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Negative Osmoregulation of the *Salmonella ompS1* Porin Gene Independently of OmpR in an *hns* Background

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The *ompS1* gene encodes a quiescent porin in *Salmonella enterica* serovars Typhi and Typhimurium. By using random *mariner* transposon mutagenesis, mutations that caused derepression of *ompS1* expression were isolated, one in *S. enterica* serovar Typhi and two in *S. enterica* serovar Typhimurium. All of them mapped in the *hns* gene in the region coding for the carboxy terminus of the H-NS nucleoid protein. The derepressed *ompS1* expression was subject to negative regulation at high osmolarity, both in the presence and in the absence of OmpR. This observation was possible due to the fact that there are two promoters: P1, which is OmpR dependent, and P2, which does not require OmpR for activation (rather, OmpR represses P2). The sequences upstream from position −88, a region previously shown to be involved in the negative regulation of *ompS1*, can form a static bend, and the integrity of this region was required for function and binding of H-NS and for osmoregulation, as determined with gene reporter fusions of different lengths and with a 31-bp deletion mutant. This is consistent with the notion that this region determines a structure required for repression. Hence, *ompS1* shares negative regulation by H-NS with other loci, such as the *bgl* operon and the *ade* gene.

Salmonella enterica serovar Typhi is the etiological agent of typhoid fever in humans (24). Its outer membrane proteins (OMPs) have an important role in triggering the host immune response (15, 21), and *S. enterica* serovar Typhimurium *ompC ompF* double mutants are attenuated for virulence in mice (2). Thus, aside from their roles in the exchange of small molecules and in participating in preserving the bacterial cell shape, the OMPs are relevant to the interaction between a bacterial pathogen and its host.

S. enterica serovar Typhi synthesizes three major OMPs that are highly abundant upon growth in standard laboratory media: the OmpC and OmpF porins and OmpA, a structural protein (29). In *S. enterica* serovar Typhi, as in *Escherichia coli*, expression of OmpC and OmpF is under the control of the *ompB* (*ompR-envZ*) locus. The relative levels of expression of OmpC and OmpF in *E. coli* are modulated by changes in osmolarity, which has been proposed to affect the level of phosphorylated OmpR (9, 20, 26). In *S. enterica* serovar Typhi, a shift in osmolarity affects only the expression of OmpF, whereas OmpC is not osmoregulated (17, 29).

Besides genes for the major porins, *E. coli* possesses porin genes that are quiescent under standard laboratory growth conditions, such as those for Lc (1), NmpC, and OmpN (27). In *Klebsiella pneumoniae*, *ompK37* is expressed at low levels under standard laboratory growth conditions but is highly expressed in β-lactam-resistant clinical isolates (6). This gene is 80% identical to *S. enterica* serovar Typhi *ompS2* and to *E. coli ompN*. Likewise, serovar Typhi *ompS1* belongs to this group of low-level-expressed porins (8).

In contrast to the *ompC* and *ompF* genes, which contain three and four OmpR-binding sites, respectively, *ompS1* possesses six OmpR-binding sites (22) (Fig. 1). *cis*-acting elements up to −310 bp upstream of the OmpR-dependent P1 transcription start site have been described. There is also a P2 promoter, which does not require OmpR for activation but which, however, is repressed in the presence of OmpR. The −310 region contains both positive and negative regulatory elements. Sequences upstream of position −88 are required for negative regulation, and sequences downstream of −88 are required for positive regulation (Fig. 1) (22). Expression of *ompS1* in the *S. enterica* serovar Typhi wild-type strain IMSS-1 was not affected by a shift in osmolarity or by other stress growth conditions that can affect porin expression, such as changes in pH and temperature (22).

The wide porin repertoire in *Salmonella* raises new questions regarding their function and regulation. In the present work, we report that the H-NS nucleoid protein is a repressor of *ompS1*. Previous studies on porin regulation reported that H-NS binds to the intergenic region of the *ompC-micF* genes, repressing them transcriptionally and interfering with the post-transcriptional negative regulation of *ompF* translation via *micF* (37). Moreover, another nucleoid protein, HU, has been found to affect the levels of OmpF (23).

MATERIALS AND METHODS

Bacterial strains, plasmids, and recombinant DNA techniques. The relevant bacterial strains and plasmids used in this study are listed in Table 1. DNA manipulations were performed according to standard protocols (33). Oligonucleotides used for amplification by PCR were provided by the Oligonucleotide Synthesis Facility at our institute and are listed in Table 2. PCRs were performed with Expand (Boehringer Mannheim Inc.) or with *Taq* DNA polymerase, according to the instructions by the manufacturer. The one-step mutagenesis procedure described by Datsenko and Wanner (3) for bacterial chromosomal genes was used to generate gene deletions and replacements for antibiotic resistance markers.

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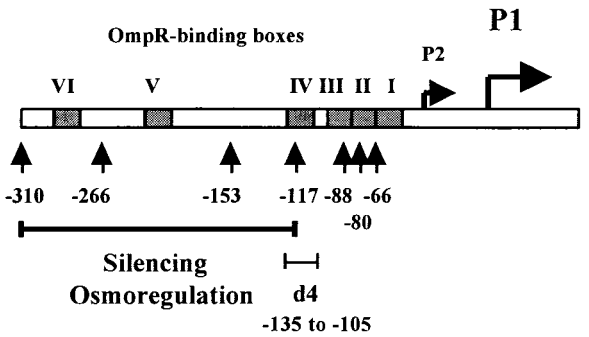

FIG. 1. The *S. enterica* serovar Typhi *ompS1* 5′ upstream regulatory region. The P1 OmpR-dependent promoter, the P2 OmpR-repressed promoter, and the six OmpR-binding boxes (I to VI), as described previously (22), are shown. The number of base pairs upstream of the P1 transcriptional start, as contained in the *ompS1-lacZ* fusions (22), is indicated throughout the region. The long horizontal bar delimits the region required for silencing and osmoregulation. The d4 bar shows the 31-bp deletion from −135 to −105.

Bacterial culture and enzymatic assays. Bacteria were grown in nutrient broth (low osmolarity) or nutrient broth plus 300 mM NaCl (high osmolarity) at 37°C and collected at mid-logarithmic phase. The culture conditions and microplate protein and β-galactosidase assays were as previously described (22).

