A novel PhoP-regulated locus encoding the cytolysin ClyA and the secreted invasin TaiA of *Salmonella enterica* serovar Typhi is involved in virulence

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Salmonella enterica serovar Typhi causes a human-restricted systemic infection called typhoid fever. We have identified a Typhi genomic region encoding two ORFs, STY1498 and STY1499, that are expressed during infection of human macrophages and organized in an operon. STY1498 corresponds to *clyA*, which encodes a pore-forming cytolysin, and STY1499 encodes a 27 kDa protein, without any attributed function, which we have named TaiA (Typhi-associated invasin A). In order to evaluate the roles of these genes in Typhi pathogenesis, isogenic Typhi strains harbouring a non-polar mutation of either *clyA* or *taiA* were constructed. In macrophages, *taiA* was involved in increasing phagocytosis, as *taiA* deletion reduced bacterial uptake, whereas *clyA* reduced or controlled bacterial growth, as *clyA* deletion enhanced Typhi survival within macrophages without affecting cytotoxicity. In epithelial cells, deletion of *taiA* had no effect on invasion, whereas deletion of *clyA* enhanced the Typhi invasion rate, and reduced cytotoxicity. Overexpression of *taiA* in Typhi or in *Escherichia coli* resulted in a higher invasion rate of epithelial cells. We have demonstrated that TaiA is secreted independently of both the *Salmonella* pathogenicity island (SPI)-1 and the SPI-2 type three secretion systems. We have shown that this operon is regulated by the virulence-associated regulator PhoP. Moreover, our results revealed that products of this operon might be involved in promoting the use of macrophages as a sheltered reservoir for Typhi and allowing long-term persistence inside the host.

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INTRODUCTION

The genus *Salmonella* is composed of two distinct species, *Salmonella bongori* and *Salmonella enterica*. While *S. bongori* is rarely involved in human infections, *S. enterica* is a major human pathogen. Out of the 2000 serovars of *S. enterica*, only a small fraction is associated with human infections (Porwollik *et al.*, 2004). Serovars Typhimurium and Enteritidis cause a localized infection, gastroenteritis, whereas serovar Typhi causes a severe systemic infection called typhoid fever, which kills an estimated 600 000 people annually (Parry *et al.*, 2002). Because Typhi is restricted to humans, Typhimurium has been used for many years to study typhoid fever pathogenesis using a murine infection model in which Typhimurium causes a systemic infection. This model has been crucial in understanding systemic infections by *Salmonella*.

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Abbreviations: DAP, diaminopimelic acid; HA, haemagglutinin; LDH, lactate dehydrogenase; OMV, outer membrane vesicle; qPCR, quantitative PCR; SCV, *Salmonella*-containing vacuole; SPI, *Salmonella* pathogenicity island; T3SS, type three secretion system.

S. enterica and *S. bongori* possess a type three secretion system (T3SS), encoded by *Salmonella* pathogenicity island 1 (SPI-1), which mediates invasion of host cells (Galan, 1999; Marcus *et al.*, 2000). Only *S. enterica* possesses a second T3SS located in SPI-2, which is required for survival inside macrophages and the infection of mammalian hosts (Ochman & Groisman, 1996; Ochman *et al.*, 1996). The T3SS injects bacterial proteins directly into the host cell and disturbs normal cell function. Induction of the SPI-1-encoded genes requires high osmolarity and low aeration, conditions present in the small intestine where the SPI-1 T3SS initiates cell invasion (Altier, 2005; Lostroh & Lee, 2001). SPI-2 T3SS genes are induced by low concentrations of magnesium and phosphate and an acidic pH (Beuzon *et al.*, 1999; Coombes *et al.*, 2004; Deiwick *et al.*, 1999). SPI-2-translocated effectors are involved in modification of the *Salmonella*-containing vacuole (SCV) and inhibition of lysosome fusion (Kuhle & Hensel, 2004). In *Salmonella*, regulation of a multitude of genes involved in intracellular survival, phagosome alteration, invasion, lipid A modification, and resistance to antimicrobial peptides, including the SPI-2 T3SS, is mediated by the PhoPQ two-component system (Prost & Miller, 2008).

Infections with Typhi are characterized by a long incubation period (7–14 days), a three-week period of symptoms, including fever and malaise, and mild intestinal inflammation (Connor & Schwartz, 2005). In contrast, Typhimurium infections in humans have a shorter incubation period (10–72 h), a shorter symptomatic period (<10 days) and produce strong intestinal inflammation (Santos *et al.*, 2001). Therefore, Typhi is likely to possess and use unique virulence factors to systemically infect humans. It has been shown that Typhi is able to survive better inside human macrophages than Typhimurium and that Typhimurium survives better inside murine macrophages than Typhi (Schwan *et al.*, 2000). The highest level of survival in macrophages seems to correlate with the host in which each serovar is able to cause a systemic infection. Survival in host macrophages is known to have a great effect on virulence (Fields *et al.*, 1986), and host macrophages have been shown to be the reservoir of *Salmonella* during systemic disease (Richter-Dahlfors *et al.*, 1997). Therefore, in the absence of an adequate animal model to study typhoid fever, it is important to focus our studies on Typhi interaction with host cells in order to better characterize its mechanisms of pathogenicity.

Transcriptomic studies of Typhi have identified a group of 117 genes that are induced continuously within infected human macrophages (Faucher *et al.*, 2006). This group includes many genes of the SPI-2 T3SS, several genes involved in antimicrobial peptide resistance and many genes with no associated function (Faucher *et al.*, 2006). Some of these genes are absent from the Typhimurium genome, including *clyA* (STY1498) and STY1499, which are two contiguous genes. Recently, these two Typhi proteins were detected under conditions that induce SPI-2, and which are thought to mimic the intracellular environment of macrophages (Ansong *et al.*, 2008).

