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Research Letter

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Peptide transporter YjiY influences the expression of the virulence gene *mgtC* to regulate biofilm formation in *Salmonella*

Kasturi Chandra[1,†], Preeti Garai[1,†], Jayanta Chatterjee[2] and Dipshikha Chakravortty[1,3,*]

[1]Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore, India, [2]Department of Molecular Biophysics Unit, Indian Institute of Science, Bangalore, India and [3]Center for Biosystem Science and Engineering, Indian Institute of Science, Bangalore, India

*Corresponding author: Tel: +91-80-22932842; Fax: +91-80-23602697; E-mail: dipa@iisc.ac.in
One sentence summary: Biofilm formation in *Salmonella* is regulated by a peptide transporter.
†These authors contributed equally to this work.
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ABSTRACT

Formation of a biofilm is one of the coping strategies of *Salmonella* against antimicrobial environmental stresses including nutrient starvation. However, the channeling of the starvation cue towards biofilm formation is not well understood. Our study shows that a carbon starvation gene, *yjiY*, coding for a peptide transporter, influences the expression of a virulence-associated gene *mgtC* in *Salmonella* to regulate biofilm formation. We demonstrate here that the mutant strain Δ*yjiY* is unable to form a biofilm due to the increased expression of *mgtC*. The upregulation of *mgtC* in the Δ*yjiY* strain correlates with the downregulation of the biofilm master regulator gene, *csgD*, and reduced levels of ATP. Our work further indicates that a *yjiY*-encoded peptide transporter may regulate the expression of *mgtC* by transporting proline peptides.

Keywords: Biofilm; *Salmonella*; peptide transporter; *mgtC*; proline; starvation

INTRODUCTION

Biofilm formation is crucial for *Salmonella* pathogenesis for resistance to the host's defenses, such as antimicrobial peptides, colonization inside the host and transmission from the asymptomatic host carrier (Prouty, Schwesinger and Gunn 2002; Rabsch *et al.* 2002; Fux *et al.* 2005; Anderson and O'Toole 2008). *Salmonella* can form a biofilm on abiotic as well as biotic substrates (Rabsch *et al.* 2002; Prouty and Gunn 2003), for example materials in contact with food items resulting in the contamination and infection of the consumer (Van Houdt and Michiels 2010). Biofilm is a major health concern as the chronic patients of *Salmonella* infection carry the pathogen in the form of biofilm (Prouty, Schwesinger and Gunn 2002; Gonzalez-Escobedo, Marshall and Gunn 2011) and become the source of drug-resistant strains (Pratap *et al.* 2012; Harish and Menezes 2011).

The transition of *Salmonella* from unicellular planktonic lifestyle to the multicellular form of a biofilm is controlled by the biofilm master regulator gene, *csgD*, which regulates the synthesis of extracellular polymeric substance (EPS) to form the biofilm matrix (Gerstel and Romling 2003; Grantcharova *et al.* 2010). *csgD* is transcribed under the cues of low osmolarity, low temperature and nutrient starvation (Stepanovic *et al.* 2004; Castelijn *et al.* 2012). The carbon catabolic profile of *Salmonella* varies from the planktonic stage to the biofilm stage, emphasizing the

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Table 1. Strains.

Strain name	Description	Reference
S. Typhimurium ATCC 14028 (STM_14028)	Wild type	Kind gift from Prof. M. Hensel (Max von Pettenkofer-Institute for Hygiene and Medizinische Mikrobiologie, Germany)
Δ*yjiY*	Knockout strain for the gene *yjiY*; Kan^r	Garai *et al.* (2016)
Δ*csgD*	Knockout strain for the gene *csgD*; Chl^r	Srinandan *et al.* (2015)
Δ*fliC*	Knockout strain for the gene *fliC*; Chl^r	Garai *et al.* (2016)
Δ*yjiY*-pQE60-*yjiY*	Complement strain for Δ*yjiY* expressing *yjiY* under the promoter of T5 present in the plasmid pQE60.	Garai *et al.* (2016)
Δ*yjiY*Δ*mgtC*	Knockout strain for the genes *yjiY* and *mgtC*; Kan^r; Chl^r	This work
Δ*yjiY*Δ*mgtC*-pUHE *mgtC*	Amp^r	Plasmid obtained as kind gift from Prof. E. A. Groisman (Yale School of Medicine); strain made for this work

relevance of metabolic status of the pathogen for biofilm formation (Kalai Chelvam *et al.* 2015). The biosynthesis and uptake of amino acids are also enhanced inside the biofilm (Hamilton *et al.* 2009) indicating that amino acids are required in these circumstances. Therefore, the formation of a biofilm by *Salmonella* is a complex process coordinated by multiple environmental and bacterial factors.

In our previous study, the carbon starvation (*cst*) genes *cstA* and *yjiY* were shown to be induced during nutrient starvation and to be involved in importing peptides in *Salmonella* (Garai *et al.* 2016). It is conceivable that such peptides may serve as the source of amino acids under biofilm-inducing conditions following starvation. Upon mining the transcriptomic data from our previous study (Garai *et al.* 2016), we found that *mgtC*, an important virulence factor, was upregulated in the absence of *yjiY*. *mgtC* is known to inhibit the synthesis of cellulose in *Salmonella* by affecting the function of bacterial F-ATPase (Pontes *et al.* 2015). As cellulose is an essential component of biofilm EPS in *Salmonella*, it raises the question of the role of *yjiY* in biofilm formation. The mutant strain Δ*yjiY* was also found to lack flagella, one of the factors required for biofilm formation in *Salmonella* (Crawford, Reeve and Gunn 2010). Hence, we chose to study the role of *yjiY*, a starvation-induced and peptide transporter-encoding gene (Kraxenberger *et al.* 2012a, Garai *et al.* 2016), in biofilm formation by *Salmonella*.

