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

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The genomic island 9 (SPI-9) from *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 *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 *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.

INTRODUCTION

The genus *Salmonella* includes two species, *Salmonella bongori* and *Salmonella enterica*, the latter containing many subspecies and serovars (Brenner *et al.*, 2000). Genome sequences of closely related *S. enterica* subsp. *enterica* (*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 *S. enterica* serovar Enteritidis (*S.* Enteritidis) are considered 'generalists' because they infect a broad range of hosts. Other serovars are host-restricted, such as *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 *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

Abbreviations: *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.

Two supplementary figures are available with the online Supplementary Material.

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 instance, 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 haemolysin (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 at.* 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 Δ*bapA* 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 OD_{600} 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 pDK3/4 plasmid as a source of the antibiotic–resistant cassettes (Table 1) (Datsenko & Wanner, 2000). The FRT scar was used to fuse the *lacZY* reporter as described (Ellermeier *et al.*, 2002) to construct transcriptional fusions with the SPI-9 genes. The *S.* Typhi Δ*rpoS*, *S.* Typhi Δ*rpoS*/pBRPOS and *S.* Typhi Δ*rpoS*/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 (Uzzau *et al.*, 2001). *S.* Typhi *rpoS*-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.

Table 1. Primers used in this study

Primers used for the Red/Swap technique (Datsenko & Wanner, 2000)	
STY2875(H1+P1)	ACCTACGCTCATAAAAAAGAATATGGCTACGGAAATTCATtgtaggctggagctgcttcg
STY2875(H2+P2)	TTCTCCAAAGTGACAAGGTCAGTTGATTTAAACGCGCTGCcatatgaatatcctccttag
STY2876(H1+P1)	GTTCGGGGCAACAAGCGGTGATATTTAAAAGGGATAAACTtgtaggctggagctgcttcg
STY2878(H2+P2)	CGGTTTTGATATCCACCGTGGCGATCATGCCTGGAACAATcatatgaatatcctccttag
SPI-9(H1+P1)	ACCTACGCTCATAAAAAAGAATATGGCTACGGAAATTCATtgtaggctggagctgcttcg
SPI-9(H2+P2)	CGGTTTTGATATCCACCGTGGCGATCATGCCTGGAACAATcatatgaatatcctccttag
Primers for epitope tagging (3xFLAG) (Uzzau *et al.*, 2001)	
STY2875-3xFLAG	AAATGATCTGTTGCAGAACAACCACCTGATAACCGGCGGT*gactacaaagaccatgacgg*
STY2875-kan	CAAATGCCAGTACGATGGCGACAGGCGCGACTCTTCCCAT*catatgaatatcctccttag*
STY2876-3xFLAG	GTTAAATCATCGCAGCATTCAATCCGTGGAGATCCAGCCA*gactacaaagaccatgacgg*
STY2876-kan	GCAACGCGATAATGACCGGCGACATGGCTGATGGCCTGCG*catatgaatatcctccttag*
STY2877-3xFLAG	GGCGCTTAACGCGGATCGGATGCAAAGTCACCGTCGGGAG*gactacaaagaccatgacgg*
STY2877-kan	CGGAAAACGCCCGTTCACGCTGAATATCGAAATCGTCCAT*catatgaatatcctccttag*
STY2878-3xFLAG	CAAACCGTTTAATCGCGCGAAAGAAGCGCTGCGCGAGCGG*gactacaaagaccatgacgg*
STY2878-kan	GCCAACCGCCCCTCGGCATGCACCTTGGGTTTCGCAAATC*catatgaatatcctccttag*
ompA-3xFLAG	AGTTAAAGGCGTTAAAGACGTGGTAACTCAGCCGCAGGCT*gactacaaagaccatgacgg*
ompA-kan	GAAAGGCGTTGCCACCCAGACCAGAGCAAAAAACCCCGCG*catatgaatatcctccttag*
Primers for mRNA detection by RT-PCR	
RT-2875L	GCCCGGTAACCTAGTCATCA
RT-2875R	GGCCGTCACGTTAATGAGTT
RT-2876L	GCTGGACAGCATCGGTAAAC
RT-2877R	TCTTTCAGGACAACGACACG
RT-2878R	GGTGAAATGGTCTCCACCAC
Sty2875C	AACCTCGATGGCGCTTACAC
Sty2876N	GGATGTAATCATTGGCAGCG
Sty2876C	TTGATGTCCGCCAGAAACTG
Sty2877N	GAACAGGCAACGCGATAATG
Sty2877C	TGCTACTTGACGAACCCAGC
Sty2878N	CAGATGCCGAGAATGAGAAA