ClyA (HlyE/SheA) is a well-characterized pore-forming cytolysin found in serovars Typhi and Paratyphi A, and in some *Escherichia coli* strains (Oscarsson *et al.*, 2002). ClyA monomers are exported in outer membrane vesicles (OMVs). *E. coli* cells expressing *clyA* are cytotoxic for mammalian cells (Lai *et al.*, 2000; Oscarsson *et al.*, 1999). A specific antibody response toward ClyA during human infection by Typhi or Paratyphi was recently reported, which indicates that ClyA is expressed *in vivo* (von Rhein *et al.*, 2006). However, direct evidence of ClyA playing a role in Typhi pathogenesis has not been reported yet. STY1499 is a putative ORF of unknown function, which we have named *taiA* (Typhi associated invasin A). Expression of this gene cluster inside human macrophages suggests an involvement in Typhi pathogenesis. To investigate this possibility, non-polar mutant strains of *clyA* and *taiA* were constructed and their interaction with human cells was characterized.

METHODS

Bacterial strains and growth conditions. Strains and plasmids used in this study are listed in Table 1. Bacteria were routinely grown

in Luria–Bertani (LB) broth. For the invasion assay or SPI-1 induction, bacteria were grown overnight in LB containing 0.3 M NaCl (LB NaCl) without aeration. For SPI-2 induction, low-phosphate, low-magnesium medium (LPM), pH 5.8, was used, as described by Coombes *et al.* (2004). N medium, pH 5.8, with 10 μ M MgCl₂ was used for PhoP activation, and N medium, pH 7.4, with 10 mM MgCl₂ was used for PhoP inactivation (Snively *et al.*, 1991). When required, antibiotics, amino acids or supplements were added at the following concentrations: 50 μ g ampicillin ml⁻¹, 50 μ g diaminopimelic acid (DAP) ml⁻¹, 34 μ g chloramphenicol ml⁻¹, 22 μ g tryptophan ml⁻¹, 22 μ g cysteine ml⁻¹ and 22 μ g arginine ml⁻¹. Transformation of bacterial strains was routinely done by using the calcium/manganese-based (CCMB) or electroporation methods, as described by O’Callaghan & Charbit (1990).

Generation of mutants and complementation. To generate non-polar mutations of *taiA* and *clyA*, the overlap-extension PCR method described by Basso *et al.* (2002) was used. For *taiA*, fragments were amplified with primers STY1499-BF and STY1499-BR (Table 2) for the 5’ end of the gene, and primers STY1499-EF and STY1499-ER for the 3’ end of the gene. These two fragments were ligated in a second PCR by using the external primers STY1499-BF and STY1499-ER. The resulting fragment, containing a 318 bp internal deletion, was digested with *Bam*HI and *Xba*I and ligated into suicide vector pMEG375 (Kaniga *et al.*, 1998). The resulting plasmid pSIF024 was conjugated from *E. coli* MGN-617 to Typhi ISP1820 by overnight plate-mating on LB with DAP. Transconjugants were selected by growth on LB plates containing chloramphenicol without DAP. Selection for double-crossover allele replacement was obtained by *sacB* counterselection on LB agar plates without NaCl and containing 5% sucrose (Kaniga *et al.*, 1991). Isogenic strain DEF061 contains a non-polar mutation of *taiA*. For deletion of *clyA*, the same method was used with primers *clyA*-BF, *clyA*-BR, *clyA*-EF and *clyA*-ER. The resulting isogenic strain DEF062, constructed with plasmid pSIF025, contains a non-polar mutation of *clyA*. Mutations were confirmed by PCR. Both mutants have growth curves in LB similar to that of the wild-type strain (data not shown). To complement these mutants, *taiA* and *clyA* were cloned separately into the low-copy-number vector pWSK29. This plasmid has been shown to have no deleterious effect on Typhi infection of host cells (Abromaitis *et al.*, 2005). *taiA* and *clyA* were amplified with *Elongase* (Invitrogen) with primers STY1499-FC, STY1499-ER and *clyA*-FC, *clyA*-ER, respectively. PCR fragments were digested with *Bam*HI and *Xba*I and ligated into pWSK29, resulting in plasmids pWSK*taiA* and pWSK*clyA*. pWSK*taiA* was transformed into the wild-type strain to investigate the effect of overexpression and into DEF061 to complement the *taiA* mutation, resulting in strains DEF074 and DEF075, respectively. To complement the *clyA* mutation, pWSK*clyA* was transformed into DEF062 to produce DEF124.

Generation of mutant strains of SPI-1 and SPI-2 T3SS.

Mutations of *invA* and *ssrB* were done by using the approach described above. Primers *invA*-BF, *invA*-BR, *invA*-EF and *invA*-ER were used to generate a mutant allele for *invA*, and primers *ssrB*-BF, *ssrB*-BR, *ssrB*-EF and *ssrB*-ER for *ssrB*. These fragments were cloned into pMEG-375, and digested by *Bam*HI and *Xba*I, resulting in plasmids pSIF072 and pSIF074, respectively. Allelic exchange was performed as described above, and mutations were confirmed by PCR. Typhi strain DEF147 corresponds to an *invA* mutant and DEF149 corresponds to a *ssrB* mutant.

Epitope tagging of *TaiA*. Primer 1499-R-2HA was designed to contain the last 22 nt (without the stop codon) of *taiA* and two haemagglutinin (HA) tag sequences. This primer was used with STY1499-FC to PCR-amplify STY1499 with its native promoter and add two HA sequences at its 3’ end. The resulting fragment was digested with *Xba*I and *Bam*HI and ligated into pWSK29 to create