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) exhibits most of the biofilm-related traits (Steenackers, Vanderleyden and Keersmaecker 2012) and thus serves as a suitable model to study biofilm formation. By using standard methods of assessment of biofilm formation, we could establish that *yjiY* is essential for biofilm formation in *S.* Typhimurium. We show here that the upregulation of *mgtC* in the Δ*yjiY* strain leads to defective biofilm formation, possibly due to a shortage of proline peptides. The mutant strain Δ*yjiY* displays downregulation of *csgD* and decrease in ATP, which adversely affect the ability to form biofilm. This study reveals one of the mechanisms by which the nutritional status of *Salmonella* dictates its pathogenesis.

MATERIALS AND METHODS

Bacterial strains

All bacterial strains used in this work are listed in Table 1 with their genetic description. *Salmonella enterica* serovar Typhimurium strain 14028 was used as the wild type strain, and was also the parental background for all the mutant strains used in this study, i.e. Δ*yjiY*, Δ*csgD*, Δ*fliC* and Δ*yjiY*Δ*mgtC*. All strains were grown and maintained in Lennox broth (LB; 0.5% NaCl, 1% casein enzyme hydrolysate and 0.5% yeast extract) at 37°C under shaking conditions.

Generation of knockout strains

All knockout strains were generated by the one-step inactivation method based on λ recombinase-mediated recombination (Datsenko and Wanner 2000). For the double knockout strain Δ*yjiY*Δ*mgtC*, the chloramphenicol resistance gene replaced *mgtC* in the genome of the kanamycin-resistant strain Δ*yjiY*. The primers used for generating knockouts are listed in Supplementary Table S1.

Growth conditions for biofilm formation

LB without NaCl, i.e. 1% casein enzyme hydrolysate and 0.5% yeast extract, was used as biofilm medium. Overnight cultures grown in LB were subcultured in 2 ml biofilm medium at the dilution of 1:100 in a 24-well plate and incubated at 28°C for 48 h without shaking. All images of biofilm in the form of a pellicle were taken with a digital camera. To check the role of the amino acid proline in biofilm formation, 0.1, 1 and 10 mM proline was added to the biofilm medium. For co-culture experiments, wild type and Δ*yjiY* cultures were inoculated together in 2 ml biofilm medium at the ratio of 1:1. Aliquots were taken at different time points of stationary growth at 28°C and plated on LB agar for the growth of both strains. Kanamycin-containing LB agar was used for selective growth of Δ*yjiY* to calculate the individual CFU values for both strains.

Confocal microscopy

Sterile coverslips (22 × 60 mm) were placed in 50 ml sterile tubes with 10 ml of biofilm medium. Cultures were inoculated for biofilm formation as mentioned previously. After 48 h of incubation, biofilm appeared on the coverslip at the liquid–air interface, in the form of a thin line spanning the width of the coverslip (22 mm). The coverslip was washed thoroughly with water to remove planktonic cells and stained with Congo red (40 mg/ml in water) for 15 min at room temperature. After washing with water, the coverslip was mounted on a slide and imaged for biofilm distribution, with a laser scanning confocal microscope (Zeiss LSM 710) using a ×40 objective. Z stacks were taken to generate a three-dimensional image. At least 15 fields were imaged per coverslip, covering almost the entire area of the biofilm. The images were analyzed using ImageJ to obtain mean fluorescence intensity of the Congo red-stained EPS. The values obtained were plotted as percentage mean fluorescence, where the mean of wild type values was considered 100%.

Scanning electron microscopy

Biofilm was allowed to form on coverslips as mentioned in the previous section. After thorough washing with water, the sample was fixed in 2.5% gluteraldehyde for 24–96 h at room temperature. Excess gluteraldehyde was removed by washing with water and the sample was dehydrated by gradient washes in increasing concentrations of 30%, 50%, 75%, 85% and 95% ethanol. The coverslip was then air dried under vacuum before coating with gold for imaging by scanning electron microscopy.

Crystal violet staining

Crystal violet staining was used to quantify the biofilm formed on the solid surface, i.e. the wall of the well of a 24-well plate. The protocol followed for biofilm formation was the same as mentioned above. After 48 h, the cultures were discarded and the plate was rinsed thoroughly with water. The biofilm, in the form of a ring on the wall, was stained with 1% crystal violet for 15 min at room temperature. The stained biofilm was destained with 70% ethanol and the intensity of color of the destained solution was quantified by measuring absorbance at 595 nm, representing the biofilm mass.

Quantitative PCR

RNA was isolated by the TRIzol method (Sigma-Aldrich) from biofilm culture after 24 h. cDNA was synthesized with Bioline MuMLV reverse transcriptase (Tetro). Quantitative PCR was carried out using SYBR Green Q-PCR kit (Thermo Fisher Scientific). The primers used are listed in Supplementary Table S1. Relative expression in Δ*yjiY* was plotted as fold change with respect to wild type (Δ*yjiY*/WT), after normalizing with the reference gene, 16S rRNA.

Intracellular ATP measurement

One milliliter of 24 h-old biofilm culture was centrifuged at 9615g for 5 min. The pellet was resuspended in 500 μl of MilliQ, vortexed and boiled at 95°C for 10 min to release the intracellular ATP. An ATP determination kit from Molecular Probes was used for the assay and the protocol was followed as per the manufacturer's instructions. Briefly, 8 μl of the prepared sample was added to 72 μl of reaction solution containing reaction buffer, DTT, luciferin and luciferase. The amount of luminescence produced was measured using a Tecan microplate reader and the corresponding amount of ATP was calculated using a standard curve, ranging from 40 to 0.075 pmol of ATP provided in the kit. The amount of ATP was normalized with the CFU value and plotted as ATP in picomoles per 10^8 CFU for each strain.

