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Contribution of the *stg* Fimbrial Operon of *Salmonella enterica* Serovar Typhi during Interaction with Human Cells▽

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Salmonella **serovars contain a wide variety of putative fimbrial systems that may contribute to colonization of specific niches.** ***Salmonella enterica*** **serovar Typhi is the etiologic agent of typhoid fever and is a pathogen specific to humans. In a previous study, we identified a gene, STY3920 (*stgC*), encoding the predicted usher of the *stg* fimbrial operon, that was expressed by serovar Typhi during infection of human macrophages. The *stg* genes are located in the *glmS-pstS* intergenic region in serovar Typhi and certain *Escherichia coli* strains, but they are absent in other *S. enterica* serovars. We cloned the *stg* fimbrial operon into a nonfimbriate *E. coli* K-12 strain and into *S. enterica* serovar Typhimurium. We demonstrated that the *stg* fimbrial operon contributed to increased adherence to human epithelial cells. Transcriptional fusion assays with serovar Typhi suggested that *stg* is preferentially expressed in minimal medium. Deletion of *stg* reduced adherence of serovar Typhi to epithelial cells. However, deletion of *stg* increased uptake of serovar Typhi by human macrophages, and overexpression of *stg* in serovar Typhi and serovar Typhimurium strains reduced phagocytosis by human macrophages. These strains survived inside macrophages as well as the wild-type parent. Although the *stgC* gene contains a premature stop codon that disrupts the expected open reading frame encoding the usher and is therefore considered a pseudogene, our results show that the *stg* operon may encode a functional fimbria. Thus, this serovar Typhi-specific fimbrial operon contributes to interactions with host cells, and further characterization is important for understanding the role of the *stg* fimbrial cluster in typhoid fever pathogenesis.**

The genus *Salmonella* is composed of two species, *Salmonella bongori* and *Salmonella enterica*. *S. enterica* comprises more than 2,400 serovars (11) and has been divided into seven subspecies (19). Subspecies I contains *S. enterica* serovars Typhi and Typhimurium and most of the other serovars that cause diseases in humans and other warm-blooded animals. Some serovars, such as serovar Typhimurium, cause disease in a variety of animals, whereas other serovars, such as serovar Typhi, cause disease in only one or a few species. Serovar Typhi is a human-specific pathogen and the etiologic agent of typhoid fever, a systemic disease, whereas serovar Typhimurium causes localized gastroenteritis in most cases of human infection. In spite of a high degree of genome homology (>90%) between serovars Typhi and Typhimurium (22, 29), the difference in the types of diseases that these serovars cause in humans, systemic and localized, respectively, suggests that one difference between these pathovars might be in the way that these closely related pathogens interact with host cells. Each of these serovars might produce or secrete distinct molecules that contribute to differences in tissue tropism. The genomes of *Salmonella* serovars Typhi and Typhimurium were completed and compared previously (22, 29). The serovar Typhi strain CT18 genome contains 601 genes located in 82 unique genomic regions that are absent from the serovar Typhimurium strain LT-2 genome (29). Thus, it is likely that serovar Typhi possesses unique genetic information that may be important for systemic spread and survival in the human host. The largest unique region in serovar Typhi is 134 kb long and was designated *Salmonella* pathogenicity island 7 (SPI-7). SPI-7 harbors the *viaB* locus encoding the Vi antigen, which is used in the current conjugated vaccine (17). Vi is a polysaccharide capsule involved in preventing interleukin-8 production, thus reducing neutrophil influx in the intestine (31, 33). The *pil* genes coding for type IV pili facilitate bacterial entry into human epithelial cells and are also located on SPI-7 (43).

After ingestion, serovar Typhi is transported to the intestinal lumen, where it adheres to and invades the small intestine. Bacteria are taken up by mononuclear cells in the intestinal lymphoid tissue, drain into the general circulation, and spread to the spleen and liver. After replication, a large number of bacteria are released into the bloodstream, which coincides with the onset of typhoid fever symptoms. In chronic carriers, bacteria can persist in the mesenteric lymph nodes, bone marrow, spleen, and gall bladder for the life of the patient. Many virulence factors may be needed and expressed during the course of infection.

Adhesion to host cells and mucosal surfaces is often considered an essential step because it allows bacteria to initiate colonization. Fimbriae or pili and other surface molecules mediate adherence via specific receptors on host cell surfaces. Genes encoding a wide variety of putative fimbriae are present in *Salmonella* serovars, but only a few *Salmonella* fimbriae have been characterized so far. These putative fimbriae may confer different binding specificities required at different steps of the infection and may be involved in host adaptation by conferring