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference or source
<i>S. enterica</i> serovar Typhi strains		
ISP1820	Wild-type Typhi	Hone <i>et al.</i> (1991)
DEF061	ISP1820 $\Delta taiA$	This study
DEF062	ISP1820 $\Delta clyA$	This study
DEF074	ISP1820 (pSIF029)	This study
DEF075	DEF061 (pSIF029)	This study
DEF124	DEF062 (pSIF051)	This study
DEF130	ISP1820 (pWSK29)	This study
DEF147	ISP1820 $\Delta invA$	This study
DEF149	ISP1820 $\Delta ssrB$	This study
DEF150	ISP1820 (pSIF069)	This study
DEF169	DEF147 (pSIF069)	This study
DEF171	DEF149 (pSIF069)	This study
DEF429	χ 8521 (pSIF069)	This study
χ 8521	ISP1820 $\Delta phoP$	R. Curtiss III
<i>E. coli</i> strains		
DH5 α	<i>supE44</i> $\Delta lacU169$ (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Invitrogen
MGN-617	SM10 λ pir <i>asd</i> <i>thi</i> <i>thr</i> <i>leu</i> <i>tonA</i> <i>lacY</i> <i>supE</i> <i>recA</i> RP4 2-Tc::Mu[λ pir] $\Delta asdA4$	Kaniga <i>et al.</i> (1998)
Plasmids		
pMEG-375	<i>sacRB</i> <i>mobRP4</i> <i>oriR6K</i> ; Cm ^r , Ap ^r	Dozois <i>et al.</i> (2003)
pWSK29	Low-copy-number cloning vector; Amp ^r	Wang & Kushner (1991)
pSIF024	pMEG-375 with flanking region of <i>taiA</i> used for <i>taiA</i> deletion	This study
pSIF025	pMEG-375 with flanking region of <i>clyA</i> used for <i>clyA</i> deletion	This study
pSIF029 (pWSK <i>taiA</i>)	pWSK29 carrying <i>taiA</i>	This study
pSIF051 (pWSK <i>clyA</i>)	pWSK29 carrying <i>clyA</i>	This study
pSIF069 (p <i>taiA</i> -2HA)	pWSK29 carrying <i>taiA</i> tagged with 2HA epitope	This study
pSIF072	pMEG-375 with flanking region of <i>invA</i> used for <i>invA</i> deletion	This study
pSIF074	pMEG-375 with flanking region of <i>ssrB</i> used for <i>ssrB</i> deletion	This study

p*taiA*-2HA. This plasmid was transformed into Typhi strain ISP1820 to generate strain DEF150. Production of a 29 kDa protein detectable by anti-HA antibody was confirmed by Western blotting. p*taiA*-2HA was also transformed into the SPI-1 (DEF147), SPI-2 (DEF149) and *phoP* (χ 8521) mutants, resulting in strains DEF169, DEF171 and DEF429, respectively.

Infection of human cultured macrophages. The human monocyte cell line THP-1 (ATCC TIB-202) was maintained in RPMI 1640 (Wisent) containing 10% (v/v) fetal calf serum (FCS) (Invitrogen), 25 mM HEPES (Wisent), 2 mM L-glutamine, 1 mM sodium pyruvate and 1% modified Eagle's medium (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 addition of 10^{-7} M phorbol 12-myristate 13-acetate for 72 h. Bacteria were grown overnight standing in LB, which corresponds to OD₆₀₀ 0.6 ($\sim 3 \times 10^8$ c.f.u. ml⁻¹), and were then added to the cell monolayer at a m.o.i. of 10:1, and centrifuged for 5 min at 800 g to synchronize bacterial uptake. After incubation for 20 min at 37 °C, the infected cells were washed three times with prewarmed PBS, pH 7.4, and the infected monolayers were either lysed (*t*₀) from the tissue-culture dishes or incubated for 2 h with medium supplemented as above containing 100 µg gentamicin ml⁻¹ (Wisent) to kill extracellular bacteria. Then, the infected monolayers were washed three times with prewarmed PBS and further incubated for an additional 46 h in the presence of fresh supplemented tissue-culture medium containing 12 µg gentami-

cin ml⁻¹. The infected cells were then washed three times with prewarmed PBS and the infected monolayers were lysed (*t*₄₈). The cells were lysed by addition of 1 ml 0.1% (w/v) sodium deoxycholate in PBS (PBS-DOC) per well, and the number of surviving bacteria was determined as c.f.u. by plating on LB agar. The c.f.u. of the bacterial inocula were determined and the bacterial uptake was expressed as the percentage of bacteria recovered at *t*₀ compared with the inoculum. The survival (fold increase) corresponds to the percentage of bacteria recovered at 48 h post-infection divided by the number of bacteria at *t*₀. Results are expressed as the mean $\pm$ SE of the replicate experiments. The Wilcoxon signed rank test was used for statistical analysis. When indicated, the macrophages were incubated 1 h prior to infection with 1 µg cytochalasin D ml⁻¹ (Sigma) to inhibit bacterial uptake, as described by Rosenshine *et al.* (1994). The addition of cytochalasin D was maintained throughout the infection.

Infection of human cultured epithelial cells. HeLa cells (ATCC CCL-2) were grown in Dulbecco's MEM (Wisent) supplemented with 10% (v/v) heat-inactivated FCS (Wisent) and 25 mM HEPES (Wisent) (complete medium). Infection was carried out as described by Elsinghorst (1994). One day before infection, cells were seeded at 2.5×10^5 cells per well in 24-well tissue-culture plates. One hour before infection, cells were washed three times with prewarmed PBS, and fresh complete medium was added to each well. Bacteria were grown overnight in LB NaCl to OD₆₀₀ 0.6 ($\sim 3 \times 10^8$ c.f.u. ml⁻¹) and added to each well at an m.o.i. of 20. 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.

