Functional Analysis of the Chaperone-Usher Fimbrial Gene Clusters of *Salmonella enterica* serovar Typhi

Karine Dufresne, Julie Saulnier-Bellemare and France Daigle*

Department of Microbiology, Infectiology and Immunology, Université de Montréal, Montreal, QC, Canada

The human-specific pathogen *Salmonella enterica* serovar Typhi causes typhoid, a major public health issue in developing countries. Several aspects of its pathogenesis are still poorly understood. *S.* Typhi possesses 14 fimbrial gene clusters including 12 chaperone-usher fimbriae (*stg, sth, bcf, fim, saf, sef, sta, stb, stc, std, ste*, and *tcf*). These fimbriae are weakly expressed in laboratory conditions and only a few are actually characterized. In this study, expression of all *S.* Typhi chaperone-usher fimbriae and their potential roles in pathogenesis such as interaction with host cells, motility, or biofilm formation were assessed. All *S.* Typhi fimbriae were better expressed in minimal broth. Each system was overexpressed and only the fimbrial gene clusters without pseudogenes demonstrated a putative major subunits of about 17 kDa on SDS-PAGE. Six of these (Fim, Saf, Sta, Stb, Std, and Tcf) also show extracellular structure by electron microscopy. The impact of fimbrial deletion in a wild-type strain or addition of each individual fimbrial system to an *S.* Typhi afimbrial strain were tested for interactions with host cells, biofilm formation and motility. Several fimbriae modified bacterial interactions with human cells (THP-1 and INT-407) and biofilm formation. However, only Fim fimbriae had a deleterious effect on motility when overexpressed. Overall, chaperone-usher fimbriae seem to be an important part of the balance between the different steps (motility, adhesion, host invasion and persistence) of *S.* Typhi pathogenesis.

Keywords: chaperone-usher, fimbriae, *S.* Typhi, infection, biofilm, pathogenesis

INTRODUCTION

*Salmonella enterica* serovar Typhi is a human-specific pathogen responsible for a systemic disease called typhoid fever. It causes ~22 million infections and 200,000 deaths annually worldwide (WHO, 2012; Qamar et al., 2015). Over the years, the number of cases has increased, but the use of antibiotics has controlled the eventual burden. However, the increased emergence of multidrug resistant *S.* Typhi strains can complicate treatment and can lead to a higher death rate (Rowe et al., 1997; Thong et al., 2000; Pokharel et al., 2006; WHO, 2012). A better understanding of *S.* Typhi pathogenesis is required to better control and treat typhoid (Obaro et al., 2017).

*Salmonella* species enter their host by the intestinal tract and cross the intestinal barrier (Clark et al., 1994). *S.* Typhi invades the human host by a variety of virulence factors such as two type III secretion systems (T3SS) encoded by *Salmonella* pathogenicity islands (SPI)−1 and−2, the
The S. Typhi genome possesses a unique repertoire of 14 putative fimbrial clusters identified by whole-genome sequencing (Humphries et al., 2003) including 12 CU fimbriae (Townsend et al., 2001). However, only 3 CU fimbriae (Stg, Sta, and Tcf) were previously studied (Forest et al., 2007; Bishop et al., 2008; Berrocal et al., 2015; Leclerc et al., 2016; Gonzales et al., 2017), mainly due to the weak expression of most of these fimbriae under laboratory conditions (Low et al., 2006; De Masi et al., 2017). Among the CU fimbriae, 5 (stg, sef, sta, ste, and tcf) are present in S. Typhi but absent in the well-studied broad-range pathogen S. Typhimurium and 5 of the fimbrial gene clusters in S. Typhi (stg, sth, bcf, sef, and ste) have at least one pseudogene. Approximately 5% of the S. Typhi genome contains pseudogenes, which were often associated with its host-specificity and may restrict S. Typhi only to the human host (Baker and Dougan, 2007). In fimbrial putative gene clusters, pseudogenes are present in the usher genes (stgC, sthC, and bcfC), but also in subunits or adhesin genes (sthE, sefA, sefD, and steA). However, S. Typhi mutants with a deletion of stg demonstrated decreased infection of cell lines, suggesting a potential function for this fimbrial cluster despite the presence of a pseudogene in the usher gene (Forest et al., 2007; Berrocal et al., 2015; Gonzales et al., 2017). Allelic variation, especially for the FimH adhesin of the Type 1 fimbria, was studied in several serovars and may also be implicated in host tropism for Salmonella (Kisiela et al., 2012; Yue et al., 2015; De Masi et al., 2017).