Random transposon mutagenesis. The *S. enterica* serovar Typhi IMSS-1 and *S. enterica* serovar Typhimurium 14028 wild-type strains were conjugated with *E. coli* SM10 λ *pir*/pFD1 harboring the *mariner* transposon (Km^r) (32). They were then grown in Luria-Bertani broth (containing, per liter, 10 g of peptone, 5 g of yeast extract, and 10 g of NaCl) or SOC broth (containing, per liter, 20 g of tryptone, 5 g of yeast extract, 0.5544 g of NaCl, 0.1864 g of KCl, 1.2038 g of $MgSO_4$, 0.9522 g of $MgCl_2$ and 3.2 g of glucose). They were then subcultured in fresh medium and incubated at 37°C with shaking until they reached mid-logarithmic growth phase. Cells were collected by centrifugation and washed once with 1× phosphate-buffered saline (containing, per liter, 8.0 g of NaCl, 0.61 g of Na_2HPO_4, 0.2 g of KH_2PO_4, and 0.2 g of KCl and adjusted to pH 7.4). They were then resuspended in 1× phosphate-buffered saline and plated onto SOC agar for incubation at 37°C overnight. Mutants derived from this conjugation were scraped and resuspended in 0.9% NaCl, plated on MacConkey agar (containing, per liter, 1.5 g of casein peptone, 17.0 g of gelatin peptone, 1.5g of meat peptone, 10.0 g of lactose, 1.5 g of bile salts, 5.0 g of NaCl, 13.5 g of agar, 0.03 g of neutral red, and 0.001 g of crystal violet [pH 7.2]), and incubated overnight at 37°C. When needed, 12 μg of tetracycline per ml or 25 μg of kanamycin per ml was used.

Localization of the *mariner* transposon insertion site in the *Salmonella* mutants. Bacterial genomic DNA was isolated by using a commercial kit (Aqua Pure genomic DNA isolation kit; Bio-Rad) and digested with either *Bam*HI,

TABLE 2. Primers used

Primer	Sequence[a]
LFhns-S	5′-caaaataaGtcgaCttgcttaaaggc-3′ (*Sal*I site)
RRhns-X	5′-cgttattctAgacgaatatgagtccga-3′ (*Xba*I site)
Mar3-2	5′-ccggggacttatcagccaacctgttat-3′
S1B-CAT5	5′-ggtcgacggatccggggaat-3′
S1S-CAT3	5′-caagcaggtcgacatcttttctgttcat-3′
ompS1-PE	5′-ttgctgcgcctgccactaataac-3′
ompA-PE	5′-tttgcgcctcgttatcatccaa-3′
310b-1	5′-tagccttttatcatttattttatc-3′
310b-2	5′-atgagttatgtgttttgatttga-3′
310b-3	5′-caaagcatcaaatacatataaaaa-3′
310b-4	5′-tgtttctatttggtttttttataatac-3′
310b-7	5′-attcttagtcacttatatatcctgtattat-3′
310b-8	5′-aatatgtagccacttcaacaaaac-3′

[a] Uppercase letter indicate changes in the primer sequence with respect to the wild type, designed to introduce restriction enzyme sites.

TABLE 1. Relevant bacterial strains and plasmids used

Strain or plasmid	Genotype and/or relevant markers	Reference or source
E. coli strains		
BL21(DE3)	F^- *ompT gal* (*dcm*) (*lon*) $hsdS_B(r_B^- m_B^-)$ *met*(DE3)	Novagen
DH5α	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1* Δ(*lacZYA-argF*)*U169* ϕ80Δ*lacZ*ΔM15	Invitrogen
SM10λ*pir*	*thi thr leu tonA lacY supE recA* RP4-2-Tc::Mu λ*pir*	18
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1* Δ(*lac-pro*) [F′*proAB lacI*^{q} *lacZ*Δ*M15* Tn*10*]	Stratagene
S. enterica serovar Typhi and Typhimurium strains		
IMSS-1	9, 12, d, Vi serotype; reference clinical strain	28
IMSS-41	IMSS-1 Δ*ompR*::Cm^r	This study
STY*hns*99	IMSS-1 *hns*99::Km^r *mariner*	This study
STY9941	STY*hns*99 Δ*ompR*::Cm^r	This study
ATCC 14028s	Wild-type *S. enterica* serovar Typhimurium	American Type Culture Collection
STM*hns*114	ATCC 14028s *hns*114::Km^r *mariner*	This study
STM*hns*127	ATCC 14028s *hns*127::Km^r *mariner*	This study
Plasmids		
pACYC184	Vector for cloning of *S. enterica* serovar Typhi *hns*, p15A; Tc^r Cm^r	New England Biolabs
pACYC184ΔTc	pACYC184 containing a *Nru*I-*Eco*RV deletion within the tetracycline resistance gene	This study
phnsty184	Vector pACYC184 carrying *S. enterica* serovar typhi wild-type *hns*; Cm^r	This study
pKD46	oriR101ts, λ Red recombinase system under p*araB* promoter; Ap^r	3
pKD3	pANTSγ derivative containing an FRT-flanked Cm^r gene from pSC140	3
pMC1871	Vector pBR322 carrying a promoterless *E. coli lacZ* gene; Tc^r	35
pT6HNS	Vector pMPM-T6Ω carrying the *E. coli hns* gene fused to His_6 under pBAD promoter	V. H. Bustamante et al., unpublished results

*Kpn*I, or *Sal*I. The fragments were cloned into pUC18 or pUC19 vector and selected for Km^r colonies. Clones containing from 1.2 to 4 kb of insert were selected for sequencing. The DNA sequence near the 3′ end of the site of insertion of the *mariner* transposon was determined with the Thermosequenase kit (United States Biochemicals), according to the instructions of the manufacturer, or at the Sequencing Facility at our institute by using the Mar3-2 primer (Table 2). The sequences obtained were compared with those currently available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST/) by using N-Blast or T-BLAST-X (National Institutes of Health) with the default parameters. The computer-assisted analysis of nucleotide and amino acid sequences was performed with the GENE WORKS (IntelliGenetics), DNA Strider 1.0 (CEA France), Amplify 1.2 (University of Wisconsin), and OLIGO 4.0 (National Biosciences Inc.) software. Graphics were constructed with Cricket Graph-III version 1.0.1 (Computer Associates International, Inc.), and radioactive gels were developed and analyzed with a PhosphorImager (Molecular Dynamics).

Cloning of the wild-type *S. enterica* serovar Typhi *hns* gene. The wild-type *S. enterica* serovar Typhi *hns* gene was amplified by PCR with Expand (Boehringer Mannheim Inc.) and cloned into the pACYC184 vector (New England Biolabs). Briefly, *S. enterica* serovar Typhi IMSS-1 genomic DNA was used for PCR amplification with LFhns-S (annealing from position −538 to −513 of the serovar Typhi *hns* translational start site) and RRhns-X (annealing from position +630 to +604 of the serovar Typhi *hns* translational termination site) primers (Table 2). The 1,584-bp PCR fragment was digested and cloned into the *Sal*I and *Xba*I sites of pACYC184, giving phnsty184. The identity of the cloned fragment was verified by sequencing.