Peptide uptake assay

The dipeptide Pro-Pro was synthesized by solid phase peptide synthesis using standard 9-fluorenyl-methoxycarbonyl-based chemistry (Chatterjee, Laufer and Kessler 2012) and 5,6-carboxyfluorescein (CFSE) was attached to the N-terminus of the peptide as described elsewhere (Garai *et al.* 2016). Purification of the peptide was carried out using RP-HPLC, with a C18 capcell-pack semiprep column, on a 20–80% acetonitrile–water gradient. The purified sample was lyophilized, dissolved in DMSO to obtain a stock solution of 1 mM and filter sterilized. From overnight bacterial culture, 5×10^7 CFU, washed twice with sterile PBS, was added to 1 ml PBS containing 50 μM of the dipeptide. After incubation at 37°C for required time points, cells were pelleted and washed with PBS. These cells were imaged using laser-scanning confocal microscopy (Zeiss LSM 710) with a ×63 oil-immersion objective. Images were analyzed using ZEN software version 2009 and fluorescent bacterial cells or CFSE positive cells were counted. The percentage of CFSE-positive cells out of total 4′,6-diamidino-2-phenylindole-stained cells was calculated.

Growth in presence of Pro-Pro peptide

For growth assessment in the presence of the peptide Pro-Pro, minimal medium was prepared containing 100 mM NaCl, 30 mM triethanolamine HCl (pH 7.1), 25 mM sodium pyruvate, 5.0 mM NH_4Cl, 2.0 mM NaH_2PO_4, 0.25 mM Na_2SO_4, 0.05 mM $MgCl_2$, 1.0 mM KCl and 1.0 μM ferric chloride and filter sterilized (Garai *et al.* 2016). Overnight cultures of all strains in LB were washed twice in PBS and finally resuspended in minimal medium. From this, 10^6 CFU was inoculated in 1 ml minimal medium containing the peptide Pro-Pro at the concentration of 50 μM and incubated at 37°C under shaking condition. Aliquots were taken at different time points and plated on LB agar. The CFU values were plotted against time to obtain the growth curve.

RESULTS

YjiY is essential for biofilm formation by *Salmonella*

The assessment of biofilm formation in bacteria is usually done in terms of pellicle formation at the liquid–air interface (Romling and Rohde 1999; Solano *et al.* 2002) when grown at 28°C and under low osmolarity (Steenackers, Vanderleyden and Keersmaecker 2012), the optimum conditions for the expression of the biofilm master regulator *csgD* in *S.* Typhimurium (Romling *et al.* 2000). The mutant strain, Δ*yjiY*, did not form a pellicle under these biofilm-inducing conditions, unlike the wild type strain (Fig. 1A). We further decided to assess the biofilm-forming ability on a solid surface using a glass coverslip as the substrate by staining the EPSs. The stability of a biofilm depends on the EPS, which help in aggregation of cells, absorption of nutrients, stabilization of the architecture of the biofilm and cell to cell communication (Flemming and Wingender 2010). EPS can be stained with Congo red, which binds to cellulose and curli fimbriae, the two major components of *Salmonella* EPS (Romling *et al.* 2000; Mohite and Patil 2014). We checked the distribution of EPS to measure the extent of biofilm formed on the glass coverslip. Confocal microscopy imaging of the biofilm matrix stained with Congo red showed less denser biofilm in the Δ*yjiY* strain (Fig. 1B). EPS help the bacteria to adhere to the substratum and hence maintain the biomass of biofilm. Electron microscopy showed that biofilm biomass or number of cells adhering to the solid surface was remarkably reduced in the Δ*yjiY* strain as compared with the wild type strain (Fig. 1C). The biofilm on the solid surface was quantified by crystal violet staining (Supplementary Fig. S1). This phenotype was successfully reversed in the complement strain, implying that *yjiY* is essential for biofilm formation by *Salmonella*. Since *yjiY* is a carbon starvation gene, we speculated that the defective biofilm formation in Δ*yjiY* may occur due to reduced growth. However, equivalent growth of wild type and Δ*yjiY* strains in individual (monoculture) and mixed cultures (co-culture) (Fig. 2A and B) indicates that Δ*yjiY* has no defect in growth and fitness, respectively, in biofilm-inducing conditions.

