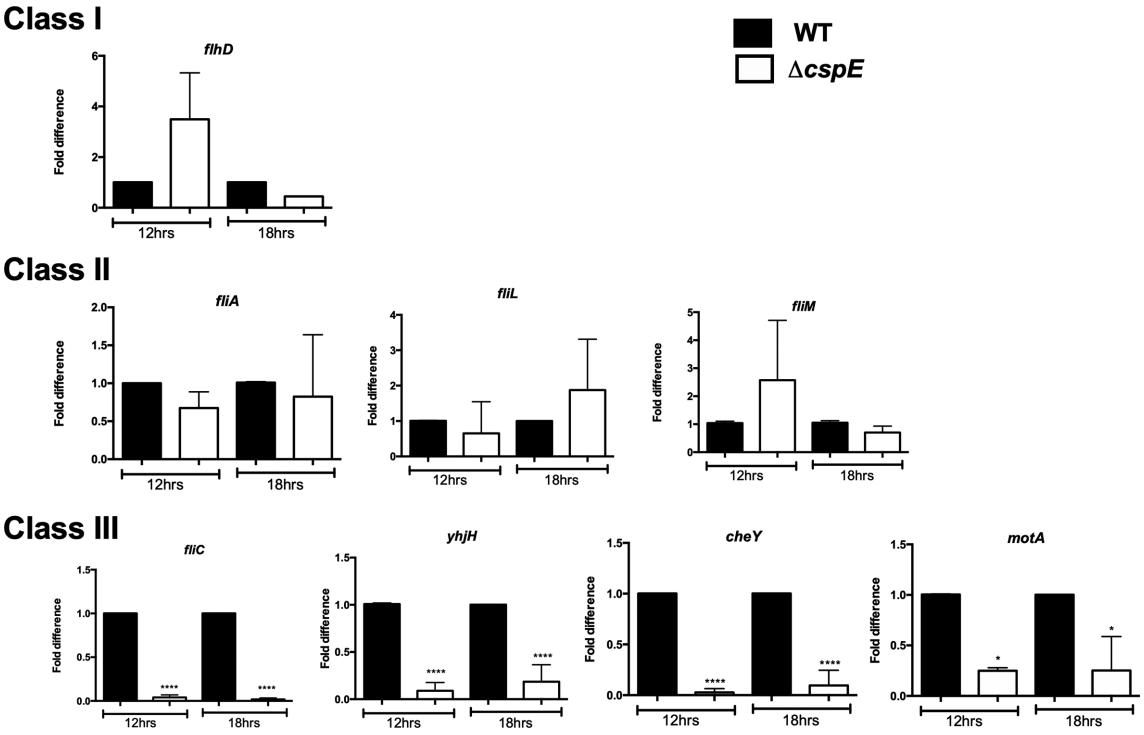
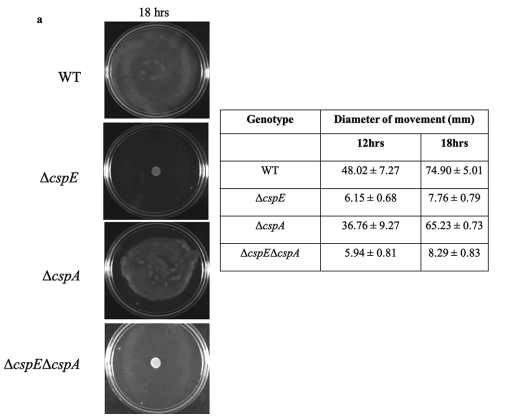
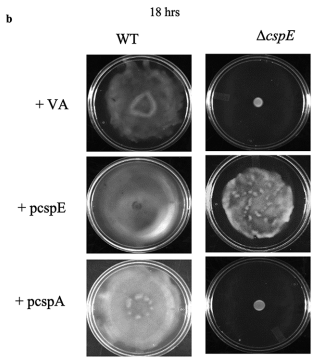


