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Role of RpoS in Fine-Tuning the Synthesis of Vi Capsular Polysaccharide in *Salmonella enterica* Serotype Typhi[▽]

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Regulation of the synthesis of Vi polysaccharide, a major virulence determinant in *Salmonella enterica* serotype Typhi, is under the control of two regulatory systems, *ompR-envZ* and *rscB-rscC*, which respond to changes in osmolarity. Some serotype Typhi strains exhibit overexpression of Vi polysaccharide, which masks clinical detection of lipopolysaccharide O antigen. This variation in Vi polysaccharide and O antigen display (VW variation) has been observed since the initial studies of serotype Typhi. In this study, we report that *rpoS* plays a role in this increased expression in Vi polysaccharide. We constructed a variety of isogenic serotype Typhi mutants that differed in their expression levels of RpoS and examined the role of the *rpoS* product in synthesis of Vi polysaccharide under different osmolarity conditions. Vi polysaccharide synthesis was also examined in serotype Typhi mutants in which the native promoter of the *rpoS* was replaced by an *araC*P_{BAD} cassette, so that the expression of *rpoS* was arabinose dependent. The RpoS$^-$ strains showed increased syntheses of Vi polysaccharide, which at low and medium osmolarities masked O antigen detection. In contrast, RpoS$^+$ strains showed lower syntheses of Vi polysaccharide, and an increased detection of O antigen was observed. During exponential growth, when *rpoS* is unstable or present at low levels, serotype Typhi RpoS$^+$ strains overexpress the Vi polysaccharide at levels comparable to those for RpoS$^-$ strains. Our results show that RpoS is another regulator of Vi polysaccharide synthesis and contributes to VW variation in serotype Typhi, which has implications for the development of recombinant attenuated *Salmonella* vaccines in humans.

Salmonella enterica serotype Typhi is a facultative intracellular pathogen that causes typhoid enteric fever exclusively in humans and is among the organisms causing the most costly human infections in terms of both morbidity and mortality (51). The mechanism responsible for the virulence of serotype Typhi is different from those of other serovars of *Salmonella*, and in this regard, serotype Typhi produces the virulence capsular (Vi) polysaccharide, which is an important virulence determinant during infection (27). Vi polysaccharide is a polymer of α-1→4-galacturonic acid with an *N*-acetyl group at position C-2 and a variable O acetylation at C-3 (69). Virtually all strains isolated from blood or bone marrow samples from patients with acute typhoid fever and from bile samples or feces from those who carry serotype Typhi in the gallbladder are found to express Vi polysaccharide antigen when tested in clinical microbiology laboratories (29, 39, 55). Vi-positive (Vi$^+$) strains were shown to be more virulent than Vi$^-$ mutant strains in experiments conducted with human volunteers (27), and the Vi$^+$ strains were resistant to complement-mediated killing and phagocytosis (61) and survived in human serum (20). In addition, the Vi$^+$ strain but not the Vi$^-$ mutant strain can multiply in the human macrophage cell line THP-1 and the mouse macrophage-like cell line J774.1 (22).

The genes required for the biosynthesis of the capsular antigen Vi are located in a 133.5-kb chromosomal region called *Salmonella* pathogenicity island 7 (SPI-7). Locus *viaB*, which encodes Vi polysaccharide in serotype Typhi, consists of 10 genes: *tviBCDE* for Vi polysaccharide biosynthesis; *vexABCDE* for the export of the Vi antigen; *tviA*, which is activated by unlinked regulators *rcsB-rcsC* (1, 28); and *ompR-envZ*, which are themselves controlled by osmolarity levels (53).

The Vi polysaccharide is expressed or overexpressed in media with low or medium osmolarities, respectively, and often covers and masks the lipopolysaccharide (LPS) O antigen (1, 71). Strains of serotype Typhi Ty2 grown in media with medium osmolarities (446 mosmol, ~170 mM NaCl [71]) exhibit high-level production of Vi antigen. When the Vi antigen is expressed, the bacteria are less adherent to and invasive into epithelial cells (71) but are more resistant to killing by macrophages (22). In high-osmolarity media (676 mosmol, ~300 mM of NaCl [71] or more, as in the intestinal lumen), the Vi antigen is not expressed (1, 53, 80), exposing the somatic antigen and liberating secretion proteins, which are stockpiled inside the bacterial cell when Vi polysaccharide is expressed at the bacterial cell surface (1). In this stage, serotype Typhi is more invasive into epithelial cells but is less resistant to killing by macrophages (22). Therefore, the Vi antigen of serotype Typhi is a negative factor for invasion but a positive factor for survival and multiplication inside macrophages (22). Since the Vi polysaccharide can block the access of antibodies to the underlying O antigen, sometimes agglutination with *Salmonella* somatic D_1 antiserum cannot be demonstrated until the bacterial cells are boiled to remove the Vi polysaccharide (11).

From initial studies on serotype Typhi, variations in Vi and O antigen detection have been observed. Observations recorded by Kauffman (33) and confirmed by Felix and Pitt (14) demonstrated the concept of VW variation in Vi and O antigen

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TABLE 1. *Salmonella* strains and relevant characteristics

Strain	Relevant characteristics	Origin	Source or reference
χ3769 serotype Typhi Ty2	Wild-type RpoS$^-$ Ap^s Cys$^-$ V form		15
χ3744 serotype Typhi ISP1820	Wild-type RpoS$^+$ Ap^s Cys$^-$ Trp$^-$ V-W form		70
χ8438 serotype Typhi Ty2	RpoS$^+$ Ap^s Cys$^-$ VW form	χ3769	This study
χ9060 serotype Typhi Ty2[a]	*rpoS*ΩAp RpoS$^-$ Ap^r Cys$^-$ V form	χ8438	This study
χ9061 serotype Typhi ISP1820[1]	*rpoS*ΩAp RpoS$^-$ Ap^r Cys$^-$ Trp$^-$ V form	χ3744	This study
χ9066 serotype Typhi ISP1820[2]	$\Delta P_{rpoS183}$::TT*araC* P_{BAD}*rpoS* Cys$^-$ Trp$^-$ VW form induced by arabinose	χ3744	This study
χ9067 serotype Typhi Ty2[b]	$\Delta P_{rpoS183}$::TT*araC* P_{BAD} *rpoS* RpoS$^-$ Cys$^-$ V form	χ3769	This study
χ9068 serotype Typhi Ty2[b]	$\Delta P_{rpoS183}$::TT*araC* P_{BAD} *rpoS* Cys$^-$ VW form induced by arabinose	χ8438	This study
χ9197 serotype Typhi Ty2	Δ*tviABCDE*10 Cys$^-$ Vi$^-$ Vi-phage^S RpoS$^-$ W form	χ3769	This study
χ9198 serotype Typhi ISP1820	Δ*tviABCDE*10 Cys$^-$ Trp$^-$ Vi$^-$ Vi-phage^S RpoS$^+$ W form	χ3744	This study
χ9336 serotype Typhi Ty2	Δ*tviABCDE*10 Cys$^-$ Vi$^-$ Vi-phage^S RpoS$^+$ W form	χ8438	This study
χ4991 serotype Typhimurium UK-1	*rpoS*ΩAp RpoS$^-$ Ap^r		47
χ7213 *E. coli* K-12	*thi-1 thr-1 leuB6 fhuA21 lacY1 glnV44* Δ*asdA4 recA1* RP42-Tc::Mu [λ*pir*] Km^r		62

[a] Mutants generated by P22HT*int* transduction.
[b] Mutants generated by conjugation and allele exchange.

relationships (74). Serotype Typhi strains inagglutinable with O antisera and agglutinable only with Vi antisera are called V-form strains, while serotype Typhi strains that lack the Vi antigen and agglutinate only with O antisera are called W-form strains (74). The VW form, which is referenced as the most common form observed in clinical laboratories, is identified when both Vi and O antigen are detected by agglutination with the respective antisera (74). Coincident with the early work of Felix and Pitt (14), and since verified by others (16, 17, 22, 75), most virulent strains of serotype Typhi were the VW form.