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Source or reference
S. enterica serovar Typhi strains		
ISP1820	Wild type	R. Curtiss III (Arizona State University)
DEF004	ISP1820 Δ*stg*	This study
DEF033	ISP1820(pSIF018)	This study
DEF064	ISP1820(pCR2.1)	This study
DEF066	DEF004(pSIF026)	This study
DEF068	ISP1820::P*stgA-lacZ*(::pSIF020)	This study
S. enterica serovar Typhimurium strains		
χ3339	Mouse-passaged isolate of SL1344 *rpsL hisG*	9
DEF047	χ3339(pSIF018)	This study
DEF048	χ3339(pCR2.1)	This study
E. coli strains		
DEF045	ORN172(pCR2.1)	This study
DEF049	ORN172(pSIF018)	This study
MGN-617	SM10 λpir *asd thi thr leu tonA lacY supE recA* RP4 2-Tc::Mu[λpir] Δ*asdA4*	15
ORN172	*thr-l leuB thi-1* Δ(*argF-lac*)*U169 xyl-7 ara-13 mtl-2 gal-6 rpsL tonA2 supE44* Δ(*fimBEACDFGH*)::*kan pilG1*	42
Plasmids		
pCR2.1	High-copy-number cloning vector, Km^r Ap^r	Invitrogen
pFUSE	*lacZYA mob*$^+$ (RP4), R6K *ori* (suicide vector), Cm^r	2
pMEG-375	*sacRB mobRP4 ori*R6K, Cm^r Ap^r	Megan Health (St. Louis, MO)
pSIF004	Suicide vector with flanking region of *stgA* in 5′ end and *stgD* in 3′ end used for *stg* deletion	This study
pSIF016	pCR2.1 carrying a 530-bp fragment of *stgA*	This study
pSIF018	pCR2.1 carrying a 5-kb fragment of *stg* (pCR2.1*stg*)	This study
pSIF020	pFUSE carrying a 530-bp fragment of *stgA*, Cm^r	This study
pSIF026	pWSK29 carrying a 5-kb fragment of *stg* (pWSK*stg*)	This study
pWSK29	Low-copy-number cloning vector, Amp^r	41

the ability to bind to specific host cells. The genome sequence of serovar Typhi contains 13 putative operons corresponding to fimbrial gene sequences, designated *bcf*, *csg* (*agf*), *fim*, *saf*, *sef*, *sta*, *stb*, *stc*, *std*, *ste*, *stg*, *sth*, and *tcf*, as well as *pil* coding for the type IV pili (29). Five of these operons, *sef*, *sta*, *ste*, *stg*, and *tcf*, and the type IV pili were not detected in serovar Typhimurium (29). In a previous study, we determined that STY3920 (*stgC*), a gene encoding the usher of the putative *stg* fimbrial operon, is absent in serovar Typhimurium and is expressed by serovar Typhi during infection of human macrophages (6). *stgC* contains a premature stop codon that disrupts the predicted open reading frame (ORF) encoding the usher, and it is therefore considered a pseudogene. As similar fimbrial clusters in *Escherichia coli* also contain genes with premature stop codons and have functional roles (7, 14, 26, 37), we hypothesized that the *stg* operon may encode functional fimbriae that contribute to the interaction of serovar Typhi with human cells. In this study, we cloned and characterized the *stg* fimbrial operon and demonstrated its role in adhesion to epithelial cells and phagocytosis by macrophages.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were routinely grown in Luria-Bertani (LB) broth at 37°C, unless indicated otherwise. When required, antibiotics, amino acids, or supplements were added at the following concentrations: kanamycin, ampicillin, and diaminopimelic acid (DAP), 50 μg/ml; chloramphenicol, 34 μg/ml; and tryptophan, cysteine, and arginine, 22 μg/ml. Transformation of bacterial strains was routinely done by using the calcium/manganese-based or electroporation method as described previously (27).

Cloning of the *stg* fimbrial operon. The *stg* operon was amplified from genomic DNA of strain ISP1820 using the Elongase enzyme mixture (Invitrogen) with primer StgA-F (5′CGGGATCCGAGATGAGAATAACGGAATA-3′) containing a BamHI restriction site (underlined) and primer StgD-R (5′GCTCTAGA CATTGATATGACTTATTTTG-3′) containing an XbaI restriction site (underlined). The 5-kb PCR product was purified and cloned into vector pCR2.1 using a TOPOXL PCR cloning kit (Invitrogen), resulting in plasmid pSIF018. The XbaI-HindIII fragment was subcloned into low-copy-number vector pWSK29 at the same restriction sites, resulting in plasmid pSIF026. The different constructs were transformed into the nonfimbriate *E. coli* K-12 Δ*fim* mutant strain ORN172 (42) or into *S. enterica* serovar Typhimurium and Typhi strains.

Adherence to human epithelial cells. The ability of *E. coli* strain ORN172 containing the *stg* operon (pSIF018) or only the vector (pCR2.1) to adhere to human epithelial cells (INT-407) was assessed. A total of 2.5×10^5 cells grown in minimal essential medium (Wysent) supplemented with 10% heat-inactivated fetal calf serum (Wysent) and 25 mM HEPES (Wysent) were seeded in 24-well tissue culture plates 24 h before the adherence assays. One hour before infection, cells were washed three times with prewarmed phosphate-buffered saline (PBS) (pH 7.4), and fresh complete medium was added to each well. Bacteria were grown overnight on LB medium plates and were resuspended in PBS to an optical density at 600 nm (OD_{600}) of 1.5 ($\sim 1.5 \times 10^9$ CFU/ml). Approximately 2.5×10^7 CFU was added to each well (multiplicity of infection [MOI], 100). The 24-well plates were then centrifuged at 1,000 × *g* for 5 min to synchronize infection, incubated at 37°C in 5% CO_2 for 90 min, and rinsed three times with PBS. PBS-0.1% deoxycholic acid sodium salt was added to each well, and samples were diluted and spread on LB medium plates for enumeration by viable