Lower case: annealing site with pKD4 (Datsenko & Wanner, 2000).
Italic lower case: annealing site with pSUB11 (Uzzau *et al.*, 2001).

Assays for β-galactosidase activity. *S.* Typhi mutant strains with *lacZY* fusions were grown under the conditions previously described and then chilled to 4 °C. β-galactosidase activity was measured by a modification of the method of Miller (Miller & Hershberger, 1984) described in Jofre *et al.* (2014). Briefly, each bacterial culture was suspended in 900 µl of Z buffer (0.6 M Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol; pH 7.0). Bacteria were permeabilized with 10 µl chloroform, 10 µl 0.1 % SDS, vortexed for 10 s and incubated at 30 °C for 10 min and 200 µl of *o*-nitrophenyl-D-galactopyranoside (4 mg ml^{-1}) was added. Reactions were stopped by the addition of 500 µl 1 M Na_2CO_3. β-galactosidase activity is expressed in Miller units, $10^3 \times (OD_{420} - 1.75 \times OD_{550})$ ml^{-1} min^{-1} OD_{600}^{-1}. All experiments were repeated at least three times. We obtained the fold induction as follows: (Miller units of tested strain)/(Miller units of parental strain grown under non-inducer conditions at the same OD_{600} as the tested strain).

RT-PCR assay. Total RNA from the strains grown under the test conditions was extracted using TRIzol reagent (Invitrogen) as described by the manufacturer. RNA was precipitated with isopropanol for 10 min at room temperature, washed with ice-cold 70 % v/v ethanol and resuspended in DEPC-treated water, prior to treatment with DNase I to remove traces of DNA. Purity of extracted RNA was determined by spectrometry. Reverse transcription was performed on 2 µg of DNase-treated RNA using Superscript II RT (Invitrogen) at 50 °C for 50 min followed by 70 °C for 10 min in 20 µl with 2.5 µM of the corresponding reverse primer (Table 1). The 1942R reverse primer for 16 s mRNA detection was used as a reference transcript. Reaction without Superscript II was performed as negative control. The cDNA (or the negative control) was amplified by PCR using Sty2875C+Sty2876N to detect cDNA between STY2875 and STY2876 ORFs; Sty2876C+Sty2877N to detect cDNA between STY2876 and STY2877 ORFs; Sty2877C +Sty2878N to detect cDNA between STY2877 and STY2878 ORFs; RT2876L+RT2877R to detect cDNA between STY2876 and STY2877 ORFs; RT-2876L+RT2878R to detect cDNA between STY2876 and STY2878 ORFs (Fig. 1c, left); RT-2875L+RT-2875R to detect STY2875 cDNA, and 1942R+18F to detect 16 s cDNA. In all cases, we used 30 cycles at 96 °C for 40 s, 60 °C for 45 s and 72 °C for 2 min, followed by a final extension step at 72 °C for 10 min. As negative control, we performed PCR after the DNAse treatment and before the reverse transcription (not shown for Fig. 1c). This procedure was done at least three times independently for each condition. All the primers are listed in Table 1.