Table 2. Primers used in this study

Primer	Sequence
STY1499-BF	CTGTAATGCCTCGCCACGATCCATGATTAAG
STY1499-BR	GCTCTAGAGCAAGCAGTTGAATCCAAAGGG
STY1499-EF	CGGGATCCCCAGAAGCTGGAATACTGCCTG
STY1499-ER	CGTGGCGAGGCATTACAGCAAGCCATTTTGG
clyA-BF	GCAGCAATAGTCACGACACCACACCATTCATAA
clyA-BR	GCTCTAGAAACCGCAGATGGGGCATTAG
clyA-EF	CGGGATCCGCTATCGGGCGTTAAAAGTACACAG
clyA-ER	TGTCGTGAGTATTGCTGCGGGCGTGATTGAAGG
invA-BF	CGGGATCCCTACAAGCATGAAATGGCAGAAC
invA-BR	AGGACAAGACTTCAATCAAGATAAGACGGCTGG
invA-EF	TGATTGAAGTCTTGCTCCTTACGTCTGTCTG
invA-ER	GCTCTAGACGCCCAGATCCATACATCATCG
ssrB-BF	CGGGATCCAAGGCTGTTTAGGTCAAATAGGGC
ssrB-BR	CTTCGGGCGGATAAGTATGTCAGGCTCGTATGCG
ssrB-EF	ATACTTATCCGCCGAAGAATGAGGTTAATAG
ssrB-ER	GCTCTAGATCGCCGATAGAATACGACATGG
STY1499-FC	GCTCTAGAGAGGCAACCACCAGCCCTGTC
clyA-FC	GCTCTAGATGTCTGGAGGTAATAGGTAAG
1499-R-2HA	CGGGATCCCTATTAAGCGTAGTCTGGGACGTCGTATGGGTAAAGAGCGT- AGTCTGGGACGTCGTATGGGTAAGATCTACGCAGGGTACGATTACTC
16s-F-qPCR	CGGGGAGGAAGGTGTTGTG
16s-R-qPCR	CAGCCCGGGGATTTCACATC
1499-F-qPCR	ATATCACCGATGCGGTGGGAATC
1499-R-qPCR	ACTTTCACCATTCCATCTTCCGGC
1498-F-qPCR	ACGGAACCGAAACAACCAGATTC
1498-R-qPCR	GCGTCTTCTTACCGTGTCTTTGTTGG
pagC-F-qPCR	TTTAATGGTTGGGCCAGCCTATCG
pagC-R-qPCR	TTAAATGTCGCCTTTACCGTGCCG
prgI-F-qPCR	CAGGTAACAGAGGCGCTGGATAAA
prgI-R-qPCR	TTACCGTGTTGATTGCGCGTTAC
sseB-F-qPCR	ATATGGCGATCATGGGAAGCTGGA
sseB-R-qPCR	TCCGTATTCCGGTTGGCGTCATTA

Cells were either lysed with 1 ml PBS-DOC to evaluate the level of adherence (t_0) or incubated for a further 90 min with complete medium containing 100 µg gentamicin ml⁻¹ to kill extracellular bacteria and assess the invasion level (t_{90}). Cells were then lysed as described above. The invasion level corresponds to the number of bacteria recovered after 90 min of gentamicin treatment compared with the number of bacteria at t_0 . Results are expressed as the mean $\pm$ SE of the replicate experiments. Statistical differences were assessed using the Wilcoxon signed rank test.

Cytotoxicity assay. Human cells (THP-1 or HeLa) were seeded in 24-well plates and infected as described above. After 48 h of infection for THP-1 cells and 90 min for HeLa cells, supernatants were collected and lactate dehydrogenase (LDH) release was evaluated with the Cytotox96 kit (Promega) according to manufacturer's instructions. LDH released is expressed as $100 \times [(\text{experimental release} - \text{spontaneous release})_{\text{test strain}} / (\text{experimental release} - \text{spontaneous release})_{\text{control strain}}]$, in which spontaneous release is the amount of LDH activity in the supernatant of uninfected cells. Results are expressed as the mean $\pm$ SE of the replicate experiments. Statistical differences were assessed using the Wilcoxon signed rank test.

RNA isolation, reverse transcription and real-time quantitative PCR (qPCR). RNA was isolated from bacteria in the supernatant of infection, and at 0, 8 and 24 h post-infection of human macrophages

(infected as described above) by using TRIzol reagents as described by the manufacturer (Invitrogen). RNA was then precipitated with 2.5 M LiCl (Ambion) for 30 min at -20°C , washed with ice-cold 75% (v/v) ethanol and resuspended in diethyl pyrocarbonate-treated water. Rigorous DNase treatment was then performed to remove any trace of DNA (DNA-free kit, Ambion). Purity of extracted RNA was verified by spectrometry and absence of contaminating DNA was confirmed by real-time quantitative PCR (qPCR) with primers 16s-F and 16s-R (data not shown). A total of 50 ng RNA was reverse-transcribed by using Superscript II (Invitrogen) with 0.5 µg random hexamers (Sigma). As a negative control, a reaction without Superscript II was also included (NRT). qPCRs were performed in a Rotor-Gene 3000 thermal cycler (Corbett Research) by using the QuantiTect SYBR Green PCR kit (Qiagen), according to manufacturer's instructions. Primers used are described in Table 2. The transcriptional level of the genes of interest under each condition was normalized against a reference gene (16S rRNA) and analysed by applying the $2^{-\Delta\Delta\text{CT}}$ method (Livak & Schmittgen, 2001). For each condition, reverse transcription was done three times independently, and the NRT sample was used as a negative control.

In vitro secretion assays. Bacteria were grown in either 5 ml LB NaCl standing or in LPM, pH 5.8, with shaking to OD₆₀₀ 0.6. Bacteria were pelleted by centrifugation at 12 000 g for 5 min at 4°C . The supernatant was collected, treated with 0.1 mM PMSF (Sigma) and

filtered through a 0.2 µm pore-size syringe filter (Fisher), and then 1.8 ml was precipitated with TCA (10 % final concentration, v/v) at 4 °C for 16 h. The TCA-insoluble fraction was collected by centrifugation, washed two times with ice-cold acetone and resuspended in an appropriate volume of SDS-PAGE loading buffer [62.5 mM Tris/HCl, pH 6.8, 10 % (v/v) glycerol, 2 % (w/v) SDS, 0.05 % (w/v) β-mercaptoethanol, 0.05 (w/v) bromophenol blue] according to the OD₆₀₀ of the original culture. When necessary, samples were neutralized with 1 µl 1.5 M Tris-HCl, pH 8.8. The bacterial pellet was also dissolved in an appropriate volume of SDS-PAGE loading buffer, according to the OD₆₀₀ of the original culture. Proteins were separated on 10 or 15 % (w/v) SDS-polyacrylamide gels and then transferred onto PVDF membranes by using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad) according to the manufacturer's instructions. Membranes were blocked overnight in Tris-buffered saline containing 0.1 % (v/v) Tween 20 (TBST) and 5 % (w/v) non-fat dried milk at 4 °C. Blots were then incubated for 1 h at room temperature with either rabbit affinity-isolated anti-HA tag (1:5000) (Sigma), rabbit anti-GroEL (1:40000) (Sigma) or rabbit affinity-purified antibodies raised against recombinant SseB (1:2000) (Coombes *et al.*, 2004) and recombinant SopB (1:2500) (Coombes *et al.*, 2005) in TBST with 2.5 % (w/v) non-fat dried milk. Peroxidase-conjugated AffiniPure goat anti-rabbit IgG (Jackson Immuno-Research Laboratories) was used as the secondary antibody at a 1:5000 dilution in TBST with 2.5 % (w/v) non-fat dried milk for 1 h at room temperature. ECL plus Western blotting detection reagent (GE Healthcare) was used to detect antibody complexes. Blot images were acquired with a Typhoon Trio scanner using the ECL+ setting (GE Healthcare).

