Regulation and production of Tcf, a cable-like fimbriae from Salmonella enterica serovar Typhi

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tcf (Typhi colonization factor) is one of the 12 putative chaperone/usher fimbrial clusters present in the Salmonella enterica serovar Typhi genome. We investigated the production, expression and regulation of tcf as well as its role during interaction with human cells. The tcf gene cluster was cloned and induced in Escherichia coli and S. Typhi, and the production of intertwined fibres similar to the Cbl (cable) pili of Burkholderia cepacia was observed on the bacterial surface by electron microscopy. In S. Typhi, tcf was expressed more after growth in M63 minimal medium than in standard Luria–Bertani medium. Analysis of the promoter region identified putative binding sites for the global regulators RcsB, ArgR and Fur. The expression of tcf was measured in isogenic strains lacking these global regulators. Under the conditions tested, the results showed that tcf expression was higher in the fur mutant and was regulated by iron concentration. Fur may regulate these fimbriae indirectly via the small RNAs RyhB1 and RyhB2. An isogenic mutant harbouring a deletion of the tcf cluster did not demonstrate any defect in adhesion or invasion of human epithelial cells, or in phagocytosis or survival in macrophages, when compared to the WT serovar Typhi strain. However, the tcf cluster contributed to adherence to human epithelial cells when introduced into E. coli. Thus, tcf genes encode functional fimbriae that can act as an adhesin and may contribute to colonization during typhoid fever.

INTRODUCTION

Bacterial adhesion to host cells represents the initial and often critical step for pathogenesis. Adhesion is commonly mediated by fimbriae, which are structures found on the bacterial surface involved in specific interactions with host cells. Usually, fimbriae are not constitutively expressed and only produced under specific environmental conditions (Neidhardt et al., 1996). Fimbrial expression can be controlled positively or negatively at the genetic level. Some regulatory mechanisms are unique to specific strains, whilst others are shared by many bacteria (Clegg et al., 2011). Some of these mechanisms include invertible DNA elements, DNA methylation, cyclic di-GMP and DNA-binding regulators (Clegg et al., 2011).

Salmonella enterica isolates are classified into >2500 serovars. Some serovars can infect different hosts, such as serovar Typhimurium (S. Typhimurium), whereas other serovars display a narrower host range or are specific to only one host species, such as serovar Typhi (S. Typhi). The latter has no known animal reservoir and is specific to humans, causing the systemic disease typhoid fever. Interestingly, each serovar of Salmonella harbours a unique combination of fimbrial gene clusters, suggesting a role for fimbriae in host adaptation (Clegg et al., 2011). Genome sequencing analysis has revealed 12 chaperone/ usher fimbrial clusters along with curli, a nucleator-dependent assembly adhesin and a type IVB pilus in the genome of S. Typhi (Parkhill et al., 2001). Little is known concerning conditions of expression and the roles of each of the different fimbrial adhesins in S. enterica. Few of these adhesion systems have been characterized so far and none have ever been visualized in S. Typhi. Five fimbrial clusters, i.e. sif, sta, ste, stg and tcf, and the type IV pilus present in S. Typhi strains are absent from the genome of S. Typhimurium, responsible for gastroenteritis in humans (Townsend et al., 2001). These S. Typhi-specific adhesion systems may be involved in its host specificity.

The tcf (Typhi colonizing factor) cluster is of particular interest. This 5.2 kb cluster consists of only four genes tcfABCD, encoding, respectively, the chaperone, the major subunit, the usher and the tip adhesin (Fig. 1a). Tcf belongs to the alternate chaperone/usher family of the x-fimbrial clade, a group of fimbriae that contains many

Abbreviations: SPI, Salmonella pathogenicity island; sRNA, small RNA.
It has been shown that tcfA is expressed at high salt concentrations but its deletion did not impair bacterial adhesion or invasion of epithelial cells (Bishop et al., 2008). Furthermore, antibodies against TcfB were detected in the blood of patients with typhoid fever (Harris et al., 2006), suggesting that these fimbriae are produced and may play a role during infection. However, the regulatory mechanisms that control the expression of the tcf genes are still unknown.