Purification of H-NS His-tagged protein. Purification of His_6-tagged H-NS protein was performed with Ni-nitrilotriacetic acid resin (QIAExpress; Qiagen) according to the instructions of the manufacturer and the modifications established by V. H. Bustamante et al. (unpublished results). Briefly, *E. coli* BL21(DE3) carrying the pT6HNS plasmid (Table 1) was grown to mid-logarithmic phase. L(+)-Arabinose (Sigma-Aldrich) was added to a final concentration of 0.1%, and the bacteria were incubated for 3 h at 30°C and 250 rpm. Cells were then pelleted by centrifugation, resuspended in urea buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl [pH 8.0]), and disrupted by sonication. The suspension was centrifuged, and the supernatant was filtered through an Ni-nitrilotriacetic acid agarose column (QIAExpress; Qiagen); the column was washed with urea buffer at pH 8.0 and urea buffer at pH 6.0, and finally the bound protein was eluted with urea buffer at pH 4.5. Fractions containing purified His_6-tagged H-NS protein were selected after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) and loaded into a Slyde-A-Lyzer 10K cassette (Pierce) for extensive dialysis, first in 50 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$–20% glycerol–0.5 M NaCl–0.1% Triton X-100–4 M urea and then in the same buffer containing 1 M urea instead of 4 M urea. Finally, dialysis was done in 30 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$–20% glycerol–240 mM NaCl–0.1% Triton X-100–3 mM EDTA.

Electrophoretic mobility shift assays. We conducted PCRs that generated *ompS1* products encompassing the length of the regulatory region harbored in representative *ompS1-lacZ* fusions of the pRO series (22), using those plasmids as templates and with oligonucleotide primers S1B-CAT5 (annealing from position 6 to 25 in the pRO plasmid series) and S1S-CAT3 (annealing from position 425 to 398 in pRO310, position 268 to 241 in pRO153, and position 203 to 176 in pRO88). The sites of primer annealing are with respect to the *Pst*I site at the beginning of the *lacZ* reporter gene. These fragments and the enteropathogenic *E. coli* (EPEC) *ler* regulatory and structural gene fragments (100 ng of each), used as positive and negative controls, respectively (Bustamante et al., unpublished), were mixed with increasing concentrations of His-tagged H-NS in the presence of 10 mM Tris-HCl, 1 mM EDTA, 80 mM NaCl, 10 mM 2-mercaptoethanol, 1 mM dithiothreitol, and 10% glycerol. They were incubated 20 min at 37°C and then separated by electrophoresis in 6% polyacrylamide gels in 1× Tris-borate-EDTA buffer. The DNA bands were visualized by staining with ethidium bromide.

Analysis of DNA static curvature. The BEND-IT software, from the International Centre for Genetic Engineering and Biotechnology website (http://www.icgeb.org/dna/bend_it.html), was used to identify regions of potential intrinsic curvature. The curvature is calculated as a vector sum of dinucleotide geometries by using the BEND algorithms of Goodsell and Dickerson (11) and is expressed as degrees per helical turn (10.5°/helical turn = 1°/bp). In addition, the mobility of PCR fragments of 152 or 153 bp was analyzed by 8% PAGE at room temperature and at 4°C in 0.5× Tris-borate-EDTA. The PCR fragments were amplified with primer pairs 310b-1 (annealing from position −303 to −280)-310b-2 (−154 to −176), 310b-3 (−226 to −203)-310b-4 (−76 to −101), and 310b-7 (−124 to −95)-310b-8 (+27 to + 4) (Table 2; see Fig. 8A).

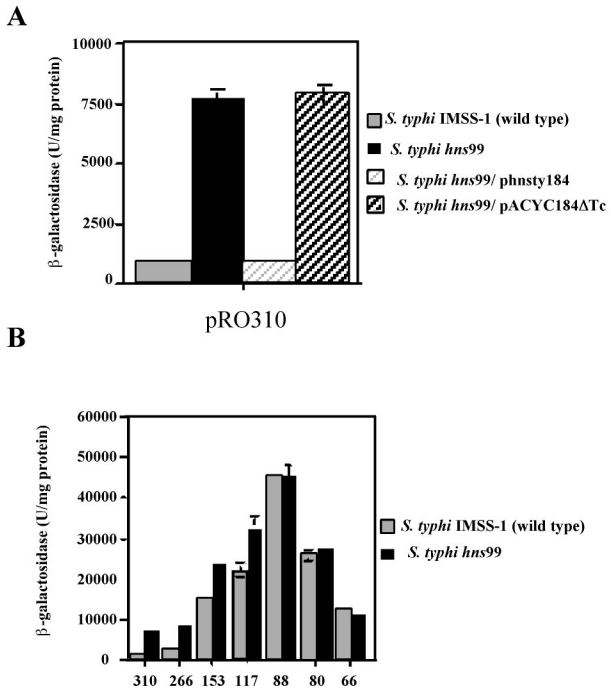

FIG. 2. Derepression of *S. enterica* serovar Typhi *ompS1* in an *hns* background. Bars indicate the specific β-galactosidase activity. (A) Expression in the *S. enterica* serovar Typhi wild-type strain and in the isogenic *hns*99 derivative. Complementation was with cloned *S. enterica* serovar Typhi wild-type *hns* or with the pACYC184ΔTc vector. (B) Expression from reporter fusions of different lengths in the *S. enterica* serovar Typhi wild-type strain and in the *hns*99 derivative. Numbers indicate the length of the *ompS1* regulatory region fused to *lacZ*, as depicted in Fig. 1. Results are the averages and standard deviations from three independent experiments. An absence of error bars indicates that the standard deviation was too small to be shown.

Primer extension analysis. One microgram of total RNA (for *ompA*) or 50 μg of total RNA (for *ompS1*, in two reaction mixtures with 25 μg each), isolated by using commercial kits (RNeasy [Qiagen] or High Pure RNA isolation kit [Roche]) was denatured at 90°C for 3 min and then slowly cooled to 45°C. The RNA was annealed with [γ-^{32}P]ATP-labeled *ompA*-PE (annealing from position +3 to −24 with respect to the *S. enterica* serovar Typhi *ompA* translational start site) or *ompS1*-PE (annealing from +57 to +35 with respect to the serovar Typhi *ompS1* translational start site). The oligonucleotide primers were extended with Moloney murine leukemia virus reverse transcriptase at 37°C for 2 h, and the extended products were collected with a Microcon-30 microconcentrator (Amicon) and analyzed by electrophoresis in urea–8% polyacrylamide gels.