TABLE 2. Comparison of the *stg* fimbrial gene products of *Salmonella* serovar Typhi with other fimbrial systems[a]

Fimbrial group	Organism	Localization	% Identity (% similarity)			
			StgA	StgB	StgC[b]	StgD
Stg	*S. bongori*	*glmS-pstS*	70.7 (81.2)	63.7 (73.7)	82.2 (89.8)	38.7 (53.6)
	Avian pathogenic *E. coli* 078 (*stg*)	*glmS-pstS*	66.5 (79.1)	54.8 (69.8)	67.1 (82.0)	36.6 (54.1)
	Enterohemorrhagic *E. coli* O157 (*lpf2*)	*glmS-pstS*	59.4 (72.8)	62.1 (76.3)[b]	73.5 (85.6)	35.4 (50.3)
	Enterohemorrhagic *E. coli* O113 (lpf_{O113})	*glmS-pstS*	66.5 (79.1)	53.6 (69.4)	67.1 (82.0)	24.7 (37.9)
Lpf	*S. enterica* serovar Typhimurium LT2	*yhjX-yhjW*	32.5 (45.7)	32.1 (53.3)	40.8 (59.2)	27.9 (45.6)
	Enterohemorrhagic *E. coli* O157 (*lpf1*)	*yhjX-yhjW*	30.5 (44.2)	33.9 (54.8)	38.8 (56.6)[b]	28.0 (44.6)
	Rabbit enteropathogenic *E. coli* O15	*yhjX-yhjW*	35.2 (48.2)	30.0 (52.2)	41.1 (58.4)	27.3 (42.2)

[a] Sequences were obtained from coliBASE (http://colibase.bham.ac.uk/).
[b] A complete ORF was used for comparison analysis.

colony counting. The results were expressed as the percentage of the initial inoculum. Statistical differences were assessed using Student's *t* test.

A similar protocol was used to test adherence of *Salmonella* and/or the isogenic *stg* mutant strains to INT-407 cells, except that bacteria were grown overnight without shaking in LB medium containing 0.3 M NaCl and an MOI of 20 was used. When indicated below, an additional 90-min incubation with 100 μg/ml gentamicin to kill extracellular bacteria was performed in order to assess the invasion level.

Generation of a single-copy *stgA-lacZ* transcriptional fusion and β-galactosidase assay. The *stgA* promoter region was amplified using the Elongase enzyme mixture (Invitrogen) and the following primers: StgA-F and StgA-R (5′AACT GCAGCCAGCAAATGCCGTTTTGTT3′). The PCR product was cloned into vector pCR2.1 using a TOPOXL PCR cloning kit (Invitrogen), resulting in plasmid pSIF016. A 530-bp fragment digested with XbaI and SpeI was purified and ligated to pFUSE digested with XbaI (2), resulting in plasmid pSIF020. Plasmid pSIF020 was confirmed to contain the *stgA* promoter in the correct orientation for *lacZ* fusion. To generate a single copy of the P*stgA-lacZ* fusion in serovar Typhi, pSIF020 was transferred by conjugation and integrated into the genome by homologous recombination as described previously (2, 3). A strain carrying a single integrated copy of P*stgA-lacZ* in ISP1820 was designated DEF068. The expression of *stg* was evaluated by β-galactosidase assays of the reporter strain DEF068 grown in different conditions. β-Galactosidase activity was measured using *o*-nitrophenyl-β-D-galactopyranoside as described previously (23).

Construction of a serovar Typhi strain with an *stg* deletion. A suicide vector for deletion of the *stg* fimbrial operon (STY3918 to STY3922) was constructed as follows. A 530-bp fragment of the 5′ end of *stgA* was generated by PCR using primers StgA-F and StgA-R, and a 482-bp fragment of the 3′ end of *stgD* was generated by PCR using primers StgD-F (5′AACTGCAGGCCGCAGAGCTG TGAAAATG3′) and StgD-R. These two fragments were ligated and cloned into the XbaI and BamHI sites of pMEG-375 (15). A resulting suicide vector containing the *stgA′-stgD′* fragment (pSIF004) was used for allelic replacement of the *stg* region. The pSIF004 suicide vector was conjugated from *E. coli* MGN-617 to serovar Typhi strain ISP1820 by overnight plate mating on LB medium with DAP. Transconjugants were selected by growth on LB medium plates containing chloramphenicol without DAP. Selection for double-crossover allele replacement was performed by *sacB* counterselection on LB agar plates without NaCl containing 5% sucrose (16). Isogenic strain DEF004 has a deletion of the *stg* region resulting from a double crossover, as determined by the absence of resistance to ampicillin and chloramphenicol encoded on the suicide vector, and the expected *stg* deletion, as confirmed by PCR (data not shown).