In this study, we characterized the production, expression, regulation and role of tcf during interaction with host cells.

We demonstrated that the tcf cluster can produce long peritrichous appendages, structurally similar to the Cbl pili that have been characterized in B. cepacia (Sajjan et al., 1995). The expression of tcf was influenced by Fur, maybe indirectly via the small RNAs (sRNAs) RhyB1 and RhyB2. The production of Tcf in an E. coli K-12 strain increased adhesion to human epithelial cells, whilst its absence in S. Typhi had little impact during the interaction with either epithelial or macrophage cells, probably due to the redundancy of adhesion systems present in S. Typhi that could compensate for the absence of Tcf.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** Strains and plasmids used in this study are described in Table 1. Bacteria were routinely grown overnight with agitation in Luria-Bertani (LB) broth at 37 °C and on LB agar plates. For experiments dependent on iron concentration, bacteria were grown overnight in M63 minimal medium (low iron) as described previously (Leclerc et al., 2013). High-iron conditions were obtained by the addition of 100 μM FeCl₃ to M63 medium. The M63 minimal medium was inoculated with 1/100 dilution of an overnight culture grown in LB washed once in M63. When required, antibiotics or supplements were added at the following concentrations: 50 μg kanamycin ml⁻¹, 50 μg ampicillin ml⁻¹, 34 μg chloramphenicol ml⁻¹, 50 μg dianaminopimelic acid ml⁻¹, 1 mM IPTG and 40 μg X-Gal ml⁻¹. Transformation of bacterial strains was done using the calcium/manganese-based or electroporation methods as described previously (O’Callaghan et al., 1990).

**Cloning of the tcf fimbrial cluster.** The tcf gene cluster was amplified from genomic DNA of S. Typhi strain ISP1820 using the Elongase enzyme mixture (Invitrogen) with primers tcfAFprom and tcfDR (Table 2). The 5.8 kb PCR product containing the predicted native promoter was purified and cloned into low-copy-number vector pWSK29 (XbaI/NorI), resulting in plasmid pSIF119. Similarly, tcfABCD was amplified starting at the predicted start codon of tcfA by Q5 High-Fidelity DNA polymerase (New England Biolabs) using primers tcf_operon_F_SacI and tcf_operon_R_XbaI, and then cloned into the IPTG-inducible vector pMMB207 (SacI/XbaI), resulting in plasmid pSIF420. The plasmids were transformed into the non-fimbriated E. coli K-12 ΔfimBEACDFGH:: Km mutant strain ORN172 (Woodall et al., 1993) or into S. Typhi.

**Induction of Tcf: SDS-PAGE and microscopy.** E. coli or S. Typhi strains harbouring the inducible vector pMMB207 with or without the tcf cluster were grown overnight in LB broth at 37 °C, then diluted 1/100 and cultured to OD₆₀₀ 0.6. The induction was done for 3 or 20 h in the presence of 1 mM IPTG. Cell surface proteins were extracted at 60 °C for 15 min. Bacteria were pelleted by centrifugation (3000 g for 10 min) and the supernatant was precipitated with 10 % trichloroacetic acid. Protein concentrations were normalized according to the OD₆₀₀ of each bacterial suspension. Proteins were loaded in a 15 % SDS-PAGE gel followed by Coomassie blue staining. The band of interest was cut from the gel, destained and after a trypsin digestion, peptides were sequenced using LC-MS/MS at the Center for Advanced Proteomics Analyses (IRIC, Université de Montréal).

For microscopy, the induced cultures were washed once with PBS and fixed overnight with 2 % glutaraldehyde. The cells were adsorbed onto nickel Formvar/carbon-coated grids for 2 min. The grids were washed three times with water. Excess liquid was removed and a drop...
of 0.1 or 0.5 % phosphotungstic acid (negative stain) was placed on
the grid. Cells were examined under a Philips CM-100 electron
microscope operated at 60 kV and images were acquired with an
AMT XR-80 CCD digital camera system (Advanced Microscopy
Techniques).