In *Salmonella*, the *rpoS* gene encodes an alternative sigma factor (σ^s/RpoS) that is the master regulator in the general stress response and is required for survival under extreme conditions (including osmotic and oxidative stress, acid shock, and transition to stationary phase) and for virulence of *S. enterica* serotype Typhimurium (6, 13, 36, 47, 48). RpoS controls the expression of the serotype Typhimurium virulence plasmid genes, *spvRABCD* (13, 47). In addition, RpoS regulates chromosomal genes required for colonization of Peyer's patches and for persistence in mice (6, 47). Serotype Typhi does not contain a virulence plasmid, and the role of *rpoS* in the virulence of this serotype is unknown. However, *rpoS* might also contribute to the virulence of this serotype because RpoS$^-$ strains of serotype Typhi are less cytotoxic than RpoS$^+$ strains, but RpoS$^-$ strains survive better inside resting THP-1 macrophages without apoptosis induction and have higher capacities for resistance in the macrophage (34).

We deduced from our observations that there is a correlation between Vi polysaccharide overexpression and the allele state of the *rpoS* gene. We report here that *rpoS* is involved in the subtle overexpression of Vi polysaccharide at osmolarities lower than 667 mosmol resulting from the addition of 300 mM NaCl to culture media. We presume that the *rpoS* gene influences the VW form described in earlier studies. Furthermore, we discuss the implication of the effect of *rpoS* on the synthesis of Vi antigen in serotype Typhi Ty2 and serotype Typhi ISP1820, which are the most useful parental strains in designing live serotype Typhi vaccines.

MATERIALS AND METHODS

***Salmonella* strains and conditions of culture.** *Salmonella* strains used in this study are listed in Table 1. The strains were routinely cultured at 37°C in LB medium (Bacto tryptone, 10 g/liter; Bacto yeast extract, 5 g/liter; NaCl, 10 g/liter) (44). Media were solidified with 1.5% (wt/vol) agar. When required, media were

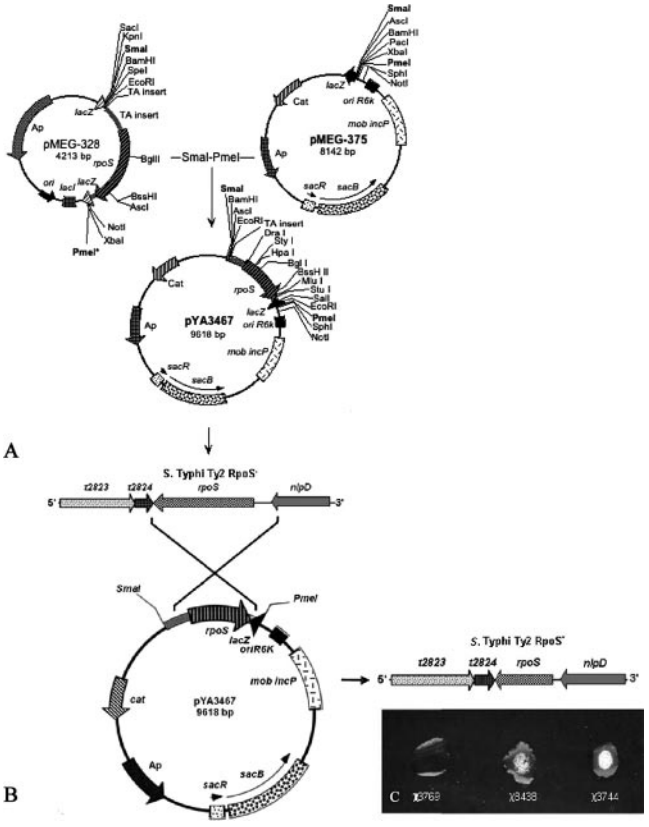

FIG. 1. Construction of suicide vector pYA3467 and generation of RpoS$^+$ strain in serotype Typhi Ty2. (A) Construction of suicide vector pYA3467 (9,618 bp) used to generate the serotype Typhi Ty2 RpoS$^+$ strain. (B) Crossover between suicide vector pYA3467 and chromosome of serotype Typhi Ty2. (C) Catalase test for determination of RpoS activity.

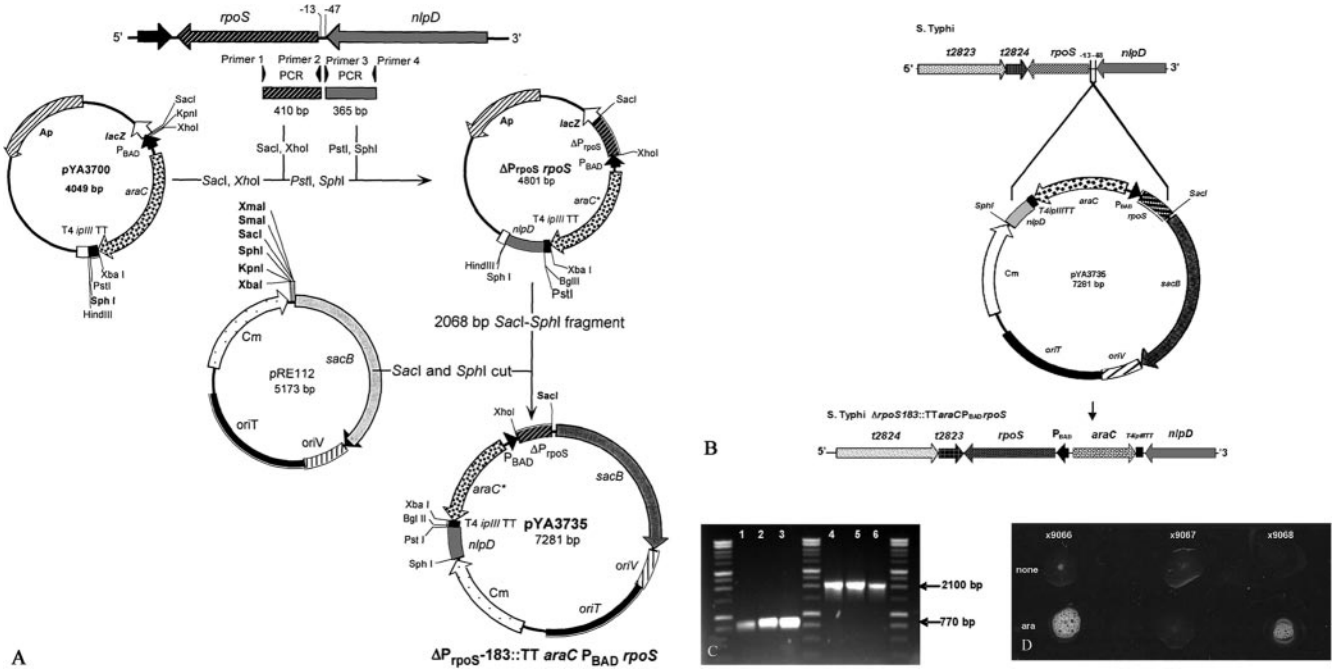

FIG. 2. Construction of suicide vector pYA3735 and generation of serotype Typhi with regulated *rpoS* expression ($\Delta P_{rpoS183}$::TT*araC*P_{BAD}*rpoS*). (A) Construction of suicide vector pYA3735 (7,281 bp) used to generate serotype Typhi $\Delta P_{rpoS183}$::TT*araC*P_{BAD}*rpoS*. (B) Crossover between suicide vector pYA3735 and chromosome of serotype Typhi. (C) Agarose gel (0.8%), a PCR product from chromosomal DNA from wild-type (770-bp) and $\Delta P_{rpoS183}$::TT*araC*P_{BAD}*rpoS* (2,100-bp) mutants. Lanes: 1, χ3744 serotype Typhi ISP1820 RpoS$^+$; 2, χ3769 serotype Typhi Ty2 RpoS$^-$; 3, χ8438 serotype Typhi Ty2 RpoS$^+$; 4, χ9066 serotype Typhi ISP1820 RpoS$^+$ $\Delta P_{rpoS183}$::TT*araC*P_{BAD}*rpoS*; 5, χ9067 serotype Typhi Ty2 RpoS$^-$ $\Delta P_{rpoS183}$::TT*araC*P_{BAD}*rpoS*; 6, χ9068 serotype Typhi Ty2 RpoS$^+$ $\Delta P_{rpoS183}$::TT*araC*P_{BAD}*rpoS*. (D) Catalase test for detection of $\Delta P_{rpoS183}$::TT*araC*P_{BAD}*rpoS* without and with growth in the presence of 0.2% Ara.

supplemented with chloramphenicol (Cm; 25 μg/ml), ampicillin (Ap; 100 μg/ml), 2,6-diaminopimelic acid (DAP; 50 μg/ml), L-arabinose (Ara; 0.2% [wt/vol]), or sucrose (5% [wt/vol]). The osmolarities of the LB agar plates were changed by adding NaCl (0, 10, 85, 150, 300, 400, and 500 mM) (71) or sucrose. The final pHs of the media were adjusted to 7.0 with NaOH before the autoclaving.

