

OmpR and LeuO Positively Regulate the *Salmonella enterica* Serovar Typhi *ompS2* Porin Gene

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The *Salmonella enterica* serovar Typhi *ompS2* gene codes for a 362-amino-acid outer membrane protein that contains motifs common to the porin superfamily. It is expressed at very low levels compared to the major OmpC and OmpF porins, as observed for *S. enterica* serovar Typhi OmpS1, *Escherichia coli* OmpN, and *Klebsiella pneumoniae* OmpK37 quiescent porins. A region of 316 bp, between nucleotides –413 and –97 upstream of the transcriptional start point, is involved in negative regulation, as its removal resulted in a 10-fold increase in *ompS2* expression in an *S. enterica* serovar Typhi wild-type strain. This enhancement in expression was not observed in isogenic mutant strains, which had specific deletions of the regulatory *ompB* (*ompR envZ*) operon. Furthermore, *ompS2* expression was substantially reduced in the presence of the OmpR D55A mutant, altered in the major phosphorylation site. Upon random mutagenesis, a mutant where the transposon had inserted into the upstream regulatory region of the gene coding for the LeuO regulator, showed an increased level of *ompS2* expression. Augmented expression of *ompS2* was also obtained upon addition of cloned *leuO* to the wild-type strain, but not in an *ompR* isogenic derivative, consistent with the notion that the transposon insertion had increased the cellular levels of LeuO and with the observed dependence on OmpR. Moreover, LeuO and OmpR bound in close proximity, but independently, to the 5' upstream regulatory region. Thus, the OmpR and LeuO regulators positively regulate *ompS2*.

Salmonella enterica serovar Typhi is the causal agent of typhoid fever in humans, affecting a wide sector of the world population (37). Typhoid fever is a systemic infection, characterized by the presence of bacteria in the liver, spleen, and bone marrow, as *S. enterica* serovar Typhi invades and survives within macrophages (6). Specific humoral and cellular immune responses are mounted against *Salmonella* outer membrane proteins (OMPs) (2, 53, 51). *Salmonella enterica* serovar Typhimurium and *S. enterica* serovar Typhi OMPs have proven to be relevant immunogens for protection of mice challenged with virulent strains (23, 35). *S. enterica* serovar Typhimurium double *ompC* and *ompF* porin mutants showed attenuated virulence (7), and *S. enterica* serovar Typhimurium porins have been observed to trigger signal transduction in host cells (17). Hence, studies on the molecular features and regulation of *Salmonella* OMPs and porins should aid in further understanding their role during bacterium-host interactions.

S. enterica serovar Typhi synthesizes three highly abundant OMPs upon growth in standard laboratory media: the OmpC and OmpF porins and the OmpA structural protein (42). Expression of the *ompC* and *ompF* genes in *S. enterica* serovar Typhi is under the control of EnvZ and OmpR, a two-component signal transduction system encoded by the *ompB* (*ompR envZ*) locus. Interestingly, a shift in osmolarity only affects OmpF expression, and OmpC levels remain constant (43, 31). In contrast, the relative levels of OmpC and OmpF expression in *E. coli* are modulated in a reciprocal manner by changes in osmolarity, which also affect the level of the phosphorylated

form of the response activator OmpR in the cell (reviewed in references 29 and 40).

In addition to its role in porin regulation, the *Salmonella ompB* operon has been implicated in the regulation of virulence. Mutants in *ompR* are attenuated in vivo, are defective in capsular Vi synthesis and in cytotoxicity towards macrophages, do not replicate within cultured macrophages, and show diminished expression from the *ssrA/B* genes in *Salmonella* pathogenicity island 2. *ompR* and *envZ* mutants are defective in the formation of *Salmonella*-induced filaments (13, 38, 28, 27, 33).

Adding to the porin repertoire of *Salmonella*, *S. enterica* serovar Typhi contains the *ompS1* gene that codes for an OMP with all of the molecular features of the OmpC/OmpF family, which is, nevertheless, expressed at very low levels compared to the major OmpC and OmpF porins. In the absence of negative upstream regulatory sequences, *ompS1* shows a hierarchical regulation, where expression occurs from a P1 promoter in the presence of OmpR or from an OmpR-independent P2 promoter that becomes activated in the absence of P1 activation (36). Here we report the isolation and initial characterization of *ompS2* from *S. enterica* serovar Typhi, which shows sequence similarity to *ompS1*. Regulation of *ompS2* was also negatively controlled by *cis*-acting sequences located upstream of the promoter, and its expression was positively regulated by the OmpR regulator as well. However, in addition, the LeuO regulator was found to be a positive regulator, and both OmpR and LeuO bound to the upstream regulatory region.

LeuO has been implicated in the bacterial response to stress; thus, it might be important for survival in natural environments outside the laboratory (14, 30). Moreover, overexpression of LeuO has been found to affect the expression of several genes

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference
Serovar Typhi strains		
IMSS-1	Mexican clinical isolate; 9,12, d:-, Vi	42
81	Serovar Typhi $\Delta ompB::km$ isogenic IMSS-1 derivative	31
IMSS-40	Serovar Typhi $\Delta ompR::km$ isogenic IMSS-1 derivative	This study
IMSS-TN103	Serovar Typhi isogenic IMSS-1 derivative containing a Tn10::Cm insertion 160 bp upstream of the first ATG and 60 bp downstream of the promoter for the <i>leuO</i> gene	This study
Plasmids		
pFM413S2	pBR322-derived plasmid, containing the whole structural <i>ompS2</i> gene plus its 5' regulatory region, up to 413 bp upstream of the transcriptional start point	This study
pFM97S2	pBR322-derivative, containing the <i>ompS2</i> gene plus the regulatory region up to position -97	This study
pFM413 to 34 series	pMC1871-derived plasmids containing translational fusions of the <i>ompS2</i> 5' regulatory region, up to 413 to 34 bp upstream of the transcriptional start point, to the <i>lacZ</i> reporter gene	This study
pMPM-A3	p15A-derived vector (Ap^r), with a multiple cloning site	32
pFM800TL	pMPMA-3-derived plasmid containing the serovar Typhi <i>leuO</i> gene together with Tn10 inserted in the upstream region	This study
pFMTrcLeuO	pFMTrc12 (p15A1 <i>lacI</i> <i>trcp</i> Ap^r)-derived plasmid containing serovar Typhi <i>leuO</i>	This study
pKD46	Red recombinase expression plasmid (Ap^r)	10
pKD4	Template plasmid for gene inactivation with Km^r	10

influenced by mutations in *hns*, the gene for the H-NS nucleoid protein. Thus, LeuO relieved silencing of the *bgl* operon, the operon involved in the utilization of certain β -glucosides (52); suppressed the effect of an *hns* mutation on *cadC*, which codes for the activator of the acid-inducible lysine decarboxylase (50); and also suppressed the negative effect of the AT4 region, the 72-bp gene silencer region located between the *leuO* gene and the *leuABCD* leucine biosynthetic operon (8, 9). Besides, LeuO has a negative effect on the expression of the small regulatory DsrA RNA involved in RpoS translation (25).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown in medium A (containing [per liter] 7 g of nutrient broth, 1 g of yeast extract, 2 g of glycerol, 3.7 g of K_2HPO_4 , and 1.3 g of KH_2PO_4). To achieve high osmolarity, NaCl was added to a final concentration of 0.3 M. Cells were collected for protein, β -galactosidase, and RNA determinations at the mid-logarithmic (optical density at 600 nm [OD_{600}], 0.4) phase of growth at 37°C. This was determined to be the point of optimum levels upon analysis of the whole growth curve (data not shown). When needed, 12 μ g of tetracycline/ml or 30 μ g of kanamycin/ml was used. Cells were collected by centrifugation and washed twice with 1 \times Z buffer (0.06 M Na_2HPO_4 , 0.04 M NaH_2PO_4 , 0.01 M KCl, 0.001 M $MgSO_4$ [pH 7]). After centrifugation, the bacterial pellet was resuspended in 1 ml of Z buffer.