Bacterial survival in human macrophages. The human monocyte cell lines THP-1 (= ATCC TIB-202) and U937 (= ATCC CRL 1593) were maintained in RPMI 1640 (Invitrogen) containing 10% fetal calf serum, 25 mM HEPES, 2 mM L-glutamine, 1% minimal essential medium nonessential amino acids (Wisent), and 1 mM sodium pyruvate (Sigma). Stock cultures of these cells were maintained as monocyte-like, nonadherent cells at 37°C in an atmosphere containing 5% CO_2. Before infection, cells were differentiated by addition of 10^{-7} M phorbol 12-myristate 13-acetate (Sigma) for 24 to 72 h. For macrophage infection assays, cells were seeded at a concentration of 5×10^5 cells per well in 24-well tissue culture dishes. Bacteria grown overnight at 37°C in static conditions were added to a cell monolayer at an MOI of 10 and centrifuged for 5 min at 1,000 × *g* to synchronize phagocytosis. After incubation for 20 min at 37°C (zero time), the infected cells were washed three times with prewarmed PBS and incubated with supplemented medium as described above containing 100 μg/ml of gentamicin to kill extracellular bacteria. The infected monolayers were either lysed from the tissue culture dishes by addition of 0.1% deoxycholic acid sodium salt in PBS or incubated further. After lysis the number of surviving bacteria was determined by bacterial plate counting (CFU). The level of phagocytosis was expressed as a percentage of the initial inoculum. The survival rate was expressed as a percentage determined by comparing the number of intracellular bacteria with the number at the previous time.

Statistical differences were assessed using Student's *t* test. Where indicated, the macrophages were incubated 1 h prior to infection with 1 μg/ml of cytochalasin D (Sigma) to inhibit bacterial uptake as described previously (32). The level of cytochalasin D was maintained throughout the infection.

RESULTS

***stg* fimbrial operon.** The *stg* fimbrial cluster has a G+C content of 49% and is a member of a distinct group of related fimbrial genes that are located in the *glmS-pstS* intergenic region (21, 39). In the sequenced genomes of *S. enterica* (including unfinished genomes) this fimbrial gene cluster has been identified only in serovar Typhi. Moreover, *stg* sequences were not detected by comparative genomic hybridization in the genomes of 140 strains belonging to many serovars of subspecies I (30; M. McClelland, personal communication). The previously described distribution of *stg* determined by Southern blotting may therefore represent cross-hybridization with other less homologous fimbrial genes (39). However, a putative fimbrial gene inserted in the *glmS-pstS* region in *S. bongori* belongs to the Stg group, and its product exhibits the highest level of identity to the predicted *stg* fimbrial gene products of serovar Typhi (Table 2). The genes encoding a number of fimbrial systems in pathogenic *E. coli* are also inserted in the *glmS-pstS* region and belong to the Stg group; these systems include the Stg (21), Lpf_{O113} (5), and Lpf2 (O-island 154) (38) systems. In addition, Lpf and related fimbriae encoded in the *yhjX-yhjW* region in *Salmonella* and *E. coli* (36, 37) exhibit some identity to the predicted *stg* gene products of serovar Typhi, but less identity than other fimbriae belonging to the Stg group (Table 2). The serovar Typhi *stg* fimbrial cluster contains five ORFs designated *stgABCC′D* as *stgC* is a predicted pseudogene and contains a premature stop codon. The *stgC* ORF may code for a 170-amino-acid (aa) protein, and a second ORF designated *stgC′* may code for a 605-aa protein. The *stgC* stop codon is present in the *stgC* sequence of serovar Typhi strain ISP1820 (data not shown), as well as in the sequenced genomes of serovar Typhi strains TY2 and CT18 (4, 29).

Adhesion of *E. coli* containing the *stg* operon. To examine the capacity of the *stg* fimbrial cluster to mediate adherence to