Here, we hypothesize that some of 12 CU fimbriae of S. Typhi are produced and involved at different steps of pathogenesis despite their poor fimbrial expression and presence of pseudogenes. The characterization of all 12 CU fimbriae of S. Typhi includes expression levels under the tested conditions, surface structure assembly, interactions with host cells, role in

FIGURE 1 | S. Typhi CU fimbrial operons organization. S. Typhi possesses 12 putative fimbrial gene clusters divided in 5 clades (γ1, γ3, γ4, α, and π) depending of the usher homologies. Stg, Sth, Bcf, and Fim are γ1 fimbriae, while Ssf and Sef are in clade γ3 and Sta, Stb, and Stc are in clade γ4. Tcf is the only representative of α clade. Std and Ste are π fimbriae. Ushers are represented by black arrow and chaperones by white arrow. No distinction is made for major/minor subunits, adhesins, or other proteins (gray arrows). Pseudogenes are marked by asterisk.
biofilm production and in motility. Overall, each of the S. Typhi fimbrial systems were found to contribute to different steps of the pathogenesis process and six of these produced visible fimbriae.

**MATERIALS AND METHODS**

**Bacterial Strains, Plasmids, and Growth Conditions**

The list of bacterial strains and vectors used in this study is given in Table S1. Bacteria were routinely grown overnight at 37°C on Luria-Bertani (LB) agar plates or with agitation in LB broth. When required, supplements or antibiotics were added at the following concentrations: 0.05–1 mM IPTG, 50 µg/ml diaminopimelic acid (DAP), 50 µg/ml kanamycin, 50 µg/ml ampicillin, and 34 µg/ml chloramphenicol. IPTG is used to induce expression of fimbrial cluster cloned into pMMB307c (Morales et al., 1991) and DAP is an amino acid that allows the maintenance of the conjugal strain MNG-617 containing an asd mutation (Kaniga et al., 1991). Transformation of bacteria was performed by using the calcium/manganese based (CCMB) or electroporation methods as previously described (O’Callaghan and Charbit, 1990).

**Cloning of Fimbrial Promoters and ß-Galactosidase Assays**

The primers used for cloning of fimbrial promoters are listed in Table S2. The promoter region upstream of each gene cluster was predicted by the Softberry software BPROM (www.softberry.com) and amplified by PCR reaction. PCR fragments between 170 and 730 bp were cloned upstream of the promoterless lacZ gene in vector pRS415. The resulting vector was transformed into S. Typhi wild-type strain. Expression of each promoter was measured by ß-galactosidase assays following growth under different culture conditions. LB was used as a classic rich laboratory medium and bacteria were inoculated in broth or on agar and incubated overnight at 37°C. M63 was used as a minimal medium and was prepared as previously described (Leclerc et al., 2013). Bacteria were inoculated in M63 broth or on M63 agar. For the induction of the T3SS encoded on SPI-1, the bacteria were grown in LB 0.3M NaCl, and incubated overnight at 37°C without agitation for low oxygenation (Lee et al., 1992; Weinstein et al., 1998). For the induction of T3SS encoded on SPI-2, the bacteria were incubated in LPM broth, pH 5.8, and incubated overnight at 37°C with agitation (Coombes et al., 2004). For each condition, ß-galactosidase activity was assessed using o-nitrophenyl-ß-D-galactopyranoside (ONPG) as described previously (Miller, 1972).

**Chromosomal Deletion of Fimbrial Gene Clusters**

The primers used for mutagenesis are listed in Table S2. Mutant strains for each fimbrial gene cluster were obtained by allelic exchange mutagenesis as previously described (Forest et al., 2007) except for the deletion of sta, that was obtained by λ red recombination system (Datsenko and Wanner, 2000). Each fimbrial deletion was verified by PCR (data not shown). The afimbrial strain resulted from the successive deletion of each of the fimbrial clusters, including the deletion of genes encoding the curli (csg) and the type IV fimbriae (pil). At least two different mutants were tested for each construction and no major phenotypic differences (growth curves and adhesion assay) were observed. Only one mutant was then selected for further experiments.