DNA techniques. DNA manipulations were performed according to standard protocols (47). Oligonucleotides used for amplification by the PCR were purchased from BioSynthesis or provided by the Oligonucleotide Synthesis Facility at our institute. PCRs were done by using AmpliTaq (Perkin Elmer) according to the manufacturer's instructions. Restriction and modification enzymes were used according to the manufacturer's instructions (Amersham, Inc., Boehringer Mannheim, New England Biolabs, or Gibco BRL).

Southern blot hybridization. Hybridization of ^{32}P -labeled DNA probes (10^8 cpm/ μ g) was done overnight in 0.1 M NaH_2PO_4 - Na_2HPO_4 (pH 7) at 65°C. Washes were performed at room temperature: first in 2 \times SSC (standard saline citrate; 1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate (SDS) for 30 min and then in 1 \times SSC-0.1% SDS for 15 min. For higher stringency, washes were done at 65°C.

***S. enterica* serovar Typhi genomic library construction and screening.** A genomic library was constructed by performing a complete SalI DNA digestion of *S. enterica* serovar Typhi IMSS-1 and cloning it into plasmid pUC18. Recombinant clones were pooled successively in groups of 10 and screened by Southern blot hybridization with radioactive *ompS1*; the pool containing the 2.3-kb band was further subdivided and probed until the desired individual plasmid, pM15, was isolated. This screening procedure avoided cross-hybridization with *Escherichia coli* host DNA, a strategy adopted previously with recombinant phage libraries (42).

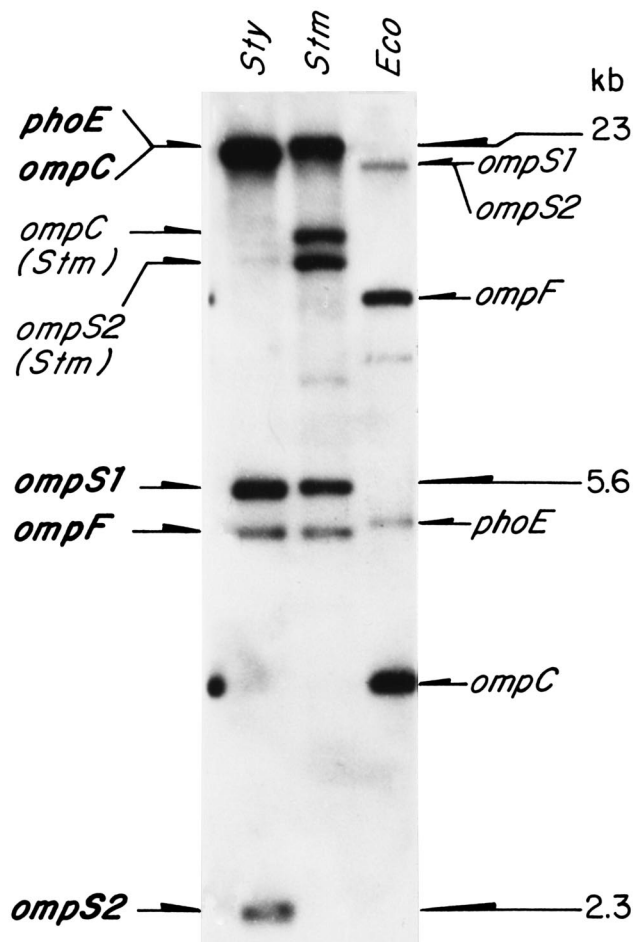


FIG. 1. Southern blot hybridization of genomic DNA from *S. enterica* serovar Typhi (*Sty*), *S. enterica* serovar Typhimurium (*Stm*), and *E. coli* (*Eco*) digested with SalI restriction endonuclease, with the *S. enterica* serovar Typhi *ompS1* gene (37) labeled with ^{32}P . The identities of the hybridizing bands for *Salmonella* genes are shown on the left, including a novel conspicuous band that corresponds to *ompS2*, and those for *E. coli* genes are shown on the right.

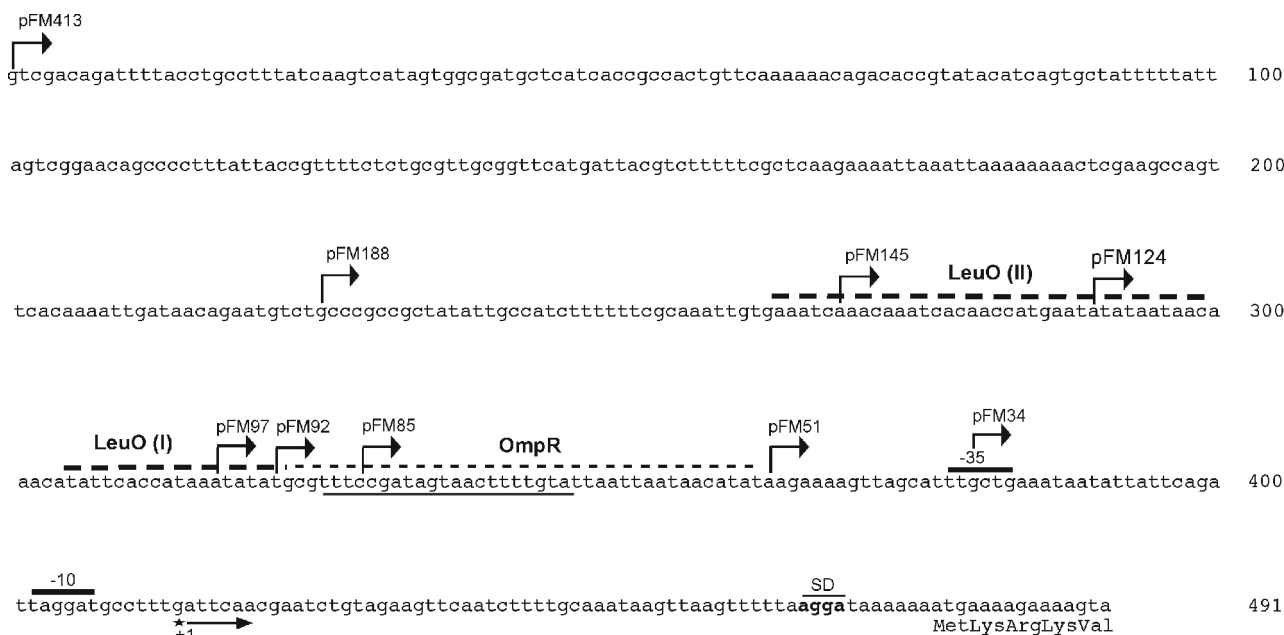


FIG. 2. Nucleotide sequence of the *S. enterica* serovar Typhi *ompS2* 5' upstream regulatory region, featuring the first five codons of the structural gene, the putative Shine-Dalgarno ribosome-binding sequences (SD), the -10 and -35 promoter sequences, and the single transcription initiation at a G residue obtained by primer extension analysis. Also shown are the boundaries for various pFM translational fusions, where the indicated segments of the regulatory region plus five codons were fused to the *lacZ* reporter gene. A sequence with similarity to the consensus OmpR-binding site (thick underline), the site for the binding of OmpR (thin dashed line), and two boxes for the binding of LeuO (I and II) (thick dashed lines) are also represented.