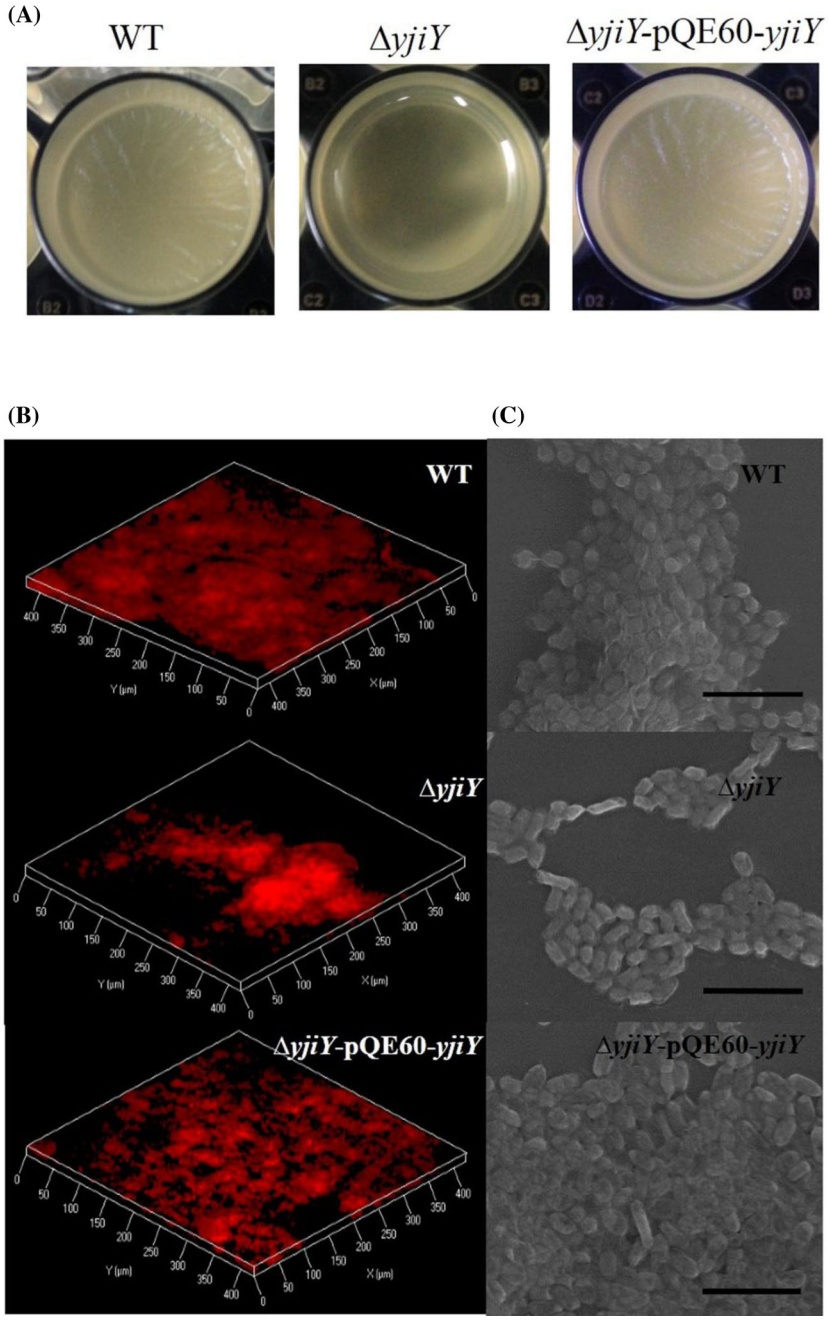

Figure 1. (**A**) Pellicle formation. Overnight cultures of the strains WT, Δ*yjiY* and Δ*yjiY*-pQE60-*yjiY* were inoculated in biofilm medium and incubated at 28°C for 48 h. Biofilm appeared in the form of pellicle. Images were taken using a digital camera. (**B**) Confocal imaging of biofilm. Overnight cultures of the strains WT, Δ*yjiY* and Δ*yjiY*-pQE60-*yjiY* were inoculated in biofilm medium containing coverslip and incubated at 28°C for 48 h. Biofilm formed on glass coverslip was stained with Congo red and imaged with a confocal microscope to generate 3D images. Scale is shown on the X- and Y-axes. (**C**) Scanning electron microscopy imaging of biofilm formed on glass coverslip. Overnight cultures of the strains WT, Δ*yjiY* and Δ*yjiY*-pQE60-*yjiY* were inoculated on biofilm medium, containing coverslip and incubated at 28°C for 48 h. Biofilm formed on the coverslip was imaged using scanning electron microscopy. Scale bar is 5 μm.

Δ*yjiY* shows alteration in the levels of factors required for biofilm formation

To understand the mechanism behind the reduced EPS production in the Δ*yjiY* strain, we looked back into the RNA microarray data from our previous work that compared the transcriptome of Δ*yjiY* with that of wild type (Garai *et al.* 2016). The virulence-associated gene *mgtC* was found to be highly upregulated in Δ*yjiY*, which was validated in LB late log phase (Supplementary Fig. S2). This held true for biofilm-inducing conditions too (Fig. 3A). The upregulation of *mgtC* explains the defect in biofilm formation by Δ*yjiY*, as in *Salmonella mgtC* is known to inhibit biosynthesis of cellulose, an important constituent of EPS (Pontes *et al.* 2015). The genes *mgtB* and *mgtA* belong to the same regulon of magnesium transport (Lee, Choi and Groisman 2014). There is a trend of increase in the expression of *mgtB*, but less than *mgtC*. Moreover, the downregulation of cellulose biosynthesis gene *bcsA* (Jonas *et al.* 2007) in the Δ*yjiY* strain (Fig. 3A) agreed