Generation of a \( \text{P}_{\text{tcfA}}-\text{lacZ} \) transcriptional fusion and
\( \beta \)-galactosidase assay. The tcfA promoter region was amplified
using the Platinum Taq DNA Polymerase enzyme mix (Invitrogen)
with tcfA promoter (EcoRI) and tcfA promoter primers. The 578 bp PCR
fragment was digested with EcoRI, purified and ligated upstream of
the promoterless lacZ gene in pRS415 (EcoRI/SmaI), resulting in
plasmid pSIF219. The tcf fusion vector was transformed in the
S. Typhi WT strain and the different isogenic mutant strains. The
expression of tcf was measured by \( \beta \)-galactosidase assay following
growth under different conditions or in different background strains.

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The human monocyte cell line THP-1 (ATCC TIB-202) was maintained in RPMI 1640 (Wisent) containing 10 % (v/v) heat-inactivated FBS (Wisent), 1 mM sodium pyruvate (Wisent) and 1 % MEM non-essential amino acids (Wisent). A stock culture of these cells was maintained as monocyte-like, non-adherent cells at 37 °C in an atmosphere containing 5 % CO2. For macrophage infection, cells were seeded at 2×10^5 cells per well in 24-well tissue culture plates 24 h before the assays. At 1 h before infection, cells were washed three times with prewarmed PBS (pH 7.4) and fresh complete medium was added to each well. S. Typhi strains were grown overnight without agitation in LB containing 0.3 M NaCl (SPI-1-inducing conditions) and added to each well at m.o.i. 20:1. E. coli strains were grown overnight without shaking in LB and added to each well at m.o.i. 100:1. The 24-well plates were then centrifuged at 1000 × g for 20 min to synchronize infection, incubated at 37 °C for 180 min (invasion) by addition of PBS/deoxycholic acid sodium salt (0.1 %), and samples were diluted and spread on LB plates for enumeration as c.f.u.

### Construction of isogenic deletion mutants of *S. Typhi*
A suicide vector was constructed for the deletion of the *tcf* fimbrial operon from *S. Typhi* strain ISP1820. A 578 bp fragment of the 5′ end of the *tcfA* gene from *S. Typhi* was generated by PCR using primers *tcfA_Fprom* and *tcfA_Rover*, and a 226 bp fragment of the 3′ end of the *tcfD* gene was generated by PCR using primers *tcfD_Fover* and *tcfD_Rover*. These two fragments were joined by overlapping the sequences in a second PCR using primers *tcfA_Fprom* and *tcfD_Rover*. The resulting fragment harbouring an internal deletion of *tcf* was cloned into the XbaI/NorI sites of pMEG-375 to create pSIF098. Allelic replacement of the *argR* region, confirmed by PCR (data not shown). A similar approach was used to create isogenic *argR* and *rcsDBC* deletion mutants. All the primers used for these constructions are described in Table 2. *Salmonella* mutants containing *fur*, *ryhB1* or *ryhB2* mutations were described previously (Leclerc et al., 2013).

### Interactions with human epithelial cells: adhesion and invasion assays
**INT-407** (Henle) cells (ATCC CCL-6) were grown in minimal essential medium (Wisent) supplemented with 10 % (v/v) heat-inactivated FBS (Wisent) and 25 mM HEPES (Wisent). For the adherence and invasion assays, 2×10^5 cells were seeded in 24-well tissue culture plates 24 h before the assays. At 1 h before infection, cells were washed three times with prewarmed PBS (pH 7.4) and fresh complete medium was added to each well. *S. Typhi* strains were grown overnight without agitation in LB containing 0.3 M NaCl (SPI-1-inducing conditions) and added to each well at m.o.i. 20:1. E. coli strains were grown overnight without shaking in LB and added to each well at m.o.i. 100:1. The 24-well plates were then centrifuged at 800 g for 5 min to synchronize infection, incubated at 37 °C in 5 % (v/v) CO2 for 90 min and rinsed three times with PBS. Extracellular bacteria were killed by an additional incubation period of 90 min with 100 µg gentamicin ml−1. Cells were lysed after 90 (adherence) or 180 min (invasion) by addition of PBS/deoxycholic acid sodium salt 0.1 %, and samples were diluted and spread on LB plates for enumeration as c.f.u.