**Cloning of the Fimbrial Gene Clusters**

The primers used for cloning of fimbrial gene clusters are listed in Table S2. Fimbrial gene clusters, with or without the promoter region, were amplified by PCR using Q5 High-Fidelity DNA polymerase (New England Biolabs). Fragments between 4.5 and 9.7 Kb were then cloned in IPTG-inducible vector pMMB207c (fimbrial gene cluster without promoter region) (Morales et al., 1991) or into the low-copy vector pWSK29 (fimbrial gene cluster with its native promoter region) (Wang and Kushner, 1991). These constructions were transformed by electroporation into the afimbrial S. Typhi strain.

**SDS-PAGE and Transmission Electron Microscopy**

S. Typhi containing the inducible vector pMMB207c with or without each fimbrial gene cluster was induced overnight at 37°C on LB plates with chloramphenicol and 50 µM IPTG. Bacteria were harvested in LB broth. For SDS-PAGE, bacteria were washed with a solution of 0.9% sodium chloride and then with a solution of 75 mM sodium chloride and 0.5 mM Tris pH 7.4. Extracellular proteins were extracted by heat treatment at 60°C during 15 min and were precipitated by addition of 10% trichloroacetic acid (Beloin et al., 2006). The concentration of proteins was normalized. The samples were loaded on SDS-PAGE 15% and stained with Coomassie R-250. The band of interest for Std and Stc (StdA and StcA) were cut from the gel, destained and digested by trypsin. Peptides were sequenced using LC-MS/MS at the Center for Advanced Proteomics Analyses (IRIC, Université de Montréal).

For electron microscopy, the 3–24 h-induced cultures were fixed with 2% glutaraldehyde for 30 min and adsorbed onto nickel formvar-carbon coated grids for 10 min and stained with 1% phosphotungstic acid. Bacteria were observed under Philips CM-100 or Hitachi H-7100 electron microscope.

**Interactions with Human Epithelial Intestinal Cells**

INT-407 (Henle) cells (ATCC CCL-6) were grown in minimal essential medium supplemented (Wisent) with 10% heat-inactivated fetal bovine serum (FBS) (Wisent) and 25 mM HEPES (Wisent). The bacterial cells were grown overnight at 37°C in LB 0.3 M NaCl with low oxygenation (without agitation). This growth condition (microaerophilic and high salt medium) induce the T3SS encoded by SPI-1 (Weinstein et al., 1998; Elhadad et al., 2016; Jiang et al., 2017). The assays were then performed as previously described with MOI of 20 (Forest et al., 2007).
Interactions with Macrophages

THP-1 cells (ATCC TIB-202) were maintained in RPMI 1640 (Wisent) supplemented with 10% heat-inactivated FBS (Wisent), 1 mM sodium pyruvate (Wisent) and 1% MEM non-essential amino acids (Wisent). The assays were then performed as previously described at a MOI of 10 (Daigle et al., 2001).

Biofilm Assays

The protocol was adapted from Ganjali Dashti et al. (2016). Bacteria were grown overnight in LB broth. The cultures were then diluted 1:10 in nutrient broth containing bile, glucose, and potassium and incubated 72 h statically at 37°C. The biomass production was determined by the crystal violet assay as described by Tremblay et al. (2015). The assay was carried out at least three times for each bacterial strain.

Motility Assays

The motility assays were as previously described (Sabbagh et al., 2012). Bacteria were grown in LB broth overnight with agitation at 37°C and diluted 1/100 prior to puncture of the agar plate for mutant strains and afimbrial strains with pWSK29 vector. For afimbrial strains with inducible vector pMMB207c, the overnight cultures were diluted 1/100 in new medium and the strains were induced with 1 mM IPTG for 3 h at OD600 value of 0.6 and then punctured in the agar plate. Bacterial strains were accompanied by the control strain on each agar plate for comparison. Plates were incubated for 16 h at 37°C. The diameter (mm) was measured and each construction was tested at least in triplicates. The results are presented as the mean ratio of the tested strain/wild-type ± SEM of the replicas.