RESULTS

Presence of *clyA* and *taiA* in bacterial genomes

BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) analysis using the sequence encoding *clyA* and *taiA* genes reveals that this gene cluster is present in *S. enterica* serovar Typhi, Paratyphi A, Javiana and Schwarzengrund, but absent in other sequenced serovars, including Typhimurium (Fig. 1a), and absent in other microbial genomes. This gene cluster possesses a GC content of 40 %, which is relatively low compared with the average of 52 % for the Typhi genome (Parkhill *et al.*, 2001). By comparative genomic hybridization, serovars Montevideo, Oranienfoburg, Sendai and Muenchen were also shown to possess these genes, although only one strain per serovar was tested (Porwollik *et al.*, 2004). The presence of *clyA*, by itself, has been observed in *E. coli*, in both pathogenic and K-12 strains (Ludwig *et al.*, 2004). BLAST analysis with the nucleotide sequence revealed that *clyA* is also present in *Shigella flexneri* serotypes 2a and 5. However, *taiA* was absent from *E. coli*, and BLAST analysis using the nucleotide sequence did not identify any homologous genes. However, BLAST analysis at the protein level using the coliBASE database (<http://colibase.bham.ac.uk/>) revealed homology to a hypothetical protein in *Yersinia* spp. Putative subcellular localization of TaiA using PSORTb was unable to predict a subcellular localization, and no putative signal peptide for secretion was identified (Gardy *et al.*, 2005).

clyA and *taiA* are organized in an operon

Possible promoters for *taiA* and *clyA* were searched for using NNPP version 2.2 with the prokaryote settings (http://www.fruitfly.org/seq_tools/promoter.html). A putative promoter sequence was detected 200 bp upstream of *taiA*, but none was found upstream of *clyA*. This suggested that *clyA* was transcribed from the *taiA* promoter and that they might be organized in an operon. The transcriptional linkage was verified by RT-PCR. PCR was performed on cDNA, and a 1.2 kb fragment which encompassed *taiA* and *clyA* was amplified with primers 1499-F and 1498-R (Fig. 1b). Therefore, *taiA* and *clyA* are co-transcribed and constitute an operon.

clyA and *taiA* are expressed in human macrophages

Transcriptomic analysis of Typhi infecting human cultured macrophages (THP-1) has shown that *taiA* and *clyA* are induced during infection (Faucher *et al.*, 2006). To confirm this finding, qPCR was performed on RNA samples from bacteria present in the supernatant of infection and from infected macrophages at 0, 8 and 24 h post-infection (Fig. 1c). The supernatant sample contained RNA from bacteria that were not associated with macrophages and was used as the control condition, as previously described for the microarray experiment (Faucher *et al.*, 2006). The 0 h time point contains RNA from bacteria associated with macrophages, either extracellularly or intracellularly. Both genes seemed to be strongly induced when the bacteria were associated with macrophages (0 h), and even more at later time points during the infection. The lower expression of *clyA* compared with *taiA* might be due to a lower mRNA stability of *clyA*, as differential mRNA stability in a polycistronic operon has been shown to be a common mechanism of post-transcriptional regulation in bacteria (Grunberg-Manago, 1999). Nonetheless, both genes have the same expression pattern, which corroborates the results indicating that these genes are co-transcribed.

TaiA, but not ClyA, is involved in macrophage uptake

Upregulation of *clyA* and *taiA* expression during early association of Typhi with human macrophages suggests that they have an effect on macrophage uptake. To test this hypothesis, mutant strains harbouring a non-polar deletion of *taiA* or *clyA* were constructed, and their contribution to uptake by human cultured macrophages (THP-1) was investigated using a gentamicin protection assay. The *clyA* deletion did not affect bacterial uptake significantly. However, deletion of *taiA* reduced bacterial association or uptake by human macrophages to 60 % ($P < 0.005$) of the wild-type value (Fig. 2a). Complementation of the *taiA* mutant with a wild-type copy of the gene on a low-copy-number plasmid restored the wild-type phenotype. In order to differentiate between bacterial association and

Fig. 1. Genetic organization and expression of *taiA* and *clyA*. (a) DNA region encoding *taiA* and *clyA* in Typhi and the corresponding DNA region in Typhimurium. (b) The transcription unit was analysed by RT-PCR. Primers used are depicted in (a): 1, 1498-R-; 2, 1498-F-; 3, 1499-R-; 4, 1499-F-qPCR. NRT, no reverse transcriptase (negative control). (c) qPCR validation of expression of *taiA* and *clyA* inside human macrophages, compared with the supernatant of infection.

phagocytosis, macrophages were pretreated with cytochalasin D before infection. Cytochalasin D is an inhibitor of host cell cytoskeletal function and blocks bacterial uptake by macrophages (Rosenshine *et al.*, 1994). In the presence of cytochalasin D, only 3–4 % of the initial bacterial inoculum was associated with macrophages, and the level

of association of the Δ *taiA* strain was similar to that of the wild-type (Fig. 2a). Taken together, these results suggest that TaiA, but not ClyA, is involved in increasing bacterial uptake by host macrophages by a mechanism independent of bacterial adhesion. Because Δ *clyA* and Δ *taiA* strains have different phenotypes, it is unlikely that the effect observed for the Δ *taiA* strain was due to a polar effect of the *taiA* mutation on *clyA* expression.

ClyA, but not TaiA, affects survival inside macrophages

The contribution of *clyA* and *taiA* to Typhi survival in human cultured macrophages (THP-1) was investigated using the gentamicin protection assay. The *taiA* deletion did not affect survival significantly, but *clyA* deletion enhanced survival of Typhi in human macrophages by 40 % ($P=0.02$) of the wild-type phenotype, after 48 h of infection (Fig. 2b). Complementation of the mutant strain with a low-copy-number plasmid harbouring *clyA* decreased survival significantly ($P=0.03$). In order to investigate the effect of ClyA production inside macrophages, a cytotoxicity assay was performed by monitoring the release of LDH, a cytolysis indicator. Macrophages infected with the Δ *clyA* strain or the complemented mutant strain released the same amount of LDH as the macrophages infected by the wild-type strain (Fig. 2c).