RESULTS

Interruption of the *Salmonella hns* gene results in derepression of *ompS1* expression. We used random transposon mutagenesis to identify putative negative effectors of *ompS1* expression. Briefly, *E. coli* SM10 λ *pir*/pFD1 (harboring the *mariner* transposase; Km^r) was conjugated with the wild-type strain *S. enterica* serovar Typhi IMSS-1 or *S. enterica* serovar Typhimurium 14028 transformed with the *ompS1-lacZ* fusion pRO310. This construct has the longest fused regulatory region (Fig. 1) and had the lowest β-galactosidase activity in both wild-type strains with respect to shorter constructs (22) (Fig. 2B). It produced white colonies on MacConkey indicator plates. From approximately 18×10^3 Km^r serovar Typhi mutants and 80×10^3 Km^r serovar Typhimurium mutants, we

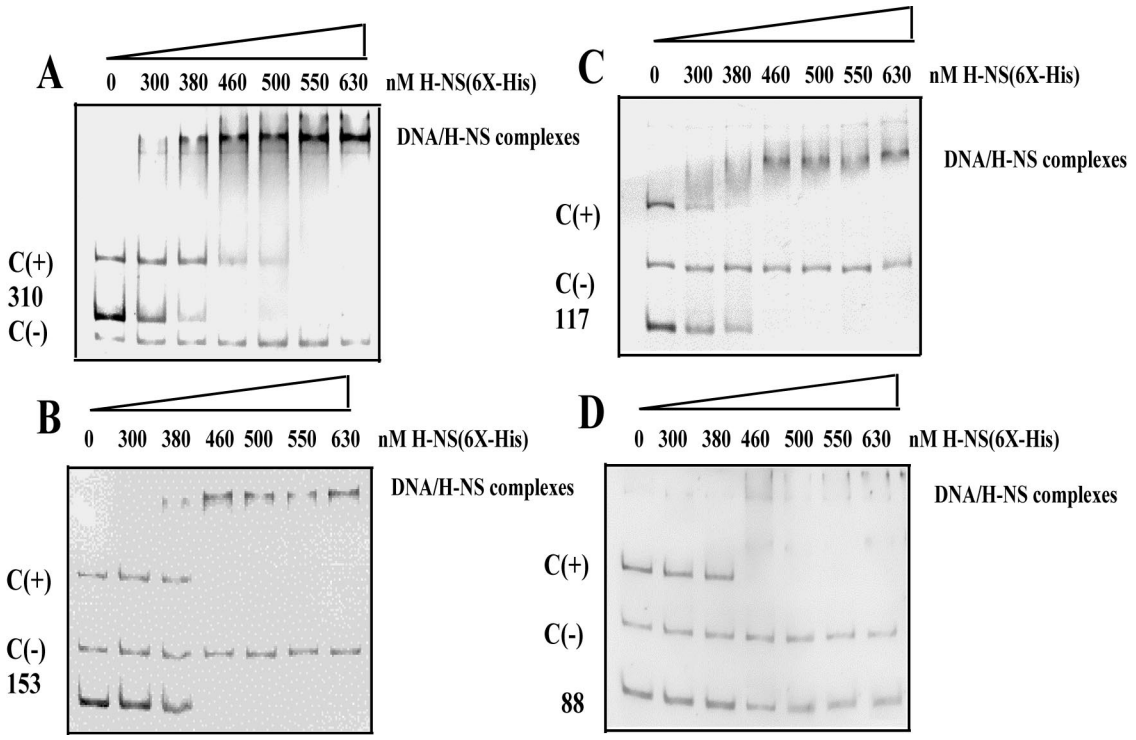

FIG. 3. Binding of H-NS to the *ompS1* upstream regulatory region. Electrophoretic mobility shift assays were carried out with the *ompS1* regulatory region (100 ng), using His_6-tagged H-NS at the indicated concentrations. C(+), is the EPEC *ler* regulatory region, used as a positive control; C(−), EPEC *ler* coding region, used as negative control. 310 (A), 153 (B), 117 (C), and 88 (D), fragments of the *ompS1* regulatory region encompassing the indicated length as shown in Fig. 1. The complexes were separated on 6% polyacrylamide gels.

were able to isolate one serovar Typhi and two serovar Typhimurium red colonies on MacConkey plates. β-Galactosidase assays on cultured samples of these mutants (Fig. 2A; see Fig. 4A) showed a 10-fold derepression of *ompS1* expression compared to that in the wild-type strains.

The DNA flanking the Km^r marker from the mutants was cloned, and the interrupted gene in all three events was identified as *hns*. All of the insertions mapped at or near the region coding for the DNA-binding domain (amino acids 89 to 119 of the 137-residue protein) (5, 7). In *S. enterica* serovar Typhi, the resistance marker inserted into the codon for amino acid 99, and in *S. enterica* serovar Typhimurium, the insertions were in codons 114 and 127 of H-NS. Thus, these mutations were designated *hns*99, *hns*114, and *hns*127, respectively.

In order to further strengthen the evidence for a role of H-NS in *ompS1* expression, we transformed either a plasmid bearing the wild-type *S. enterica* serovar Typhi *hns* gene or the pACYC184ΔTc vector plasmid into the mutants. Expression of cloned *hns* was under the control of ca. 500 bp of its own regulatory region, and the clone does not contain any flanking gene upstream or downstream. Upon complementation, *ompS1* expression was restored to low levels close to those observed in the wild-type strain (Fig. 2A). Such complementation was not observed with the pACYC184ΔTc vector plasmid.

We also analyzed the length of the *ompS1* regulatory sequence needed for the effect by H-NS. The fusions containing between bp −310 and −117 (Fig. 1) were positively affected by interruption of *hns*, whereas fusions from −88 and downstream were not affected by the *hns* interruption (Fig. 2B). Thus, the region upstream of position −88 was required for negative regulation by H-NS.

The shorter the fusions, the higher was the activity in both the wild-type and *hns* backgrounds, in agreement with the notion that as the fusions are shortened, there is a gradual release of an appropriate structure for repression.

H-NS binds to the upstream regulatory region of *ompS1*. In order to test whether H-NS directly interacted with the *ompS1* regulatory region, we conducted electrophoretic mobility shift assays with PCR fragments encompassing the regulatory region contained in four representative *ompS1-lacZ* fusions (Fig. 3). We were able to detect binding of H-NS to the fragments spanning bp −310, −153, and −117 upstream of the P1 promoter (Fig. 3A to C). In contrast, with a −88 upstream fragment, no shift in mobility was observed (Fig. 3D). All fragments contained 21 bp into the structural gene on their 3′ end. These data were consistent with the results of *ompS1* expression, where fusions upstream of bp −88 were derepressed in *Salmonella hns* strains (Fig. 2B).

