SPI-9 of \textit{Salmonella enterica} serovar Typhi is constituted by an operon positively regulated by RpoS and contributes to adherence to epithelial cells in culture

Juan C. Velásquez, Alejandro A. Hidalgo, Nicolás Villagra, Carlos A. Santiviago, Guido C. Mora and Juan A. Fuentes

The genomic island 9 (SPI-9) from \textit{Salmonella enterica} serovar Typhi (S. Typhi) carries three ORFs (STY2876, STY2877, STY2878) presenting 98\% identity with a type 1 secretory apparatus (T1SS), and a single ORF (STY2875) similar to a large RTX-like protein exhibiting repeated Ig domains. BapA, the \textit{Salmonella enterica} serovar Enteritidis orthologous to S. Typhi STY2875, has been associated with biofilm formation, and is described as a virulence factor in mice. Preliminary \textit{in silico} analyses revealed that S. Typhi STY2875 ORF has a 600 bp deletion compared with S. Enteritidis bapA, suggesting that S. Typhi STY2875 might be non-functional. At present, SPI-9 has not been studied in S. Typhi. We found that the genes constituting SPI-9 are arranged in an operon whose promoter was up-regulated in high osmolarity and low pH in a RpoS-dependent manner. All the proteins encoded by S. Typhi SPI-9 were located at the membrane fraction, consistent with their putative role as T1SS. Furthermore, SPI-9 contributed to adherence of S. Typhi to epithelial cells when bacteria were grown under high osmolarity or low pH. Under the test conditions, S. Typhi SPI-9 did not participate in biofilm formation. SPI-9 is functional in S. Typhi and encodes an adhesin induced under conditions normally found in the intestine, such as high osmolarity. Hence, this is an example of a locus that might be designated a pseudogene by computational approaches but not by direct biological assays.

\textbf{INTRODUCTION}

The genus \textit{Salmonella} includes two species, \textit{Salmonella bongori} and \textit{Salmonella enterica}, the latter containing many subspecies and serovars (Brenner et al., 2000). Genome sequences of closely related \textit{S. enterica} subsp. \textit{enterica} (\textit{S. enterica}) serovars share more than 90\% identity at the nucleotide level (Chan et al., 2003). Nevertheless, each serovar presents specific features, including differences in host specificity (McClelland et al., 2001; Parkhill et al., 2001). Some serovars, such as \textit{S. enterica} serovar Enteritidis (\textit{S. Enteritidis}) are considered ‘generalists’ because they infect a broad range of hosts. Other serovars are host-restricted, such as \textit{S. enterica} serovar Typhi (S. Typhi), a human-restricted pathogen that causes typhoid fever (Barrow & Duchet-Suchaux, 1997; Parkhill et al., 2001). The evolution of the different \textit{S. enterica} features might have occurred by acquisition of new genes through horizontal transfer, loss of genetic information by deletions or pseudogene formation, or by a combination of these mechanisms (Hacker & Carniel, 2001; Moran & Plague, 2004). The newly acquired genes are usually clustered in specific genomic regions termed genomic islands. Because they promote genetic variability, genomic islands play an

\textbf{Abbreviations:} \textit{S. Typhi, Salmonella enterica serovar Typhi; S. Enteritidis, Salmonella enterica serovar Enteritidis; S. enterica, Salmonella enterica; S. bongori, Salmonella bongori; SPI, Salmonella pathogenicity island; T1SS, Type one secretion system; gDNA, genomic DNA.}

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important role in microbial evolution (Hentschel & Hacker, 2001; Hsiao et al., 2005). Pathogenicity islands correspond to a subset of genomic islands encoding functions related to enhancing virulence. In *S. enterica*, 24 *Salmonella* pathogenicity islands have (SPI-1 to SPI-24) been described to date (Hayward et al., 2014; Pezoa et al., 2014; Urrutia et al., 2014).