Statistical Analysis

Statistical analysis was performed on GraphPad Prism. Two-tailed unpaired Student’s t-test was applied on data sets for each construction compared to their control data. P < 0.001 was considered extremely significant (**); P < 0.01 was considered very significant (*) and P < 0.05 was considered significant (*).
FIGURE 2 | Expression of S. Typhi ISP1820 CU fimbrial promoters. Expression of upstream predicted promoter region was assessed by β-galactosidase assay. Six growth conditions mimicking infection were evaluated. M63 broth (white bars) and agar (light gray bars) were tested as minimal media. LB broth (dark gray bars) and agar (black bars) were presented as rich media. Media inducing SPI-1 (wide striped bars) or −2 (slim striped bars) T3SS were used. Results are expressed as the mean ± SEM of at least three distinct experiments performed in duplicates.

FIGURE 3 | Fimbrial major subunits in extracellular structures extract. Bacteria were harvested from a LB agar plate supplemented with 50 µM IPTG and treated to extract the extracellular structures. (A) The control (pMMB) and 7 fimbrial gene clusters without pseudogene were migrated together on polyacrylamide gel and stained with Coomassie blue as well for (B) the control and the 5 fimbrial gene clusters with pseudogene. Asterisks are placed at the right of the proteinaceous bands considered as fimbrial subunits.
Uptake and Survival within Macrophages

Interaction of S. Typhi with THP-1 macrophages was assessed for phagocytosis (t = 0) and for survival after 24 h (Figure 6A). Deletion of stg and stb significantly increased phagocytosis levels compared to the wild-type (up to 160 and 155%, respectively) while deletion of bcf, saf, and tcf decreased it to 88, 65, and 87% respectively. Deletion of bcf increased survival to 126% of the wild-type strain, whereas deletion of stc and std resulted in decreased survival to 68 and 48% respectively compared to the wild-type strain. Regarding the addition of individual fimbria to the afimbrial strain, Fim and Stb present the most relevant phenotypes (Figure 6B): Fim fimbriae increased phagocytosis (259%) and survival (600%), whereas Stb decreased phagocytosis (10%) and survival (2%).

Fimbriae and Motility

The role of fimbriae on motility was tested on LB 0.3% agar plates. Either the deletion of one fimbrial system or the addition of individual fimbrial systems to the afimbrial strain did not show any significant phenotype compared to the wild-type control, as the same level of swimming motility was observed (data not shown). However, when each fimbrial system was overexpressed, the Fim fimbriae (pMMBfim) drastically decreased the swimming to 52.3 ± 3.2% of the control. By contrast, none of the other fimbrial systems showed a significant difference when compared to the control strain (data not shown).

Role of Fimbriae on Biofilm Production

The role of fimbriae for biofilm formation was tested by crystal violet coloration assays. First, the consequence of the deletion of individual fimbrial systems was evaluated (Figure 7). Deletion of stg, bcf, saf or std decreased biofilm production to between 70 and 85% of the wild-type production. Introduction of each individual fimbrial systems to the afimbrial strain was also evaluated (Figure 7). Addition of Stg, Sth, Bcf, and Ste reduced biofilm production to 72–88% of the control and addition of Stb increased biofilm production to 128%. We then tested the effect of fimbrial overexpression by induction of the fimbrial cluster (Figure 7). Fim, Sth, Std, and Tcf increased biofilm production to levels between 140 and 180% of the control strain, whereas Stg had decreased biofilm production to 68%.

DISCUSSION

S. Typhi possesses 12 different chaperone/usher fimbriae that belong to 5 fimbrial clades (γ1, γ2, γ4, π, and α) based on usher homologies (Nuccio and Bäumler, 2007). These fimbriae were identified by whole-genome sequencing and most are considered to be putative, as it is not known under what conditions they may be expressed or they have not been characterized or visualized yet (Baker and Dougan, 2007). Here, we investigated the expression and characterization of all 12 of the S. Typhi CU fimbriae. We evaluated the expression of fimbrial promoters, production of fimbrial proteins and functional production of fimbriae. We also characterized the effects of fimbrial deletions or phenotypes due to introduction of specific fimbrial gene clusters to an afimbrial strain on interaction with host epithelial cells and macrophages, bacterial motility, and biofilm formation.