### Infection of cultured macrophages: survival assays
The human monocyte cell line THP-1 (ATCC TIB-202) was maintained in RPMI 1640 (Wisent) containing 10 % (v/v) heat-inactivated FBS (Wisent), 1 mM sodium pyruvate (Wisent) and 1 % MEM non-essential amino acids (Wisent). A stock culture of these cells was maintained as monocyte-like, non-adherent cells at 37 °C in an atmosphere containing 5 % (v/v) CO2. For macrophage infection, cells were seeded at 5×10^5 cells per well in 24-well tissue-culture dishes and differentiated by the addition of 10−4 M phorbol 12-myristate 13-acetate (Sigma) for 48 h. Macrophage infection was performed as described previously unless otherwise specified (Daigle et al., 2001). Briefly,
bacteria were grown overnight without agitation in LB to OD600 0.6 (SPI-2-inducing condition). Bacteria were added to the cell monolayer at m.o.i. 10:1 and plates were centrifuged for 5 min at 800 g to synchronize bacterial uptake. After 20 min incubation at 37 °C, extracellular bacteria were removed by washing cells three times with prewarmed PBS and the infected monolayers were either lysed with PBS/deoxycholic acid sodium salt 0.1% (0 h) or incubated for 2 h in media containing 100 μg gentamicin ml⁻¹ (Wisent) to kill extracellular bacteria, and then with 12 μg gentamicin ml⁻¹ for the rest of the experiment. The number of surviving bacteria was determined as c.f.u. by plating on LB agar.

Statistical analyses. Statistical differences were assessed using Student’s unpaired t-test.

RESULTS

Tcf production

The tcfABCD cluster (Fig. 1a) was cloned into the IPTG-inducible plasmid pMMB207 (pSIF420) as fimbriae are often poorly expressed in vitro (Forest et al., 2007; Humphries et al., 2003; Korea et al., 2010). Tcf was first expressed in a non-fimbriated E. coli strain in order to avoid the redundancy of the multiple systems in S. Typhi. After induction, cell surface extracts obtained by heat shock to detach bacterial surface proteins revealed a specific band of ~15 kDa in the strain harbouring the tcfABCD fimbrial genes (Fig. 1b). This band was not visible in cell surface extracts of a strain harbouring the empty vector. Accordingly, we tested the production in S. Typhi and similar results were obtained. However, higher levels of cell lysis were observed and only 3 h induction was necessary to visualize the 15 kDa band (Fig. 1c). The 15 kDa band was extracted and sent for amino acid sequencing. MS analyses identified TcfB and sent for amino acid sequencing. MS analyses identified TcfB, covering 148/191 aa, representing 100 % of the predicted mature protein (148 aa).

The production of extracellular fimbrial structures on the bacterial surface was investigated by transmission electron microscopy (Fig. 2). Induced expression of Tcf resulted in the production of elongated fimbriate-like structures on the E. coli bacterial surface (Fig. 2a), whereas no similar structure was observed on the surface of the strain harbouring the empty vector (Fig. 2b). When Tcf was induced in S. Typhi, abundant and long extracellular appendages were observed (Fig. 2c). The peritrichous filaments were intertwined, forming bundles (Fig. 2d) and a network of fibres between bacteria (Fig. 2e). These fimbriae closely resembled Cbl pili of B. cepacia (Sajjan et al., 1995).

Expression of tcf

To investigate tcf expression, a transcriptional fusion between the tcfA promoter and the lacZ gene coding for β-galactosidase was constructed using the plasmid pRS415 and introduced into S. Typhi. The effect of the growth phase on tcf expression was studied with bacteria grown in LB medium. β-Galactosidase expression was low during the exponential phase, and increased in the stationary phase and on solid medium (Fig. 3). The expression reached its maximum level after 24 h incubation (overnight shaking). tcf expression was also evaluated during growth conditions used for SPI-1 induction (invasion) (overnight in LB containing 0.3 M NaCl without agglutitation) or for SPI-2 induction (survival in macrophages) (stationary, overnight in LB without agglutitation). Under these conditions, tcf expression levels were similar to the late exponential phase with agglutitation. Finally, growth in minimal medium (M63, overnight shaking) was tested and gave the highest tcf expression levels, and was used for the rest of the study (Fig. 3).