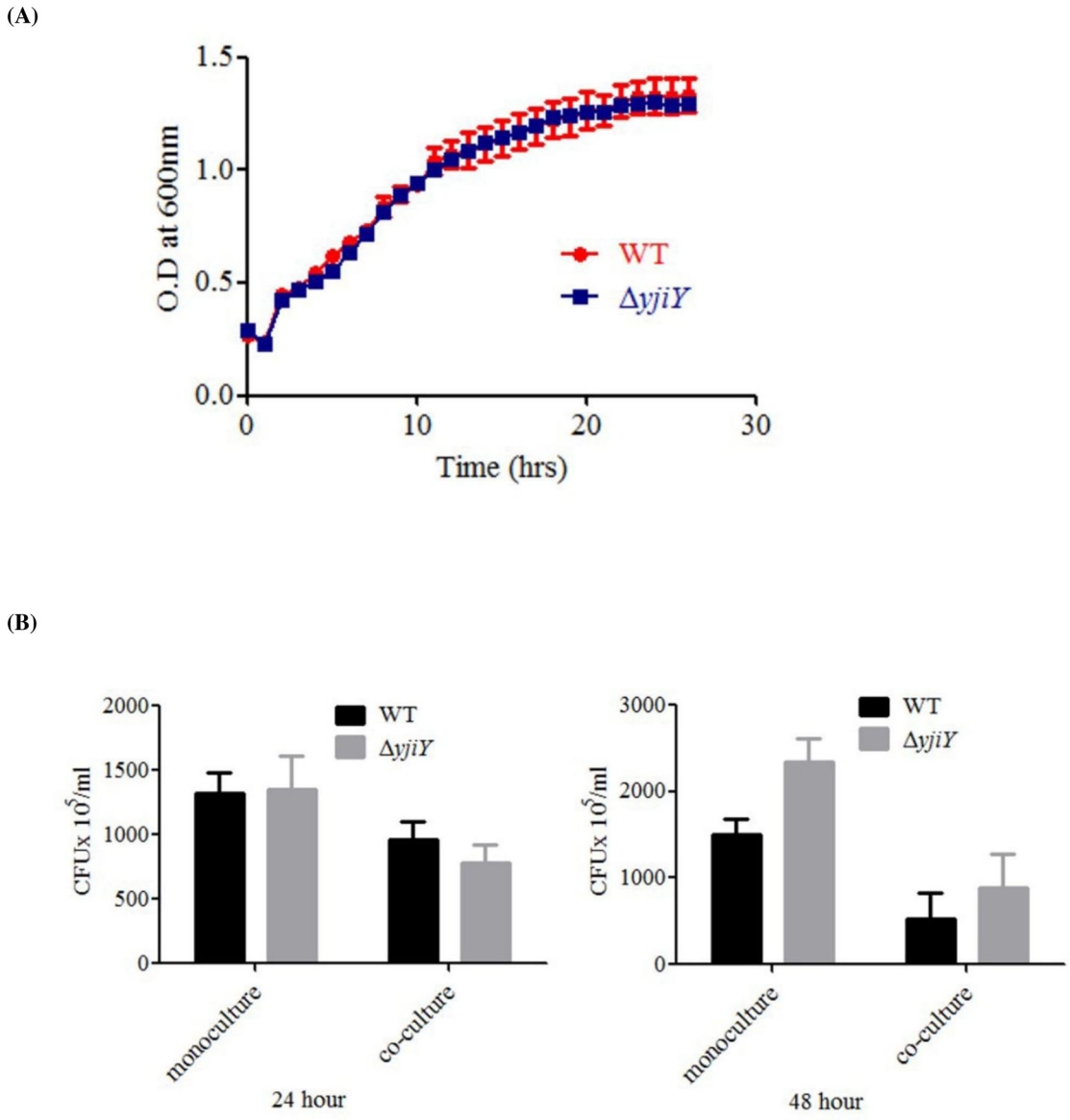

Figure 2. Growth in biofilm medium. (**A**) Growth in shaking conditions. The strains were grown in biofilm medium and at different time points, the OD was measured at 600 nm. (**B**) Growth in static culture. After 24 and 48 h of stationary growth in biofilm medium, strains were plated on agar to obtain CFU values. For co-culture, both strains were inoculated at 1:1 ratio in the same well. At least three biological replicates were used for each experiment. Student's *t*-test was used to calculate the statistical significance.

with the upregulation of *mgtC*. The decreased production of cellulose also results in a higher growth rate (Pontes *et al.* 2015), which can explain the trend, though not significant, of increased CFU values of the Δ*yjiY* strain after 48 h (Fig. 2B). However, in the strain Δ*yjiY*, the expression of the biofilm master regulator, *csgD* (Gerstel and Romling 2003; Grantcharova *et al.* 2010), which also regulates *bcsA* (Jonas *et al.* 2007), was found to be reduced (Fig. 3A). The gene *bapA* contributes to pellicle formation and is regulated by *csgD* (Jonas *et al.* 2007). Hence, the downregulation of *bapA* could be a result of reduced expression of *csgD*. This implies that lack of cellulose may not be the only reason for this phenotype.

The increase in the expression of *mgtC* is linked with decreased production of ATP, as *mgtC* inhibits the function of bacterial F-ATPase (Lee, Pontes and Groisman 2013). ATP is required during biofilm production (Liu *et al.* 2014), as it contributes to EPS production for the formation of cellular aggregates (Jiang and Liu 2012). We measured the level of ATP after 24 h of growth under biofilm-inducing conditions. Twenty-four hours was chosen as the time point to monitor the step prior to EPS synthesis as well as to avoid EPS in the samples for RNA isolation and ATP determination. The amount of ATP was indeed found to be significantly lower in Δ*yjiY* than the wild type strain (Fig. 3B).

Upregulation of *mgtC* is responsible for inhibiting biofilm formation in Δ*yjiY*

To establish the role of *mgtC* in defective synthesis of EPS in Δ*yjiY*, the gene *mgtC* was deleted from the genome of Δ*yjiY*. The double knockout strain Δ*yjiY*Δ*mgtC* was tested for biofilm forming ability on a glass coverslip. Confocal imaging of Congo red-stained biofilm showed remarkably increased amount of EPS