Characterization of serotype Typhi strains. The serotype Typhi strains were characterized for type I fimbriae in static broth cultures (35, 49) and for motility in motility medium (bioMérieux, Marcy I'Etoile, France). The presence of LPS was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis when visualized by silver staining (23, 72). Plasmid profiles were verified by alkaline lysis and agarose gel (0.5%) electrophoresis (31, 63). Fermentation patterns of various carbohydrates and production of H_2S were determined by using the API 20E system (bioMérieux, Marcy I'Etoile, France). The serotype Typhi RpoS$^-$ strains gave API 20E code number 640454057 (a good identification as serotype Typhi [97.4%] and *Salmonella* spp. [1.8%]), whereas the RpoS$^+$ strains gave API 20E code number 440454057 (an excellent identification as serotype Typhi [99.9%] and *S. enterica* serovar Choleraesuis [0.1%]). The expression levels of the Vi, O_9 (serotype Typhi-specific), D_1 (O_1, O_9, and O_{12}), and H_d antigens of serotype Typhi strains were evaluated by the growth of strains on LB agar plates with different osmolarities after 18 to 24 h.

Construction of serotype Typhi Ty2 RpoS$^+$. The recombinant suicide vector pYA3467 (9.6 kb), carrying the *rpoS* gene from serotype Typhi ISP1820 (993 bp) between flanking regions (338 bp 5′ and 83 bp 3′) of the *rpoS* (σ^{s2}/RpoS$^-$) allele in serotype Typhi Ty2 (χ3769), was constructed as follows (Fig. 1A). The *rpoS* allele from serotype Typhi Ty2 represents a frameshift mutation caused by a guanosine insertion at position 1313 that modified the amino acid sequence of the C-terminal part of σ^s/RpoS, resulting in a protein of 384 amino acids rather than 330 (58). The SmaI/PmeI fragment, harboring 338 bp of the 5′-end upper region, 993 bp of the *rpoS* gene, an extra 83 bp of the 3′-end region, and ~93 bp of multiple cloning sites (a total of ~1,450 bp), was inserted into the SmaI/PmeI sites of pMEG-375 to generate pYA3467 (Fig. 1A). To construct serotype Typhi Ty2 RpoS$^+$, the suicide plasmid pYA3467 was conjugationally transferred from *Escherichia coli* χ7213 (32) to serotype Typhi Ty2 strain χ3769 in LB broth supplemented with DAP (67). Strains containing single-crossover plasmid insertions (serotype Typhi Ty2 *rpoS*::pYA3467) (Fig. 1B) were isolated on LB agar plates containing Cm without DAP. Loss of the suicide vector after the second recombination between homologous regions (i.e., allelic exchange) was selected by using the *sacB*-based sucrose sensitivity counterselection system (18). The colonies were screened for Cm^s and for positive catalase activity evidence by bubble production after addition of H_2O_2 (Fig. 1C). In particular, *rpoS* mutants are impaired in the ability to resist H_2O_2 during stationary phase. Indeed, two of the catalases produced by *Salmonella* (KatE and KatN) are expressed in stationary phase under the control of *rpoS* (3, 60). To observe the presence of *rpoS* mutations, we added H_2O_2 to the bacterial colony mass removed from LB agar. The extent of bubbling indicated absence or reduction of catalase production in strains with differing *rpoS* genotypes (57). The presence of the RpoS$^+$ insertion in χ8438 serotype Typhi Ty2 RpoS$^+$ was confirmed by a catalase activity assay (Fig. 1C).

Construction of serotype Typhi *rpoS* mutants. Strains χ9060 and χ9061 were constructed by P22HT*int*-mediated transduction of χ8438 and χ3744, respectively, to introduce the inactive *rpoS* allele (*rpoS*ΩAp; truncated RpoS) from χ4991 (47). The bacteriophage P22HT*int* was propagated in the serotype Typhimurium χ4991 donor strain by standard methods (65). Plaque assays were performed to determine phage titers. The concentrated phage lysate was prepared, using χ4991 as the host, as described by Santander and Robeson (64). Serotype Typhi was infected with a multiplicity of infection of 10 (phage/recipient). Transductants were selected on LB agar containing Ap. Green indicator plates (54) and Evans blue uridine indicator plates (68) were used to confirm that transductants were phage-free. The serotype Typhi *rpoS*ΩAp strains were screened for Ap^r and absence of bubbling after addition of H_2O_2 and expression of a truncated RpoS protein by Western blot analysis (see Fig. 3).

Construction of serotype Typhi mutants with regulatable expression of *rpoS*. The recombinant suicide vector pYA3735 (7.3 kb), carrying a TT*araC*P_{BAD} (1,329-bp) insertion between flanking regions (410 bp 5′ and 365 bp 3′) deleting a 35-bp promoter sequence (*rpoS* −13 to −48), was constructed as follows (Fig. 2A). PCR primers 5′-TGCGAGCTCCTCGACTGCACGGATAAGCCCC-3′ (primer 1) and 5′-CCGCTCGAGAGGAGCCACCTTATGCAGATTA-3′ (primer 2) were designed to amplify the *rpoS* flanking region (410 bp). A SacI site was included in primer 1 (underlined), and an XhoI site was included in primer 2 (underlined). The *nlpD* flanking region (365 bp) was amplified, mediated by

primers 5′-CATCTGCAGCTGGTTCCGCCGCTTTA TCGCTG-3′ (primer 3) and 5′-ACATGCATGCGGATGGCAAAGTGATCGAAA-3′ (primer 4). A PstI site was included in primer 3 (underlined), and an SphI site was included in primer 4 (underlined). The flanking regions were amplified from serotype Typhi Ty2 χ3769 and were cloned into pYA3700 between the TT*araC*P_{BAD} insertion (Fig. 2A). The resultant plasmid (designated pΔP_{rpoS}*rpoS*, 4,801 bp), carrying the TT*araC*P_{BAD} cassette between the flanking regions, was digested with SacI and SphI to yield a 2,068-bp fragment that was cloned into pRE112 digested with SacI and SphI (12). The resulting suicide vector was designated pYA3735. To construct serotype Typhi $\Delta P_{rpoS183}$::TT*araC*P_{BAD}*rpo*S mutants, the suicide plasmid pYA3735 was conjugationally transferred from *Escherichia coli* χ7213 (32) to serotype Typhi strains in LB supplemented with DAP (67). The strains containing single-crossover plasmid insertions were isolated on LB agar plates containing Cm without DAP (Fig. 2B). Loss of the suicide vector after the second recombination between homologous regions (i.e., allelic exchange) was selected by using the *sacB*-based sucrose sensitivity counterselection system (18). The colonies were screened for Cm^s and for positive catalase activity with H_2O_2 (bubble production) only when grown in the presence of 0.2% Ara (Fig. 2D). The presence of the $\Delta P_{rpoS183}$::TT*araC*P_{BAD}*rpoS* insertion in serotype Typhi strains was confirmed by PCR amplification of DNA fragments bigger than those amplified from the parental strains. The P_{rpoS} primer set (primers 1 and 4) amplified 770- and 2,100-bp DNA fragments from the chromosomal DNA templates of serotype Typhi (parental) and the serotype Typhi ($\Delta P_{rpoS183}$::TT*araC*P_{BAD}*rpoS*) mutant, respectively (Fig. 2C). The regulation of the *rpoS* gene by *araC*P_{BAD} was confirmed using a catalase activity assay for cells grown with and without 0.2% Ara (Fig. 2D).