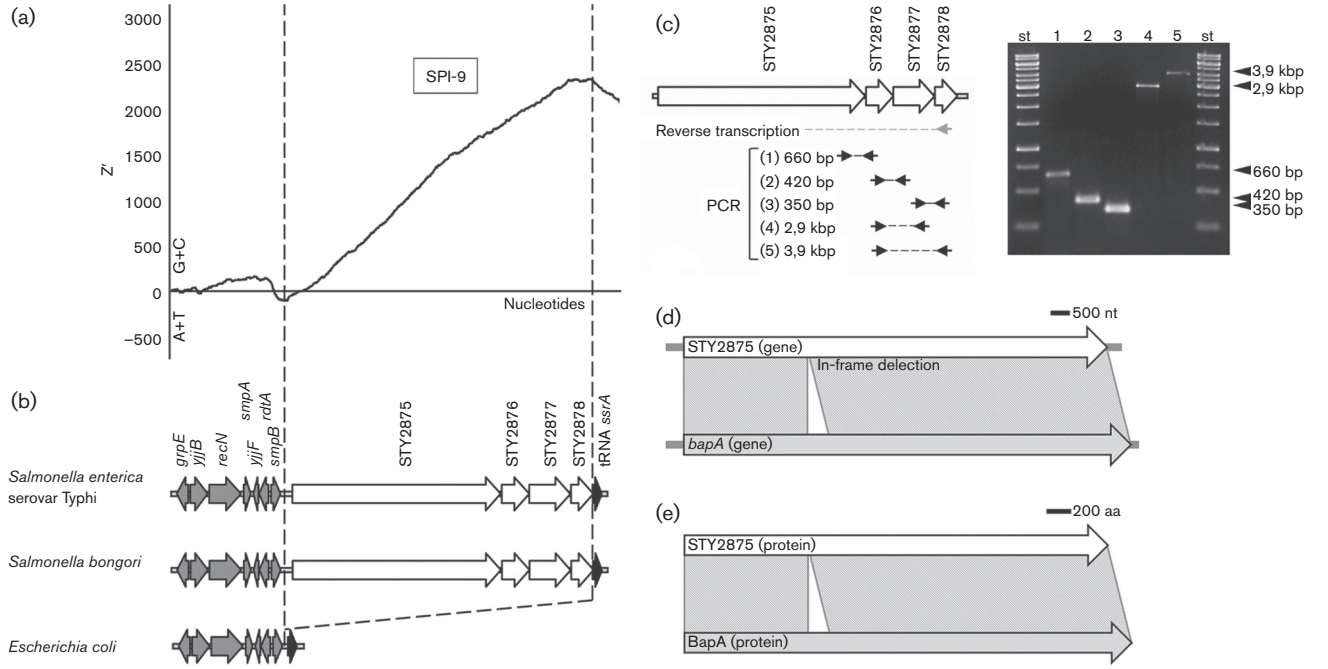

Fig. 1. SPI-9 is a genomic island found in *Salmonella*, consisting of the STY2875-STY2876-STY2877-STY2878 operon. (a) G+C content assessed by the Z' number (Zhang & Zhang, 2004). A positive slope defines a G+C–rich zone; a negative slope denotes an A+T–rich zone. Abrupt changes in the slope define the limits of genomic islands. (b) *In silico* analyses showing the presence of SPI-9 in *Salmonella enterica* (e.g. *S. enterica* serovar Typhi STH2370) and *Salmonella bongori* 12149. SPI-9 is absent from *E. coli* K12. White arrows: SPI-9 ORFs; grey arrows: ORFs outside the SPI-9; black arrows: tRNA *ssrA*. (c) The SPI-9 ORFs constitute an operon in *S. enterica* serovar Typhi STH2370. Left: location of the primer used to perform reverse transcription (grey) and the combination of primers to perform PCR (black) with their expected amplicons (bp). Right: RT-PCR performed on total RNA extracted after culturing *S. enterica* serovar Typhi STH2370 to the stationary phase. The numbers above the lanes represent the combination of the primers used. St: molecular weight standard. (d) Nucleotide alignment between STY2875 from *S.* Typhi STH2370 and *bapA* from *S.* Enteritidis PT4. STY2875 presents a large in-frame deletion (600 bp). (e) Predicted protein alignment between STY2875 from *S.* Typhi STH2370 and BapA from *S.* Enteritidis PT4. The actual sequences and alignments are shown in Figs S1 and S2, available in the online Supplementary Material). nt: nucleotides; aa: amino acids.