*S. enterica* infection begins with ingestion of contaminated water or food. Some environmental conditions in the intestine, such as high osmolarity or low pH, induce the expression of SPI-1 genes and other virulence–related genes (Altier, 2005; Jofre et al., 2014). The products of several of these genes mediate adherence and/or invasion of intestinal epithelial cells (Galán, 2001). A subset of *S. enterica* serovars, such as *S. Typhi* in humans, can enter the host bloodstream, disseminate and survive inside macrophages. Most of these steps depend wholly on the expression of SPI-2 genes (Ochman et al., 1996). SPI-2 genes are usually induced by depletion of nutrients or oxidative stress, conditions normally found at this stage of the infection (Ochman et al., 1996). Besides SPI-1 and SPI-2, the participation of other SPIs in *S. Typhi* infection has been described. For example, SPI-3 participates in survival inside macrophages (Retamal et al., 2009); SPI-7 encodes the Vi capsular antigen (Bueno et al., 2004); SPI-18 encodes the HlyE hemolysin (Fuentes et al., 2008); and SPI-24 encodes the intestinal adhesin ShdA (Urrutia et al., 2014). In contrast, other regions referred to as SPIs have not been characterized in *S. Typhi* with respect to virulence. Parkhill et al. identified a *S. Typhi* genomic region termed SPI-9 (16 kb) (Parkhill et al., 2001), but its role in virulence has not been addressed. This region carries three ORFs (STY2876, STY2877, STY2878) presenting identity (98%) with a type 1 secretory apparatus and a single ORF (STY2875) similar to a large RTX-like protein exhibiting repeated Ig domains (Parkhill et al., 2001). Type 1 secretion systems (T1SS) are widespread among Gram-negative bacteria. These systems achieve secretion in a single step directly from the bacterial cytoplasm to the extracellular milieu. The translocation machinery is composed of three indispensable membrane proteins: (1) the translocator, an ABC-transporter providing energy through ATP hydrolysis (and perhaps the initial channel across the inner membrane); (2) a multimeric membrane fusion protein (MFP) spanning the initial part of the periplasm and forming a continuous channel to the surface; and (3) an outer trimeric membrane protein connected to the translocator by the MFP (Dinh et al., 1994; Holland et al., 2005). The T1SS is capable of transporting polypeptides of up 800 kDa across the cell envelope (Holland et al., 2005). The secretion signal is usually located at the C-terminal end of the secreted protein and exhibits no cleavage during secretion (Delepelaire, 2004).

BapA, orthologous to *S. Typhi* STY2875 and located in the corresponding *S. Enteritidis* SPI-9, has been associated with biofilm formation (Latasa et al., 2005). Accordingly, the expression of BapA is coordinated with genes encoding curli fimbriae and cellulose (Latasa et al., 2005). In addition, BapA seems to contribute to *S. Enteritidis* virulence since mice orally inoculated with *S. Enteritidis ΔbapA* survive longer compared to those inoculated with the WT strain (Latasa et al., 2005). In *silico* analyses revealed that *S. Typhi* STY2875 ORF presents a 600 bp deletion compared with *S. Enteritidis bapA*, suggesting that STY2875 might be non-functional (i.e. a pseudogene).

In this manuscript, we characterized SPI-9 in *S. Typhi*. We found that all the genes constituting SPI-9 are arranged in an operon whose expression is increased under high osmolarity and low pH in a RpoS-dependent way. All SPI-9 encoded proteins are located at the membrane fraction, consistent with their putative role as a T1SS. Furthermore, STY2875 can be considered an adhesin that contributes to adherence to epithelial cells when bacteria were previously grown under high osmolarity or low pH.

**METHODS**

**Bacterial strains, media and culture conditions.** *Salmonella enterica* serovar *Typhi* STH2370 (*S. Typhi* STH2370) was obtained from the Infectious Diseases Hospital Lucio Córdova, Chile (Valenzuela et al., 2014). *S. Typhi* STH2370 and derivatives were grown routinely in liquid culture using Luria Bertani (LB) medium (Bacto peptone, 10 g l⁻¹; Bacto yeast extract, 5 g l⁻¹; NaCl, 5 g l⁻¹) at 37 °C, with aeration, or in microaerophilic conditions by adding an overlay of 500 µl of sterile mineral oil as a barrier to oxygen. When required, the medium was supplemented with kanamycin (50 µg ml⁻¹), chloramphenicol (20 µg ml⁻¹), ampicillin (50 mg ml⁻¹) or gentamicin (20 µg ml⁻¹). The media were solidified by adding agar (15 g l⁻¹).

To determine the effect of pH, bacteria were grown in citrate-buffered LB broth (pH 5.0); for the effect of high osmolarity, bacteria were grown in 400 mM NaCl phosphate-buffered LB broth (pH 7.0). As a reference non-inducer condition, phosphate-buffered LB broth (pH 7.0) was used. In all cases, bacteria were grown in a stationary phase culture to OD₅₆₀ of 0.5 at 37 °C with shaking for aeration. Details on the growth conditions have been previously described (Fuentes et al., 2009; Jofre et al., 2014).