Regulation of tcf expression

To understand the regulation involved in tcf transcription, bioinformatics analysis of the tcfA promoter region was performed. Putative binding sites for H-NS, RcsB, Fur and ArgR were identified using the bacterial promoter analysis software Softberry Bprom (www.softberry.com) (Fig. 4a). Putative regulation by the global regulators RcsB, Fur and ArgR was evaluated by generating three isogenic S. Typhi deletion mutants of rcsDBC, fur and argR. H-NS deletion is pleiotropic and was not investigated. The tcfA–lacZ reporter plasmid was transformed into the mutant strains and β-galactosidase activity was measured from strains grown overnight in M63 with agglutitation. No difference in tcf expression was observed when comparing the argR mutant and the rcsDBC mutant with the WT strain (Fig. 4b). However, the transcription of tcf was significantly influenced by Fur. The expression of tcf significantly increased in the fur mutant in comparison with the WT strain. Therefore, these results suggested that the presence of the iron regulator Fur inhibited the expression of the tcf fimbrial cluster.

Role of iron, Fur and the sRNAs RyhB1/RyhB2 in regulation of tcf expression

As the Fur regulator is activated by iron and the regulation of its target genes is usually iron-dependent, the regulation of tcf by iron levels (high and low) was investigated in the WT and fur mutant strains. The expression of tcf was lower under high-iron conditions for each strain (Fig. 5a). This result was in accordance with the Fur-dependent repression of tcf expression, because Fur binding activity occurs under iron-rich conditions (Escolar et al., 1999). Tcf expression was lower in the WT strain under both high- and low-iron conditions (Fig. 5a). Interestingly, Tcf expression was significantly higher in the fur mutant under low-iron conditions, where fur is usually inactive.

The tcf cluster is absent in S. Typhimurium and in E. coli, but Fur is conserved in these strains. To determine whether Fur regulation of tcf was specific to S. Typhi, the tcfA–lacZ reporter was used in E. coli.
reporter plasmid was transformed into *S. Typhimurium* and *E. coli* K-12 and their isogenic fur mutants. β-Galactosidase production was measured from strains grown overnight in M63. The expression of tcf was significantly higher in the fur mutant of *Salmonella*, but not in the fur mutant of *E. coli* (Fig. 5b).

Fur has been shown to indirectly regulate gene transcription through the repression of RNA RyhB in *E. coli* (Massé & Gottesman, 2002). As there are two homologues of RyhB in *Salmonella*, i.e. RyhB1 (RfrA) and RyhB2 (RfrB), we investigated the role of each of these sRNAs in tcf expression. Isogenic mutants ryhB1 and/or ryhB2 were generated in the WT *S. Typhi* strain and in the fur mutant. The tcfA-lacZ reporter plasmid was transformed into the mutant strains and β-galactosidase production was measured from strains grown overnight in M63. No difference in tcf expression was observed when comparing the ryhB1 or ryhB2 mutant strains to the WT strain (Fig. 6). Interestingly, tcf expression significantly decreased with the loss of both sRNAs compared with the WT strain (Fig. 6). tcf expression also significantly decreased with the loss of each sRNA in the fur mutant. No regulation was observed with the deletion of all three regulators (the triple mutant), as the level of expression of tcf was not significantly different from that of the WT (Fig. 6). Thus, the sRNAs RyhB1 and RyhB2 seemed to play a role in the expression of the tcf fimbrial cluster.