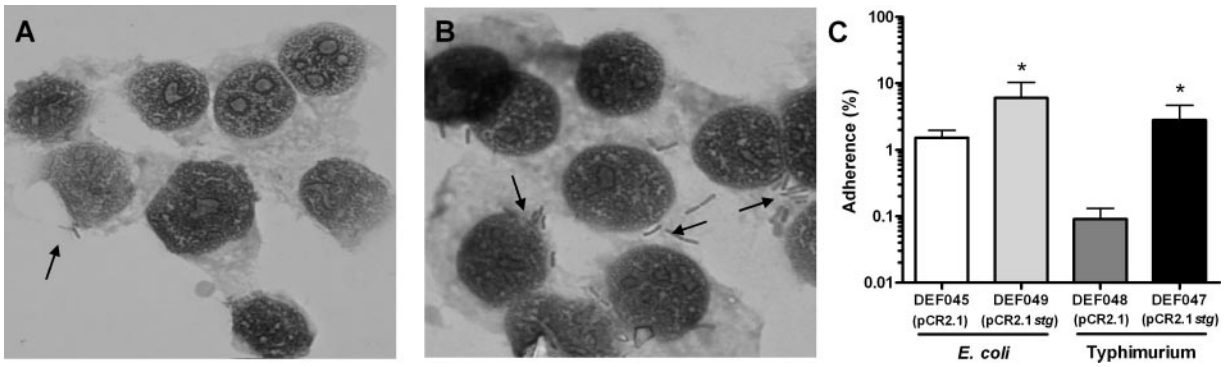

FIG. 1. Adherence and expression of the *stg* fimbrial operon by *E. coli* and *S. enterica* serovar Typhimurium. (A and B) Adherence of *E. coli* strain ORN172 to human epithelial cells (INT-407) containing the vector (pCR2.1) (DEF045) (A) or the *stg* genes (pSIF018) (DEF049) (B). Slides were stained with 5% Giemsa stain. Bacteria are indicated by arrows. (C) Percentage of the initial inoculum associated with epithelial cells after 90 min of incubation for *E. coli* and serovar Typhimurium carrying the *stg* operon (DEF047) or the control vector (DEF048). All assays were conducted in duplicate and repeated independently at least three times. The results are expressed as the means ± standard errors of the replicate experiments. An asterisk indicates that there is a significant difference between the strain containing the control vector and the strain containing the *stg* operon ($P < 0.005$).

INT-407 cells, the *stg* operon was cloned in different vectors and transformed into *E. coli* strain ORN172. ORN172 is an *E. coli* K-12 noninvasive strain with a deletion in the *fim* operon that does not express type 1 fimbriae and is commonly used to study adherence conferred by recombinant fimbrial systems (42). *E. coli* ORN172 cells containing the vector alone (pCR2.1) adhered poorly between the cells or without pattern on the cell surface and were often isolated (Fig. 1A). However, ORN172 cells containing *stg* (pSIF018) adhered in aggregates or clusters on the cell surface (Fig. 1B). The introduction of *stg* into *E. coli* conferred a significantly higher level of adhesion to epithelial cells, which was threefold higher than that of the strain harboring the vector alone (Fig. 1C). A higher level of adhesion was also observed when a low-copy-number vector (pSIF026) was used (data not shown).

Adhesion of serovar Typhimurium containing the *stg* operon. As the *stg* fimbrial operon is absent in serovar Typhimurium, we used this serovar to establish whether *stg* could contribute to adherence to INT-407 cells by a heterogeneous *Salmonella* serovar. Serovar Typhimurium strain χ3339 harboring *stg* (pSIF018) exhibited a significantly higher level of adhesion to INT-407 cells, which was 30-fold higher than that of the strain harboring the vector alone (pCR2.1) (Fig. 1C). As salmonellae are able to invade epithelial cells, the level of invasion was also determined by a gentamicin protection assay. An invasion level similar to that exhibited by the wild-type parent harboring only the vector was observed (data not shown).

***stg* expression in serovar Typhi.** To study the expression of the *stg* fimbrial operon in the native serovar Typhi strain, an *stgA*::*lacZ* fusion was inserted into the chromosome of strain ISP1820, generating strain DEF068. Strain DEF068 was used to determine the influence of a number of in vitro growth conditions on *stg* expression. The expression of the promoter fusion was determined for bacteria grown in LB medium from early log phase to stationary phase. β-Galactosidase expression increased from early to stationary phase, following overnight growth in LB medium (Fig. 2). The β-galactosidase expression following growth on LB agar was nearly twofold higher (54 U) than the expression following overnight growth in LB broth (29 U) (Fig. 2). The highest levels of β-galactosidase expression were observed following overnight growth in minimal medium (M9-glucose) (76 U) (Fig. 2). Expression in conditions that mimic those encountered during invasion and infection of host cells was also studied. The effect of the sodium chloride concentration in the medium was evaluated, as this concentration represents a condition that can influence cell invasion by *Salmonella* (1, 8). The effect of iron availability and pH on *stg* expression was also evaluated. Changes in these conditions did not result in any significant changes in β-galactosidase expression (data not shown).

***stg* contributes to adherence of serovar Typhi to epithelial cells.** We assessed whether *stg* contributes to adherence of serovar Typhi to INT-407 cells by constructing an isogenic Δ*stgABCC'D* mutant by allelic exchange. The mutated strain, DEF004, exhibited a significantly lower level of adherence (80% of the wild-type strain adherence) (Fig. 3A). A level of adherence significantly higher than that of the wild-type strain was observed when the *stg* mutant was complemented with the *stg* genes on a low-copy-number vector (pSIF026) (Fig. 3A). In

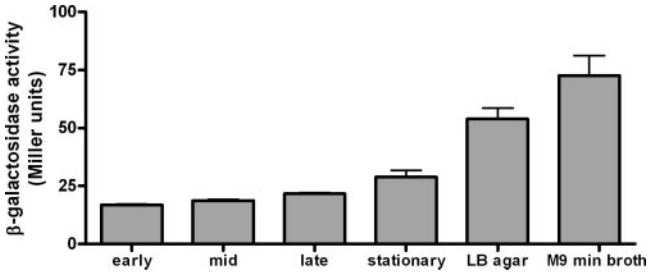

FIG. 2. *stg* expression in serovar Typhi: β-galactosidase activity expressed from the P*stgA*::*lacZ* fusion in serovar Typhi (DEF068) in different growth conditions. Bacteria were grown in LB medium with agitation to early log phase (OD_{600}, 0.3), mid-log phase (OD_{600}, 0.6), late log phase (OD_{600}, 0.9), and stationary phase (overnight), on LB agar, and in M9-glucose broth (M9 min broth) (overnight). The error bars indicate standard deviations.