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 H_2O 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 control 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 chemioluminescence (ECL, Amersham 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, $MgCl_2$ 10 mM), sonicated for 100 s on ice and supplemented with phenylmethylsulfonyl 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 & LaBaer, 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.* Choleraesuis 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

a previously described method (Stepanovic *et al.*, 2004). The wells were filled with 180 µl 400 mM NaCl phosphate-buffered LB broth (pH 7.0). Twenty µl of overnight bacterial culture were added to each well (n=8). The negative control wells contained LB only. The plates were incubated aerobically for 24 h at 37 °C. The content of the plate was then poured off and the wells washed three times with 250 µl of sterile distilled water and air-dried. The microplates were stained with 250 µl of crystal violet per well used for Gram staining (Gram-color Staining Set for Microscopy; Merck) for 5 min. The excess was rinsed off with distilled water. After the microplates were air-dried, the dye bound to the adherent cells was resolubilized with 250 µl of 33 % (v/v) glacial acetic acid per well. The optical density of each well was measured at 570 nm.

Adherence assays of HEp-2 epithelial cells. HEp-2 and Caco-2 monolayers were obtained by seeding 5×10^4 cells per each well of a 96-well plate. Cells were cultured to confluence at 37 °C in a 5 % CO_2/95 % air mixture in 100 µL of RPMI-10 % FBS or RPMI-20 % FBS medium for HEp-2 or Caco-2, respectively. Tested bacterial strains were grown in micro-aerophilic conditions as described earlier to OD_{600} of 0.2−0.3, in LB pH 7.0 (control), LB pH 5.0 or LB pH 7.0 400 mM NaCl. The strains were used to infect HEp-2 or Caco-2 monolayers at a multiplicity of infection (MOI) of 100 : 1. Infected eukaryotic cells were incubated 1 h prior to washing the cells five times with sterile PBS to remove non-adherent bacteria. Cell monolayers were disrupted by adding sterile deoxycholate 0.5 %. Adherent bacteria were determined by bacterial plate counting (CFU), and expressed as a percentage of the initial inoculum. In all cases, experiments were performed in at least three full biological replicates, each time in triplicate.

RESULTS

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 chromosome, 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; Juhas *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 (OD_{600}=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 STY2876, 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 (OD_{600}=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 & Slauch, 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