**Fig. 2.** Transmission electron micrographs of Tcf fimbriae. Visualization of bacterial surface structures by transmission electron microscopy. Images after 20 h growth in LB with 1 mM IPTG of (a) *E. coli* ORN172 (pMMB207-tcfABCD), (b) *E. coli* (pMMB207), (c–e) *S. Typhi* (pMMB207-tcfABCD) and (f) *S. Typhi* (pMMB207). Bar, 500 nm.
Fig. 3. tcf expression in S. Typhi. β-Galactosidase activity was measured in strains containing a transcriptional fusion between the tcfA promoter and the lacZ gene. The S. Typhi WT strain harbouring this fusion was grown in LB medium with agitation to the early exponential phase (OD₆₀₀ 0.3), mid-exponential phase (OD₆₀₀ 0.6), late exponential phase (OD₆₀₀ 0.9), stationary phase (OD₆₀₀ 1.2) and overnight (overnight). This strain was also grown overnight without agitation (static) in LB (SPI-2-inducing conditions) and in LB containing 0.3 M NaCl (SPI-1-inducing condition), on LB agar (solid medium) and on M63 minimal medium (overnight with agitation). Results are expressed as mean ± SEM of three experiments performed in triplicate.

Fig. 4. tcf regulation in S. Typhi. Regulatory region of the tcfA promoter. (a) The 578 bp sequence used in the tcfA–lacZ fusion including 473 bp upstream of the tcfA gene and 105 bp after the start codon (sequence in red). The promoter region of tcfA contains putative binding sites for RcsB, Fur, H-NS and ArgR (underlined). (b) β-Galactosidase activity was measured from PₜcfA–lacZ transcriptional fusion. S. Typhi WT strain and the isogenic fur, argR and rcsDBC mutants harbouring this fusion were grown in M63 medium with agitation (overnight shaking). All assays were conducted in triplicate and repeated independently at least three times. The results are expressed as mean ± SEM of the replicate experiments. *Significant difference between the mutant and WT strains (P<0.05).
Role of *Tcf* during interaction with host cells

As fimbriae are commonly known to play an important role in association with host epithelial cells, we investigated the potential role of the *tcf* fimbrial cluster during interaction with INT-407 epithelial cells. The effect of complete deletion of the *tcfABCD* fimbrial cluster on adherence and invasion was assessed by determining the number of bacteria associated with epithelial cells after 90 or 180 min of co-culture. No difference was observed in the number of bacteria associated with epithelial cells during adherence or invasion assays between the *tcf* mutant and the WT strain (Fig. 7a). As other adhesion systems may compensate for the absence of *Tcf* in *S. Typhi*, the complete *tcf* cluster was cloned under its native promoter in the low-copy-number plasmid pWSK29, and the construct was transformed into non-fimbriated *E. coli* K-12 strain ORN172. The introduction of *tcf* into *E. coli* conferred a significantly higher level of adherence to epithelial cells (*P*=0.0078), which was two-fold higher than the level of adherence of the strain containing the empty vector (Fig. 7b).

The role of *Tcf* was also investigated during the interaction of *S. Typhi* with human macrophages, as *S. Typhi* survives within these cells during typhoid fever. Phagocytosis was measured after 20 min (0 h) and survival after 2 and 24 h of interaction between the bacteria and cells. Slight differences in the amounts of bacteria recovered at each time point were observed between the WT and Δ*tcfABCD* mutant (Fig. 7c). However, none of these effects were statistically significant.

Collectively, these results indicated that under the *in vitro* conditions tested, *Tcf* had a limited role during *S. Typhi* interaction with host cells but that the *tcf* fimbrial cluster encoded functional fimbriae playing a role in adherence when expressed in a non-fimbriated *E. coli* strain.

**DISCUSSION**

*S. Typhi* possesses 12 putative chaperone/usher loci, few of these fimbrial systems having been characterized to date. In this study, we demonstrated that the *tcf* fimbrial cluster
can produce functional fimbriae. To the best of our knowledge, this is the first time that a fimbrillar structure of *S. Typhi* has been visualized on the bacterial surface by microscopy. *Tcf* produced unusual, long, intertwined appendages similar to the Cbl pilus of *B. cepacia* (Sajjan *et al.*, 1995). Cbl pilus belong to the z-fimbrial class (Nuccio & Baumler, 2007). Phylogenetic analyses have shown that *Tcf* and Cbl are predicted to be more closely related to each other than to other members of the z-fimbriae, such as CS and CFA/I fimbriae (Anantha *et al.*, 2004; Nuccio & Baumler, 2007). *TcfB* shares 44 % identity and 64 % similarity to CblA from *B. cepacia* strain BC7. Furthermore, the unusual cable structures of *Tcf* and Cbl are distinct from thinner, more typical fimbrial morphologies observed for CS1 and CFA/I fimbriae.