Fig. 6. *cspE* deletion lowers transcription of class III flagellar genes. qPCR of transcripts of representative genes of class I, class II and class III of the flagellar regulon. Cells from 12 h and 18 h swarming plates of WT and Δ*cspE* were used. In the panels, values for WT at the representative time points were normalized to one and fold differences was compared. The data is representative of three independent experiments and presented as mean±SD. **P*<0.05, *****P*<0.0001

exist, which utilize the EPS of the EPS-producers. Often these cheaters may outcompete the EPS producers owing to their preferable growth closer to the substratum [68], expending less energy in producing essential products [69] etc. In the present context, we enquired whether Δ*cspE* or Δ*cspA* could behave as a 'cheater' in competition with the WT. Δ*cspA* manifested robust competitive biofilm formation, since it reduced the biofilm of the WT, when present in equal number. However, the day 5 biofilm imaging observations indicated that Δ*cspE* was incapacitated in biofilm formation (Fig. 5).



Genotype	Diameter of movement (mm)	
	12hrs	18hrs
WT	48.02 ± 7.27	74.90 ± 5.01
Δ*cspE*	6.15 ± 0.68	7.76 ± 0.79
Δ*cspA*	36.76 ± 9.27	65.23 ± 0.73
Δ*cspE*Δ*cspA*	5.94 ± 0.81	8.29 ± 0.83



Genotype		Diameter of movement (mm)	
		12hrs	18hrs
+VA	WT	50.07 ± 5.83	73.49 ± 3.81
	Δ*cspE*	6.21 ± .48	7.82 ± 0.56
+pcspE	WT	47.96 ± 8.51	74.33 ± 4.80
	Δ*cspE*	37.20 ± 10.82	62.07 ± 2.02
+pcspA	WT	42.67 ± 10.94	73.06 ± 4.43
	Δ*cspE*	7.23 ± 0.38	7.95 ± 0.82

Fig. 7. Deletion of *cspE*, but not *cspA*, reduces swimming and abolishes swarming motility in *S.* Typhimurium. (a) Swarming (0.5% Agar +0.5% glucose; 12 and 18 h) motility of indicated strains are shown. (b) Swarming motility of WT/VA and Δ*cspE*/VA with *cspE* complementation (WT/pcspE, Δ*cspE*/pcspE) and *cspA* over-expression (WT/pcspA, Δ*cspE*/pcspA). Overnight grown cultures were inoculated on the agar plates and images were acquired at the respective time-points. Data is representative of three independent experiments and presented as mean±SEM.

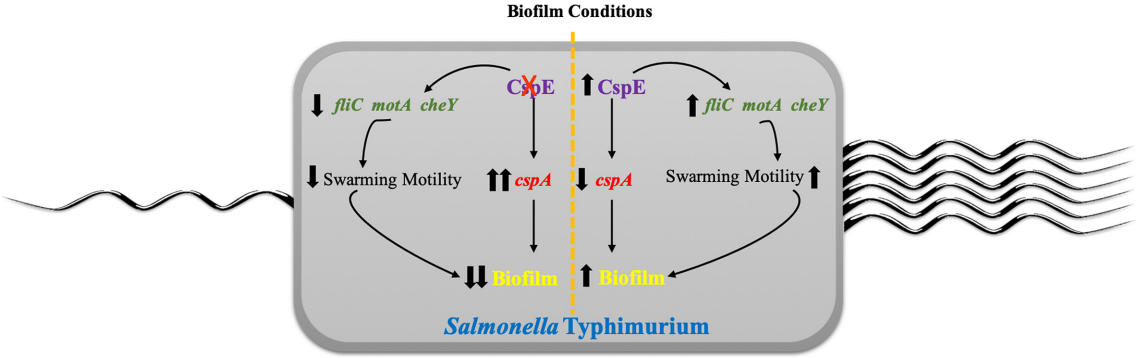


Fig. 8. *S.* Typhimurium CspE is essential for biofilm formation and motility. CspE regulates biofilm formation through the down-regulation of another cold shock protein, CspA. However, its regulation of swarming motilityis independent of CspA.