Cloning of the *ompS2* structural gene. The 2.3-kb Sall insert from pM15, containing the entire structural *ompS2* gene plus a 5' regulatory region encompassing 413 bp upstream of the transcriptional start site, was cloned into pBR322 to generate pFM413S2. pFM97S2, carrying the entire *ompS2* structural gene plus 97 bp upstream of the transcriptional start site, was constructed by PCR amplification with pFM413S2 as the template and the following oligonucleotides: 5'-ATATATGCGTTTTCCGATAGTAAC-3' (complementary to the *ompS2* regulatory region) and 5'-TCTGTGCCACGGGATCCGTGCGTCTGTGTTT-3' (complementary to the 3'-terminal portion of the structural gene and generating a BamHI restriction site). The amplified fragment was cloned into pBR322, cut with BamHI and NruI.

Nucleotide and amino acid sequence analysis. The entire 2.3-kb Sall insert from pM15 was sequenced by the dideoxy chain termination method (48) and deposited in the EMBL database. The GAP program (version 10) of the Computer Genetics Group (Madison, Wis.) was utilized for nucleotide and amino acid sequence alignments. Porin sequences were retrieved from the GenBank database. The *S. enterica* serovar Typhimurium and *Salmonella enterica* serovar Paratyphi *ompS2* sequences were retrieved from the database of the Washington University School of Medicine Genome Sequencing Center.

Construction of translational *ompS1-lacZ* reporter fusions. PCR was used for generating plasmids pFM413 and pFM266 from pFM413S2. Thus, oligonucleotides 5'-GTCGACAGATTTTACCTGCC-3' and 5'-ATTACGCTTTTTTCGCTCAA-3' were used, respectively, for amplifying from the 5' upstream region; and oligonucleotide 5'-TACTTTTCTTTTCATTTTTT-3' was used for the 3' downstream portion. The regulatory regions were then cloned into the SmaI site of the pMC1871 fusion vector (Pharmacia) (49). Construction of the shorter pFM188, 145, 124, 97, 92, 85, 51, and 34 derivatives was done by digesting pFM266 with EcoRI, doing a time course digestion with Bal31, blunt-ending with Klenow polymerase, digesting with SauI, and ligating into pMC1871 cut with SauI and SmaI. Fusions were sequenced to determine their 5' end points.

Primer extension analysis. Ten micrograms of total RNA, isolated by using a commercial kit (RNeasy; Qiagen), from a sample collected after the culture reached an optical density at 600 nm (OD_{600}) of 0.4 was linearized at 93°C for 3 min and then slowly cooled down to 42°C to favor annealing with a 32 P-labeled oligonucleotide complementary to the 5' terminus of the *lacZ* gene (5'-GCCA GGGTTTTCCCAGTCACGACG-3'). The oligonucleotide primer was extended with reverse transcriptase, and the extended product was collected with a Microcon-10 microconcentrator (Amicon) and analyzed by electrophoresis in 8%

polyacrylamide-urea gels by following established procedures (3, 36). Samples for electrophoresis were quantified by OD_{260} .

Microplate protein and β -galactosidase determinations. Protein concentration determined by the Lowry method and β -galactosidase activity were adapted as a microtiter plate assay (31, 36).

OMP purification and electrophoresis. OMPs were prepared as a Triton X-100 insoluble fraction and scaled down to a mini-prep level as described previously (44). They were separated by SDS-12% polyacrylamide gel electrophoresis (12% PAGE) and visualized by staining with Coomassie brilliant blue. Relative abundance was determined by densitometric scanning (Alpha Imager UV5.04; Alpha Innotech Corp.).

Disruption of *S. enterica* serovar Typhi *ompR*. The procedure for the one-step inactivation of bacterial chromosomal genes by Datsenko and Wanner (10) with the pKD46 Red recombinase plasmid was used. For the nonpolar deletion of *ompR*, the specific H1 and H2 homologue extension sequences used were *ompR5'-P1* (5'-GAGAATTATAAGATTCTGGTGGTTGATGACGATATGC GTCTGTGTAGGCTGGAGCTGCTTC-3') and *ompR3'-P2* (5'-GACGTAGC CCAGGCCCATACGGTCTGAATATAACGCGGCATATGAATATCCTC CTTAGTCC-3'), respectively, joined to the P1 and P2 priming sequences specific for the Km^r cassette gene from pKD4.

***S. enterica* serovar Typhi transposon mutagenesis.** Transposon Tn10, contained in plasmid pBSL181 (1), was used to perform random mutagenesis on *S. enterica* serovar Typhi IMSS-1 carrying *lacZ* fusion plasmid pFM413. An exponential bacterial culture ($OD_{550} = 0.8$) of 500 ml was concentrated to 5 ml for electroporation in a Bio-Rad electroporator (*E. coli* Pulser) by following the manufacturer's instructions. Three hundred microliters of the concentrated culture were electroporated with 8 μ g of pBSL181. The cells were then placed in 1 ml of medium S.O.C. (Invitrogen) plus 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) to induce the transposase for 1 h at 37°C. Selection was in NCE medium (3.94 g of KH_2PO_4 , 6.46 g of $K_2HPO_4 \cdot 3H_2O$, 43.75 g of $NaNH_4HPO_4 \cdot 4H_2O$) plus 0.2% lactose, 0.1% tryptophan, 0.3% cysteine HCl H_2O , 80 μ g of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal)/ml, 30 μ g of chloramphenicol/ml, and 12 μ g of tetracycline/ml. Three transformants, which showed a dark blue phenotype indicative of an increase in pFM413 *lacZ* expression, were obtained from the screening of a total of 30,000 transformants. *S. enterica* serovar Typhi lacks β -galactosidase activity; thus, the screening relied not only on color development but also on enhanced growth in minimal medium.

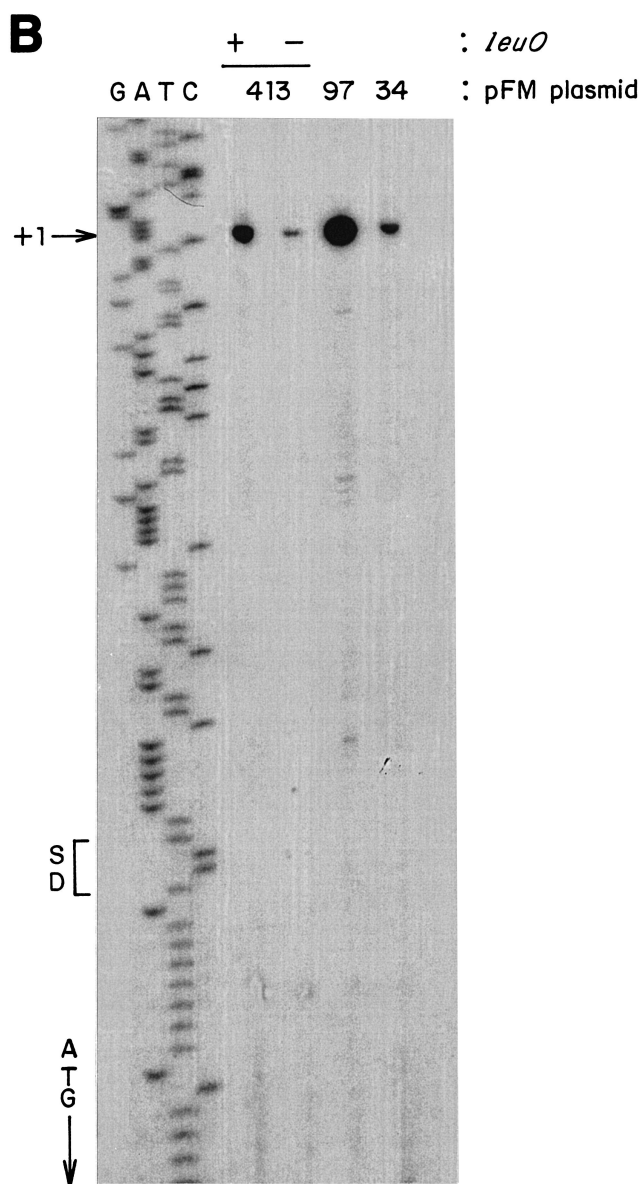


FIG. 3—Continued.