One way to obtain information concerning *Tcf* fimbral expression is to use a *tcfA*–*lacZ* transcriptional fusion on a vector to measure the transcription levels of the *tcf* genes and to detect slight changes in gene expression. *tcfA* was expressed during growth in LB, with the highest level obtained in the late stationary phase (overnight), which also corresponds to conditions under which nutrients, including iron, begin to be depleted. High expression in the stationary phase was observed previously for several fimbriae of *E. coli* O157 : H7 (Low *et al.*, 2006), as well as for Stg of *S. Typhi* (Forest *et al.*, 2007). The highest expression of *tcfA* was after overnight growth in a minimal medium (M63). A previous study showed that *tcfA* expression was low in the stationary phase using reverse transcription PCR, and it was enhanced when bacteria were grown overnight without agitation with high salt concentrations (SPI-1-inducing conditions) (Bishop *et al.*, 2008). Our results showed that the transcription of *tcfA* was not increased under SPI-1-inducing conditions and was not influenced by the addition of salt when grown overnight without agitation. The differences in *tcf* expression in these two studies may be due to the methods used (reverse transcription PCR compared to transcriptional fusion) and/or the strains used (BRD948 compared with ISP1820). Overall, we have shown that the *S. Typhi* *tcf* cluster can be expressed during *in vitro* growth conditions.

Regulation of gene expression is usually achieved by the binding of regulatory proteins in the region of the promoter, and mutations in this region can affect transcription by altering the affinity of a protein (regulator or RNA polymerase) to its binding site. Analysis of the regulatory region of *tcfA* identified putative binding sites for the regulators H-NS, ArgR, RcsB and Fur (Fig. 4a). H-NS is known to repress the transcription of many fimbriae (Corcoran & Dorman, 2009; Korea *et al.*, 2010; Müller *et al.*, 2006; Nicholson & Low, 2000; Torres *et al.*, 2007; White-Ziegler *et al.*, 2000) in *E. coli* and *Salmonella*, including the Tcf homologue CS1 fimbriae (Murphree *et al.*, 1997). As H-NS regulation was expected and the phenotype of the H-NS mutant is pleiotropic, its role was not investigated further. ArgR is a repressor of arginine metabolism
genes and is involved in Xer-mediated recombination as an accessory factor (Paul & Summers, 2004). Its role as a regulator of fimbrial expression has not been demonstrated, and the absence of ArgR had no effect on the transcription of tcfA. Rcs was shown to be involved in the regulation of fimbrial gene expression in different bacteria, in particular in the repression of the pil genes coding for the S. Typhi type IVB pili (Lee et al., 2006; Lehti et al., 2012). The absence of the Rcs global regulatory system did not alter tcf expression under the conditions we investigated (Fig. 4b). Fur represents a global regulator activated by iron and is involved in iron homeostasis (Escolar et al., 1999). Interestingly, both homologues of Tcf, i.e. Cbl and CFA/I, were also shown to be repressed by iron (Karjalainen et al., 1991; Tomich & Mohr, 2004), suggesting the involvement of the iron regulator Fur. Our data showed that Fur represses tcf expression (Fig. 4b), whereas low iron conditions increased it. As fur is inactivated under low iron availability (Escolar et al., 1999), these results correlated with the increased expression of tcf in the fur mutant. Interestingly, the regulation of tcf by Fur was observed in S. Typhimurium and S. Typhi, but not in E. coli, although Fur is highly conserved between these genera, suggesting an indirect mechanism of regulation by Fur that is specific to Salmonella.