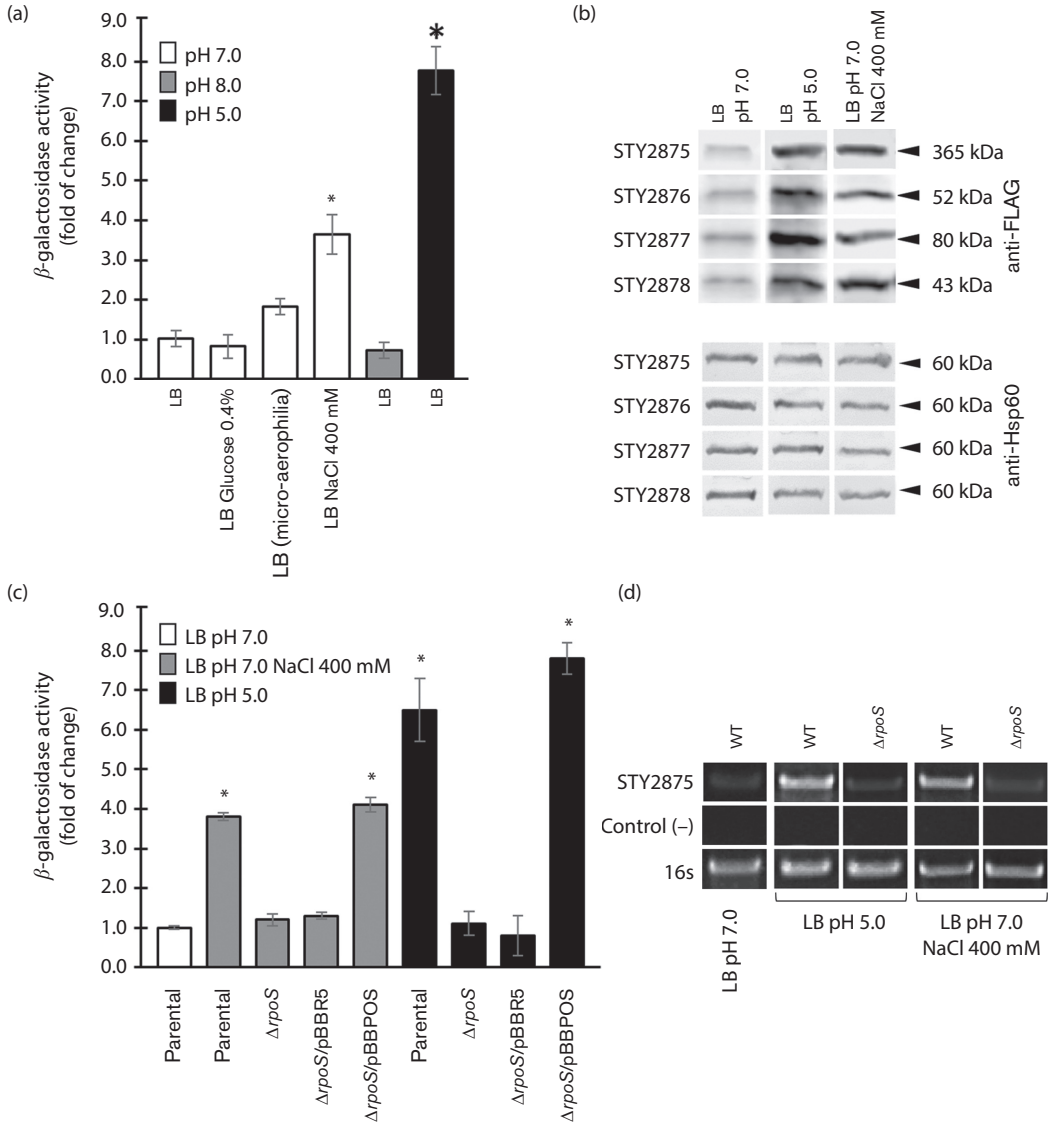

Fig. 2. Expression of *S.* Typhi SPI-9 ORFs was induced under low pH and high osmolarity in an RpoS-dependent manner. (a) *S.* Typhi ΔSTY2875 :: *lacZY* was grown to logarithmic phase (OD_{600} of 0.5) under different culture conditions prior to determining β-galactosidase activity. LB at pH 7.0 (LB) was used as a reference growth condition. (b) Immunodetection of the epitope-tagged (3x×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) β-galactosidase activity of *S.* Typhi ΔSTY2875 :: *lacZY* (Parental) and *S.* Typhi ΔSTY2875 :: *lacZY* Δ*rpoS* :: cam (Δ*rpoS*). To complement the Δ*rpoS* strain, the pBRPOS (pBBR5 :: *rpoS*) plasmid was used (Jofre *et al.*, 2014). pBRR5 corresponds to the empty vector (Jofre *et al.*, 2014). (d) RT-PCR assay performed to detect STY2875 mRNA and 16 s mRNA (control) from *S.* Typhi (WT) or *S.* Typhi Δ*rpoS* :: FRT (Δ*rpoS*) (Jofre *et al.*, 2014) grown to logarithmic phase (OD_{600} 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.