**Construction of *S. Typhi* mutant strains.** Mutant strains carrying deletion and/or substitution of the STY2875, STY2876, STY2877 and/or STY2878 genes by resistance cassettes (aph: resistance to kanamycin) or an FRT scar were constructed using the Red/Swap method (Datsenko & Wanner, 2000). PCR primers (60 nt) were synthesized with 40 nt of homology to the target gene at the 5' end of each primer and 20 nt at the 3'-end aligning with pDK34 plasmid as a source of the antibiotic–resistant cassettes (Table 1) (Datsenko & Wanner, 2000). The FRT scar was used to fuse the lacZ/Y reporter as described (Ellermeier et al., 2002) to construct transcriptional fusions with the SPI-9 genes. The *S. Typhi* ΔrpoS, *S. Typhi* ΔrpoS/pBRPOS and *S. Typhi* ΔrpoS/pBBR5 mutants were previously reported (Fuentes et al., 2009; Jofre et al., 2014). *S. Typhi* STH2370 STY2875-3xFLAG, *S. Typhi* STY2876-3xFLAG, *S. Typhi* STY2877-3xFLAG, *S. Typhi* STY2878-3xFLAG, *S. Typhi* ompA-3xFLAG and *S. Typhi* impX-3xFLAG mutants were constructed using the primers listed in Table 1 as previously described (Uzau et al., 2001). *S. Typhi* rpsO-3xFLAG and *S. Typhi* impX-3xFLAG strains have been previously reported (Bucarey et al., 2006; Fuentes et al., 2009; Jofre et al., 2014). All the double mutants were constructed by electrotransformation with genomic DNA (gDNA) from single mutants as described (Toro et al., 1998). The presence of each substitution was confirmed by PCR using primers complementary to the DNA genome flanking the sites of substitution.
were stopped by the addition of 500 µl 1 M NaCl. 

Activity is expressed in Miller units, 10

pended in DEPC-treated water, prior to treatment with DNase I to

room temperature, washed with ice-cold 70 % v/v ethanol and resus-

dition was extracted using TRIzol reagent (Invitrogen) as described by

RT-PCR assay.

SPI-Assays for b-galactosidase activity. S. Typhi mutant strains with

lucY fusions were grown under the conditions previously described and

then chilled to 4 °C. b-Galactosidase activity was measured by a

modification of the method of Miller (Miller & Hershberger, 1984)
described in Jofre et al. (2001). Briefly, each bacterial culture was sus-
pended in 900 µl of Z buffer (0.6 M Na

-D-galactopyranoside (4 mg ml

Total RNA from the strains grown under the test con-

ditions was extracted using TRIzol reagent (Invitrogen) as described by

Primers for epitope tagging (3xFLAG) (Uzzau et al., 2001)

Primers for mRNA detection by RT-PCR

Primers used for the Red/Swap technique (Datsenko & Wanner, 2000)

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Lower case: annealing site with pKD4 (Datsenko & Wanner, 2000).
Italic lower case: annealing site with pKD4 (Uzzau et al., 2001).

http://mic.microbiologyresearch.org 1369
3xFLAG fusion proteins were immunodetected using anti-FLAG M2 mAbs from Sigma. Strains carrying the epitope-tagged gene were grown in 2 ml cultures under the conditions previously described. Bacterial pellets from 2 ml were resuspended in 100 µl of H tagged gene were grown in 2 ml cultures under the conditions previously described (Lobos & Mora, 1991). Briefly, bacteria were cultured in LB pH 7.0 400 mM NaCl to exponential phase without aeration. The cultures were centrifuged at 13 000 r.p.m. for 45 min at 4°C. At this point, the pellet corresponds to the total membrane fraction (including outer and inner membrane), whereas the supernatant corresponds to the cytoplasmic fraction. The proteins in the cytoplasmic fraction were precipitated with phenylmethylsulfonly fluoride 2 mM. The sample was centrifuged at 3000 r.p.m., and then mixed with 100 µl of Laemmli lysis buffer (Laemmli, 1970). Suspensions were incubated at 100°C for 5–10 min, centrifuged to remove cell debris, and 10 µg resolved by SDS-PAGE. Proteins were transferred to poly(vinylidene difluoride) membranes (stained with Ponceau S to confirm the protein load) and probed with mAbs (1 : 1.000) and horseradish peroxidase-conjugated goat antimouse IgG [1 : 5000 (Sigma)]. As controls, we used primary mouse anti-Hsp60 mAbs (1 : 10 000), subsequently probed with a secondary horseradish peroxidase-conjugated goat antimouse IgG [1 : 5000 (Sigma)] to detect the Hsp60 protein (60 KDa). In all cases, detection was by enhanced chemiluminescence (ECL, Amer sham Pharmacia).

**Immunoblotting.** 3xFLAG fusion proteins were immunodetected using anti-FLAG M2 mAbs from Sigma. Strains carrying the epitope-tagged gene were grown in 2 ml cultures under the conditions previously described. Bacterial pellets from 2 ml were resuspended in 100 µl of H2O and mixed with 100 µl of Laemmli lysis buffer (Laemmli, 1970). Suspensions were incubated at 100°C for 5–10 min, centrifuged to remove cell debris, and 10 µg resolved by SDS-PAGE. Proteins were transferred to poly(vinylidene difluoride) membranes (stained with Ponceau S to confirm the protein load) and probed with mAbs (1 : 1.000) and horseradish peroxidase-conjugated goat antimouse IgG [1 : 5000 (Sigma)]. As controls, we used primary mouse anti-Hsp60 mAbs (1 : 10 000), subsequently probed with a secondary horseradish peroxidase-conjugated goat antimouse IgG [1 : 5000 (Sigma)] to detect the Hsp60 protein (60 KDa). In all cases, detection was by enhanced chemiluminescence (ECL, Amer sham Pharmacia).