Fur represses transcription of the sRNAs RyhB1 and RyhB2 in S. Typhi (Leclerc et al., 2013). Therefore, Fur might indirectly repress tcf expression by repressing these sRNAs, as loss of fur or growth in low iron concomitantly increases expression of both sRNAs (RyhB1 and RyhB2) and tcf. Furthermore, we have shown that the double rhyhB1/rhyhB2 mutant exhibited significantly less tcf promoter activity compared with the WT strain (Fig. 6). In fact, the sRNAs RyhB1 and RyhB2 appear to play a role as positive regulators of tcf. However, it is difficult to determine if the regulation of tcf expression by these sRNAs is by direct or indirect mechanisms. As there is only one RyhB in E. coli, it could explain why there was no regulation of tcf observed and why the regulation was specific to Salmonella, as both sRNAs may be needed for regulation of tcf (Fig. 6). This may also suggest that some other system (absent in E. coli) is affected in Salmonella and changes the regulation of Tcf. More studies are needed to investigate and better understand the regulation mechanism of tcf.

We have also characterized the role of the tcf cluster of S. Typhi during the interaction with host cells. As fimbriae are normally used to mediate adherence to host cells, we compared the interaction of the WT and isogenic tcf deletion mutant strains with epithelial cells. No significant difference was observed between the adhesion and invasion levels of both strains (Fig. 7a), confirming previous experiments (Bishop et al., 2008). Some Salmonella fimbrial clusters were shown to be involved in the interaction with macrophages in different in vitro experiments and in long-term infection processes in different in vivo models (Edwards et al., 2000; Forest et al., 2007; Klumpp & Fuchs, 2007; Lawley et al., 2006; van der Velden et al., 1998). Thus, we also evaluated the role of the tcf mutant in the interaction with human cultured macrophages, and despite the slight variations in the number of bacteria recovered during the infection for the WT and the tcf mutant strains, no statistically significant difference was observed (Fig. 7c). Thus, the absence of the tcf cluster did not have any effect during interaction with host cells. Redundancy of fimbrial systems may explain the absence of a phenotype, or the presence of iron in the tissue culture medium (in the serum) may repress tcf expression.

However, it is common for bacteria to possess multiple virulence factors that have functional similarity. Redundancy is usually seen during the adhesion process of bacteria, which can use multiple adhesins to increase their interaction with host cells (Virji, 2009). Therefore, it is possible that the true function of Tcf was masked by the presence of another system during the interaction with epithelial cells and macrophages, as S. Typhi harbours 12 different fimbrial clusters as well as many other putative adhesins (Forest & Daigle, 2012; Townsend et al., 2001). Although the Tcf fimbrial cluster is dispensable for the interaction of S. Typhi with epithelial and macrophage cells under the conditions described here, we cannot discount a role for Tcf in human infection as antibodies against TcfB were detected following typhoid fever in human serum (Harris et al., 2006).

In order to avoid redundancy, the tcf cluster was cloned under its native promoter and transformed in a non-fimbriated E. coli strain. The adhesion to epithelial cells was significantly higher in the presence of tcf (Fig. 7b). Adhesion phenotype mediated by fimbrial genes expressed in E. coli strains was observed for other Salmonella fimbriae, such as Stg and Sef (Forest et al., 2007; Rank et al., 2009), confirming that fimbriae can contribute to the interaction with host cells.

As fimbriae are exposed to the immune system they are candidates for vaccine development, and this is why it is important to understand their regulation and the role played by each fimbrial operon. To the best of our knowledge, this is the first report demonstrating that the Tcf fimbrial cluster of S. Typhi is functional and, when expressed, produces unusual morphology similar to the Cbl pili of B. cepacia. Furthermore, Fur and the sRNAs RyhB1 and RyhB2 play a role in Tcf regulation. Further research is needed to demonstrate the specific mechanism of tcf expression in vitro and during the infection process.

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Salmonella enterica

subunits encoded by 11 Salmo nella enterica

class 5 adhesive fimbriae of enterotoxigenic Escherichia coli

functional relationships of colonization factor antigen I and other


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