Osmoregulation of *ompS1* expression in *Salmonella hns* mutants. In the *S. enterica* serovar Typhi IMSS-1 wild-type strain, the low level of *ompS1* expression was not affected by changes in osmolarity (22). Nevertheless, we decided to test the effect on the derepressed *ompS1* activity in the *hns* background in response to osmolarity. Indeed, the derepressed *ompS1* expression was osmoregulated, as it decreased at increasing osmolarity in both *S. enterica* serovar Typhimurium and *S. enterica* serovar Typhi (Fig. 4A). This

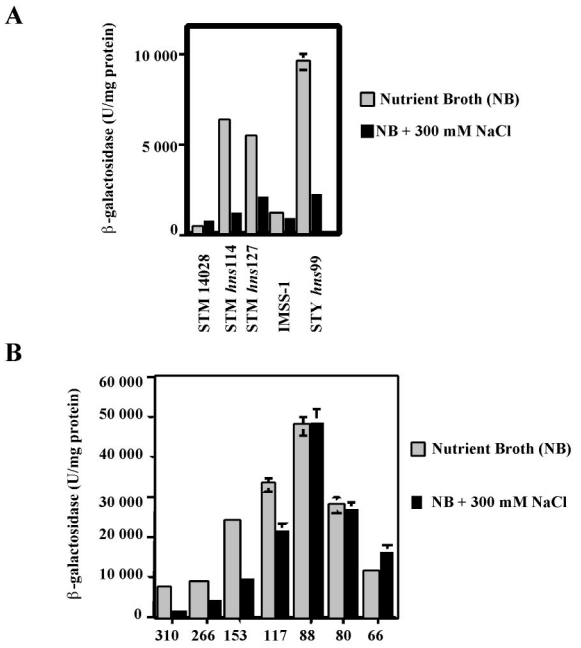

FIG. 4. Osmoregulation of *ompS1* in *Salmonella hns*. (A) Expression in *S. enterica* serovar Typhi (STY) and in *S. enterica* serovar Typhimurium (STM) wild-type strains and in their isogenic *hns* derivatives, as assessed with the bp −310 *lacZ* reporter fusion, at low (NB) and high (NB + 300 mM NaCl) osmolarities. (B) Expression in *S. enterica* serovar Typhi *hns*99 of *ompS1-lacZ* fusions of different lengths (as shown in Fig. 1) at low and high osmolarities. Error bars indicate standard deviations.

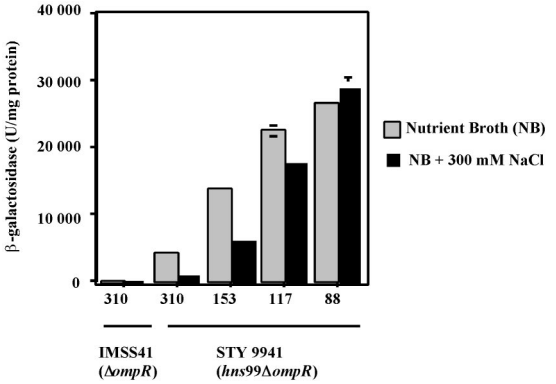

FIG. 5. Osmoregulation in *S. enterica* serovar Typhi 9941 (*hns*99 Δ*ompR*). Expression of the *ompS1-lacZ* fusions constructs of different lengths in *S. enterica* serovar Typhi IMSS-41 Δ*ompR* and STY9941 *hns*99 Δ*ompR* at low (NB) and high (NB + 300 mM NaCl) osmolarities is shown. The number below each bar indicates the length of the *ompS1* regulatory region fused to *lacZ*. Error bars indicate standard deviations.

osmoregulation was gradually abolished upon deletion of the region upstream of position −88. The decrease in expression varied, as it ranged from nearly fourfold in the −310 fusion (ca. 8,000 U of β-galactosidase in low-osmolarity medium versus ca. 1,900 U in high-osmolarity medium) to less than twofold in the −117 fusion (from ca. 32,000 U to ca. 20, 000 U), with no effect in the −88 and shorter fusions (Fig. 4B). This dependence on the length of the regulatory region was similar to that observed for the derepressing effect in the *hns* background (Fig. 2B).

Since OmpR is required for osmoregulation of the *ompC* and *ompF* major porin genes (26), we tested whether osmoregulation of *ompS1* in the *hns* mutants was dependent on OmpR: this was possible because there is a P2 promoter that does not require OmpR for activation (22) (Fig. 1). Expression of *ompS1* was derepressed in *S. enterica* serovar Typhi 9941 (*hns*99 Δ*ompR* double mutant) (Fig. 5) with respect to the *ompR* strain, and it also decreased at high osmolarity, i.e., even in the absence of OmpR. Fusions containing from bp −310 and up to bp −117 diminished their expression at high osmolarity, whereas no decrease in expression was seen with the −88 construct (Fig. 5). The pattern of expression was remarkably similar to that obtained in the presence of OmpR (Fig. 4B). Indeed, the decrease in expression ranged from almost fourfold in the −310 fusion (ca. 4,100 U of β-galactosidase in low-osmolarity medium versus ca. 1,100 U in high-osmolarity medium) to less than twofold in the −117 fusion (from ca. 22,000 to 17,000 U) (Fig. 5). Hence, the region upstream of bp −88 was also determinant for osmoregulation in the absence of OmpR, as it was for H-NS binding and silencing (Fig. 2 to 4). Again, these data are consistent with the proposal that the upstream region determines an appropriate structure for repression, which occurs at high osmolarity in the absence of H-NS or at both low and high osmolarity in the presence of H-NS.

The identity of the promoters affected by the *hns* interruption was addressed by primer extension analysis (Fig. 6). The expression of chromosomal *ompS1* was not detected in the *S. enterica* serovar Typhi wild-type strain, and transcription from the P1 OmpR-dependent promoter was indeed derepressed in the *hns*99 mutant. This activity was lower at high osmolarity. In the double *hns*99 Δ*ompR* mutant, the OmpR activation-independent P2 promoter was derepressed, as no activity was detected in a Δ*ompR* mutant, and its activity was lower at high osmolarity. Furthermore, *ompA* transcriptional activity was not affected either by the genetic background or by changes in osmolarity. It is worth noting that the promoter strength for P1 was less than 2% the level for *ompA*, even under derepression in the *hns* background. This was based on the densitometric scan of the autoradiogram and taking into account that the total amount of RNA used to detect the transcriptional start sites for *ompS1* was 50 μg, whereas for *ompA* it was only 1 μg. Moreover, P2 showed one-half of the promoter strength of P1 under derepression in the *hns*99 Δ*ompR* background (Fig. 6). These results on the activity of the native chromosomal genes reflect the differences in activity observed with the *lacZ* fusion studies (Fig. 4B and 5).