**Subcellular fractionation.** Subcellular fractionation was performed by a modification of a method previously described (Lobos & Mora, 1991). Briefly, bacteria were cultured in LB pH 7.0 400 mM NaCl to exponential phase without aeration. The cultures were centrifuged at 3000 g for 15 min at 4°C. The supernatant was discarded and the pellet was resuspended in lysis buffer (Tris-HCl 10 mM pH 8.0, MgCl2 10 mM), sonicated for 100 s on ice and supplemented with phenylmethylsulfonly fluoride 2 mM. The sample was centrifuged at 3000 g for 15 min at 4°C. The pellet was discarded. The supernatant was centrifuged at 13 000 r.p.m. for 45 min at 4°C. At this point, the pellet corresponds to the total membrane fraction (including outer and inner membrane), whereas the supernatant corresponds to the cytoplasmic fraction. The proteins in the cytoplasmic fraction were precipitated with 10% trichloroacetic acid (Link & Labrec, 2011).

**In silico analyses.** Comparative sequence analyses were made with the SPI-9 sequences available at http://www.ncbi.nlm.nih.gov/ (S. Typhi strains STH2370, Ty2, Ty21a CT18; S. Typhimurium strains 14028 s, LT2, DT104, SL1344; S. Paratyphi A strain 9150; S. Enteritidis strain PT4; S. Choleræusis SC-B67; S. Gallinarum strain 287/91; Salmonella bongori; and E. coli K12). Sequences were analysed using BLAST alignment and tools available at http://www.ncbi.nlm.nih.gov/, with visual inspection to improve the results. Subcellular localization was analysed using PSORTb (Yu et al., 2010). To determine de Z’ number, we followed an algorithm previously described (Zhang & Zhang, 2004).

**Determination of biofilm formation.** Quantification of biofilm production in a 96-well flat-bottomed polystyrene microplate was based on...
SPI-9 is a genomic island found in Salmonella enterica and Salmonella bongori, but absent from Escherichia coli

Parkhill et al. found several S. Typhi genomic regions absent from the E. coli K12 genomic region, including a region named SPI-9 (16 kb) (Parkhill et al., 2001). To characterize SPI-9 in S. Typhi, we analysed the G+C content as previously described (Zhang & Zhang, 2004). Fig. 1(a) shows the Z' number (Zhang & Zhang, 2004), with the accumulated G+C in SPI-9 and neighbouring regions. A positive slope represents G+C accumulation whereas a negative slope represents A+T accumulation. S. Typhi SPI-9 presented 57% G+C, marking a difference with the rest of the S. Typhi chromosome (52% G+C). Similar to other genomic islands (Bueno et al., 2004), S. Typhi SPI-9 is located adjacent to a tRNA gene (ssrA) (Fig. 1b). We were unable to detect other features commonly associated with new genomic islands (Che et al., 2014; Juhás et al., 2009), such as the presence of flanking direct repeats, mobility loci or instability, suggesting that SPI-9 is a more ancient island. In silico analyses revealed that SPI-9 is present in all the Salmonella enterica serovars studied, including S. Typhi strains STH2370, Ty2, Ty21a and CT18; S. Typhimurium strains 14028 s, LT2, DT104 and SL1344; S. Paratyphi A strain 9150; S. Enteritidis strain PT4; S. Choleraesuis strain SC-B67; and S. Gallinarum strain 287/91 (data not shown). Finally, SPI-9 is also present in Salmonella bongori, but absent from E. coli K12 (Fig. 1b). From these results, we concluded that SPI-9 exhibits features normally associated with ancient genomic islands.

SPI-9 ORFs constitute an operon in S. Typhi STH2370

Preliminary experiments indicated that expression of the ORFs found in S. Typhi STH2370 SPI-9 increased during the stationary phase (data not shown). Moreover, we observed that, in all cases, we obtained similar changes in the expression of all ORFs under the test conditions. This result prompted us to test whether SPI-9 ORFs constitute an operon. For this, we extracted RNA from S. Typhi STH2370 grown to stationary phase (OD600=1.4). Then, we synthesized cDNA using a reverse primer located at the STY2878 ORFs (Fig. 1c, left, grey arrow). As negative control, we performed PCR after the DNase treatment and before the reverse transcription (not shown). Finally, we detected cDNA by PCR using the primers listed in Table 1 and depicted in Fig. 1c (left, black arrows). As shown in Fig. 1c (right), we obtained the expected amplicons with all primer combinations, indicating that STY2875 and STY2876, as well as STY2877 and STY2878, are transcribed in a polycistronic mRNA. From these results, we inferred that SPI-9 ORFs (i.e. STY2875, STY2876, STY2877 and STY2878) constitute an operon in S. Typhi STH2370.