To identify downstream regulators of CspE during biofilm formation, more specifically for the role of any other CSP; transcript analysis revealed a significant and kinetic up-regulation of CspA (Fig. 3a) in Δ*cspE*. It has been previously reported in *E. coli* that CspE negatively regulates CspA [62]. The probable mechanism of transcriptional repression of CspA by CspE involves physical binding of CspE to the cold box region which is located in the 5' proximal part of 5′-UTR of *cspA* mRNA. The CspE mediated repression of CspA can be further contributed by increased promoter-proximal pausing of RNA polymerase, just downstream of the *cspA* cold box sequence [62, 70]. Also, a study showed that CspA, CspB and CspD clustered together, based on PCA analysis of RNA ligand profiling indicating that these proteins might bind to similar ligands [27]. CspD in *E. coli* has been shown to be associated with biofilm and persister cell formation [71] and high levels of the protein is toxic to the cells [61]. However, in our study we did not see any changes in CspD or CspB transcripts. Therefore, we focused on delineating the functional roles of CspA by means of gene deletion and multicopy over-expression studies. The single deletion of CspA (Δ*cspA*) did not affect biofilm growth of *S.* Typhimurium; however the double deletion strain, i.e. Δ*cspE*Δ*cspA*, exhibited higher biofilm formation compared to Δ*cspE* (Fig. 4a). Although differences were observed in biofilm formation in these four strains (Fig. 4), there was no difference in growth in LB (Fig. 3b). Also, multicopy over-expression of CspA reduced the biofilm of the WT strain (Fig. 4b). Juxtaposing the observations, it appears from Fig. 1 that under biofilm conditions, the only CSP that is positively regulated is CspE while the other CSPs appeared down-regulated. Following which we observed attenuation in biofilm formation upon deletion of *cspE* (Fig. 2a). To elucidate the roles of other CSP in this context we observed that the CspA transcripts were up-regulated in the Δ*cspE* strain (Fig. 3a). To specifically characterize the role of CspA we performed deletion studies. While the single deletion of *cspA* (Δ*cspA*) did not have any effect on biofilm formation, the deletion of *cspA* in the background of Δ*cspE* had a beneficial effect, indicating a negative role of CspA in biofilm regulation (Fig. 4a). This conclusion was verified with the over-expression study of CspA, which reduced biofilm formation in the WT strain (Fig. 4b). Therefore, we believe that in *S.* Typhimurium 14028 s strain, up-regulation of CspE and down-regulation of CspA is an essential early step in biofilm formation.

In an attempt to identify regulators influencing the biofilm formation, we tested for the rdar morphotype components. The rdar morphotype assay in *Salmonella* is a convenient and robust way to detect the EPS components curli and cellulose [72]. None of these factors showed any difference between the WT and Δ*cspE* (Fig. S1 a–c). Apart from the EPS, which has been reported to be essential for the substrate binding and microcolony formation, motility also plays an essential role. Biofilm formation is considered a stress response wherein bacteria swim away from stressful regions to form aggregates to protect from the stressful environment, further reiterating the necessity of motility to form biofilms [73]. Although motility and biofilm formation represent two opposing lifestyles [74], the transition between these is not stark. There are common molecular determinants such as flagella which assist in facilitating the gradual switch from motile to non-motile state. In the initial attachment and colonisation phase of biofilm formation, flagella mediated motility is important to enable the surface attachment. In *E. coli*, it has been observed that cells lacking flagella due to *fliC* deletion in or having inactive flagella due to a deletion in different *mot* genes are compromised during the early stages of biofilm formation. In fact, flagellar genes are indispensable for establishing the initial attachment and movement of bacterial cells on a surface [67, 75]. In case of *Salmonella*, *fliC* deletion mutants are impaired in their capacity to attach on cholesterol coated surfaces [76], broiler skins [77] or plant cell wall surfaces [78]. It is likely that motility is required until the bacteria encounters a surface, and then aggregates, to form a biofilm, thus changing the lifestyle from motile to lower motility. In the absence of motility, cells are unable to encounter a proper surface or even form aggregates in the initial stages [27, 74]. The transition from motility to lower motility entails the reduction of flagella mediated motility [67]. A major mode of motility regulation is by the transcriptional modulation of