Construction of serotype Typhi Vi^- strains. Additionally, serotype Typhi Vi^- strains (Δ*tviABCDE10*) were constructed as controls. Deletion of the *tviA*, *tviB*, *tviC*, *tviD*, and *tviE* genes (Δ*tviABCDE10*) in the *viaB* loci of the serotype Typhi strains was performed by allelic exchange. The recombinant suicide vector pYA4009 (5.9 kb), carrying the flanking regions (361 bp 5′ and 422 bp 3′), was constructed as follows. PCR primers 5′-ACATGCATGCGAACGGTATTACT GTCAGTCACAAG-3′ (primer 1) and 5′-TGCGACCTCATGAAAAAAATCA TCATATTACTA-3′ (primer 2) were designed to amplify the upstream *tviA* flanking region (361 bp). An SphI site was included in primer 1 (underlined), and a SacI site was included in primer 2 (underlined). The *vexA* flanking region (422 bp) was amplified by primers 5′-TGCGAGCTCGAAGTCTCCTTATGCTGAA ATAAC-3′ (primer 3) and 5′-TCCCCCGGGCAGATTATTTCAAATACGAT TAGG-3′ (primer 4). A SacI site was included in primer 3 (underlined), and a SmaI site was included in primer 4. The flanking regions were amplified from serotype Typhi Ty2 χ3769 and were cloned into pRE112 digested with SphI and SmaI (12). The resulting suicide vector was designated pYA4009. The *tviABCDE10* mutant strains were constructed by allelic exchange and the sucrose sensitivity counterselection system (18), as described above. The deletion was confirmed by PCR and corroborated by the sensitivity of the mutant to the Vi-II phage and the lack of agglutination with Vi antisera.

Agglutination assays. Agglutination tests were performed on glass microscope slides by mixing 50 μl of antisera against Vi, O_9, D_1 (O_1, O_9, and O_{12}), and H_d (Difco Laboratories, Detroit, MI) with suspensions of single colonies. The reactions were visualized by phase contrast microscopy at 10× magnification. When the assays for O_9 and D_1 antigens were negative, the cells were boiled for 20 min and cooled prior to addition of O antisera for detection of O antigens (39, 53).

Western blot analysis. The strains were grown in 3 ml of LB medium with different NaCl concentrations at 37°C with aeration. The samples were collected when the absorbance values reached 1.5 (optical density at 600 nm, 1.5; ~1×10^9 CFU/ml), which corresponds to stationary growth phase, known to stimulate *rpoS* expression. One milliliter of culture was collected and centrifuged at 10,500 × *g* for 10 min. The supernatant was discarded, and the total bacterial proteins were suspended in 150 μl of loading buffer and boiled for 10 min. The total proteins were normalized by a nanodrop spectrophotometer (ND-1000; NanoDrop) at 25 mg/μl, separated by 10% (wt/vol) sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membranes (63). Fat-free milk powder solution (5% [wt/vol]) was used for blocking. The membrane was incubated with a primary anti-mouse RpoS monoclonal antibody (1:1,000) (Neoclone), anti-rabbit H_d polyclonal (1:5,000) (Difco), or anti-rabbit GroEL polyclonal (1:10,000) (Sigma) for 1 h at room temperature, washed three times with phosphate-buffered saline supplemented with Tween (0.05%), and then incubated with a 1:10,000 dilution of alkaline phosphatase-conjugated anti-mouse immunoglobulin G (Sigma) or anti-rabbit immunoglobulin G (Sigma). Color was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate.

Rocket immune electrophoresis. The measurement of Vi polysaccharide expression was visualized by rocket immune electrophoresis (38, 73) adapted for Vi antigen. The strains were grown under the same conditions described above for the Western blot assays. One milliliter of culture was centrifuged at 10,500 × *g* for 10 min. The supernatant was discarded, and the total bacterial pellet was washed one time and resuspended in 150 μl of barbital buffer (60 mM, pH 8.6) (Sigma). The cells were disintegrated by sonication on ice until the suspension became clarified. Total protein concentration was used to normalize the samples (25 mg/μl). Agarose (1% [wt/vol]) was prepared in barbital buffer, melted, and adjusted to 55°C. The Vi antiserum (Sigma) was mixed with the warm agarose to make 1% Vi antiserum (vol/vol) gels. The gel was prepared in mini plates (Bio-Rad) 1.5 mm thick. After being cooled, the gel was placed in a Gel-bond membrane (3 M). Holes with 3-mm diameters were made 10 mm from the bottom of the gel, with 3 mm of distance between them. An aliquot of 20 μl of disrupted bacteria was placed in the holes. The electrophoresis was run from the negative to the positive pole at 10 V/cm for 3 h in barbital buffer. Paper filters were used as conducting bridges. Following the run, the gels were removed from the membrane and placed in NaCl (0.2 M) overnight. The gel was washed with distilled water for 1 h and placed back in the Gel-bond membrane. The gels were dried at 30°C and stained with a solution of Coomassie blue (0.5%) in acetic

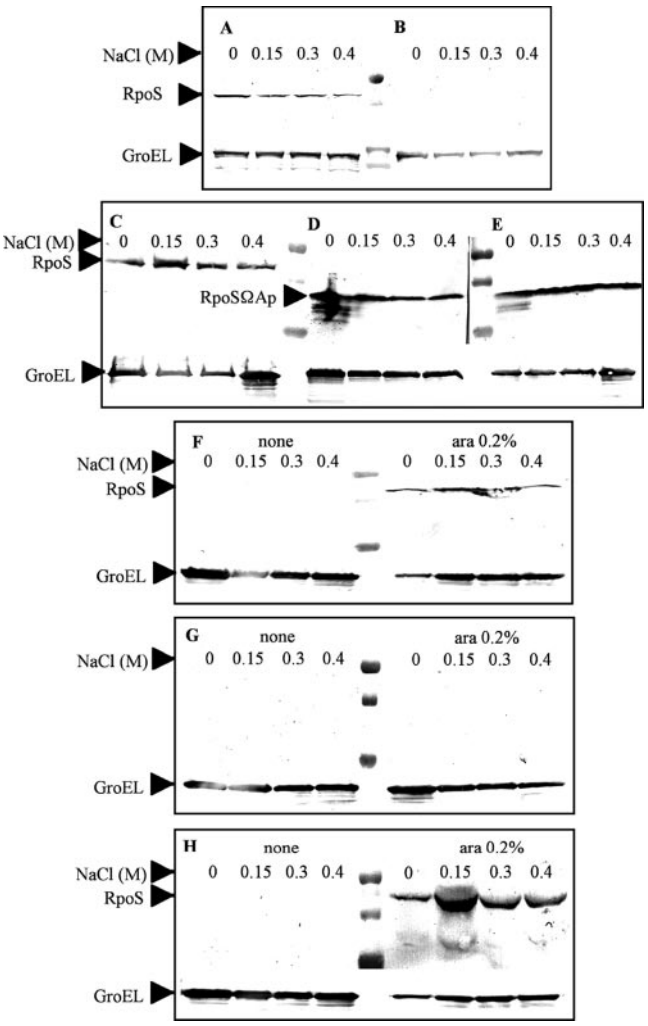

FIG. 3. Evaluation of RpoS expression at different osmolarities by Western blot analysis. (A) χ3744 serotype Typhi ISP1820 $RpoS^+$. (B) χ3769 serotype Typhi Ty2 $RpoS^-$. (C) χ8438 serotype Typhi Ty2 $RpoS^+$. (D) χ9060 serotype Typhi Ty2 *rpoS*ΩAp $RpoS^-$. (E) χ9061 serotype Typhi ISP1820 *rpoS*ΩAp $RpoS^-$. (F to H) Assays conducted in the absence and presence of arabinose (0.2%). (F) χ9066 serotype Typhi ISP1820 $RpoS^+$ $\Delta P_{rpoS183}$::TT*araC*P_{BAD}*rpoS*. (G) χ9067 serotype Typhi Ty2 $RpoS^-$ $\Delta P_{rpoS183}$::TT*araC*P_{BAD}*rpoS*. (H) χ9068 serotype Typhi Ty2 $RpoS^+$ $\Delta P_{rpoS183}$::TT*araC*P_{BAD}*rpoS*.