RESULTS

Cloning and characterization of the *ompS2* gene. Hybridization of either the *S. enterica* serovar Typhi *ompC* (42), *ompS1* (16), *ompF* (EMBL accession no. X89757; formerly *ompS3*), or *phoE* (EMBL accession no. X74595) ³²P-labeled structural genes to Southern blots of genomic *S. enterica* serovar Typhi DNA, cut with the *SalI* restriction endonuclease, rendered a 2.3-kb band aside from the known *ompS1* 5.6-kb band (16). This 2.3-kb band was conspicuous, as it did not correspond to other known porin genes: it was particularly evident upon hybridization with *S. enterica* serovar Typhi *ompS1* (Fig. 1).

Thus, the 2.3-kb *SalI* fragment containing the *S. enterica* serovar Typhi *ompS2* gene was isolated from a recombinant *S. enterica* serovar Typhi genomic library constructed in pUC18, as described in Materials and Methods. Analysis of the nucle-

otide sequence of the 2.3-kb fragment allowed the identification of an open reading frame (ORF) coding for a 362-amino-acid mature protein (40,043 Da) plus a putative 21-amino-acid leader peptide. Homology searches revealed that the nucleotide sequence of this ORF shares the following identities with other porin genes: *Klebsiella pneumoniae kpn37*, 77%; *E. coli ompN*, 76%; *S. enterica* serovar Typhi *ompS1*, 72%; *E. coli ompC*, 72%; *S. enterica* serovar Typhi *ompC*, 71%; *S. enterica* serovar Typhi *ompF*, 66%; *E. coli ompF*, 66%. This gene, thus named *ompS2*, shares 98 and 90% identity with its counterparts in *S. enterica* serovar Typhimurium and *S. enterica* serovar Paratyphi A, respectively. Furthermore, Southern blot hybridization with *S. enterica* serovar Typhi *ompS2* as a probe rendered a strong hybridization signal with a 2.3-kb *SalI* fragment not only with *S. enterica* serovar Typhimurium and *S. enterica* serovar Paratyphi A but also with various other salmonellae DNA (data not shown), indicative of its high conservation. Hence, the hybridizing bands labeled *ompS1* and *ompS2* actually correspond to a truncated ORF (36) and to the *ompN* gene, respectively, in *E. coli* (Fig. 1).

Analysis of the deduced amino acid sequence showed that the OmpS2 protein maintains the pattern of alternate variable (loop) and conserved (β -strand) regions characteristic of the porin superfamily (12, 24), with the conservation of the Arg-37, Arg-75, Asp-106, and Arg-126 that have been implicated in determining pore permeability (4, 34). The similarities and identities of the OmpS2 sequence with the amino acid sequences from other porins are as follows: *K. pneumoniae* Kpn37, 87 and 83%; *E. coli* OmpN, 84 and 84%; *S. enterica* serovar Typhi OmpS1, 76 and 72%; *S. enterica* serovar Typhi OmpC, 76 and 71%; *E. coli* OmpC, 73 and 69%; *S. enterica* serovar Typhi OmpF, 68 and 64%; *E. coli* OmpF, 68 and 64%.

The highest identity of *S. enterica* serovar Typhi *ompS2* outside of the salmonellae was with the *K. pneumoniae kpn37* and *E. coli ompN* genes and the respective proteins (12, 41). Nevertheless, *ompS2* in *S. enterica* serovar Typhi is located downstream of the *rstA* gene and upstream of an ORF, for which no homology was found to a characterized gene. On the other hand, *ompN* in *E. coli* is not located at an equivalent site: it maps at centisome 30.9 between the *ynaF* and *ydbK* loci, while the *rstA-rstB* operon is located at centisome 36.21 (Entrez Genome, National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?db=Genome]).

***ompS2* expression.** The *ompS2* regulatory region (Fig. 2) shares only 34 to 38% identity with those from other porin genes, including *kpn37* and *ompN*, its putative counterparts in *K. pneumoniae* and *E. coli*, respectively (12, 41). In contrast, for instance, the regulatory regions of the *ompC* and *ompF* homologues in *S. enterica* serovar Typhi and *E. coli* share 75 and 77% identity, respectively.

A 491-bp DNA segment, encompassing 413 bp upstream of the first transcribed nucleotide, plus 63 transcribed and non-translated nucleotides (leader mRNA) and the first 5 codons of the structural gene, was used to generate a translational fusion to the *lacZ* reporter gene, thus constituting plasmid pFM413 (Fig. 2; Table 1). This region corresponds to the 5' upstream sequence present in plasmid pFM413S2, which in addition, contains the whole structural *ompS2* gene (Table 1). Similarly, a series of plasmids containing shorter portions of the regula-

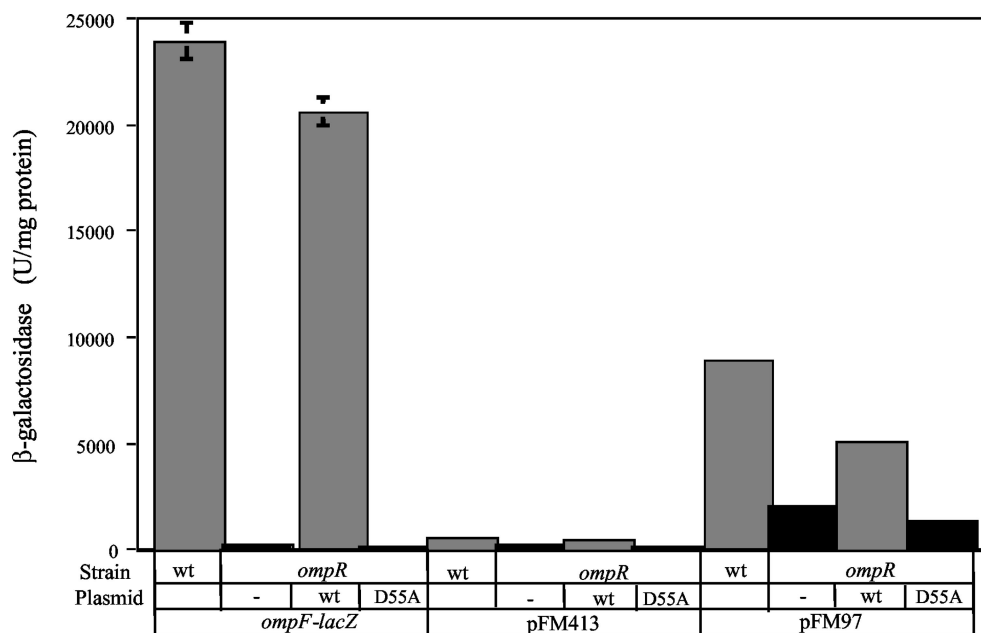


FIG. 4. Effect of the OmpR D55A mutant on *ompS2* activity. Activities are shown for an *S. enterica* serovar Typhi *ompF-lacZ* fusion, used as a control, and of pFM413 and pFM97 in either the IMSS-1 wild type (wt) or in the isogenic *S. enterica* serovar Typhi IMSS-40 *ompR* derivative ($\Delta ompR::Km$), not complemented (-) or complemented with either a plasmid harboring the wild-type (wt) OmpR gene or the D55A mutant gene, mutated in the codon for the main phosphorylation site (11).

tory region were generated, as described in Materials and Methods (Fig. 2; Table 1).