Deletion of a region from bp −135 to −105 results in loss of osmoregulation in *Salmonella hns*. In order to further verify the role of the *ompS1* upstream regulatory region in negative regulation, we analyzed the expression of a *lacZ* fusion containing the bp −310 region with a deletion of 31 bp (from −135 to −105), the pRO310d4 fusion (Fig. 1) (22). We measured expression of the pRO310d4 fusion in *S. enterica* serovar Typhi IMSS-1 (wild type) and in its isogenic Δ*ompR* (IMSS-41), *hns* (*hns*99), and *hns*99 Δ*ompR* (STY9941) mutants (Fig. 7A). As

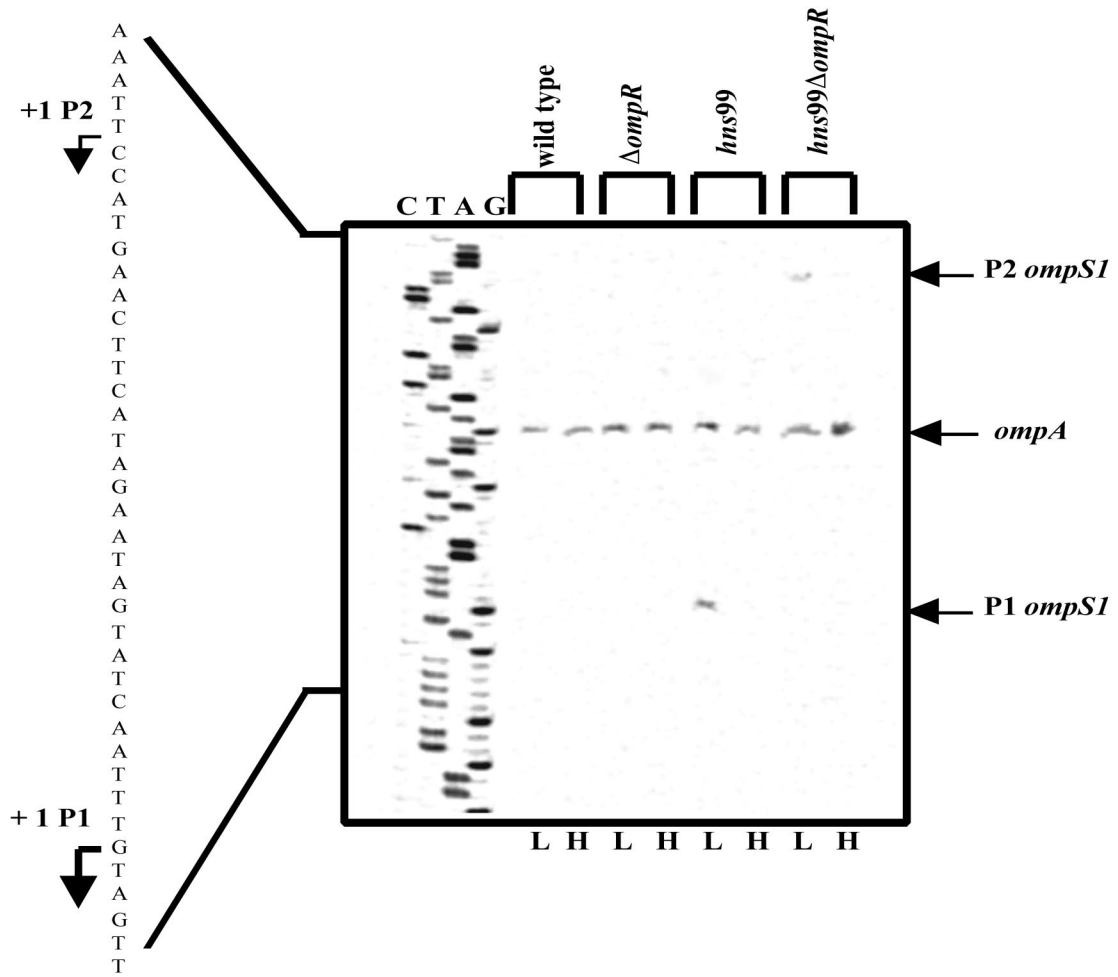

FIG. 6. *ompS1* chromosomal promoter activity at low (L) and high (H) osmolarities in *S. enterica* serovar Typhi strains, as assessed by primer extension. IMSS-1 is the wild-type strain, IMSS-41 is the isogenic Δ*ompR* derivative, *hns*99 is the isogenic transposon mutant, and STY9941 (*hns*99 Δ*ompR*) is the isogenic double mutant. The DNA ladder sequence for *ompS1* is shown on the left. The bands corresponding to the transcriptional start sites of the OmpR-dependent P1 promoter, the P2 OmpR-repressed promoter, and the *ompA* gene are marked. The amounts of total RNA used were 50 μg for the *ompS1* transcriptional start site and 1 μg for *ompA*.

observed previously (22), the mere deletion of this region results in a 20-fold increase in activity in the wild type. Moreover, no osmoregulation was observed in the *hns*99 or *hns*99 Δ*ompR* background, in contrast to that seen with pRO310 (Fig. 4 and 5). Even though some increase was observed in the *hns* strains, this effect was much lower than the 10-fold observed for pRO310 (Fig. 2A and 5).

The notion that the integrity of the −310 region is needed for full H-NS binding and thus derepression in an *hns* background was also shown by electrophoretic mobility shift assays with H-NS. As can be seen in Fig. 7B, the deletion from position −135 to −105 resulted in a lowering of the affinity of H-NS compared to the full −310 region.

The 5′region of *ompS1* is intrinsically curved. It has been proposed that H-NS preferentially binds to curved DNA (16) and that negative osmoregulation of *ompF* involves a DNA loop structure (14). Hence, the possibility that the 5′ upstream region of *ompS1* might acquire a curved configuration was tested. Three circularly permuted DNA fragments encompassing the upstream regulatory region, present in pRO310, were analyzed by PAGE at 4°C, where curved fragments migrate anomalously compared to at room temperature. The more centered the curvature on a given fragment, the slower the migration (39). As can be seen in Fig. 8C, at 4°C fragment 1 (position −303 to −154) migrated the slowest, fragment 2 (−226 to −76) had an intermediate migration, and fragment 3 (−124 to +27) had the fastest migration. In contrast, all three fragments had the same migration at room temperature (Fig. 8B). This is consistent with the presence of a curved region at the center of fragment 1 and close to the center of fragment 2.

The curvatures predicted by analysis in silico were more prominent at positions −230 and −149, having values of 13 and 11°/10.5 bp of helical turn, respectively. This is consistent with the electrophoretic data. The same in silico analysis on the *E. coli ompF* 5′ regulatory region rendered values of 11 and 12, near those for OmpR-binding box F3 and the integration host factor-binding sites, where regions of bent DNA have been postulated (14).