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 (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

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* Δ*rpoS* :: 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 Δ*rpoS* 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 Δ*rpoS* 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 Δ*rpoS* mutant (Fig. 2c). To directly detect the STY2875 transcript, and to confirm our previous results, we performed RT-PCR using RNA extracted from the *S.* Typhi STH2370 wild-type and the Δ*rpoS* derivative grown to logarithmic phase (OD_{600}=0.5) in LB pH 5.0, LB pH 7.0 400 mM NaCl or LB pH 7.0 (control). 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 detecting STY2875 by PCR. The STY2875 transcript was significantly induced under low pH and high osmolarity only when *rpoS* was present, supporting our previous results (Fig. 2d). In contrast, amplification of cDNA with 16 S-specific primers showed that no significant differences were observed among the amounts of RNA under different growth conditions (control) (Fig. 2d).

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 (OD_{600}=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 (OD_{600}=1.4) in LB pH 7.0, was used as control (Fig. 3).

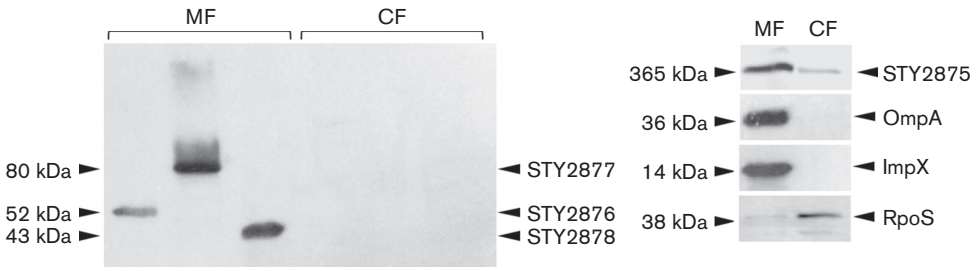

Fig. 3. *S.* Typhi SPI-9–encoded proteins are found in the membrane fraction. Immunodetection of epitope-tagged (3xFLAG) proteins STY2875 (right), STY2876, STY2876, STY2877 and STY2878 (left) on the membrane fraction (MF; outer membrane and inner membrane), and cytoplasmic fraction (CF). Immunodetection 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) as fractionation control is shown (right). An additional band of approximately 36 kDA found for RpoS was previously reported (Jofre *et al.*, 2014).

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. 1e). 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 (OD_{600}=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 (OD_{600}=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 (Bueno *et al.*, 2004) and *tsx-impX* (Bucarey *et al.*, 2006) in *S. enterica*, emphasizing the importance of a concerted regulation of newly acquired functions.