SPI-9 ORFs of S. Typhi STH2370 are induced under low pH and high osmolarity in an RpoS-dependent manner

To study conditions that could affect the expression of the SPI-9 operon, we used a single-copy, chromosomal transcriptional lac fusion to STY2875, the first gene of the operon, to explore conditions that might affect the expression of the genes belonging to SPI-9. Thus, we constructed the S. Typhi STH2370 ΔSTY2875::lacZY mutant by replacing an internal segment of the STY2875 ORFs by a lac reporter (lacZY) as previously described (Ellermeier et al., 2002). This strain was cultured to logarithmic phase (OD600=0.5) under different conditions previously associated with different stages of the infection process, including the presence of glucose, micro-aerophilic conditions, high osmolarity and changes in pH (Bajaj et al., 1996; Ellermeier & Slach, 2007; Eriksson et al., 2003; Hansen-Wester & Hensel, 2001; Rhen & Dorman, 2005; Rychlik & Barrow, 2005). As shown in Fig. 2a, low pH and high osmolarity increased β-galactosidase activity associated with the expression of STY2875, whereas the other conditions exerted no significant effects, compared with LB at pH 7.0.

To determine whether these conditions also increase the amount of the proteins encoded by SPI-9, we constructed the S. Typhi STY2875-3xFLAG, S. Typhi STY2876-3xFLAG, S. Typhi STY2877-3xFLAG and S. Typhi STY2878-3xFLAG strains by placing a 3xFLAG at the C termini of the respective ORFs. This procedure allowed the subsequent detection
of the FLAG-tagged proteins by Western blotting as previously described (Uzzau et al., 2001). Therefore, these strains were grown under low pH or high osmolarity to logarithmic phase \( (\text{OD}_{600}=0.5) \) and 20 µg of total protein were resolved in a 15% polyacrylamide-SDS gel. Proteins were transferred onto a poly(vinylidene difluoride) membrane and probed with anti-FLAG M2 mAb (Sigma). We detected Hsp60 as load control. Fig. 2b shows that bacteria grown under low pH or high osmolarity exhibited an increased amount of all SPI-9 encoded proteins in comparison with

Fig. 2. Expression of S. Typhi SPI-9 ORFs was induced under low pH and high osmolarity in an RpoS-dependent manner. (a) S. Typhi \( \Delta STY2875::\lacZY \) was grown to logarithmic phase \( (\text{OD}_{600}\text{ of }0.5) \) under different culture conditions prior to determining \( \beta \)-galactosidase activity. LB at pH 7.0 (LB) was used as a reference growth condition. (b) Immunodetection of the epitope-tagged \( (3\times \text{FLAG}) \) proteins STY2875, STY2876, STY2876, STY2877 and STY2878 obtained from bacteria previously grown in LB pH 7.0, LB pH 5.0 or LB pH 7.0 NaCl 400 mM. As load control, we detected Hsp60. (c) \( \beta \)-galactosidase activity of S. Typhi \( \Delta STY2875::\lacZY \) (Parental) and S. Typhi \( \Delta STY2875::\lacZY \Delta rpoS::\text{cam} \) \( (\Delta rpoS) \). To complement the \( \Delta rpoS \) strain, the pBRPOS \( (\text{pBBR}5::rpoS) \) plasmid was used (Jofre et al., 2014). pBR5 corresponds to the empty vector (Jofre et al., 2014). (d) RT-PCR assay performed to detect STY2875 mRNA and 16s mRNA (control) from S. Typhi (WT) or S. Typhi \( \Delta rpoS::\text{FRT} \) \( (\Delta rpoS) \) grown to logarithmic phase \( (\text{OD}_{600}\text{ of }0.5) \) in LB pH 7.0, LB pH 5.0 or LB pH 7.0 NaCl 400 mM. The purified RNA was used to synthesize cDNA for the SPI-9 operon using the same reverse primer shown in Fig. 1c (right, grey arrow) prior to performing PCR to detect STY2875. All experiments were performed in three full biological replicates, each time in technical triplicate. *\( P<0.05.\)
bacteria grown in LB at pH 7.0 (control), supporting the results obtained with the β-galactosidase assays (Fig. 2a) and reinforcing the results showing that SPI-9 corresponds to an operon (Fig. 1c). Thus, low pH and high osmolarity induce the expression of SPI-9 genes at the transcriptional level.