Fimbriae are poorly expressed when grown under laboratory conditions. A study from Kröger et al. (2013) compared 22 infection-mimicking conditions by an RNA-seq-based analysis of S. Typhimurium SL1344 and revealed that fimbrial genes, including the 7 CU fimbrial operons in common with S. Typhi, were not or poorly expressed. To determine the expression of S.
Typhi CU fimbrial promoters, the use of a multi-copy reporter gene fusion (pRS415) with each fimbrial promoter was required to obtain a sufficient β-galactosidase activity, as a chromosomal lacZ fusion did not provide a sufficient level of expression for quantification of Miller units (data not shown). Six growth conditions were tested: rich (LB), minimal (M63), both in liquid (broth) and solid (agar), and SPI-1 and−2 T3SSs induction conditions (Figure 2). As the niche of S. Typhi is restricted to the human body, only 37°C was tested. Interestingly, one specific fimbrial promoter from each of the different CU clades was dominant (sth for γ₁, saf for γ₃, stc for γ₄, and std for π), suggesting a role for each fimbrial clade in S. Typhi. Also,
the majority of the fimbrial systems had a unique expression pattern, except for stg and ste (Table 1). The highest expression for each fimbrial promoter was obtained during growth in minimal medium. Low nutrient conditions may be encountered by S. Typhi during colonization of liver and spleen, in the blood, or in the gall bladder, corresponding to different sites where fimbriae may be needed (Gonzalez-Escobedo et al., 2011; Keestra-Gounder et al., 2015). SPI-1 T3SS-inducing condition was usually the condition with the lowest expression, except for stc and std which demonstrated their lowest on M63 agar. In S. Typhimurium, the balance between adherence (fimbriae), invasion (SPI-1 T3SS) and motility (flagella) is usually finely regulated. The induction of one of these elements can therefore result in a decrease in expression of the other factors (Clegg and Hughes, 2002; Saini et al., 2010; Baxter and Jones, 2015). Otherwise, other SPI-1 inducing conditions might be more optimal for the expression of the studied fimbriae and might reflect more accurately interaction with epithelial cells.
As fimbriae are poorly expressed in laboratory conditions, the functional assembly of putative fimbrial operons of *S. Typhi* was induced and detection of the major subunit proteins were visualized for Fim, Saf, Sta, Stb, Stc, Std, and Tcf systems. No specific bands were observed for putative fimbrial gene clusters that contain pseudogenes. This could be explained by lack of functional assembly for these fimbriae. Fim, Saf, Sta, Stb, Std, and Tcf fimbriae were also visualized by TEM and demonstrated differences in morphology and distribution on the surface of cells which may imply difference in pathogenesis functions (Figure 4). Fimbrial gene clusters with pseudogenes (*stg, sth, bcf, sef, and ste*) and Stc fimbriae were not observed by TEM. This can be due to their detachment during the grid preparation or to the absence of functional assembly. Most of the pseudogenes are located in usher or subunits genes that may prevent the formation of the fimbriae by avoiding the transport of the subunits through the outer-membrane or the fimbriae formation. However, fimbrial operons with pseudogenes may be functional by complementing the non-functional usher by another fimbrial usher or by using a suppressor tRNAs to bypass stop codon to form a functional usher or subunit (Berrocal et al., 2015). The presence of pseudogenes may still impact on pathogenesis as some of the fimbrial proteins may be exported in the environment or fixed to the surface in absence of full-length fimbriae, acting similarly to afimbrial adhesin.

The potential roles of the different CU fimbriae were investigated by testing effects of the individual deletion of these systems from the wild-type strain or by introduction of each system to an afimbrial *S. Typhi* strain on bacterial interactions with host cells (INT-407 and THP-1) (Figures 5–6, Table 1), on motility, and on biofilm formation (Figure 7). Interestingly, each fimbria seems to have a role in invasion as deletion of one of them or individual addition of fimbriae in afimbrial strain decrease invasion to epithelial cells compared to their control. This implies that fimbriae may affect invasion by regulation of the SPI-1 T3SS or that they are critical for the initial contact with host cells to allow a stable interaction between the T3SS and the host. A combination of multiple fimbriae may be important for the optimal contact between the bacteria and the epithelial cells. Another hypothesis is that the SPI-1 T3SS or other adhesins at the surface of the bacteria are affected by the deletion of fimbriae and may confer lower invasion of epithelial cells. The functionality of the T3SS may be verified by a secretion assay in further studies and the presence of different adhesins could be determined by Western Blot. There was no difference in motility, except for induction of Fim, whereas most of fimbriae had variable effects (positive or negative) on biofilm formation, except for Sef and Sta. Specific results for each fimbrial system are discussed in the next sections.