Involvement of *taiA* and *clyA* during infection of human epithelial cells

Since TaiA seems to be involved in invasion of macrophages, and a published study has reported that purified *E. coli* ClyA is cytotoxic to epithelial cells (Wai *et al.*, 2003), we investigated the role of TaiA and ClyA during invasion of epithelial cells. Surprisingly, the level of invasion of epithelial cells was similar for the Δ *taiA* strain and the wild-type strain (Fig. 3a). Interestingly, when an additional copy of *taiA* was added to the wild-type strain, a much higher level of invasion was observed, compared with the wild-type strain harbouring the empty vector (Fig. 4a). The Δ *clyA* strain was two times more invasive ($P=0.03$) than the wild-type strain (Fig. 3a). In addition, the Δ *clyA* strain was 25 % less cytotoxic ($P=0.015$) towards epithelial cells than the wild-type strain (Fig. 3b). Complementation restored the wild-type phenotype.

TaiA enhances *E. coli* invasion of HeLa

As TaiA was involved in Typhi uptake by host cells, we next investigated the effect of *taiA* addition to non-invasive *E. coli* during interaction with epithelial cells. Therefore, *E. coli* DH5 α was transformed with pWSK*taiA* and pWSK29 and invasion assays were performed. Similarly to the results obtained with Typhi, *E. coli* harbouring pWSK*taiA* was two times more invasive ($P=0.03$) than *E. coli* harbouring the empty vector (Fig. 4b). No difference was observed for the initial adhesion levels (data not shown).

Fig. 2. Interaction of $\Delta taiA$ and $\Delta clyA$ strains with human macrophages. (a) Typhi uptake by macrophages was investigated by comparing the number of bacteria at 0 h with the number of bacteria added to the well. The effect of *taiA* mutation on bacterial uptake was confirmed by treatment of macrophages with cytochalasin D (CD), an inhibitor of actin polymerization. (b) Typhi replication inside human macrophages was investigated by comparing the number of bacteria at 48 h post-infection with the number of bacteria at 0 h (fold increase). (c) Cytotoxicity of $\Delta clyA$ was investigated by quantifying LDH release at 48 h post-infection. Results are shown as a percentage of the control value for each replicate; see text for details. Experiments were replicated at least three times independently; NS, not significant.

TaiA is a novel secreted protein

To allow detection of TaiA by Western blotting, the protein was tagged at its C-terminal end with two HA epitopes and cloned in a low-copy-number vector with its native promoter. The HA tag has been proven useful for studying

protein secretion, and C-terminal tags are usually well tolerated (Uzzau *et al.*, 2001). The presence of TaiA-2HA in the supernatant of bacteria grown in conditions known to induce expression of genes involved in invasion of host

Fig. 3. Interaction of $\Delta taiA$ and $\Delta clyA$ strains with human epithelial cells. (a) Typhi invasion of epithelial cells was investigated by comparing the number of bacteria at 90 min with the number of bacteria at 0 h. (b) Cytotoxicity was investigated by quantifying LDH release at 90 min post-infection. Results are shown as a percentage of the control value for each replicate; see text for details. Experiments were replicated at least three times independently; NS, not significant.

Fig. 4. Effect of *taiA* overexpression during interaction with epithelial cells. HeLa cells were infected with Typhi (a) or *E. coli* (b) harbouring the empty vector (pWSK29) or pWSKtaiA, and the invasion rate was determined. Results are shown as a percentage of the control value for each replicate; see text for details. Experiments were replicated at least three times independently.

cells was investigated (LB NaCl) (Lostroh & Lee, 2001). A 29 kDa protein was detected by anti-HA antibodies in the supernatant fraction and in the pellet fraction of the strain harbouring *ptaiA*-2HA, but not in those of the strain harbouring the vector alone (Fig. 5a, lanes 1 and 2). The cytoplasmic GroEL protein was not detected in the supernatant fraction, indicating that it is unlikely that TaiA-2HA was detected because of cytoplasmic leakage. Therefore, TaiA is a novel secreted protein of Typhi involved in uptake by host cells.

TaiA is secreted independently of SPI-1 or SPI-2 T3SS

Because TaiA seems to be involved in early interaction with human macrophages and because it is secreted in SPI-1-inducing conditions (LB NaCl), we investigated whether TaiA secretion was mediated by the SPI-1 T3SS. This was achieved by monitoring secretion of TaiA-2HA in a $\Delta invA$ strain, which is unable to assemble a functional SPI-1 T3SS (Sukhan *et al.*, 2001). As expected, the Typhi $\Delta invA$ strain

was less able to invade human epithelial cells (data not shown) and was not able to secrete SopB (Fig. 5a, lane 3). TaiA-2HA was still secreted by a $\Delta invA$ strain, indicating that its secretion in SPI-1-inducing media was independent of SPI-1 T3SS (Fig. 5a, lane 3).

Since TaiA was not secreted by SPI-1 T3SS and because it was involved in Typhi interaction with human macrophages, it may be secreted by SPI-2 T3SS, which is used to translocate bacterial effectors into host macrophages; therefore, the secretion of TaiA by the SPI-2 T3SS was investigated. Under SPI-2-inducing conditions, a 29 kDa protein, detected by anti-HA tag antibodies, was produced by bacteria harbouring *ptaiA*-2HA (Fig. 5b). However, this protein was detected only in the bacterial pellet and not in the culture supernatant (Fig. 5b). As a control for SPI-2 secretion, SseB was detected in the culture supernatant of Typhi strains harbouring the vector alone or *ptaiA*-2HA, but was absent in the $\Delta ssrB$ strain (Fig. 5b, lane 3). GroEL was used as a control for bacterial cytoplasm leakage and was not detected in the culture supernatant. Our data show that TaiA is not secreted under SPI-2-inducing conditions, and it is therefore unlikely that TaiA is secreted by the SPI-2 T3SS.