As can be seen in Fig. 3A, expression in wild-type *S. enterica* serovar Typhi increased more than 10-fold upon removal of 317 bp, between positions -413 and -97, as shown by the relative activities directed by the fusion plasmids pFM413 and pFM97. Hence, this region contains negative-control elements that maintain low levels of *ompS2* expression. On the other hand, the absence of 12 residues in pFM85 resulted in a significant decrease in expression (about fourfold) in comparison with pFM97 (Fig. 3). Thus, a relevant region for positive control of expression is located downstream of position -97. Figure 3B depicts the mapping of the *ompS2* promoter by primer extension from reporter plasmids. Accordingly, expression is the lowest from pFM413 and increases substantially upon removal of the 317 bp upstream of -97, in pFM97, or by the addition of the plasmid carrying the cloned *leuO* gene, *ptrleuO* (see below). No other promoter was detected downstream of the major transcriptional start (+1). The relative increase in expression in pFM34 is from the only promoter and is likely due to the formation of a -35-like box upon fusion of the plasmid sequences and the *ompS2* regulatory sequences, i.e., TTCCC/GCTGAAA (consensus residues are underlined).

Dependence of *ompS2* expression on OmpR. As observed in Fig. 3, most of the active *ompS2-lacZ* fusions (particularly pFM97) in the wild-type strain showed lower levels of expression in the isogenic *S. enterica* serovar Typhi 81 mutant strain ($\Delta ompB$), similar to those for pFM85. Furthermore, *ompS2* full expression from the most-active pFM97 construct was dependent on the presence of wild-type OmpR (Fig. 4). This OmpR-dependent activation required the main OmpR phosphorylation site, since a significant lowering in reporter gene

activity was observed in the presence of the OmpR D55A variant (11). The fact that there is a residual expression both in the absence of OmpR and in the presence of the OmpR D55A derivative reflects the promoter strength in the absence of OmpR and of the upstream negative regulatory region.

OmpS2 is an OMP. Figure 5 illustrates the presence of OmpS2 in the outer membrane upon expression from plasmid pFM97S2 (Table 1), which contains the *ompS2* structural gene expressed under a regulatory region encompassing 97 bp upstream of the transcriptional start point. This region directed the highest *ompS2-lacZ* activity (Fig. 3). In contrast, no expression was observed from plasmid pFM413S2 (Fig. 5), where the structural gene was under the control of 413 bp upstream of the transcriptional start, also in accord with the translational fusion studies (Fig. 3). The amount of OmpS2 in the outer membrane was determined by densitometric scanning relative to the corresponding OmpA protein (S2/A). Similarly, the amount of OmpF was determined in some of the lanes where the osmolarity was changed (F/A) (Fig. 5, bottom). No effect by osmolarity was observed on OmpS2 expression from pFM97S2, compared with the expected lowering of expression at high osmolarity for OmpF (43). The same result was obtained when expression from the pFM97 *ompS2-lacZ* fusion was assessed at low and high osmolarity (data not shown).

In accordance with the general pattern of the *lacZ* fusion (Fig. 3) and transcriptional data obtained by primer extension (data not shown), expression of the OmpS2 protein from pFM97S2 was lowered in an *S. enterica* serovar Typhi $\Delta ompB$ background (Fig. 5).

LeuO positively regulates *ompS2*. In a search for additional proteins involved in the regulation of *ompS2*, random mu-

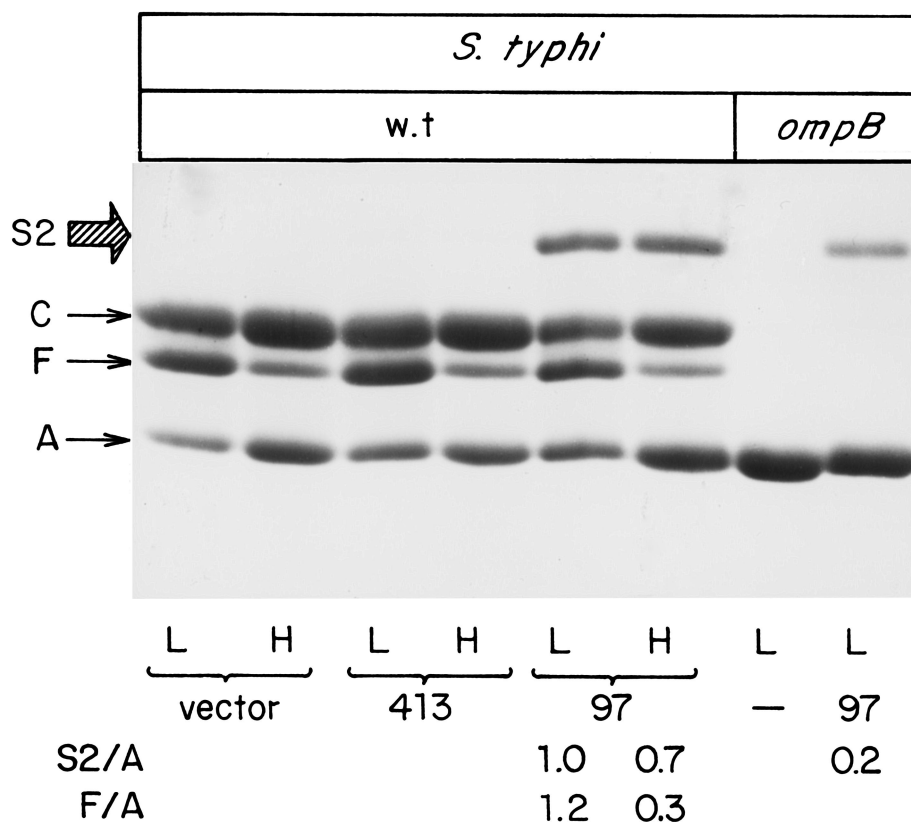


FIG. 5. Expression of the OmpS2 protein. PAGE profile of OMP preparations from wild-type (wt) *S. enterica* serovar Typhi, grown at either low (L) or high (H) osmolarity, and harboring either the pBR322 vector (vector), pFM413S2 (413; containing the structural *ompS2* gene under the control of a 413-bp regulatory region), or pFM97S2 (97; *ompS2* under a 97-bp regulatory region), *S. enterica* serovar Typhi $\Delta ompB::Km$, containing either no plasmid (-) or pFM97S2 (97), at low osmolarity. The major OMPs OmpC, OmpF, and OmpA are indicated as C, F, and A, respectively. The relative abundance of the OmpS2 (S2) or OmpF (F) protein with respect to OmpA (A) is shown for some lanes and was obtained by densitometric analysis.

tagenesis of wild-type *S. enterica* serovar Typhi IMSS-1 with transposon Tn10 rendered 3 transformants, from a total of 30,000, with increased expression of reporter plasmid pFM413. In all three, the transposon inserted upstream of the *leuO* gene, which codes for the LeuO regulator, a member of the LysR family of regulatory proteins (21).

In one mutant, IMSSTN103, the site for Tn10 insertion was 160 bp upstream of the first ATG and 60 bp downstream of the promoter, as determined by nucleotide sequence analysis (data not shown). The β -galactosidase activity was quantified for the pFM413 reporter plasmid (Fig. 6A), and the expression of the OmpS2 protein from the chromosomal gene was observed upon electrophoresis of OMP preparations (Fig. 6B). Interestingly, expression was higher at high osmolarity in both instances. The amount of OmpS2 in the outer membrane was quantified by densitometric scanning, relative to the corresponding OmpA protein (S2/A). As a control, the amount of OmpF (F/A) was lower at high osmolarity in the absence of cloned *leuO* (Fig. 6B), as expected (43). With respect to the lowering of OmpC and OmpF in the outer membrane in IMSSTN103 at high osmolarity (Fig. 6B), it appears to be due to a posttranslational effect, as the expression of *ompC-lacZ* and *ompF-lacZ* translational fusions in IMSSTN103 was not affected compared to the wild type (data not shown).