The highest expressed promoter was *saf*. Saf fimbriae were also visualized on TEM. Those fimbriae are conserved among *S. enterica* subspecies *enterica* (ssp. I). No noticeable phenotype during cell infection, motility or biofilm formation has been previously reported (Folkesson et al., 1999; Humphries et al.,...
TABLE 1 | Summary of fimbrial expression and pathogenesis phenotypes.

| Fimbriae | Promoter expression | INT–407 infection | THP–1 infection | Motility | Biofilm formation |
|----------|---------------------|------------------|----------------|----------|-----------------|
|          |                     | Adherence | Invasion | Uptake | Survival | Overexpression | Deletion | Addition | Overexpression |
| M63 broth | M63 agar | LB broth | LB agar | SPI–1 | SPI–2 | Deletion | Addition | Deletion | Addition | Deletion | Addition | Overexpression | Deletion | Addition | Overexpression |
| 1       | 2           | 3           | 4         | 5       | 6       | 1          | 2          | 3          | 4        | 5         | 6       | 1           | 2          | 3          | 4           | 5       | 6       |

Table 1: Summary of fimbrial expression and pathogenesis phenotypes.

2003). However, S. Typhi Saf fimbriae were strongly expressed inside human macrophages (Faucher et al., 2006). The main effect observed for the saf deletion mutant of ISP1820 was decreased phagocytosis, invasion and biofilm formation (Table 1). Also, the addition of the saf gene cluster to the afimbrial strain decreased adherence and invasion to epithelial cells. In summary, Saf fimbriae are the most expressed fimbriae in S. Typhi in conditions tested, demonstrated specificity for macrophages and may be implicated in biofilm formation.

Std and Stc are highly expressed and the only systems with an expression level in SPI-1 T3SS inducing conditions that was not their lowest (Table 1). They also share similarities in infection of host cells, with a decrease of survival in macrophages, when expressed, and in biofilm formation when overexpressed. Std and Stc fimbriae are widely distributed in Salmonella enterica ssp. I, forming the core fimbriae with Csg (curli fimbriae), Fim, Sth, Bcf, and Stb (Dufresne, 2017). Interestingly, S. Typhimurium both Std and Stc fimbriae were recognized to be involved in intestinal persistence in mice (Weening et al., 2005). The role of these two fimbriae for invasion and survival could be generalized to other serovars of S. enterica ssp. I and their affinity could be for the intestinal epithelial cells.