Fig. 5. TaiA is secreted independently of both T3SSs. Bacteria expressing TaiA with a double HA-tag at its C-terminal were used to investigate TaiA secretion. Bacteria harbouring the vector alone were used as a negative control. Bacteria were grown in (a) LB NaCl overnight standing (SPI-1-inducing condition) or (b) LPM, pH 5.8, aerobically (SPI-2-inducing condition). Proteins in the supernatant (S) and bacterial pellet (P) were harvested and subjected to Western blotting. Isogenic $\Delta invA$ and $\Delta ssrB$ strains were used as negative controls for SPI-1 and SPI-2 T3SS-mediated secretion, respectively. See text for details.

taiA and *clyA* expression is regulated by PhoP

The PhoPQ two-component system regulates expression of many intracellular genes (Bijlsma & Groisman, 2005; Groisman, 2001). Since *taiA* and *clyA* are involved during infection of human macrophages, this raises the possibility that PhoPQ regulates their expression. Therefore, we compared the expression of *taiA* and *clyA* in the wild-type strain and in a *phoP* mutant in growth conditions known to activate (low magnesium) or inactivate PhoP (high magnesium) (Garcia Vescovi *et al.*, 1996). In the wild-type strain, when PhoP was activated, *taiA* and *clyA* were 100- and 35-fold more expressed, respectively (Fig. 6a). However, their expression was much lower in the *phoP* mutant (Fig. 6a). Under these growth conditions, *taiA* and *clyA* expression was similar to that of *pagC*, a PhoPQ-activated gene. TaiA-2HA was highly produced under PhoP-activating conditions, completely abolished in the $\Delta phoP$ strain and barely detectable when grown in conditions in which PhoP is inactive, as visualized by Western blotting (data not shown). However, TaiA-2HA was not secreted under these conditions. We then investigated by qPCR the expression of *taiA* and *clyA* in the wild-type strain and in a *phoP* mutant during infection of THP-1 macrophages 24 h post-infection. *pagC* was downregulated almost 100-fold in the *phoP* mutant, validating this method for investigating gene regulation by PhoP inside macrophages (Fig. 6b). A 10-fold reduction of both *taiA* and *clyA* expression was observed in the *phoP* mutant (Fig. 6b). It is well known that PhoPQ also regulates the SPI-2 T3SS, which may explain why *taiA* and *clyA* have intracellular expression profiles similar to those of some SPI-2 T3SS genes. However, induction of SPI-2

Fig. 6. PhoP regulates expression of *taiA* and *clyA*. (a) Real-time qPCR was used to compare expression of *taiA* and *clyA* in conditions in which PhoP is known to be active (low concentration of magnesium, low pH) or inactive (high concentration of magnesium, pH 7.4) in the wild-type strain and in a $\Delta phoP$ (DEF429) strain. *pagC* and *prgI* are known to be activated and repressed, respectively, by the active form of PhoP, and were used as controls. (b) Real-Time qPCR was used to compare the expression of *taiA* and *clyA* in the wild-type strain and in a $\Delta phoP$ (DEF429) or $\Delta ssrB$ (DEF149) strain at 24 h post-infection of human macrophages. The fold change represents the level of normalized expression in the mutant strain compared with the wild-type strain. *pagC* and *sseB* were used as controls for PhoP- and SsrB-dependent expression, respectively. See Methods for details.

T3SS structural and effector genes require the *ssrA/ssrB* two-component system, which is also controlled by the PhoPQ system (Hensel *et al.*, 1998; Waterman & Holden, 2003). To investigate a possible regulation of *taiA* and *clyA* by SsrB, we compared expression of these genes in the wild-type and in a $\Delta ssrB$ isogenic strain, 24 h post-infection of human macrophages. Expression of *taiA* and *clyA* in a $\Delta ssrB$ strain was similar to their expression level in the wild-type strain background (Fig. 6b). As expected, the SPI-2-encoded effector *sseB* was strongly repressed in a $\Delta ssrB$ strain. Relative expression values for *taiA* and *clyA* between the $\Delta phoP$ and $\Delta ssrB$ strains were significantly different ($P \leq 0.05$). Thus, *taiA* and *clyA* are part of the PhoP regulon but not the SsrB regulon.

DISCUSSION

The goal of this study was to assess the implication of a gene cluster, composed of two ORFs named *taiA* and *clyA* that were upregulated during macrophage infection, during Typhi interaction with host cells. We first demonstrated by transcriptional analysis that *taiA* and *clyA* are co-transcribed, since a 1.2 kb cDNA overlapping both genes was amplified by PCR (Fig. 1b). Moreover, both genes show the same induction profiles during infection of human macrophages (Fig. 1c). The expression profile obtained by qPCR correlates well with the results obtained in the transcriptomic study of Typhi inside human macrophages (Faucher *et al.*, 2006).

Typhi ClyA contributed to cytotoxicity in epithelial cells (Fig. 3b), as has been shown for *E. coli* ClyA (Wai *et al.*, 2003). At the intestinal phase of the infection, ClyA might be useful to lyse epithelial cells to allow deep tissue infection. However, ClyA reduced long-term survival (48 h post-infection) of Typhi inside human macrophages, because the mutant strain showed an increased survival rate, without affecting cytotoxicity (Fig. 2b, c). The mechanisms underlying differences in the interaction of ClyA with epithelial cells and macrophages are currently unknown and will necessitate more tests using different epithelial and macrophage cell lines. ClyA insertion into the SCV membrane could create a pore that could alter the SCV content, which may in turn affect the survival of Typhi. The ClyA pore is at least 35 Å (0.35 nm) wide, which is sufficiently large to allow passage of small compounds such as maltose (Oscarsson *et al.*, 2002; Tzokov *et al.*, 2006). One may therefore hypothesize that the formation of pores in the SCV by ClyA affects the concentration of ions and small molecules inside the SCV. This may occur either by leakage from the host cell cytoplasm into the SCV or by leakage in the opposite direction. We can also hypothesize that the cytolysin is secreted from the intracellular compartment of infected cells, and will affect adjacent epithelial cells, as shown for the typhoid toxin CdtB (Spano *et al.*, 2008).