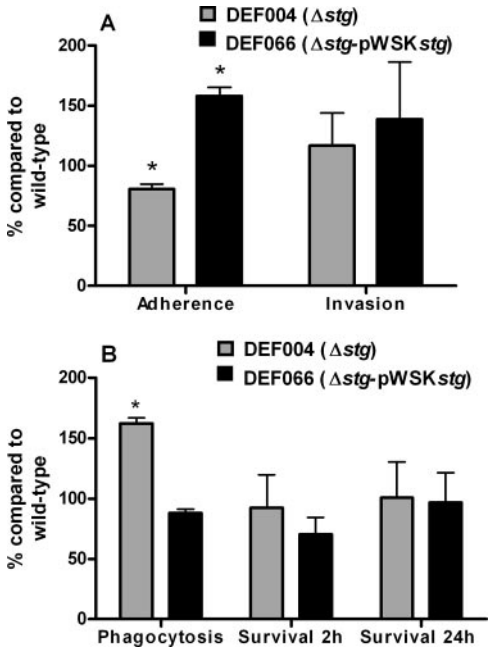

FIG. 3. Role of *stg* in the interaction of serovar Typhi with human cells: capacity of the wild-type strain, the *stg* mutant (DEF004), and the complemented strain (DEF066) to adhere to and invade INT-407 cells (A) or to survive within THP-1 macrophage-like cells (B). All assays were conducted in duplicate and repeated independently at least three times. The results are expressed as the means ± standard errors of the replicate experiments. Significant differences ($P < 0.005$) in adherence or phagocytosis between the mutant and the wild-type strain of serovar Typhi are indicated by asterisks. The values for percent recovery were normalized to the wild-type control value, which was defined as 100% at each time point.

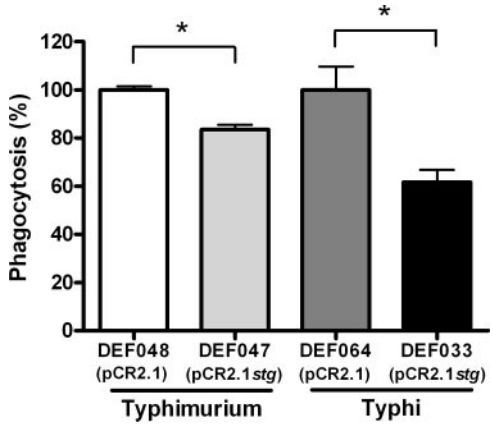

FIG. 4. Effect of overexpression of *stg* on phagocytosis. Serovar Typhimurium carrying the *stg* cluster (DEF047) or the control vector (DEF048) and serovar Typhi carrying the *stg* cluster (DEF033) or the control vector (DEF064) were incubated with THP-1 macrophage-like cells. The percentage of the initial inoculum associated with cells after 120 min of incubation is indicated. All assays were conducted in duplicate and repeated independently at least three times. The results are expressed as the means ± standard errors of the replicate experiments. An asterisk indicates that there is a significant difference in phagocytosis between the wild-type strain containing the vector alone and the strain with the *stg* operon ($P < 0.05$).

spite of the lower level of adherence of the mutant, its level of invasion was higher than that of the wild-type parent, but not significantly higher (Fig. 3A).

Loss of *stg* results in increased phagocytosis of serovar Typhi by macrophages. As survival in macrophages plays an essential role in systemic infection by *Salmonella*, we characterized the interaction of the isogenic *stg* mutant with human macrophages. The wild-type strain and the mutant were used to infect human macrophage-like cells, and the numbers of bacteria present after phagocytosis at 2 and 24 h postinfection were determined. The mutant showed a significantly higher level of phagocytosis than the wild-type strain (Fig. 3B). The levels of bacterial survival at 2 or 24 h postinfection were similar for both the *stg* mutant and the wild-type strain (Fig. 3B). Complementation of the *stg* mutant with *stg* on a low-copy-number vector (pSIF026) restored the wild-type phagocytosis phenotype (Fig. 3B).

Role of *stg* in macrophage interactions. As bacterial uptake of the *stg* mutant by macrophages was altered, we wanted to evaluate the effect of *stg* overexpression on phagocytosis. The uptake of both serovar Typhi strain ISP1820 and serovar Typhimurium strain χ3339 harboring *stg* (pSIF018) on a multicopy vector was significantly lower than the uptake of the bacterial strain harboring the vector alone (pCR2.1) (Fig. 4). This lower level of phagocytosis was also observed using macrophage-like U937 cells (data not shown). Then, in order to differentiate between the initial levels of bacteria associated with or internalized by macrophages, we used an inhibitor of cytoskeletal function, cytochalasin D, to block bacterial uptake. In the presence of cytochalasin D, less then 2% of the initial inoculum was associated with macrophages. The percentages of serovar Typhi that were associated with macrophages were similar when *stg* was present at a high copy number and when the wild-type harboring the vector alone was used (Fig. 5). In addition, the *stg* mutant also showed a level of association with macrophages similar to that of the wild-type strain when bac-