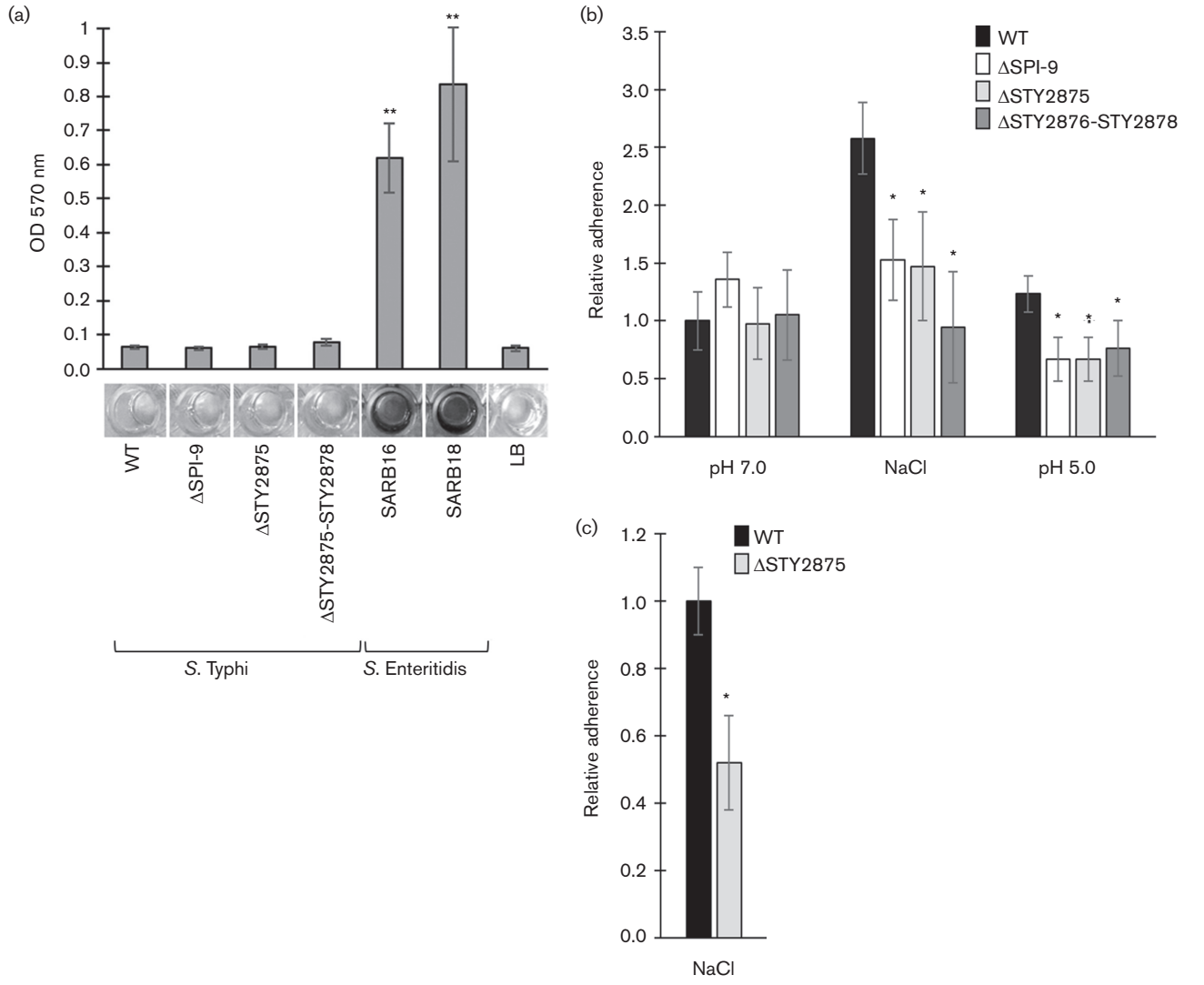

Fig. 4. *S.* Typhi SPI-9 contributes to adherence to HEp-2 epithelial cells. (a) Biofilm formation assessed by crystal violet staining. Bacteria were grown in LB pH 7.0 400 mM NaCl to stationary phase and seeded in wells prior to incubating for 24 h at 37 °C. Biofilm was revealed by staining with crystal violet (OD 570 nm) (wells placed down the bars, representative experiment). *S.* Enteritidis SARB16 and SARB18 were used as positive controls. LB alone was used as negative control. The experiments were performed in three full biological replicates, with each time-point replicated 12 times (12 wells). $^{**}P<0.01$ (ANOVA) compared to *S.* Typhi WT. (b) Adherence comparing *S.* Typhi STH2370 (WT), *S.* Typhi ΔSPI-9 (i.e. ΔSTY2875-STY2878), *S.* Typhi ΔSTY2875 and *S.* Typhi ΔSTY2876-STY2878 in HEp-2. Bacteria were grown in LB pH 7.0, LB pH 7.0 400 mM NaCl or LB pH 5.0. Data are expressed relative to the WT previously grown in LB pH 7.0. The experiments were performed in three full biological replicates, each time in technical triplicate. The figure shows a representative experiment. (c) Adherence comparing *S.* Typhi STH2370 (WT) and *S.* Typhi ΔSTY2875 in Caco-2. Bacteria were previously grown in LB pH 7.0 400 mM NaCl. Data are expressed relative to the WT. The experiments were performed in three full biological replicates, each time in technical triplicate. The figure shows a representative experiment. $^{*}P<0.05$ (ANOVA) compared to the WT in the corresponding group.

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 ΔSTY2875 is similar to that observed for *S.* Typhi SPI-9 and *S.* Typhi ΔSTY2876-STY2878, 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.

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