Table 1. CspE and CspA dependent biofilm and motility phenotypes in *S.* Typhimurium

Deletion strains						
	WT		Δ*cspE*	Δ*cspA*	Δ*cspE*Δ*cspA*	
Biofilm	++		–	++	+/-	
Swarming Motility	++		–	++	–	
Over-expression strains						
	Vector control		*cspE* over-expression		*cspA* over-expression	
	WT/ VA	Δ*cspE*/VA	WT/pcspE	Δ*cspE*/pcspE	WT/pcspA	Δ*cspE*/pcspA
Biofilm	++	–	++	+	–	–
Swarming Motility	++	–	++	++	++	–

In all, except one qPCR graph there is a normalization step of the WT to one. Changes in other strains have been calculated as fold differences to the WT and graphed. In Fig. 1 where the absolute values of the qPCR changes have been mentioned, the antilog of $-\Delta\Delta C_T$ was not calculated. We intended to show the pattern of gene regulation in each strain at various time points and hence the normalization step was avoided.

the flagellar genes [51] which are arranged into three hierarchies of regulons, namely I, II and III. Mutations in the major genes of the flagellar regulon have been shown to severely attenuate biofilm formation [75]. Further regulations occur at the levels of chemotactic response [79] or regulation of the speed of the flagellar motor [80]. In the *S.* Typhimurium biofilm, sessility and motility are inversely regulated by the c-di-GMP signaling network via the PilZ domain containing proteins BcsA (cellulose synthase) and YcgR (regulator of flagellar rotation) [81, 82].

Functional regulators of the flagellar genes are relatively poorly understood. The roles of CspE as a plausible regulator was determined in terms of the transcript levels of the flagellar genes, during the CspE mediated biofilm formation pathway. The class I and II flagellar genes appeared similar in the 5 day old WT and Δ*cspE*. However, the class III flagellar gene transcripts were significantly lesser in Δ*cspE* (Fig. 6). The class III flagellar genes form the structure and motor of functional flagella. In the absence of the major flagellin protein FliC and the motor protein MotA, it is possible that Δ*cspE* contains fewer flagella, which affects motility, thereby attenuating biofilm formation. To address the functional effect of changes in the amounts of the flagellar genes, both swimming and swarming motility studies were performed. Swimming motility is essentially the movement of a single bacterium in a liquid environment using rotating flagella. On the other hand, swarming motility is characterized by surface movement of hyperflagellated, multicellular groups of bacteria, often requiring a chemotactic signal [74]. Experiments in 0.3% agar which represents swimming motility, demonstrated slower swimming motility at the initial time-point, in Δ*cspE*. However, this difference was overcome at the late time-point (Fig S2). In the context of swarming motility, Δ*cspE* and Δ*cspE*Δ*cspA* showed a non-motile phenotype (Fig. 7a). In an attempt to invoke the role of CspE and CspA, we utilized the multi-copy over expression systems. While CspE complementation rescued the attenuated swarming motility of Δ*cspE,* the over-expression of CspA had no effect (Fig. 7b). These observations indicated that the swarming motility pathway was a CspE-dependent but CspA-independent (Fig. 8) (Table 1).

Biofilms are also associated with antimicrobial resistance [83], combating host defense [84], impeding desiccation [85] and effects of disinfectants [86]. They pose major problems in healthcare [87], agriculture [88] and industrial settings [89]. In addition, they represent a source of food contamination events caused by spoilage due to pathogenic microbes which has received a great deal of attention. Research activities are mainly focused on elucidating the biotic and abiotic factors that influence biofilm formation and maturation, and identifying, developing, and validating novel strategies for their control. Overall, the present study sheds light on a novel regulator and roles of *S.* Typhimurium encoded CspE, in regulating biofilm through the regulation of swarming motility. It also identifies a novel role of another CSP, viz. CspA, which behaves as a negative regulator in the biofilm pathway.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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