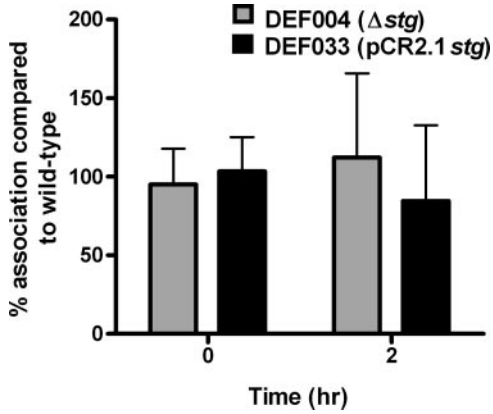

FIG. 5. Role of *stg* fimbrial operon in bacterial association with macrophages. Bacterial uptake was inhibited with cytochalasin D, and the numbers of bacteria with *stg* (DEF033) and without *stg* (DEF004) associated with macrophages were compared. All assays were conducted in duplicate and repeated independently at least three times. The values for percent recovery were normalized to the wild-type control value, which was defined as 100% at each time point. The results are expressed as the means ± standard errors of the replicate experiments.

terial uptake was inhibited by cytochalasin D (Fig. 5). Since the levels of association with macrophages were similar in cytochalasin D-treated cells regardless of the presence of *stg*, these results indicate that the *stg* fimbrial system contributes to a reduction in internalization of serovar Typhi by macrophages.

DISCUSSION

Bacterial adhesion to host cells is often considered an essential step for colonization. Adhesion is mediated via surface molecules, including fimbriae or pili. Many gene clusters corresponding to fimbrial systems are present in the genomes of *S. enterica*. However, only a few systems have been characterized, and only the *fim* cluster coding for type 1 fimbriae was detected after in vitro growth of serovar Typhimurium at 37°C in static broth (13). A combination of fimbrial systems may be responsible for the differences in binding and host specificities observed for different *Salmonella* serovars. Serovar Typhi is restricted to humans and harbors 13 putative fimbrial systems and a type IV pilus (29). We have previously found that *stg* is transcribed by serovar Typhi within macrophages (6). In *S. enterica*, the *stg* fimbrial cluster located in the *glmS-pstS* region is present only in serovar Typhi (30).

The *stg* gene cluster was suggested to be nonfunctional since the predicted ORF for the putative usher gene *stgC* contains an internal stop codon and is classified as a pseudogene (29, 39). Mutations in genes encoding assembly proteins, such as the usher, result in the absence of fimbriae from the bacterial surface (18). The fimbrial usher protein family consists of a group of large proteins (800 to 900 aa) present in the outer membranes of gram-negative bacteria (40). The usher acts in the assembly process together with a periplasmic fimbrial chaperone protein. Phylogenetic analyses suggest that the chaperone and the usher, in general, evolved in parallel from their evolutionary precursor proteins (40). In bacteria expressing numerous fimbriae, each fimbrial system typically encodes a specific periplasmic chaperone protein and outer membrane usher protein (24, 34). However, fimbrial expression may be possible using complementary fimbrial proteins from other clusters. This is likely to occur with the LP fimbria-encoding *lpf1* cluster of *E. coli* O157:H7. This cluster contains a stop codon in the predicted usher-encoding gene which results in two ORFs, *lpf1C* (368 aa predicted) and *lpf1C′* (443 aa predicted) (37). The cloned *lpf1* gene cluster produced detectable fimbriae, and these fimbriae contributed to microcolony formation, demonstrating that this system was therefore functional (37). The aims of our study were to characterize the *stg* fimbrial cluster and determine if this fimbrial cluster was functional despite the presence of a predicted pseudogene which comprises two ORFs, *stgC* (170 aa predicted) and *stgC′* (605 aa predicted), that may act as the usher gene. To circumvent the effect of the premature stop codon in the StgC usher gene, it is possible that other fimbrial ushers present in the cell may function for Stg; otherwise, the truncated StgC usher may be functional (24).

An increased level of association to epithelial cells was observed when the *stg* fimbrial cluster was cloned into a nonfimbriated *E. coli* strain (Fig. 1). We were unable to visualize any filamentous structures by transmission electron microscopy with negative staining. Other related fimbriae were also difficult to visualize and/or detect (26, 37, 38). Thus far, no studies have detected these fimbriae using wild-type strains, and fimbrial proteins or structures were detected only using an afimbrial recombinant *E. coli* strain and either multicopy or inducible vectors (21, 26, 37, 38). We were also unable to detect StgA when *stg* genes were cloned on a multicopy vector in *E. coli* or in *Salmonella* by Western blotting using an anti-StgA from *E. coli* (21). One explanation for the lack of fimbrial structures despite an adhesion phenotype may be that some export and partial assembly of the Stg protein occurs, which results in an adhesin that is not filamentous. Stg and related fimbriae exhibit a low level of transcription in vitro (26, 35, 37). This may also explain why these fimbriae are not readily detected in vitro. In serovar Typhi, using an *stgA-lacZ* single-copy fusion, a low level of *stg* expression was also detected in different growth conditions. The highest levels of *stg* expression were obtained when bacteria were grown in minimal medium or on solid medium (Fig. 2), and they were not influenced by the presence of salts or iron. The low level of fimbrial gene expression observed during in vitro growth of serovar Typhi is similar to results obtained with serovar Typhimurium (13). In serovar Typhimurium, which contains 13 fimbrial operons (22), only type 1 fimbriae were expressed in vitro at 37°C. Similarly, the majority (11/15) of fimbrial clusters in *E. coli* O157:H7 were not expressed under the majority of the conditions tested in vitro (20). It is currently not known why expression of many fimbrial systems is suppressed in vitro.