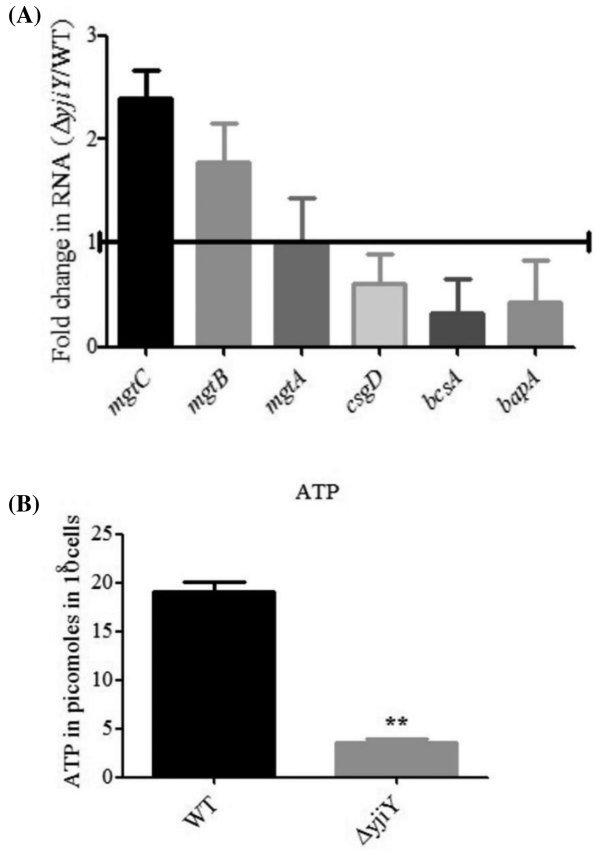

Figure 3. Level of biofilm forming factors. (**A**) QPCR. RNA was isolated from WT and Δ*yjiY* strains after 24 h of growth in biofilm medium and subjected to QPCR for the indicated genes. Fold change in the RNA level in Δ*yjiY* with respect to WT was plotted. The value above and below 1 on Y-axis imply upregulation and downregulation, respectively. (**B**) ATP measurement. Intracellular ATP was measured from 24 h-old biofilm culture. Statistical analysis was performed by Student's *t*-test (**$P < 0.005$). At least two biological replicates were used for each experiment.

in the double knockout strain as compared with Δ*yjiY*, which reverted upon overexpressing *mgtC* (Fig. 4A and B). The strain Δ*csgD* was used as a negative control (Fig. 4A and B and Supplementary Fig. S1), as it cannot synthesize the EPS components required for biofilm formation (Srinandan *et al.* 2015). The EPS content in the double knockout strain was still less than the wild type, probably due to parallel regulation by *csgD* (Fig. 5C).

As a flagellum is important for the initial stage of biofilm formation (Karatan and Watnick 2009) and Δ*yjiY* is reported to be aflagellate (Garai *et al.* 2016), we wanted to know if the absence of flagella was leading to defective biofilm formation in the Δ*yjiY* strain. We used the strain Δ*fliC*, which lacks the structural subunit of flagella, flagellin or FliC (Bonifield and Hughes 2003; Garai *et al.* 2016), for bioflm formation. The lack of flagella did not alter the ability to form biofilm on a glass coverslip (Fig. 4A and B and Supplementary Fig. S1). Therefore, the inability of the Δ*yjiY* strain to form a biofilm seemed to be independent of the presence of flagella. Hence, we conclude that the upregulation of *mgtC* contributes to the abrogation of EPS production and thereby biofilm formation in the Δ*yjiY* strain.

YjiY may regulate *mgtC* expression by transporting peptides of proline

One of the cues behind induction of *mgtC* transcription is known to be the shortage of the amino acid proline (Lee, Choi and Groisman 2014). However, Δ*yjiY* could not resume its biofilm-forming ability in the presence of proline (Fig. 5A and Supplementary Fig. S3A). This may be due to the unavailability of proline transporters in liquid medium, as they are majorly expressed during desiccation, in contrast to EPS genes (Finn *et al.* 2013). Based on the reported role of the protein YjiY in peptide transport in *Salmonella*, we hypothesized that YjiY might transport peptides of proline and thereby regulate *mgtC* expression. We carried out a fluorescently labeled peptide uptake assay (Garai *et al.* 2016) for the dipeptide of proline (Pro-Pro). With increasing incubation time, the Δ*yjiY* strain indeed showed significantly lower uptake than wild type (Fig. 5B). These proline peptides are probably required only for biofilm formation and not nutrition, as the mutant did not display any defect in growth when the Pro-Pro dipeptide was provided as the sole source of energy (Supplementary Fig. S3B). Proline also regulates the transcription of *mgtB* (Lee, Choi and Groisman 2014), which was indeed found to be moderately upregulated (Fig. 3A). Nevertheless, the result of this assay indirectly supports our hypothesis that YjiY may regulate the expression of *mgtC* by transporting proline peptides (Fig. 5C).

DISCUSSION

Salmonella has diverged from its evolutionarily close relatives such as *E. coli* by acquiring specific virulence-associated genes and developing a harmonious relationship between its metabolism and virulence. The carbon starvation (*cst*) genes *cstA* and *yjiY* have already been established as an example of the cross connection between metabolism and virulence in *Salmonella* (Garai *et al.* 2016). Our finding that the peptide transporter-encoding carbon starvation gene, *yjiY*, is essential for biofilm formation by *Salmonella* is an additional evidence in this context. *Salmonella* is known to express several peptide transporters to escape from nutrient starvation (Gibson, Price and Higgins 1984; Hiles, Powell and Higgins 1987; Olson *et al.* 1991; Garai *et al.* 2016), a common stress that leads to biofilm formation (Stepanovic *et al.* 2004; Castelijn *et al.* 2012). We have taken one step further in understanding how starvation is associated with biofilm formation by deciphering the link between starvation gene *yjiY* and virulence gene *mgtC*. The additional inference that *yjiY* is involved in regulating transcription of *csgD*, the master regulator for biofilm formation in *Salmonella* (Steenackers H, Vanderleyden and Keersmaecker 2012), is strengthened by the reported correlation between the expression of *csgD* and *yjiY* during the stationary phase of growth (Karatan and Watnick 2009; Kraxenberger *et al.* 2012b). However, the association between *csgD* and *mgtC* in *yjiY*-dependent biofilm formation remains to be investigated.