It is worth noting that it has been previously observed that the insertion of a Tn10 derivative, 26 or 19 bp upstream of the *leuO* ORF, caused a negative effect on the expression of the *rpoS* gene or a positive effect on the expression of the *bgl* operon, respectively. Hence, it was postulated that *leuO* expression increased due to the activity of the P_{out} promoter from the IS10 element, and therefore, the LeuO regulator exerted an effect on either *rpoS* or *bgl*, respectively (25, 52).

To test the idea that *ompS2* expression was due to an increase in expression of *leuO* in IMSSTN103, the *S. enterica* serovar Typhi *leuO* gene together with the upstream Tn10 insertion was amplified by PCR and cloned to render pFM800TL (Table 1). Transformation of wild-type *S. enterica* serovar Typhi IMSS-1 with pFM800TL, harboring pFM413, resulted in a substantial increase in *ompS2-lacZ* reporter activity, being higher at high osmolarity (Fig. 7A), similar to what was observed in the IMSSTN103 insertion mutant (Fig. 6). This effect did not occur in the isogenic *ompR* derivative IMSS-40 (Fig. 7A). Thus, besides supporting the notion that the increase in *ompS2* expression was the result of augmented levels of LeuO, the dependence on the OmpR transcriptional regulator was further substantiated.

To observe the effect of osmolarity on *ompS2* expression in the absence of the transposon upstream of *leuO*, the structural

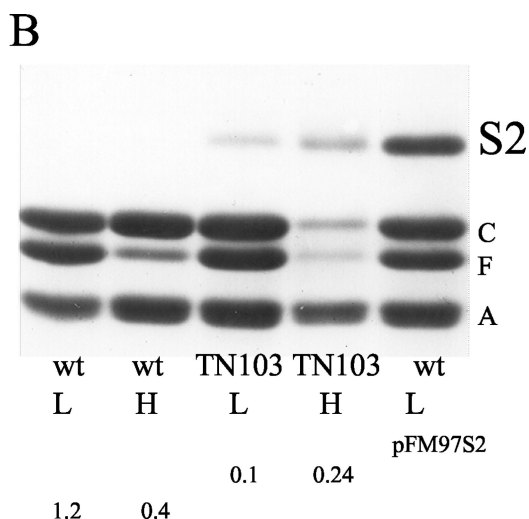
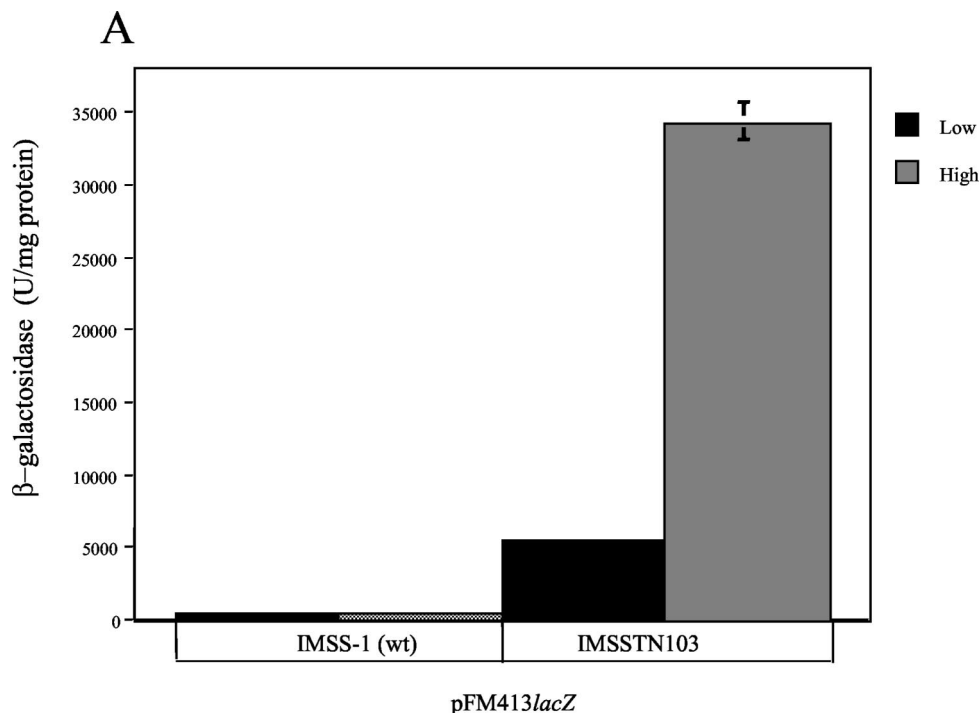


FIG. 6. LeuO participates in the regulation of *ompS2*. (A) Activity of *ompS2* from pFM413, both in *S. enterica* serovar Typhi IMSS-1 (wild type [wt]) and in the IMSSTN103 insertion mutant (Tn10::leuO), at low (black bars) and high (gray bars) osmolarity. (B) PAGE profile of OMP preparations from *S. enterica* serovar Typhi IMSS-1 (wt) and from the IMSSTN103 insertion mutant (TN103) at low (L) and high (H) osmolarity, depicting the expression of OmpS2 (S2) from the chromosomal gene. The expression of OmpS2 from pFM97S2 was included as a control marker. The major OMPs OmpC, OmpF, and OmpA are indicated as C, F, and A, respectively. The relative abundance of the OmpS2 (S2) or OmpF (F) protein with respect to OmpA (A) is shown for some lanes and was obtained by densitometric analysis.

leuO wild-type gene was amplified by PCR and cloned into vector plasmid pFMTrc12 under the control of the *trc* promoter to render pFMTrcleuO (see Materials and Methods). Again, the activity of the pFM413 reporter gene was increased in the wild type upon transformation with pFMTrcleuO, and the level of expression was higher at high than at low osmo-

larity (Fig. 7B), in a similar manner as observed when *leuO* was cloned together with the upstream inserted transposon (Fig. 7A) or in the IMSSTN103 insertion mutant (Fig. 6).

Binding of LeuO and OmpR-P to the *ompS2* 5' regulatory region. The experiments described above indicated that both the LeuO and the OmpR regulators were involved in the positive regulation of *ompS2* expression. Thus, to determine whether LeuO and OmpR directly interacted with the 5' upstream regulatory region of *ompS2*, DNA footprinting experiments were performed. As can be seen in Fig. 8, protection with LeuO was obtained at 0.03 μ M and higher, upstream of position -91 at two boxes, named LeuO (I) and LeuO (II) (also shown in Fig. 2). In the presence of OmpR-P, LeuO also bound to both boxes I and II, and there was an enhanced sensitivity to DNase digestion at -118, indicative of a change in conformation upon binding of both regulators (Fig. 8). OmpR-P bound at 0.8 μ M and above, at and around a 20-bp sequence with similarity to the OmpR-binding F1 and C1 type boxes (20) (Fig. 8; also Fig. 2). Interestingly, OmpR-P bound at 0.2 μ M and above, in the presence of LeuO.