TABLE 2. O_9 and Vi slide agglutination reactions of serotype Typhi strains grown on LB agar (pH 7) supplemented with different amounts of NaCl at 37°C overnight (18 to 24 h)[a]

Strain	Reaction for indicated antigen and NaCl concn (mM)[a]													
	0		10		85		150		300		400		500	
	O_9	Vi	O_9	Vi	O_9	Vi	O_9	Vi	O_9	Vi	O_9	Vi	O_9	Vi
χ3769 RpoS$^-$	−	+++	−	+++	−	+++	−	+++	++	+++	+++	+	+++	−
χ3744 RpoS$^+$	++	++	+++	++	++	++	++	++	+++	−	+++	−	+++	−
χ8438 RpoS$^+$	++	++	+++	++	++	++	+++	++	+++	−	+++	−	+++	−
χ9060 RpoS$^-$	−	+++	−	+++	−	+++	−	+++	++	+++	+++	+	+++	−
χ9061 RpoS$^-$	−	+++	−	+++	−	+++	−	+++	++	+++	+++	+	+++	−
χ9066 RpoS$^-$	−	+++	−	+++	−	+++	−	+++	++	+++	+++	±	+++	−
χ9067 RpoS$^-$	−	+++	−	+++	−	+++	−	+++	++	+++	+++	+	+++	−
χ9068 RpoS$^-$	−	+++	−	+++	−	+++	−	+++	++	+++	+++	+	+++	−
χ9066 RpoS^{+b}	++	++	+++	++	++	++	+++	++	++	++	+++	−	+++	−
χ9067 RpoS^{-b}	−	+++	−	++	−	+++	−	+++	−	+++	+++	−	+++	−
χ9068 RpoS^{+b}	++	++	+++	++	++	++	++	++	+++	+	+++	−	+++	−

[a] O_9 agglutination reactions were carried out without prior boiling of cells. The degrees of agglutination ranged from not detectable (−) to weak (+) to strong (+++); ± and ++ indicate intermediate degrees.
[b] Strains grown with 0.2% arabinose to induce *rpoS* (see text for details).

acid-methanol-water (1:5:5 [vol/vol/vol]) at 37°C for 10 min. The excess stain was removed by washing the gels with the same solution without Coomassie blue. Purified serotype Typhi Vi polysaccharide vaccine (Aventis Pasteur, Lyon, France) was used as a standard for determination of the Vi antigen synthesis of the serotype Typhi strains grown at different osmolarities.

Evaluation of Vi antigen expression in serotype Typhi during growth. The strains were grown in LB media with 150 mM of NaCl with aeration at 37°C. The growth was monitored by measurement of absorbance at 600 nm. The samples, taken at different times, were normalized to an optical density at 600 nm of 0.8. Then, 1 ml of culture in duplicate (proteins and Vi antigen samples) was centrifuged at 10,500 × *g* for 10 min. The supernatant was discarded, and the sample was frozen at −70°C overnight. Western blot assays were performed for RpoS and GroEL as explained above. Evaluation of Vi antigen was determined by rocket immune electrophoresis as described above.

RESULTS

Strain construction. Since in preliminary studies we observed that production of Vi antigen in serotype Typhi strains differed depending on the RpoS phenotype, we constructed a variety of strains that differed in their RpoS expression levels to further understand the relationship between this sigma factor, Vi capsule production, and osmolarity. To be sure that the results observed with serotype Typhi Ty2 strain χ3769, which has an *rpoS* mutation (57), were due to the *rpoS* mutation, we first restored this strain to the RpoS$^+$ phenotype to yield χ8438. We then generated RpoS$^-$ derivatives of the RpoS$^+$ Ty2 (χ8438) and ISP1820 (χ3744) strains by insertion of *rpoS*ΩAp and selection for the Ap cassette to yield χ9060 and χ9061, respectively. The strains were Ap^r and catalase negative and presented an expression of a truncated RpoS due to the Ap insertion cassette (Fig. 3). *rpoS* mutants with regulatable expression of *rpoS* were also constructed by deleting P_{rpoS} and replacing it with an *araC*P_{BAD} cassette. The presence of RpoS$^+$, *rpoS*ΩAp, and Δ$P_{rpoS183}$::TT*araC*P_{BAD}*rpoS* mutations was verified by testing for resistance to Ap, sensitivity to Cm, and formation of bubbles from H_2O_2 by PCR and by Western blot analysis as described in Materials and Methods. All mutants conserved the phenotypes of the original strains, including smooth LPS, fimbrial type I production, antigenic composition, and auxotrophic markers.

RpoS expression. Western blot assays were used to determine the RpoS expression levels during stationary phase growth under different osmolarity conditions. The expression levels of RpoS were relatively the same under all osmolarity conditions (Fig. 3). RpoS$^-$ strains χ3769 and χ9067 did not show expression of RpoS. The *rpoS*ΩAp strains, which express a truncated RpoS, showed lower-molecular-weight proteins than wild-type RpoS, as expected (Fig. 3D to E). The strains with regulatable *rpoS* showed expression of RpoS only when grown in the presence of 0.2% arabinose (Fig. 3F to H; see also Table 3).

Effect of *rpoS* on Vi synthesis. Since the osmolarity of the growth media influences the synthesis of Vi antigen, we examined strains of different *rpoS* genotypes in media with different osmolarities for levels of Vi antigen synthesis. The RpoS$^+$ strains grown at osmolarities less than 676 mosmol (~300 mM NaCl [71]) showed overexpression of Vi antigen sufficient to cover the somatic O antigen. The phenotypically O antigen-negative strains were boiled, and the O antigen was subsequently detected in all cases. When RpoS$^-$ strains were grown in media with 300 mM NaCl (676 mosmol) or more, the expression of Vi polysaccharide was less or null and the O antigen was exposed. Under this condition (>300 mM NaCl), RpoS$^+$ strains have the same synthesis of the Vi antigen and the O antigens are exposed (Table 2). The RpoS$^+$ strains and regulatable *rpoS* mutants grown with Ara (0.2%) did not overexpress the Vi polysaccharide, and both Vi and O antigens were detected at osmolarities lower than 676 mosmol. However, at 676 mosmol or higher osmolarities, Vi synthesis was almost null in these strains (Table 2).