Since we observed that low pH, high osmolarity (Fig. 2a, b) and stationary phase (data not shown) induced the expression of SPI-9 genes, we studied the role of RpoS, a sigma factor involved in gene regulation under those conditions (Hengge-Aronis, 2000). Therefore, we constructed the S. Typhi ΔSTY2875::lacZY ΔrpoS::cam double mutant as previously described (Toro et al., 1998). As shown in Fig. 2a, c, STY2875-associated β-galactosidase activity was increased under low pH and high osmolarity. Nevertheless, this effect was abolished in the ΔrpoS mutant, indicating that induction of the STY2875 transcription is dependent on RpoS under these conditions. The introduction of a plasmid encoding the rpoS gene (pBRPOS) (Jofre et al., 2014) into the ΔrpoS mutant fully restored the levels of STY2875-associated β-galactosidase activity, exhibiting similar values to those of the parental strain. The presence of the vector alone (pBBR5) produced no changes in the similar values to those of the parental strain. The presence of the vector alone (pBBR5) produced no changes in the

Taken together, these results show that transcription of the SPI-9 operon genes is increased under low pH and high osmolarity in an RpoS-dependent manner.

### Proteins encoded by S. Typhi SPI-9 are located at the membrane fraction

In silico analysis, using the PSORTb software, could not predict the subcellular localization of STY2875. According to this same analysis, the subcellular localization of STY2876 might correspond to the outer membrane fraction, whereas the subcellular localization of STY2877 and STY2878 might correspond to the cytoplasmic fraction. STY2876 exhibits 98% identity with a TolC family outer membrane protein (T1SS); STY2877 presents 98% identity with an ATP-binding protein (T1SS) normally found in the inner membrane; and STY2878 presents 98% identity with an HlyD family membrane fusion protein (T1SS). Considering that the type 1 secretory apparatus is normally located at the membrane fraction, we performed subcellular fractionation to determine the actual subcellular localization of the proteins encoded by S. Typhi SPI-9. For that, the S. Typhi STY2875-3xFLAG, S. Typhi STY2876-3xFLAG, S. Typhi STY2877-3xFLAG and S. Typhi STY2878-3xFLAG strains were cultured to stationary phase (OD₆₀₀=1.4) in LB pH 7.0 400 mM NaCl without shaking prior to fractionating the membrane fraction (outer and inner membrane) and the cytoplasmic fraction. Proteins were detected using Western blot as described above. Proteins STY2876, STY2877 and STY2878 were found in the membrane fraction, consistent with their predicted function as a type 1 secretory apparatus (Fig. 3). Furthermore, STY2875 was mainly found in the membrane fraction (Fig. 3). We were unable to detect STY2875 in the supernatant fraction (data not shown). Immunodetection of epitope-tagged (3xFLAG) proteins OmpA (outer membrane protein), ImpX (inner membrane protein) (Bucarey et al., 2006) and RpoS (cytoplasmic protein) (Jofre et al., 2014), obtained from bacteria cultured to stationary phase (OD₆₀₀=1.4) in LB pH 7.0, was used as control (Fig. 3).

Fig. 3. S. Typhi SPI-9-encoded proteins are found in the membrane fraction. Immunodetection of epitope-tagged (3xFLAG) proteins STY2875 (right), STY2876, STY2878, STY2877 and STY2878 (left) on the membrane fraction (MF; outer membrane and inner membrane), and cytoplasmic fraction (CF). Immunodetection of epitope-tagged (3xFLAG) proteins OmpA (outer membrane protein), ImpX (inner membrane protein) (Bucarey et al., 2006) and RpoS (cytoplasmic protein) (Jofre et al., 2014), obtained from bacteria cultured to stationary phase (OD₆₀₀=1.4) in LB pH 7.0, was used as control (Fig. 3).
SPI-9 contributes to adherence to epithelial cell lines

Previous studies have described S. Enteritidis BapA, orthologous to S. Typhi STY2875, as associated with biofilm formation. In addition, BapA participates in virulence contributing to gut colonization as determined by co-infection with the respective WT in ileal loop experiments (Latasa et al., 2005). In silico analyses revealed that the S. Typhi STY2875 gene presents a 600 bp deletion compared with the orthologous bapA found in S. Enteritidis, suggesting that STY2875 might be non-functional (Fig. 1d); although this deletion exerts no effect in the reading frame as determined by the protein alignment (Fig. 1c). Since in-frame deletions can generate new functional alleles (Urrutia et al., 2014), we hypothesized that S. Typhi STY2875 is functional. To test this hypothesis, we determined the contribution of SPI-9 to biofilm formation in S. Typhi. For that, we cultured S. Typhi and derivatives in LB pH 7.0 400 mM NaCl to stationary phase (OD600=1.4) prior to seeding wells and incubating for 24 h at 37 °C. As shown in Fig. 2, high osmolarity positively contributes to the production of proteins encoded by ORFs in SPI-9. We tested biofilm production of two strains of S. Enteritidis from the SARB collection (SARB16 and SARB18) as positive controls, whereas LB alone was used a negative control. To detect biofilm formation, we revealed attached bacteria with crystal violet (see Methods). As shown in Fig. 4a, the two strains of S. Enteritidis efficiently formed biofilms. In contrast, S. Typhi and its derivative mutants were unable to form biofilms. The same results were obtained using bacteria cultured in LB pH 5.0 or in LB pH 7.0 (data not shown).