DISCUSSION

The recent interest in quiescent porins, such as *ompS1* in *S. enterica* serovar Typhi (16, 36), *nmpC* and *ompN* in *E. coli* (41), and *ompK37* in *K. pneumoniae* (12), has opened many questions regarding their role in bacterial physiology. It is of interest that the *S. enterica* serovar Typhi *ompS2* gene shows a high identity with *K. pneumoniae ompK37* (12) and with *E. coli ompN* (41) and that all three genes contain complete ORFs that are strongly down-regulated, although there is much less conservation in their regulatory regions. In contrast, the counterpart of *S. enterica* serovar Typhi *ompS1* is truncated in

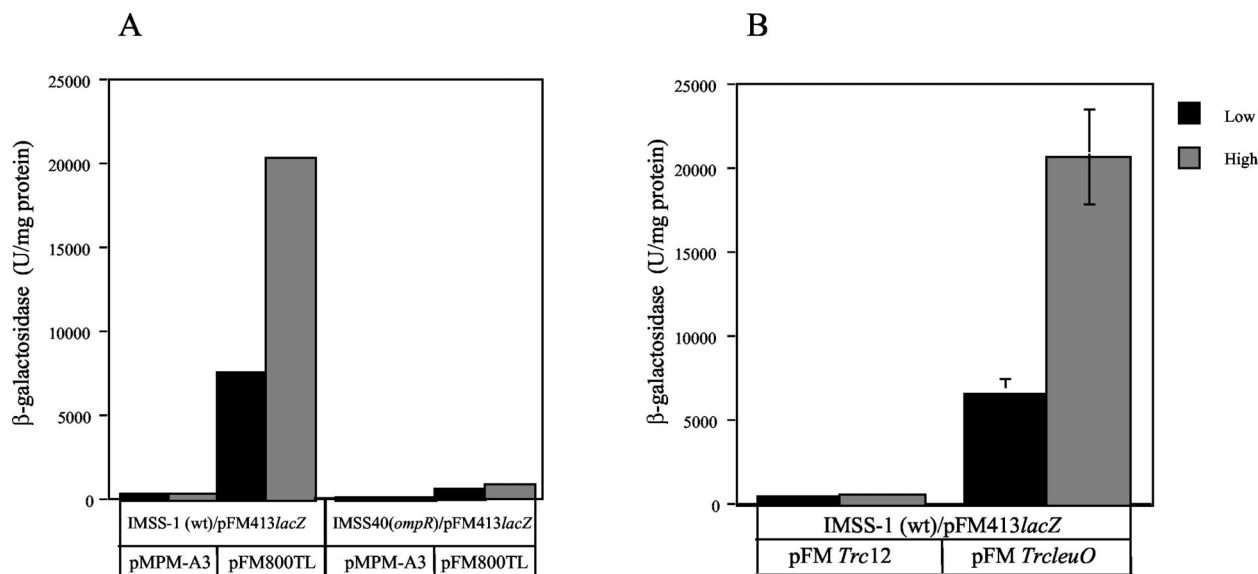


FIG. 7. Induction of *ompS2* expression in the presence of cloned *leuO*. β -Galactosidase-specific activity from pFM413 is shown. (A) *S. enterica* serovar Typhi IMSS-1 (wild type [wt]) or the isogenic IMSS-40 $\Delta ompR$ derivative, transformed with either the plasmid vector (pMPPM-A3) or pFM800TL, harboring the cloned *leuO* gene with the Tn10 insertion in the upstream region, at low (black) and high (gray) osmolarity. (B) *S. enterica* serovar Typhi IMSS-1, transformed with the pFM*Trc12* vector plasmid or with the pFM*TrcleuO* plasmid, carrying *leuO* under the control of the *trc* promoter, at low (black) and high (gray) osmolarity.

E. coli, an observation that opens thoughts on the evolutionary circumstances that caused its loss of function (36). The porin properties of *E. coli* OmpN have been found to resemble those of *E. coli* OmpC (41); hence, even though some variation in size and in anion or cation selectivity has been observed among the pores formed by *E. coli* and *S. enterica* serovar Typhimurium, no clear functional difference has yet been established between them, and it is not readily apparent why a bacterium synthesizes a variety of structurally related porins (5, 18, 26).

Studies with various translational *lacZ* fusions, containing different portions of the *ompS2* 5' regulatory region (Table 1; Fig. 2 and 3), allowed the identification of both a negative-control region located between positions -413 and -97 and a positive-control region located downstream from position -97. It is worth noting the similarities with the *ompS1* gene, which also presents a negative and positive mode of regulation and where expression was increased upon removal of distal 5' upstream portions and lowered when 5'-proximal sequences were removed (36). With respect to the region for negative control (from -413 to -97), it could be determining a necessary structure for negative regulation or it could be a region for the binding of an unknown negative effector or both.

Expression of *S. enterica* serovar Typhi *ompS2* was lowered in isogenic $\Delta ompB$ and $\Delta ompR$ strains (Fig. 3 to 5 and 7), thus pointing towards a role of the EnvZ/OmpR two-component signal transduction system in *ompS2* expression, similar to what has been observed for *S. enterica* serovar Typhi *ompS1* (16, 36). Interestingly, overlapping the positive control region, between positions -88 and -69, there is a 20-bp box which is 50% identical to the high-affinity F1 and C1 type boxes found in the *E. coli ompF* and *ompC* regulatory regions: 5'-TTTCC GATAGTAACTTTTGT-3' (Fig. 2 and 8) (19, 39, 22, 20). (In all cases, underlining shows the most conserved residues.) The box contains the GXXAC central motif of the F1, F2, C1, and

C2 boxes plus other F1 and C1 consensus nucleotides (underlined).

Interestingly, OmpR-P protected this 20-bp box from DNase digestion, together with a downstream segment that contains the motif 5'-ACATAT-3', which is also present in the 3' portion of the F1 and C1 boxes and in the *Salmonella ssrA* A1 box, although in the latter two it is 5'-ACATCT-3' and 5'-ACAA TTT-3', respectively (15, 20) (Fig. 2 and 8). Moreover, the 3' portion of the *ompS2* 20-bp box contains the motif 5'-ACTT TTG-3', which is present in the 5' portion of the F1 and C1 boxes, although in the latter it is 5'-ACATTTTG-3' (20) (Fig. 2). The role of these motifs and of their distribution towards the binding of OmpR-P is certainly a matter of further research. In this respect, in the footprinting experiments, OmpR-P bound to the *ompS2* regulatory region at 0.8 μ M (Fig. 8), similar to what was observed for the footprinting of OmpR-P on *ssrA* box A1, which has been considered just below the highest affinity F1 and C1 boxes (15).

Moreover, the LeuO activator of the LysR family was identified (21) as a regulator for *ompS2*. Upon random mutagenesis, a mutant with increased expression of *ompS2* was found where the Tn10 element had been inserted upstream of the *leuO* structural gene and downstream of its native promoter (Fig. 6). The increase in *leuO* expression upon the upstream insertion of a Tn10 element has been previously observed and substantiated in a similar manner as done here, upon analysis of collections of random mutants to establish regulators for the *bgl* operon and for the DsrA small regulatory RNA (25, 52). In this respect, *ompS2* expression substantially increased in wild-type *S. enterica* serovar Typhi upon transformation with cloned *leuO*, either together with the upstream inserted Tn10 element (pFM800TL) (Fig. 7A) or under the control of the *trc* promoter (pFM*TrcleuO*) (Fig. 7B). This is consistent with the