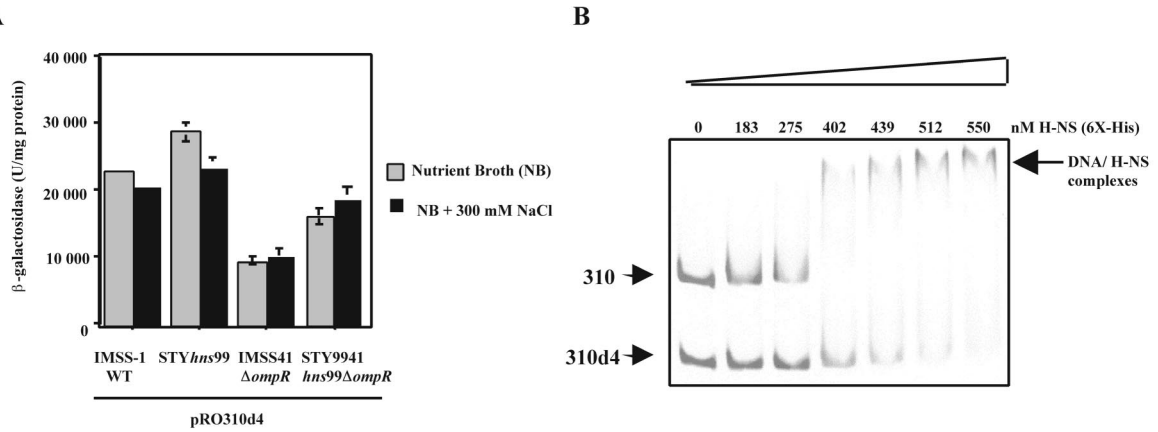

FIG. 7. Effect of the deletion in the *ompS1* regulatory region of 31 bp, from −135 to −105. (A) Expression of the pRO310d4 fusion in *S. enterica* serovar Typhi IMSS-1 (wild type), IMSS-41 Δ*ompR*, *hns*99, and STY9941 *hns*99 Δ*ompR* at low (NB) and high (NB + 300 mM NaCl) osmolarities. Error bars indicate standard deviations. (B) Comparison of the binding of H-NS to PCR fragments representing the −310 and −310d4 fusions. The nanomolar final concentrations of His_6-tagged H-NS are indicated at the top of the gel.

DISCUSSION

The *Salmonella ompS1* gene belongs to the porin superfamily. Its function and the in vitro signals that lead to the induction of its expression remain unknown so far. Nevertheless, it appears to have a role in the *Salmonella* life cycle, since mutations causing defects in swarming motility and biofilm formation have been found to map in *S. enterica* serovar Typhimurium *ompS1* (19, 38). Moreover, the fact that a serovar Typhimurium *ompS1* mutant is less virulent in mice (O. Rodríguez-Morales et al., unpublished results) points towards a role in pathogenicity. In contrast to the case for the *K. pneumoniae* minor porin OmpK37, which is expressed in the absence of the major porins (6), OmpS1 expression was not enhanced in an *S. enterica* serovar Typhi *ompC ompF* double mutant grown in standard laboratory media (data not shown).

The regulation of *ompS1* shares several features with that of the quiescent *E. coli bgl* operon, which codes for the uptake and utilization of β-glucosides, and with that of a quiescent adenine deaminase gene, *yicP (ade)*, recently discovered in *E. coli*. First, the mechanisms for their induction in a wild-type background, and thus their putative role in nature, have remained elusive. Second, their expression is negatively affected by the H-NS nucleoid protein; i.e., they are activated when *hns* is mutated. Moreover, derepression of *bgl* has also been observed by deletions in the upstream silencer. Accordingly, integration of insertion elements in the upstream silencer regions has contributed to the derepression of both the *bgl* and *ade* loci (4, 25, 31, 34, 36).

Previously we showed that *ompS1* contains two *cis*-acting regions: one from bp −310 to −88 upstream of the transcriptional start site of the P1 promoter, which is involved in silencing; and other downstream from bp −88, which is necessary for the positive control of *ompS1* expression (22) (Fig. 1). In order to elucidate the mechanism that maintains a low level of expression of *ompS1*, random transposon mutagenesis was performed in *S. enterica* serovar Typhi and *S. enterica* serovar Typhimurium. We found mutations in *hns* that resulted in the derepression of *ompS1* (Fig. 2A and 4A). All of the transposon insertions mapped to the coding region for the carboxy terminus, at or near the DNA-binding domain. Consistently, in another study, a Tn*phoA*-generated mutation in *S. enterica* serovar Typhimurium strain C5 was also found to map at the 3′end of *hns* (12).

In *S. enterica* serovar Typhi, fusions containing sequences upstream of position −88 showed higher levels of expression in the *hns* background with respect to the wild type, whereas those at and downstream of −88 were not affected (Fig. 2B). Moreover, H-NS bound to promoter fragments including sequences up to −117 and further upstream but not to a promoter fragment which includes DNA up to position −88 (Fig. 3). The result that derepression of the −310 construct in *hns*99 did not reach the levels attained by the −88 construct and that gradual derepression was observed as the constructs were shortened (Fig. 2B) suggests that the sequences upstream of this position could form a DNA structure that promotes silencing.

In the *S. enterica* serovar Typhi IMSS-1 wild-type strain, *ompS1* expression was not affected by a shift in osmolarity, leading to the proposal that *ompS1* was a nonosmoregulated porin gene (22). The lack of osmoregulation was also observed for *ompS1* with the *lacZ* fusions in wild-type *S. enterica* serovar Typhimurium 14028 (Fig. 4A). However, when *ompS1* expression was derepressed in the *hns* mutants, in either serovar Typhi or serovar Typhimurium, it was negatively regulated in high-osmolarity medium (Fig. 4A). The analysis of *ompS1-lacZ* fusions encompassing different lengths of the regulatory region showed that the negative regulatory region needed for H-NS repression, i.e., upstream of bp −88 (Fig. 2B), was also required for osmoregulation (Fig. 4B). Furthermore, osmoregulation occurred in the absence of OmpR (Fig. 5) and also required this sequence. The ratio of *ompS1* expression at low osmolarity to that at high osmolarity in the *hns*99 and *hns*99 Δ*ompR* strains was almost identical for all of the fusions (Figs. 4B and 5). Hence, both *ompS1* promoters share a common mechanism that leads to lower expression at high osmolarity in the absence of H-NS function. It appears that a repressing structure is formed under these conditions, which is also promoted in the presence of H-NS.