Next, we assessed the contribution of SPI-9 to adherence in HEp-2, an epithelial cell line commonly used to study adherence and invasion of S. enterica (Urrutia et al., 2014). For that, S. Typhi WT, S. Typhi ΔSPI-9 (i.e. ΔSTY2875-STY2878), S. Typhi ΔSTY2875 and S. Typhi ΔSTY2876-STY2878 were grown under micro-aerophilic conditions to early logarithmic phase (OD600=0.2–0.3) in LB pH 7.0 (control), LB pH 5.0 or LB pH 7.0 400 mM NaCl prior to performing adherence tests in HEp-2 epithelial cells. As shown in Fig. 4b, SPI-9 seemed to be dispensable when bacteria were previously cultured in LB pH 7.0. In contrast, SPI-9 contributed to cell adherence when bacteria were previously cultured in LB pH 5.0 or in LB pH 7.0 400 mM NaCl, consistent with the expression results showing that SPI-9 operon is induced under these conditions (Fig. 2). Furthermore, the impaired adherence among S. Typhi ΔSPI-9, S. Typhi ΔSTY2875 and S. Typhi ΔSTY2876-STY2878 is indistinguishable and independent of the culture conditions (Fig. 4b). This result shows that all SPI-9 genes (i.e. the putative effector protein STY2875 and the putative type 1 secretion apparatus encoded by STY2876, STY2877 and STY2878) contribute to adherence. When we tested the adherence in Caco-2 cells, other epithelial cell lines also worked with S. enterica (Wang et al., 2016), and we observed that S. Typhi ΔSTY2875 also presented attachment defects when previously cultured in LB pH 7.0 400 mM NaCl (Fig. 4c), supporting our conclusions.

DISCUSSION

We showed here that SPI-9 can be found in strains from the two species of Salmonella, S. enterica and S. bongori. In addition, SPI-9 is constituted by an operon formed by four genes transcriptionally up-regulated under high osmolarity and low pH in a RpoS-dependent manner. Furthermore, the encoded proteins are found in the membrane fraction and participate in adherence to epithelial cells.

It has been proposed that SPIs can be classified into four groups, according to their distribution among different species and Salmonella serovars. Islands found in both S. bongori and S. enterica (such as SPI-1 and SPI-9) were likely acquired by Salmonella before speciation. Islands found in S. enterica, but not in S. bongori (such as SPI-2), were likely acquired by Salmonella after speciation. Islands found in some, but not all, S. enterica serovars, and absent from S. bongori (such as SPI-18), were likely acquired after the speciation of the genus Salmonella, and during early stages of the diversification of S. enterica serovars (Fuentes et al., 2008). Unstable islands found in only a small subset of serovars are likely products of more recent (and, in some cases, ongoing) horizontal transfer events, such as SPI-7 (Bueno et al., 2004). This hypothesis is supported by the fact that more ancient islands, like SPI-9, appear to have lost trans-acting genes and cis-acting sites required for their mobility, remaining stable within their host genomes. We speculate that SPI-9, as well as SPI-1, were acquired before speciation and conserved to increase the fitness inside the intestine. Furthermore, the presence of SPI-9 in S. bongori, a species restricted to the intestines mainly because of the lack of SPI-2 (Hansen-Wester et al., 2004), supports the idea of the intestinal role of SPI-9.

Comparing SPI-9 among S. enterica serovars revealed that the putative type 1 secretion apparatus is highly conserved, whereas some differences are found with the putative ‘effector’ protein (i.e. STY2875). Even if S. Typhi STY2875 presents a large in-frame deletion (600 bp) compared with S. Enteritidis BapA, STY2875 is functional and participates in adherence to eukaryotic cells. Other large proteins of high molecular weight, such as S. Typhi ShdA, are also functional despite the presence of large in-frame deletions (Urrutia et al., 2014).