The role of proline in inducing expression of *mgtC* has already been reported (Lee, Choi and Groisman 2014) and our work extends its role to biofilm formation in *Salmonella*. The observation that the Pro-Pro dipeptide did not contribute to growth of *Salmonella* suggests that proline or peptides of proline contribute specifically to biofilm formation. Interestingly, the growth of Δ*yjiY* was unaffected by a reduced level of ATP, probably due to the channeling of ATP towards EPS synthesis instead of planktonic growth. The concentration of ATP obtained for the wild

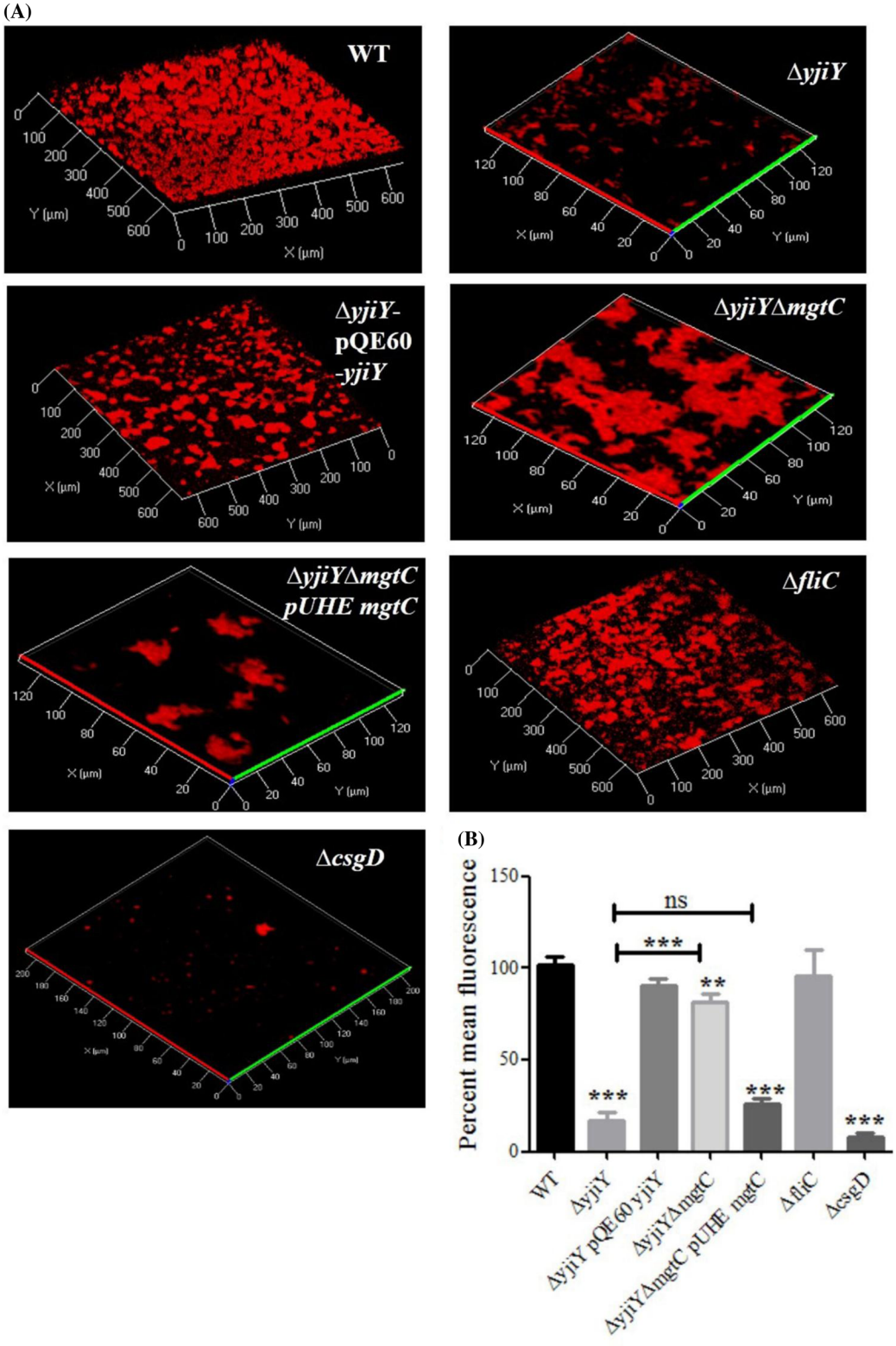

Figure 4. (**A**) Confocal imaging of biofilm. Overnight cultures of the strains indicated were inoculated on biofilm medium-containing coverslip and incubated at 28°C for 48 h. Biofilm formed on glass coverslip was stained with Congo red and imaged with confocal microscope to generate 3D images. Scale is shown on the X- and Y-axes. (**B**) Quantification of confocal images. The mean fluorescence of all images was quantified using ImageJ and plotted as percentage mean fluorescence normalized to WT. Statistical analysis was performed by Student's *t*-test, for comparison of each strain with WT (**$P < 0.005$, ***$P < 0.001$).