To make sure that the effects of osmolarity and *rpoS* genotype/expression on Vi antigen synthesis and detection of O antigen were not due to a consequence of using NaCl, identical evaluations were made using sucrose to alter osmolarity. These results are essentially identical to those presented in Table 2, with the exception that osmolarities higher than 676 mosmol did not result in substantial Vi antigen synthesis for masking O antigen detection (Table 2), whereas sucrose did cause Vi

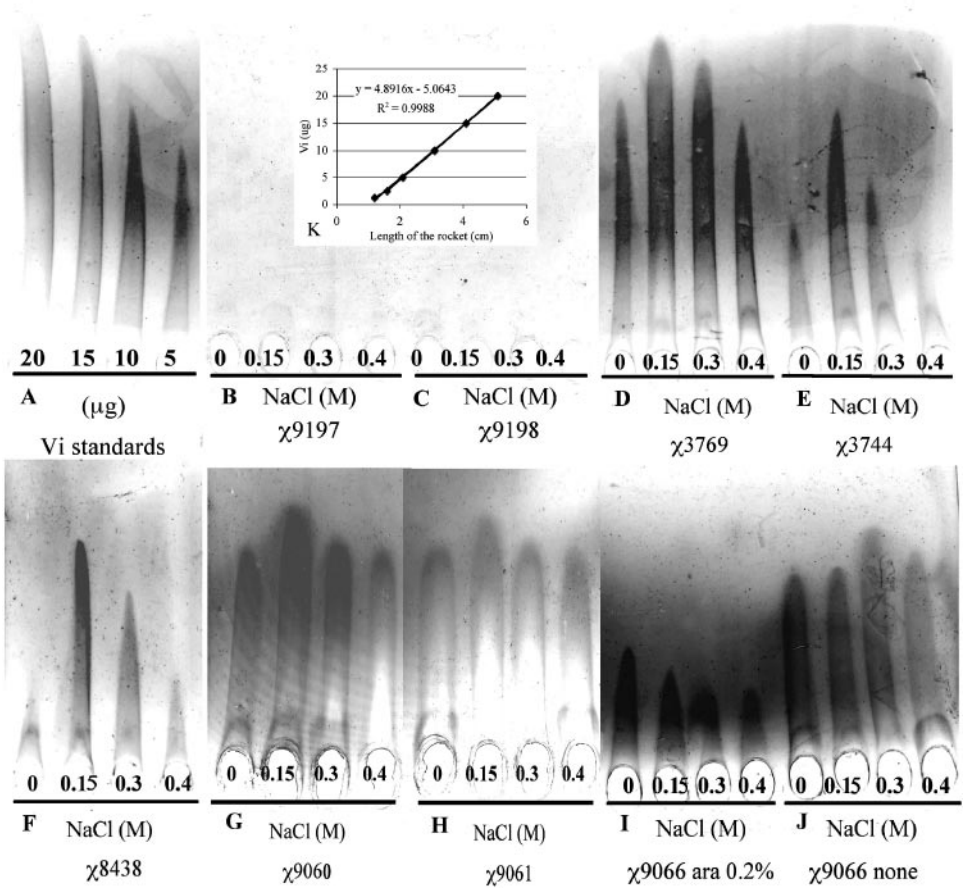

FIG. 4. Evaluation of the effect of RpoS on the synthesis of Vi polysaccharide in serotype Typhi. (A) Standards of Vi antigen. (B) Negative control, χ9197 serotype Typhi Ty2 RpoS$^-$ Δ*tviAEBCD10*. (C) Negative control, χ9198 serotype Typhi ISP1820 RpoS$^+$ Δ*tviAEBCD10*. (D) χ3744 serotype Typhi ISP1820 RpoS$^+$. (E) χ3769 serotype Typhi Ty2 RpoS$^-$. (F) χ8438 serotype Typhi Ty2 RpoS$^+$. (G) χ9060 serotype Typhi Ty2 *rpoS*ΩAp. (H) χ9061 serotype Typhi ISP1820 *rpoS*ΩAp. (I) Noninduced χ9066 serotype Typhi $\Delta P_{rpoS183}$::TT*araC*P_{BAD}*rpoS* RpoS. (J) χ9067 serotype Typhi Ty2 RpoS$^-$ $\Delta P_{rpoS183}$::TT*araC*P_{BAD}*rpoS* RpoS induced by 0.2% arabinose. The strains were grown in LB media with 0, 0.15, 0.3, and 0.4 mM of NaCl. (K) Relation between Vi antigen concentration (μg) and length of rocket (cm).

antigen synthesis that masked O antigen detection (data not shown). This effect of sucrose was more pronounced in strains that were RpoS$^-$ than in strains that were RpoS$^+$.

Pickard et al. (53) showed that in serotype Typhi vaccine strains, Vi antigen synthesis is regulated by the *ompB* locus and is dependent on osmolarity. These authors also observed that serotype Typhi Ty2 and serotype Typhi ISP1820 have different levels of Vi antigen synthesis when these strains were grown at different osmolarities. We observed the same results in these different strains of serotype Typhi (Table 2). However, Pickard et al. (53) explained the differences among the Vi antigen syntheses in these strains by the different times of isolation. On the other hand, an *rpoS* frameshift mutation in serotype Typhi Ty2 has been previously reported (58). These references and the results reported in this study suggest that the *rpoS* allelic state, one of the major differences between serotype Typhi Ty2 and serotype Typhi ISP1820, is responsible for the subtle down-regulation of Vi polysaccharide synthesis at osmolarities lower than 676 mosmol (300 mM of NaCl).

The rocket immune electrophoresis assays indicated that RpoS$^+$ strains down-regulated Vi antigen expression. The major production of Vi polysaccharide in RpoS$^+$ strains was at 150 mM of NaCl, even in the presence of RpoS, but these strains never exhibited expression levels higher than those of RpoS$^-$ strains (Fig. 4 and Table 3). The RpoS$^-$ strains demonstrated overexpression of Vi polysaccharide at all osmolarities tested. However, RpoS$^-$ strains showed regulation of Vi polysaccharide in response to osmolarity changes (Fig. 4 and Tables 2 and 3), but with a substantial increase in Vi synthesis (Fig. 4 and Table 3). These results indicated that RpoS down-regulated the Vi polysaccharide expression in serotype Typhi.

Both RpoS$^+$ and RpoS$^-$ strains showed regulation of Vi polysaccharide in response to changes in osmolarity, suggesting that the repression of Vi polysaccharide synthesis in response to osmolarity is not under the control of RpoS (1, 53). However, RpoS may help in the repression of Vi polysaccharide at high and low osmolarities, conditions under which *rpoS* is induced (6, 43).

Effects of *rpoS* on Vi antigen synthesis and H_d detection. The *fliC* gene is present only in serotype Typhi strains and encodes one type of flagellin, designated H_d (1). Serotype Typhi GIFU10007 appeared to require intrinsic, intact motility for

TABLE 3. Synthesis of Vi polysaccharide (μg of Vi/500 mg of total serotype Typhi protein) of serotype Typhi strains grown on LB agar (pH 7) supplemented with different amounts of NaCl at 37°C[a]

NaCl concn (mM)	Vi antigen concn (μg/mg) for indicated strain				
	χ3769 RopS$^-$	χ3744 RpoS$^+$	χ8438 RpoS$^+$	χ9060 RpoS$^-$	χ9061 RpoS$^-$
0	14.01 ± 0.01	3.74 ± 0.03	0.31 ± 0.4	10.59 ± 0.04	9.61 ± 0.06
150	15.48 ± 0.03	11.08 ± 0.05	8.14 ± 0.09	14.01 ± 0.05	13.52 ± 0.03
300	14.01 ± 0.06	5.70 ± 0.04	5.20 ± 0.07	14.01 ± 0.03	13.03 ± 0.08
400	10.10 ± 0.06	0.80 ± 0.2	0.80 ± 0.3	14.01 ± 0.07	13.03 ± 0.05

[a] The rocket lengths were transformed to the Vi antigen concentrations by the following equation: Vi (μg) = 4.8916 × (rocket length [cm]) − 5.0643. The standard deviations correspond to two independent experiments.

invading cultured epithelial cells, as nonmotile mutants were not invasive (42). The production of the H_d flagellin in serotype Typhi, as well as that of Vi antigen, is modulated by the RcsB-RcsC regulatory system in response to changes in the osmolarity of the growth medium (1). We examined H_d synthesis in strains of different *rpoS* genotypes in media with different osmolarities. Synthesis of the H_d flagellar antigen in RpoS$^+$ strains was detected at all osmolarities tested. In contrast, the H_d flagellar antigen was not detected in RpoS$^-$ strains at osmolarities lower than 10 mM of NaCl (Table 4). At osmolarities higher than 10 mM NaCl, the H_d antigen was detected with a strong reaction in RpoS$^-$ strains, the same as in RpoS$^+$ strains. However, when the RpoS$^-$ strains were grown at 0 and 10 mM NaCl and then boiled, the H_d antigen was detected. These results show that the Vi antigen also covers and masks the H_d antigen at low osmolarities in RpoS$^-$ strains. These results collectively suggest that the *rpoS* gene causes a subtle down-regulation of Vi polysaccharide synthesis in serotype Typhi.