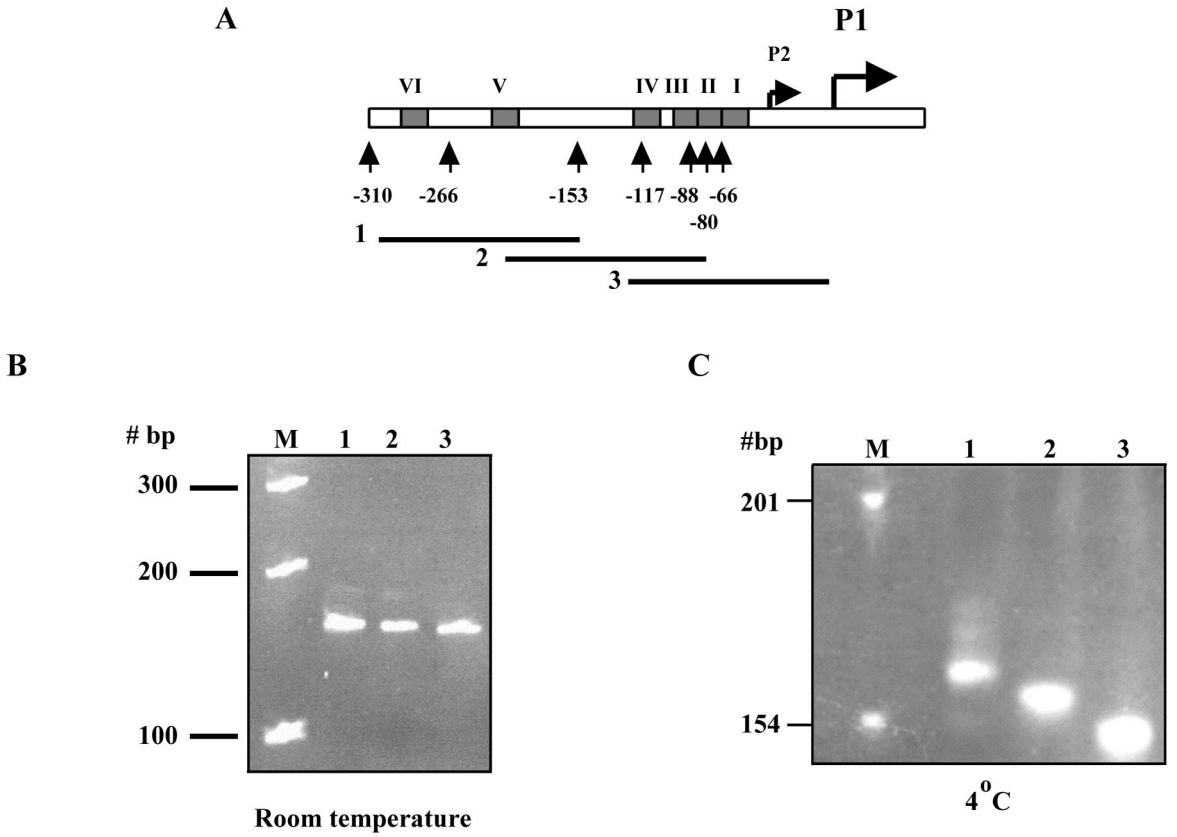

FIG. 8. The *ompS1* 5′ upstream region is statically curved. (A) Schematic diagram showing the *ompS1* 5′ region. Numbers indicate the length of the regulatory region contained in each *lacZ* fusion. The six OmpR-binding boxes are represented as filled rectangles. Lines 1, 2, and 3, PCR fragments encompassing from −303 to −154, −226 to −76, and −124 to +27, respectively, of the *ompS1* 5′ region. (B and C) PCR fragments 1, 2, and 3 separated by 8% PAGE at room temperature(B) or at 4°C (C). Lanes M, molecular size markers.

As observed with *ompF*, *ompS1* expression decreased at high osmolarity when derepressed in an *hns* strain (Fig. 4B and 5). In this respect, the *ompF* 5′ upstream regulatory region has a role in osmoregulation (14, 30). Using the pRO310d4 fusion, which contains the entire bp −310 regulatory region with a 31-bp deletion (from −135 to −105) (Fig. 1), we observed that *ompS1* expression was derepressed 20-fold and was not significantly affected in either the *hns*99 or the *hns*99 Δ*ompR* mutant; that is, it no longer responded to changes in osmolarity independently of the presence or absence of OmpR (Fig. 7A). Moreover, the binding affinity of H-NS to the 310d4 regulatory region was lower than that observed for the whole −310 region, although it was still higher than that for the −88 region (Fig. 3A and D and 7B). Thus, the integrity of the −310 regulatory region was required for full H-NS repression and osmoregulation. In this regard, the region upstream of bp −88 has an intrinsic curvature, as assessed both in silico and by DNA bending electrophoretic assays (Fig. 8C). Such curvature could determine a structure needed for repression in the presence of H-NS or at high osmolarity in the *hns* background.

Because of the fact that *ompS1* expression in the *Salmonella hns* mutants did not reach the level attained in the shortened, most active pRO88 fusion, the presence of additional negative effectors aside from H-NS cannot be excluded. Thus, we can envision at least three scenarios to explain the negative regulation of *ompS1* expression by high osmolarity. First, as mentioned above, changes in the DNA structure of the regulatory region could hinder expression, possibly by blocking access of the RNA polymerase. Another possibility would occur if H-NS is produced as a stable, truncated protein in our *Salmonella* mutants. In this regard, the participation of the nucleoid protein StpA as an adaptor for truncated H-NS in repressing the expression of the *proU* gene and the *bgl* locus in *E. coli* has been illustrated (10). Hence, the binding of a putative complex formed by truncated H-NS and StpA to the *ompS1* upstream regulatory region could be enhanced at high osmolarity, leading to the observed decrease in *ompS1* expression. Finally, the action of another putative negative effector could be increased at high osmolarity.

Thus, the characterization of the state of the H-NS protein in our mutants, analysis of *ompS1* expression in an *hns stpA* double mutant at both low and high osmolarity, and a search for other negative effectors by mutagenesis in *hns* mutants are experiments that should help in further defining the mechanism involved in the negative regulation of *ompS1* expression.

Hence, our present model to explain the strict negative regulation of *ompS1* implies the initial binding of H-NS at or near the −135 to −105 region, thus allowing the formation of a repressing loop by nucleation of H-NS to the rest of the regulatory region. Such a repressing loop would involve the entire

regulatory region, not only hindering the access of OmpR and thus preventing activation of P1 but also impeding transcription from P2, which otherwise would have been activated once OmpR could not activate P1. At high osmolarity, in the absence of H-NS function, the repressing loop again would be formed by local changes in DNA structure.

It is worth noting that the promoter strength of the native *ompS1* gene on the chromosome is relatively low compared to that of a highly expressed outer membrane protein gene such as *ompA* (Fig. 6). This likely reflects the low abundance of the OmpS1 porin even under derepression in the *hns* strains, an idea that is further confirmed by the lack of detectable OmpS1 in outer membrane protein preparations separated by PAGE and stained with Coomassie brilliant blue (data not shown).

The function of nucleoid-associated proteins, such as H-NS, remains elusive despite extensive efforts towards understanding their physiological role. Moreover, whether osmolarity is a true regulatory signal for *ompS1* in nature or whether it mimics another signal in the environment is an open question. *ompS1* belongs to the pleiotropic H-NS regulatory circuit, where nearly one-third of the genes affected by H-NS are involved in cell envelope physiology and structure, consistent with the notion that genes regulated by H-NS are generally associated with bacterial adaptations to environmental stress (13).

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