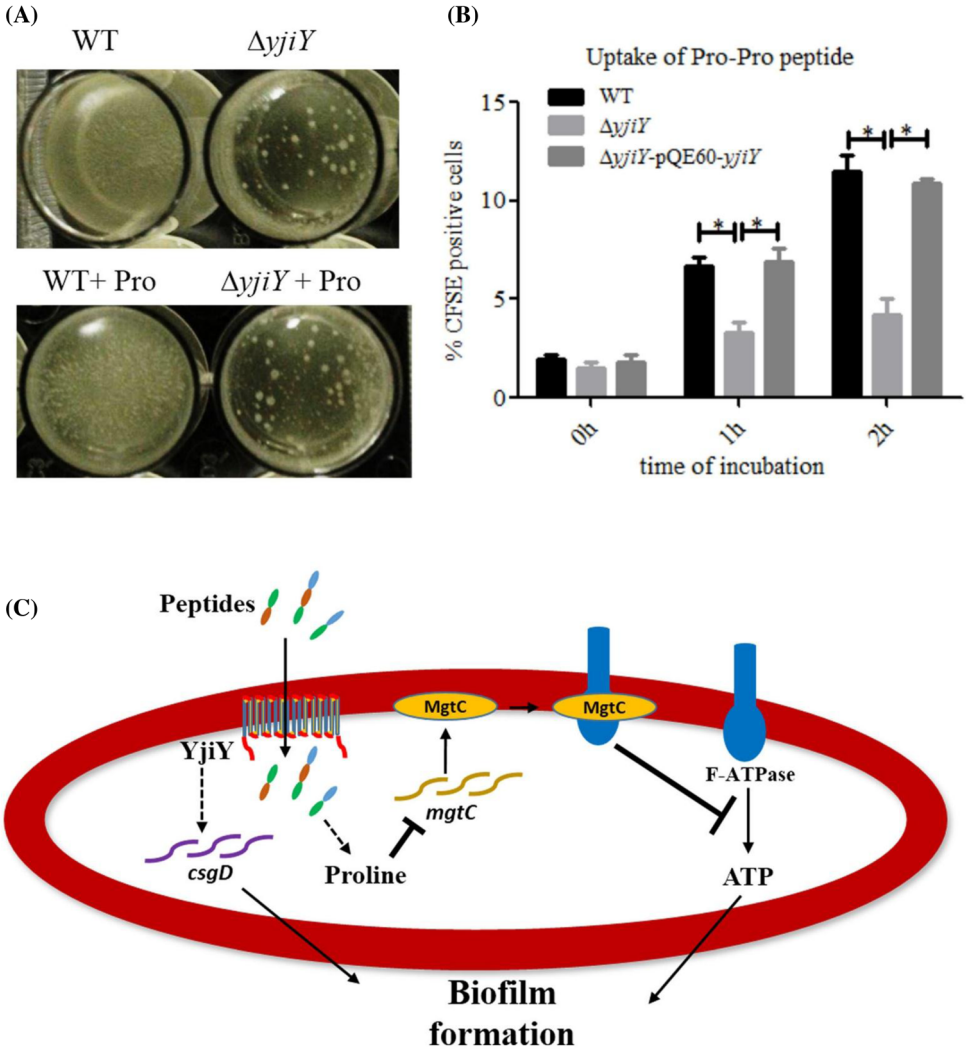

Figure 5. (**A**) Biofilm in presence of proline. Overnight cultures of the strains WT and Δ*yjiY* were inoculated in biofilm medium with or without proline (0.1 mM). After incubation at 28°C for 48 h, biofilm appeared in the form of pellicle. Images were taken using a digital camera. (**B**) Fluorescent peptide uptake assay. The strains WT, Δ*yjiY* and Δ*yjiY*-pQE60-*yjiY* were grown in presence of 50 μM CFSE-labelled dipeptide Pro-Pro (PP). After 2 h, the number of CFSE-positive cells was evaluated by confocal microscopy. Percentage of CFSE-positive cells out of total number of cells was calculated. The data shown are a compilation of two biological replicates and statistical analysis was performed using Student's *t*-test. (**C**) Hypothetical model. This model describes the effect of YjiY on *mgtC* and *csgD* to regulate biofilm formation. YjiY imports peptides from the surroundings of *Salmonella* including peptides of proline. These peptides may serve as the source of proline, which inhibits the transcription of *mgtC*. The shortage of proline in Δ*yjiY* removes this inhibition and enables the expression of *mgtC*. The inner membrane protein MgtC binds to F-ATPase to regulate the synthesis of ATP and thereby biofilm formation. YjiY can also regulate the transcription of *csgD* to affect biofilm formation by unknown mechanism. Dashed arrows represent unknown mechanisms.

type strain matches with the intracellular ATP level of *Salmonella* during amino acid starvation (Shioi *et al.* 1982). Thus, biofilm-inducing conditions may include shortage of amino acids. Despite providing proline exogenously, biofilm formation could not be rescued, probably due to the absence of proline transporters in biofilm-inducing liquid culture conditions (Finn *et al.* 2013). This may also mean that proline peptides act as the major source of proline under these conditions.

The overexpression of *mgtC* was not reflected in the virulence of the Δ*yjiY* strain in the systemic disease model of mouse (Garai *et al.* 2016) as expected (Pontes *et al.* 2015), possibly due to the difference in dosage and route of infection. Although *mgtC* and *mgtB* are transcribed together as part of an operon (Alix and Blanc-Potard 2008; Lee, Choi and Groisman 2014), *mgtC* showed higher expression in Δ*yjiY*, in agreement with our previous microarray data (Garai *et al.* 2016), indicating that these biofilm-forming conditions interfere with the expression of the complete operon.

In conclusion, this study reveals the presence of a starvation-responsive system for biofilm formation by *Salmonella*. The peptide transporter YjiY possibly supplements proline in the form of proline peptides. In the absence of YjiY, shortage of proline leads to upregulation of *mgtC*, which is unfavorable for biofilm formation (Fig. 5C). Although nutrient starvation is meant to direct the process of biofilm formation, this study indicates that the presence of certain nutrients is essential. The transported peptides not only replenish the amino acids, but can also signal the pathogen to initiate biofilm formation by avoiding expressing a virulence-associated gene. This study adds to our understanding of the connection of nutritional status of *Salmonella* with its success as a pathogen and how the activity of a virulence gene could be occasionally detrimental for adaptation to stress.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

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Author's contribution

KC and PG contributed equally in the development of the manuscript. The idea was conceived by PG and DC. Experiments were planned by PG, KC, JC and DC. KC carried out experiments majorly. PG and JC contributed to experiments too. PG wrote the manuscript majorly with the help of KC, JC and DC. DC supervised the project.

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