Although we observed similar results for masking detection of the H_d flagellar antigen with growth in media by adding sucrose to increase osmolarity, this masking in RpoS$^-$ strains was observed at much higher concentrations when sucrose was used (data not shown) than when NaCl was used to alter osmolarity (Table 4).

Flagellar synthesis is dependent on the *rcsB-rcsC* loci, which decrease the syntheses of flagella at low osmolarities (1). Western blot analysis showed that H_d flagellin synthesis was decreased under low-osmolarity conditions, as expected (Fig. 5). Similar results were obtained for *rpoS* arabinose-inducible mutants in the presence or absence of arabinose (Fig. 5). Expression levels of flagella were considerably lower when the strains were grown at 0 mM of NaCl, but flagella were still detected by agglutination in RpoS$^+$ strains. However, in RpoS$^-$ strains, flagella were not detected, due to overexpression of the Vi polysaccharide (Table 4).

TABLE 4. H_d flagellar antigen slide agglutination reactions of serotype Typhi strains grown on LB agar (pH 7) supplemented with different amounts of NaCl at 37°C overnight (18 to 24 h)[a]

Strain	Reaction for indicated NaCl concn (mM)						
	0	10	85	150	300	400	500
χ3769 RpoS$^-$	−	−	+++	+++	+++	+++	+++
χ3744 RpoS$^+$	++	+++	+++	+++	+++	+++	+++
χ8438 RpoS$^+$	++	+++	+++	+++	+++	+++	+++
χ9060 RpoS$^-$	−	−	+++	+++	+++	+++	+++
χ9061 RpoS$^-$	−	−	+++	+++	+++	+++	+++
χ9066 RpoS$^-$	−	±	+++	+++	+++	+++	+++
χ9067 RpoS$^-$	−	−	+++	+++	+++	+++	+++
χ9068 RpoS$^-$	−	−	+++	+++	+++	+++	+++
χ9066 RpoS^{+b}	++	+++	+++	+++	+++	+++	+++
χ9067 RpoS^{-b}	−	+	+++	+++	+++	+++	+++
χ9068 RpoS^{+b}	++	+++	+++	+++	+++	+++	+++

[a] The degrees of agglutination ranged from not detectable (−) to weak (+) to strong (+++); ± and ++ indicate intermediate degrees.
[b] Strains grown with 0.2% arabinose to induce *rpoS* (see text for details).

Evaluation of Vi polysaccharide synthesis in serotype Typhi RpoS$^+$ during growth. RpoS is a key factor in the stress response during the transition from the exponential growth phase to the stationary growth phase (43). RpoS is expressed at low levels or unstable at exponential growth phase in *E. coli* (30, 46). Thus, Vi antigen could have different expression levels in exponential phase, when RpoS is not present or is present at low levels, and in stationary phase in serotype Typhi, when RpoS is maximally expressed. We tested the effect of growth phase on the synthesis of Vi antigen in serotype Typhi RpoS$^+$. RpoS was not detected in early exponential phase (Fig. 6) but was detected at different levels from mid-exponential-growth-phase cultures and evidently expressed at stationary phase (Fig. 6), since bubble production in the catalase test was positive only in stationary phase. In concordance with RpoS expression levels, Vi polysaccharide synthesis was decreased (Fig. 6). These results indicated that serotype Typhi RpoS$^+$ overexpressed the Vi antigen during the early growth phase, when RpoS is not expressed or expressed at low levels. This indicates that RpoS is the cause of the VW and V variation in serotype Typhi. RpoS$^-$ strains overexpress the Vi polysaccharide without RpoS regulation, which causes a permanent V form. RpoS$^+$ strains exhibit both forms, the V form during the early exponential growth, when RpoS is not expressed or expressed at low levels, and the VW form when RpoS is expressed. On the other hand, SPI-7, where the *viaB* locus is located, is an unstable genetic element in serotype Typhi (4) and strains that have lost this pathogenic island exhibit the W form (Vi$^-$). Thus, this genetic instability could be the cause of the W form.

DISCUSSION

The expression of capsular polysaccharide by some *Enterobacteriaceae* is highly regulated in response to different environments, being activated by certain signals and suppressed by others (77). Vi capsular expression in serotype Typhi is subject to regulation by at least two separate two-component systems, *rcsB-rcsC* (in the *viaA* locus) (1) and *ompR-envZ* (in the *ompB*

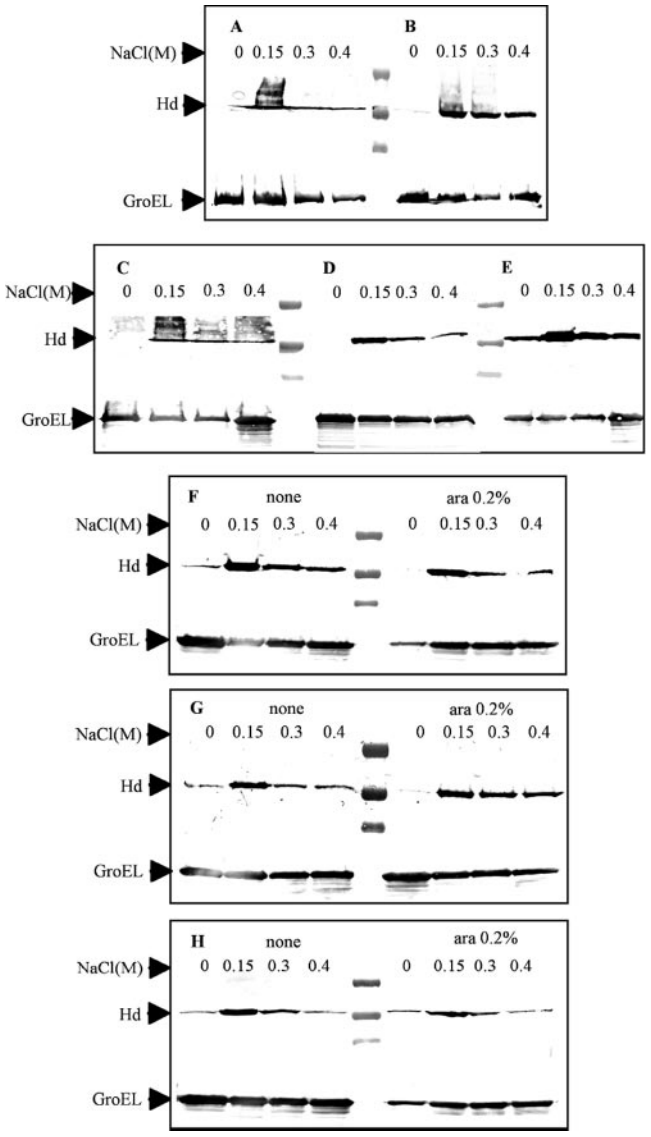

FIG. 5. Evaluation of H_d (serotype Typhi flagella factor) expression at different osmolarities by Western blot analysis. (A) χ3744 serotype Typhi ISP1820 $RpoS^+$. (B) χ3769 serotype Typhi Ty2 $RpoS^-$. (C) χ8438 serotype Typhi Ty2 $RpoS^+$. (D) χ9060 serotype Typhi Ty2 *rpoS*ΩAp $RpoS^-$. (E) χ9061 serotype Typhi ISP1820 *rpoS*ΩAp $RpoS^-$. (F to H) Assays conducted in the absence and presence of arabinose (0.2%). (F) χ9066 serotype Typhi ISP1820 $RpoS^+$ $\Delta P_{rpoS183}$::TT*araC*P_{BAD}*rpoS*. (G) χ9067 serotype Typhi Ty2 $RpoS^-$ $\Delta P_{rpoS183}$::TT*araC*P_{BAD}*rpoS*. (H) χ9068 serotype Typhi Ty2 $RpoS^+$ $\Delta P_{rpoS183}$::TT*araC*P_{BAD}*rpoS*.