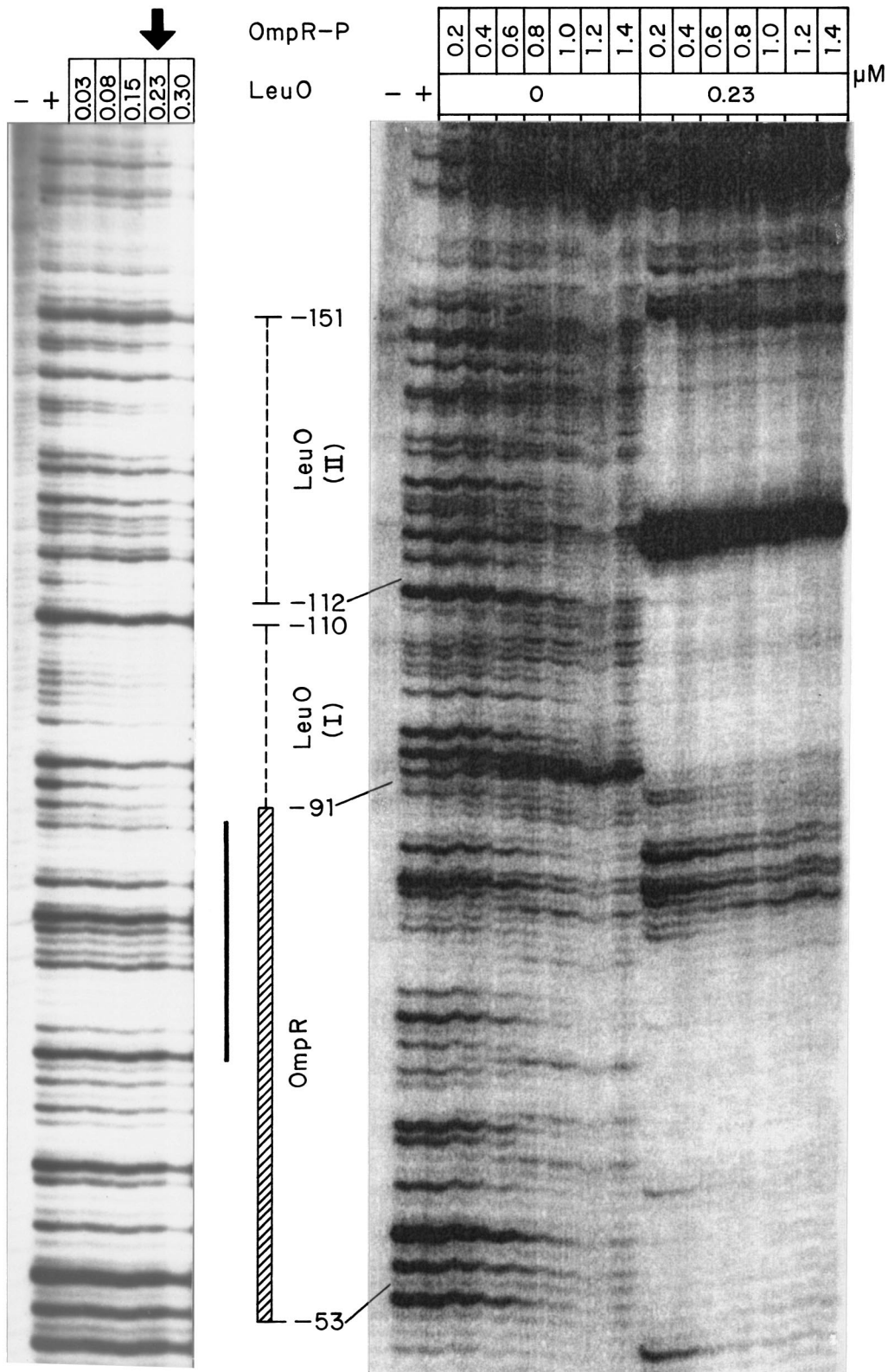


FIG. 8. DNA footprinting at the 5' regulatory region of *ompS2* with LeuO and OmpR-P. On the left panel, the DNase-minus/protein-minus lane is shown with a minus sign (-) and the DNase-plus/protein-minus lane is depicted with a plus sign (+). The following lanes contained 0.03, 0.08, 0.15, 0.23 (vertical arrow), and 0.30 μ M LeuO, respectively. The right panel also contains a DNase-minus/protein-minus lane (-) and a DNase-plus/protein-minus lane (+), followed by seven lanes with increasing concentrations of OmpR-P (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4 μ M). The next lanes contain the same increasing concentrations of OmpR-P but in the presence of 0.23 μ M LeuO. Binding boxes LeuO (I) and LeuO (II) are shown, as is the binding region for OmpR. The solid black bar represents the 20-bp sequence with similarity to the OmpR-binding F1 and C1 type boxes.

notion that an increase in the expression of LeuO causes an increase in *ompS2* expression.

LeuO bound to the 5' upstream regulatory region of *ompS2* in two defined boxes, LeuO (I) and LeuO (II), at 0.03 μ M and higher (Fig. 2 and 8). The lowest concentration is similar to the concentrations of LeuO used to protect the 25-bp AT7 box, which is part of the AT4 box, upstream of *leuO* and involved in LeuO binding, as defined in another footprinting study (9). Interestingly, Box II shares the motif 5'-AATCACA-3' with the AT7 box and the 5'-AATCA-3' motif with motif III, which is required for the LeuO-mediated negative effect on *dsrA* expression, as assessed with gene reporter fusions (46). Box I has a similar 5'-ATTCACCA-3' motif to the one in box II (Fig. 2 and 8). Interestingly, as can be seen in Fig. 8, in the presence of LeuO there was an increased the affinity of OmpR-P for the regulatory region.

Our present model for *ompS2* expression implies that LeuO relieves repression by disrupting either a structure in the region from -413 to -97 or by antagonizing the effect of an unknown repressor that binds to such region or both. Thus, OmpR-P would then be allowed to bind at and around the consensus OmpR-binding box, permitting *ompS2* expression mostly at high osmolarity, where there is a higher abundance of OmpR-P (41). This is based on the following observations. The addition of an active cloned *leuO* gene allowed expression of the pFM413 construct at the level of the pFM97 construct, i.e., it had the same effect as the removal of the -413 to -97 region (Fig. 3). This was observed at low osmolarity, although expression increased at high osmolarity (Fig. 6 and 7). Furthermore, LeuO and OmpR-P bound in close proximity at the 5' regulatory region (Fig. 2 and 8), and the activity rendered by pFM97 required phosphorylation at the major OmpR D55 residue (Fig. 4).

It is of interest that the effect of osmolarity was not observed in the expression of the OmpS2 protein under a -97 bp regulatory region (Fig. 5), yet it was evident upon expression from the chromosomal gene in the IMSSTN103 mutant (Fig. 6B) and from the full-length regulatory region reporter fusion (Fig. 6A and 7). This implies that OmpR-P is especially relevant in the presence of a full-length regulatory region and of the LeuO regulator. Hence, when the -413 to -97 region is absent, even relatively low concentrations of OmpR-P at low osmolarity would be sufficient for full expression. Thus, it is possible that osmolarity is affecting the structure of the -413 to -97 region, allowing for higher expression at high osmolarity. In this respect, no osmoregulation of *leuO* expression was observed by primer extension studies on pFMTr*leuO* (data not shown), in support of a specific effect of osmolarity on *ompS2* expression.

Hence, there are several similarities between *ompS1* and *ompS2* regarding their mode of expression. They both have an upstream negative regulatory region: in *ompS1* it encompasses bp -310 to -88 upstream of the first transcribed nucleotide (36), and in *ompS2* it encompasses bp -413 to -97. Also, they are both positively regulated by OmpR-P. On the other hand, a positive regulator such as LeuO has only been found for *ompS2*, although there is the distinct possibility that there is such a regulator for *ompS1*.

Certainly, the precise aspects of the mechanism of *ompS2* activation and repression remain to be solved, together with the environmental cues that trigger *ompS2* expression. In this

respect, the stoichiometry of the OmpR-P and LeuO interactions with the *ompS2* regulatory region, as well as the possible involvement of the H-NS nucleoid protein in repression should be assessed, together with the physiological conditions that affect the expression of both *leuO* and *ompS2*. Recently, a homologue of *ompS2* has been implicated in the pathogenesis of *Edwardsiella tarda* in fish (45), suggestive of signals in the host that trigger its expression.

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