stg and ste promoters exhibited the same pattern of expression but not at same level, stg expression was moderate whereas ste expression was lower (Table 1). Deletion of stg from S. Typhi ISP1820 increased phagocytosis in macrophages and is consistent with Forest et al. (2007) as they used S. Typhi ISP1820 and THP-1 macrophages as well. Berrocal et al. (2015) however obtained results that loss of stg decreased phagocytosis, but they used S. Typhi STH2370, a Chilean isolate, and a different macrophage cell line. Stg has deleterious effects on biofilm formation, regardless of its deletion, addition or overexpression. The deleterious effect of the deletion of stg suggests that this fimbria is involved in biofilm formation. However, the effect of addition or overexpression of Stg in the afimbrial strain suggests that other fimbriae must modulate action of Stg and are required for establishing a mature biofilm. Addition of the Ste fimbriae also reduced biofilm formation. These two fimbriae have pseudogenes in their operon and are not present in S. Typhimurium. Despite the presence of pseudogenes and the absence of fimbrial assembly suggesting these gene clusters may not be functional, these fimbrial systems contribute during bacterial interactions with host cells and biofilm formation.
Fim and Stb fimbriae had poor expression in any growth conditions tested. However, they showed major phenotypes in most of the assays. Deletion of *fim* decreased adherence and invasion to epithelial cells, while addition of Fim to the afimbrial strain also decreased adherence and invasion to INT-407 cells. When added to the afimbrial strain, Fim increased phagocytosis and survival in macrophages. Different studies involved FimH, the adhesin of Fim fimbriae, as an important marker of host-specificity. For *S. Typhi*, the presence of a valine in position 223 of FimH seems to be determinant for the binding of human cell lines (Kisiela et al., 2012; Yue et al., 2015). The defect in swimming may be due by the decrease in growth rate or by overexpression of the Fim fimbriae. Fim fimbriae may be a major player of *S. Typhi* pathogenesis and may regulate other fimbriae and virulence factors like flagella, LPS or T3SSs. Stb may be involved in long-term persistence by biofilm formation and may regulate negatively early stages of infection like host cell interactions. The balance between motility, invasion and persistence is critical for a successful infection, and Stb seems to inhibit invasion in favor of persistence. The inhibition of interaction with macrophages by Stb fimbriae could be physical: Stb fimbriae are long and cover the majority of the surface of the bacteria, which may interfere with recognition molecules for receptors on macrophages, and influence the interaction with host cells. Stb and Fim may have opposing roles on macrophages/bacteria interaction and, in a wild-type strain, may counteract the action of each other at different steps to lead to a successful infection.

Expression of the *tcf* promoter was low in every condition tested. However, Tcf fimbriae were previously visualized and are similar to cable (Cbl) pili of *Burkholderia cepacia*, which are in the same CU clade (α) and share more genetic homologies than other members of the α clade (Leclerc et al., 2016). Deletion of *tcf* decreased phagocytosis by macrophages, but does not affect survival. Overexpression of Tcf fimbriae increased biofilm formation. Tcf may play a role in late stages of infection, from the interaction with macrophages to persistence in gall bladder.

Deletion of the *sef* gene cluster decreases adherence and invasion of epithelial cells. However, its presence in an afimbrial strain did not allow recovery of the control levels. Despite the presence of a pseudogene in its operon, *sef* influenced epithelial cell interactions.

Overall, CU fimbriae act as redundant systems on different aspects of *S. Typhi* pathogenesis, such as bacteria/host cell interactions and biofilm formation. In order to reflect more accurately the *S. Typhi* pathogenesis, fimbriae should be also studied in interactions with other host cells targets such as splenocytes, hepatocytes, and cells from the gall bladder. Some fimbriae are similar in their functions and usually have reverse level of expression in a matter of balanced expression (Supplementary Figure 1). The similarity between certain fimbriae could be a form of functional redundancy. Fimbrial production is energetically demanding for the bacteria and the balance of expression between few fimbriae of similar function and presence of pseudogenes could be a way to prevent unnecessary redundancy and preserve *S. Typhi* energy. A tightly regulated equilibrium between stages of pathogenicity of *S. Typhi* is important for a successful infection of the host. The presence of fimbriae at the surface of the bacteria is confirmed for Fim, Saf, Sta, Sib, Std, and Tcf. Each of these fimbriae affect invasion of epithelial cells and phagocytosis by macrophages, although Std only affected survival in macrophages. Fimbriae such as Stc and gene clusters containing pseudogenes (*bcf, stg, ste*, and *sef*) also contributed to different aspects of infection based on our investigation. Each of these systems had an impact on interactions with epithelial cells, although *sef* and *ste* did not alter macrophage interactions. Further, they all had an effect on biofilm formation, except for *sef* and *sta*. Sta and Sth do not present particular function in the case of *S. Typhi*. Sta could be involved in a process of human-specificity, as it is only present in host-restricted strains. For Sth, it may lose its function in *S. Typhi* as it could have an importance in adhesion to abiotic surfaces in other serovars. Fimbriae are critical components of the equilibrium between stages of pathogenesis and further research is needed to more fully understand their complex role for *S. Typhi* and the pathogenesis of typhoid fever.

**AUTHORS CONTRIBUTIONS**

KD and FD: Designed the research; KD and JS-B: Proceed to the experiments; KD and FD: Analyzed the data; KD: Drafted the manuscript; KD, FD, and JS-B: Revised the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2018.00026/full#supplementary-material

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