Because host macrophages are the reservoir of *Salmonella* during systemic infection (Richter-Dahlfors *et al.*, 1997), expression of *clyA* by Typhi inside human macrophages may lead to reduced bacterial growth and persistent infection of human macrophages during the systemic phase of infection. Growth control inside host cells by intracellular pathogens is a new concept, and some mechanisms have been reported (Tierrez & Garcia-del Portillo, 2005). SciS, a Typhimurium homologue of *Legionella pneumophila* IcmF, has been shown to reduce intracellular growth in macrophages. Interestingly, loss of *sciS* attenuates virulence of Typhimurium in mice (Parsons & Heffron, 2005). Controlled growth in host cells has also been linked to chronic infection (Monack *et al.*, 2004; Tierrez & Garcia-del Portillo, 2005). This growth control might explain the longer incubation period of Typhi with respect to Typhimurium.

As macrophages are the reservoir of *Salmonella*, increasing bacterial uptake by these cells may increase the probability of establishing a systemic infection. This function might be mediated in part by TaiA, because deletion of *taiA* reduced macrophage uptake (Fig. 2a). Reduction in bacterial uptake does not seem to be caused by a reduction in bacterial adhesion or association with human macrophages, since there was no difference in cell association between the wild-type and the Δ *taiA* strain following uptake inhibition by cytochalasin D treatment (Fig. 2a). Therefore, TaiA seems to increase macrophage phagocytic activity. This activity seems restricted to macrophages, since the *taiA* deletion did not impair invasion of epithelial cells (Fig. 3a), but additional copies of *taiA* enhanced the invasion rate (Fig. 4). Nevertheless, our results indicate that TaiA is a novel invasin of Typhi.

Production of TaiA in cell pellets was observed in SPI-1-, SPI-2- and PhoP-inducing media, but secretion of TaiA was only detected under SPI-1-inducing conditions. TaiA secretion was found to be independent of both T3SSs by Western blotting. It was surprising to note secretion of TaiA in SPI-1-inducing conditions, because these conditions are not usually associated with functions involved in interaction with host macrophages. However, there are a number of studies implicating the SPI-1 T3SS during infection of macrophages, and SPI-1-translocated effectors are involved in *S. enterica* interaction with macrophages. For example, the SPI-1 T3SS causes early macrophages apoptosis, a function attributed to SipB (Chen *et al.*, 1996; Fink & Cookson, 2006; Hersh *et al.*, 1999; Lundberg *et al.*, 1999). These studies suggest that SPI-1 T3SS-inducing conditions exist during early interaction with human macrophages. However, our data do not rule out secretion of TaiA under other conditions. Moreover, differential secretion of TaiA suggests regulation at the post-transcriptional level by an unknown mechanism that will require more investigation. Detection of TaiA in the supernatant fraction or the cell fraction at the initial time point of macrophage infection (t_0) by Western blotting was unsuccessful, probably because the TaiA concentration was too low (data not shown). Nevertheless, TaiA is a novel secreted protein independent of both T3SS and, possibly, of the Sec pathway, because no signal peptide was detected in the N-terminal portion of the protein. Because ClyA is exported by OMVs (Tzokov *et al.*, 2006; Wai *et al.*, 2003), one may hypothesize that TaiA also uses this export mechanism and that these proteins interact together in the OMVs. These possibilities are currently under investigation.

The involvement of *taiA* and *clyA* during interaction of Typhi with human macrophages prompted us to investigate a possible regulation by the PhoPQ two-component system, which regulates a number of virulence factors important for *Salmonella* survival inside human macrophages (Bijlsma & Groisman, 2005; Groisman, 2001). Expression of *taiA* and *clyA* was higher under PhoP-activating conditions and reduced in a *phoP* mutant.

Similar results were obtained during infection of human macrophages, where both genes were regulated by PhoP (Fig. 6). To rule out a possible regulation by the downstream two-component system SsrA/SsrB, qPCR analysis was also performed on an *ssrB* mutant strain during infection. However, SsrB does not seem to be involved in regulation of *taiA* and *clyA* (Fig. 6). Our results show that *taiA* and *clyA* are novel members of the PhoP regulon. Regulation of the *taiA* invasin by PhoP may seem to contradict its well-accepted function as a regulator of virulence genes involved in intracellular survival, such as the SPI-2 T3SS. However, using LB broth, a condition not usually recognized to support expression of genes involved in intracellular survival, it has been shown that *pag* genes, controlled by PhoP, and some SPI-2 effectors are expressed (Belden & Miller, 1994; Bustamante *et al.*, 2008). Moreover, it was recently shown that two genes encoded on SPI-1 (*orgBC*) are activated by PhoP during growth in LB NaCl, a condition known to induce SPI-1 genes (Aguirre *et al.*, 2006). Therefore, our finding that PhoP regulates *taiA*, which is secreted under SPI-1-inducing conditions, suggests a double control of expression that may reflect a sequential requirement for this protein. The TaiA invasin will be expressed and accumulated under PhoP-activated conditions and then secreted under SPI-1-inducing conditions. However, we cannot rule out that the effect of PhoP is indirect, and we are currently investigating in more detail the mechanism of regulation. It has recently been shown that ClyA is regulated by SlyA in Typhi (von Rhein *et al.*, 2009). SlyA has been shown to be induced via the PhoPQ two-component system following internalization of bacteria by macrophages (Buchmeier *et al.*, 1997; Norte *et al.*, 2003; Shi *et al.*, 2004), and it has been shown that SlyA enhances overall transcription of PhoP-activated loci (Song *et al.*, 2008). Interestingly, a large number of SlyA-dependent genes are also controlled by the PhoPQ system (Navarre *et al.*, 2005). We hypothesize that the *taiA*–*clyA* gene cluster is also under co-regulation by SlyA and PhoP. Some PhoP-regulated genes are conserved among enterobacteria and others are specific to *Salmonella* (Lejona *et al.*, 2003). *taiA* and *clyA* represent PhoP-regulated genes that are specific to a limited number of *Salmonella* serovars.

This study shows that *taiA* and *clyA* are co-regulated by PhoP and seem to have complementary functions. TaiA is a novel secreted invasin, which increases bacterial uptake by human macrophages, and ClyA reduces bacterial growth within these cells, which might result in an increased use of macrophages as a sheltered environment. This in turn may promote persistent infection of the host, which is a key feature of typhoid fever.

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