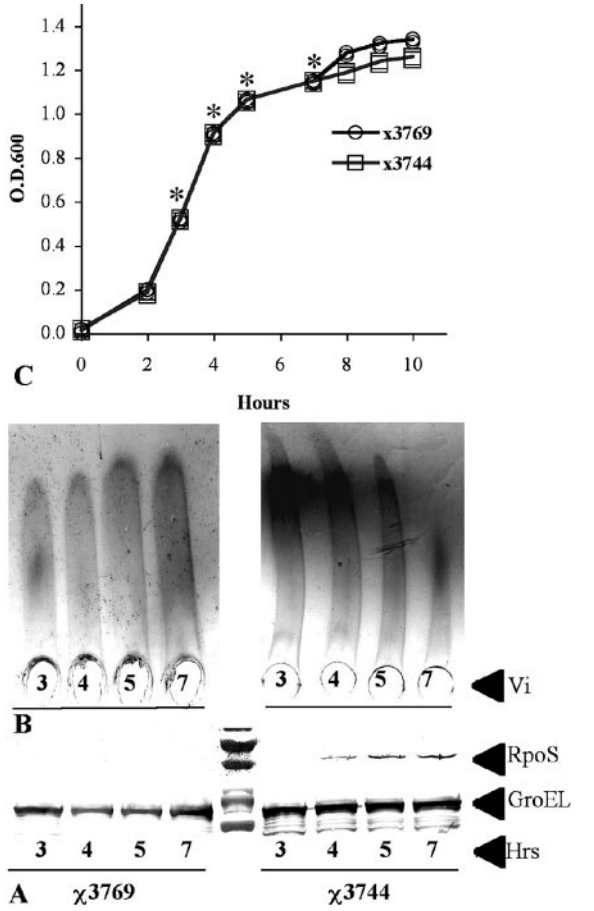

FIG. 6. Evaluation of Vi polysaccharide synthesis in serotype Typhi $RpoS^+$ during growth. (A) RpoS expression during the growth curve. GroEL was used as a control. The strains were grown in LB medium with 150 mM of NaCl. (B) Vi polysaccharide expression during the growth. (C) Growth curve. *, the samples collected at each point were normalized as described in the text.

locus) (53). TviA (VipR), a positive regulator that activates its own promoter in the *viaB* locus, interacts with RcsB, independently of RcsA and Lon protease, to promote optimal transcription of genes involved in Vi antigen synthesis (1, 53, 76, 77). Vi is expressed preferentially at low and medium osmolarities. Such environments might include aqueous environments, which are thought to contain not more than 60 mM NaCl, and certain extracellular environments, such as blood and the cell cytoplasm, where the osmolarities are equivalent to 150 mM of NaCl (310 mosmol) (45, 37, 71). The latter environment is especially relevant to that encountered by serotype Typhi in the infected host. It is possible that this preferential expression of Vi polysaccharide at low and medium osmolarities serves to protect bacterial cells from the complement-mediated actions of the O antigen-specific antibody (61). Recent studies with serotype Typhi Ty2 grown under optimal conditions for Vi polysaccharide expression showed that Vi polysaccharide reduced TLR-dependent interleukin-8 production in human colonic tissue explants, suggesting that the scarcity of neutrophils in intestinal infiltrations of typhoid fever patients is due to the Vi polysaccharide (56). In contrast, RcsB under low-osmolarity conditions, acting in association with the *viaB* locus-encoded TviA protein, negatively controls the transcription of *iagA*, *invF*, and *sipB* (encoding proteins involved in cell invasion) (1).

At high osmolarities, such as those in the intestinal lumen (with values believed to be equivalent to 300 mM of NaCl and greater [71]), the transcriptions of *iagA*, *invF*, and *sipB* are markedly increased, whereas the transcriptions of genes involved in Vi biosynthesis are markedly decreased (1). We observed that the synthesis of Vi antigen decreased as the osmo-

larity increased (Table 2 and Fig. 4), and when the osmolarity reached 676 mosmol (300 mM of NaCl), the Vi antigen no longer covered the O antigen (Table 2). Zhao et al. (80) showed that at 300 mM or more of NaCl, serotype Typhi GIFU1007 had null expression of the Vi antigen and exhibited a high invasion index in epithelial cells, together with a high secretion of SipC protein (80). Part of the stress response in the bacterial cell is the induction of the master stress response regulator protein RpoS (6, 43). High osmolarity is one of the environmental signals that induce *rpoS* (6, 43). In accordance with these findings, our results showed that RpoS down-regulates Vi polysaccharide synthesis, which could have importance for the outcome of infection. On the other hand, serotype Typhi Ty2, which was isolated in 1918 (15) and is RpoS$^-$ (58), exhibited maximal adherence to and invasiveness into tissue culture cells when grown in media with 300 mM NaCl (676 mosmol) (71). We observed that the Vi polysaccharide did not cover the O antigen when either RpoS$^-$ or RpoS$^+$ strains were grown in media with ≥300 mM of NaCl. However, at osmolarities lower than those produced by 300 mM NaCl (676 mosmol), the Vi antigen was overexpressed and the O antigens were covered and masked in RpoS$^-$ strains but not RpoS$^+$ strains, which may affect the invasiveness. In addition, at osmolarities lower than 676 mosmol, adherence to and invasiveness into tissue culture cells have been found to be minimal in serotype Typhi Ty2 (71). On other hand, studies comparing serotype Typhi, serotype Typhimurium, and *S. enterica* serotype Dublin strains showed that serotype Typhi, in this case ISP1820, is much more adherent to and invasive into Int-407 and murine MODE-K cell lines when grown in media with differing osmolarities, in contrast to the RpoS$^-$ serotype Typhi Ty2 strain (79).

Additionally, our results imply that the *rpoS* gene is responsible for the VW variation in the display of Vi and O antigens in serotype Typhi as described in early studies (15, 74). The VW form, with a dual display of Vi and O antigens, is most commonly displayed by serotype Typhi strains from infected individuals, and the majority of such clinical isolates are also RpoS$^+$ (58).

Serotype Typhi ISP1820, which is RpoS$^+$, is more virulent in humans than serotype Typhi Ty2, which is RpoS$^-$ (70). In concordance, serotype Typhi CVD906, a derivate of the serotype Typhi field isolate ISP1820 with deletion mutations in *aroC* and *aroD*, was highly immunogenic but caused fever and other adverse reactions in humans (26), in contrast to the serotype Typhi CVD908 Ty2 Δ*aroC* Δ*aroD* strain, which is RpoS$^-$ (25). The live typhoid vaccine Ty21a, which is an RpoS$^-$, Δ*galE*, and Vi$^-$ (59) derivate of serotype Typhi Ty2 (58), has been evaluated in several clinical trials and found to be well tolerated, although only modestly immunogenic, requiring three or four doses to confer protection (40, 41, 66, 78). Furthermore, the live typhoid vaccine Ty21a has yielded poor results when used as a live recombinant serotype Typhi vaccine (RAStyVs) expressing protective antigens against a diversity of pathogens (2, 9, 10, 21, 24, 50). The *rpoS* mutation could affect the immunogenicities of recombinant vaccines (1). It is thus possible that this poor immunogenicity may be the result of the *rpoS* mutation in Ty2 and its derivatives rather than Vi antigen deletion. On the other hand, the overexpression of Vi antigen in Ty2 could decrease adherence to and invasion into intestinal tissues necessary to colonize more internal lymphoid effector tissues (2). In serotype Typhimurium, it has been demonstrated that chromosomal RpoS-regulated genes are necessary for invasion into and colonization of the gut-associated lymphoid tissue (47). In accord with this, *rpoS* mutants of serotype Typhimurium, with or without the virulence plasmid that possesses RpoS-regulated genes that play no role in the colonization of the gut-associated lymphoid tissue (19, 52), exhibit diminished immunogenicities (5, 7). These results collectively imply that serotype Typhi with RpoS$^+$ genotype, with or without the ability to produce the Vi capsular antigen, might be superior to RpoS$^-$ strains as a vector in the development of recombinant attenuated *Salmonella* vaccines for humans (7, 8). This hypothesis requires further testing.

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