While they are an advantage to the bacterium for colonization of the host, fimbrial proteins at the bacterial surface may become a disadvantage, as they are easily exposed targets for the host immune system. Hence, tight regulation of fimbrial expression may be necessary during host infection. The induction of expression of fimbrial antigens during infection of mice with serovar Typhimurium was previously shown by seroconversion against most fimbriae (12). In typhoid fever patients, antibodies to three fimbrial systems, Tcf, Stb, and Csg, were detected (10). Nevertheless, we have previously detected the *stgC′* transcript during infection of macrophages (6). The optimal conditions for expression of Stg may not have been found yet, and we need to further investigate its regulation, but our results are consistent with the hypothesis that the *stg* fimbrial operon may be important for the initial interaction with host cells.

When the *stg* operon was deleted from serovar Typhi, a lower level of bacterial association with INT-407 cells was observed (Fig. 3A). Further, a higher level of bacterial association with epithelial cells was observed when the *stg* mutant was complemented by the *stg* fimbrial cluster. In addition, an increased level of association with epithelial cells was observed when the *stg* gene cluster was introduced into *E. coli* and *S. enterica* serovar Typhimurium, in which *stg* is absent (Fig. 1C). These results implicate the *stg* fimbrial operon in host cell interaction. The *stg* operon and the type IV pili are the only serovar Typhi determinants identified so far that confer adherence to human epithelial cells (43). Redundancy of virulence determinants is not uncommon. Wild-type virulent serovar Typhi strains lacking SPI-7, which harbor type IV pili, have been isolated (25), suggesting that the *stg* fimbrial operon may confer adhesion to host cells in Δ*pil* strains. The *stg* fimbrial cluster may represent an additional system for host intestinal

colonization. Many functions have been associated with fimbriae related to Stg. In avian pathogenic *E. coli*, Stg contributes to the colonization of avian respiratory tissues (21). In *E. coli* O157:H7, long-term persistence in sheep and pigs was associated with the presence of Lpf1 and Lpf2 (14), which also influenced intestinal tissue tropism (7). In rabbit enteropathogenic *E. coli*, Lpf_{R141} is involved in initial colonization (26).

Although loss of *stg* genes reduced the adherence of serovar Typhi to epithelial cells, a higher level of phagocytosis was observed with the *stg* mutant (Fig. 3B). Further, a lower level of phagocytosis was observed when *stg* was overexpressed in serovar Typhi, as well as in serovar Typhimurium (Fig. 4). The higher level of phagocytosis in the absence of the *stg* genes may have been caused by increased exposure of different bacterial surface proteins that are more readily recognized by macrophages, thus enhancing macrophage association. To rule out this possibility, bacterial association with macrophages was assessed in the presence of cytochalasin D, an inhibitor of actin polymerization, which mediates uptake of bacteria. The numbers of bacteria associated with cytochalasin D-treated macrophages were similar for the wild-type strain, the *stg* mutant strain, and a strain overexpressing *stg* (Fig. 5). Thus, the higher level of phagocytosis observed with the mutant was not the result of increased exposure of other proteins on the bacterial surface that may have increased association with phagocytes. Similarly, the lower level of phagocytosis observed when the *stg* fimbrial cluster was overexpressed was not due to a decrease in the association with macrophages but was likely due to a specific reduction in phagocytic activity. By contrast, type IV pili increased entry of serovar Typhi in macrophages (28). This suggests that Stg and type IV pili use different interaction mechanisms with host cells. The level of invasion of INT-407 cells and the intracellular survival in human macrophages of strains with *stg* or the mutant were similar to the results for the wild-type strain even when bacterial uptake by macrophages was inhibited (Fig. 3 and 5). This favors the hypothesis that the presence of the *stg* genes may be involved primarily in initial contact with host cells. It is possible that the *stg* fimbrial operon may promote inhibition of phagocytosis in order to evade inflammatory cells of the intestine so that the bacteria can invade deeper tissue.

The data presented in this paper demonstrate that the *stg* gene cluster of serovar Typhi expresses a functional and serovar-specific adhesin. The *stg* gene cluster potentially contributes to the initial stages of typhoid fever pathogenesis by mediating adherence of serovar Typhi to host epithelial cells and by inhibiting phagocytosis. It is important to understand this inhibition mechanism, to characterize the regulation, expression, and production of Stg in vivo, and to determine if Stg possesses a specific host cell receptor that may be a potential target for the prevention of typhoid fever.

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