In this work, we found that SPI-9 constitutes an operon. T1SS are normally clustered in operons (e.g. hlyCABD in E. coli, raxSTAB in Xanthomonas oryzae) (Bielaszewska et al., 2014; Ronald, 2014), supporting the idea that S. Typhi SPI-9 genes could indeed encode a T1SS. Furthermore, other genomic islands are constituted by operons, such as SPI-18 (Faucher et al., 2009; Fuentes et al., 2008), type IV pilus encoded in SPI-7 (Buenc et al., 2004) and tsx-impX (Bucarey et al., 2006) in S. enterica, emphasizing the importance of a concerted regulation of newly acquired functions.
We also found that the SPI-9 genes are induced under high osmolarity and low pH in a RpoS-dependent manner in *S. Typhi*. It has been reported that several genes that participate in the intestinal processes (such as SPI-1 genes) are expressed under high osmolarity, a condition normally found inside the gut (Bajaj *et al.*, 1996; Ellermeier & Slauch, 2007; Fuentes *et al.*, 2008; Jofre *et al.*, 2014; Rhen & Dorman, 2005; Rychlik & Barrow, 2005). On the other hand, when *S. enterica* enters a host, it senses a sudden drop of pH in the stomach. The presence of low pH in the stomach can activate
the expression of genes involved in the subsequent intestinal interaction (Rychlik & Barrow, 2005). Furthermore, the induction of the S. Typhi SPI-9 genes under high osmolarity and low pH depends on RpoS. RpoS is a sigma factor that regulates many genes involved in adaptation, especially in the intestine and in virulence genes, including hlyE (Fuentes et al., 2008, 2009; Jofre et al., 2014; Rychlik & Barrow, 2005). Transcriptomic studies revealed that S. Typhi SPI-9 genes are not induced inside macrophages (Faucher et al., 2006), suggesting that SPI-9 does not participate in the establishment of a systemic disease.

In silico analysis showed that S. Typhi SPI-9 is constituted by three ORFs with high identity (98%) with a T1SS, and a large ORF encoding a putative protein presenting repeated sequences (STY2875). STY2876, STY2877 and STY2878 are located at the membrane fraction, supporting their role as structural component of a T1SS. In addition, STY2875 is found in the membrane fraction. It has been reported that an ORF adjacent to genes encoding a T1SS and belonging to the same operon usually encodes a protein that must be exported into the extracellular milieu to exert its function, such as Vibrio cholerae rtxA and E. coli hlyA (Bakkes et al., 2010; Boardman et al., 2007). Nevertheless, other proteins secreted by the T1SS remain attached to the bacterial surface. Examples include Pseudomonas fluorescens LapA and Staphylococcus aureus V329, two proteins involved in biofilm formation (Lasa & Penades, 2006). In S. enterica, SPI-4 encodes a giant non-fimbrial adhesin (SiIE) that remains attached to the bacterial surface after export by a T1SS (Gerlach et al., 2007). The same was reported for BapA, a S. Enteritidis gene orthologous to STY2875 (Latasa et al., 2005). Apparently, the proteins exported by a T1SS involved in adherence must remain attached to bacteria to mediate a physical interaction with epithelial cells.

S. Typhi SPI-9 apparently does not contribute to biofilm formation under the test conditions. On the other hand, S. Enteritidis BapA has been shown to contribute to colonization of epithelial cells as assessed by co-infection experiments in ligated ileal loops (Latasa et al., 2005). Accordingly, we found that STY2875 contributed to adherence to epithelial cells when bacteria were previously cultured under high osmolarity or low pH, consistently with the expression assays showing that those conditions induce the expression of SPI-9 genes. Moreover, the impaired adherence observed in S. Typhi ASTY2875 is similar to that observed for S. Typhi SPI-9 and S. Typhi ASTY2876-ASTY2878, suggesting that STY2875 is specifically secreted through the hypothetical T1SS encoded by STY2876, STY2877 and STY2878. In addition to STY2875, other large proteins presenting repeated Ig domains also participate in cell adherence, including SiIE encoded in SPI-4 (Gerlach et al., 2007). Ca^{2+} ions bound by conserved D residues within the Ig domains stabilized protein and facilitate secretion (Barlag & Hensel, 2015). Furthermore, despite the deletion presented by STY2875 with respect to BapA, STY2875 appears to be functional. This fact emphasizes the need for experimental research to unequivocally determine whether a gene is a pseudogene, as previously postulated (Urrutia et al., 2014).

To summarize, we found that SPI-9 contributes to adherence to epithelial cells. In addition, SPI-9 is constituted by an operon (STY2875-STY2875) whose expression is induced under high osmolarity and low pH in a RpoS-dependent manner. Finally, we propose that SPI-9 encodes an adhesin and a dedicated type 1 secretion apparatus. This is an example of a gene that would be inferred as defective by bioinformatics, but is demonstrated to have (at least partial) physiological function.

REFERENCES


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