JOURNAL OF BACTERIOLOGY, Oct. 2003, p. 5901–5905 Vol. 185, No. 19
0021-9193/03/$08.00+0 DOI: 10.1128/JB.185.19.5901–5905.2003

Global Regulation of the *Salmonella enterica* Serovar Typhimurium Major Porin, OmpD

Carlos A. Santiviago,[1] Cecilia S. Toro,[2] Alejandro A. Hidalgo,[1] Philip Youderian,[3] and Guido C. Mora[1]*

Unidad de Microbiología, Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile,[1] and Programa de Microbiología y Micología, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile,[2] Santiago, Chile, and Department of Biology, Texas A&M University, College Station, Texas 83843-3052[3]

Received 31 March 2003/Accepted 9 July 2003

The OmpD porin is the most abundant outer membrane protein in *Salmonella enterica* serovar Typhimurium and represents about 1% of total cell protein. Unlike the case with the less abundant OmpC and OmpF porins, the stoichiometry of OmpD in the outer membrane does not change in response to changes in osmolarity. The abundance of OmpD increases in response to anaerobiosis and decreases in response to low pH, conditions encountered by serovar Typhimurium during the infection of its murine host. By constructing an operon fusion of the *lacZY* genes with the *ompD* promoter, we show that the abundance of OmpD in the outer membrane is regulated primarily at the level of transcription and is subject to catabolite repression. In response to anaerobiosis, the abundance of OmpD in the outer membrane also appears to be controlled posttranscriptionally by a function dependent on Fnr.

The *Salmonella enterica* serovars Typhi and Typhimurium cause lethal systemic infections in humans and mice, respectively. Serovar Typhi causes typhoid fever in humans, which kills more than 600,000 people annually (18). Because these serovars are close genetic relatives, the study of the pathogenic mechanisms by which serovar Typhimurium causes a lethal infection in mice serve as a model for understanding the pathogenic mechanisms of serovar Typhi in its exclusive human host.

Although these serovars are closely related, the determination of the complete genome sequences of strains of serovar Typhi (19) and serovar Typhimurium (13) show that about 20% of their genes are different and unique to each species. These differences are clustered mainly into islets and islands of genes that represent insertions or substitutions in one genome sequence with respect to the other (2).

Recently we have shown that serovar Typhi has a deletion of an islet of genes present in serovar Typhimurium and in all other serovars of *S. enterica* that we have tested. This region, which maps at minute 33.7 on the serovar Typhimurium chromosome, includes two adjacent genes, *ompD* and *yddG*, which are not present on the serovar Typhi genome (21, 22). The *ompD* gene encodes the most abundant protein in the outer membrane, a porin similar in primary amino acid sequence to the major porins OmpC, OmpF, and PhoE. Together, OmpD, OmpC, and OmpF account for about 1×10^5 to 2×10^5 porin molecules per cell (17). Under favorable growth conditions, the OmpD porin represents about half of the porins and has a stoichiometry comparable to that of the ribosome. The *yddG* gene encodes an inner membrane protein that is a member of the drug/metabolite transporter superfamily of proteins. We have shown that both *ompD* and *yddG* are required for resistance to methyl viologen. These results suggest that YddG may function together with OmpD in the efflux of methyl viologen from the cytoplasm to the extracellular space and that the porins may play critical roles in the efflux of toxic substrates, as well as in the influx of substrates across the outer membrane (21).

Like *Escherichia coli* K-12, both serovar Typhimurium and serovar Typhi also have OmpC and OmpF as abundant proteins in their outer membranes. The *E. coli* and serovar Typhimurium OmpF and OmpC porins are regulated reciprocally by the osmolarity of the growth medium. With high osmolarity, OmpC is more abundant in the bacterial outer membrane, whereas OmpF is less abundant; the converse is true at low osmolarity (5, 9). Regulation of the relative abundance of OmpC and OmpF depends on the *ompB* locus, which includes the genes *envZ* and *ompR*, encoding a sensor kinase and its cognate response regulator, respectively. The phosphorylated form of OmpR binds sites within the *ompF* and *ompC* promoters to regulate the transcription of these genes (6, 15).

To understand how the expression of the OmpD porin is regulated in wild-type serovar Typhimurium strain LT2, we began by examining environmental factors known to be involved in the regulation of expression of the major porins of *E. coli* K-12, including osmolarity, oxygen availability, and low pH (1, 5, 16). To extend our analysis, we also made mutants of LT2 defective in potential regulators of *ompD* expression. We constructed isogenic derivatives of LT2 carrying combinations of mutations in the *fnr* (formerly *oxrA*), *ompD*, *envZ*, *cya*, and *crp* genes by generalized transduction with P22 HT105/1 *int201* (23), using donor strains MST2944 (*ompD159*::Tn*10 galE496*), MST2400 (*envZ1005*::Mu*d*-P), MST2970 (*fnr2*::Tn*10 leu485*), MST2596 (*cya961*::Tn*10*), and MST2882 (*crp773*::Tn*10*). (A significant part of this work was presented as the undergradu-

* Corresponding author. Mailing address: Unidad de Microbiología, Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Alameda 340, Santiago, Chile. Phone: (56) 2686 2847. Fax: (56) 2222 5515. E-mail: gmora@genes.bio.puc.cl.

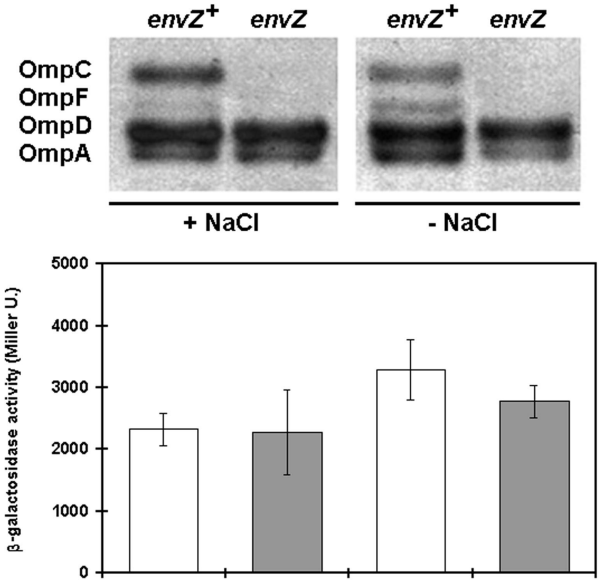

FIG. 1. The abundance of OmpD in the outer membrane of serovar Typhimurium is not affected by changes in osmolarity or by a mutation in the *envZ* gene. At the top, the gel shows that otherwise isogenic *envZ*$^+$ and *envZ1005*::Mu*d*-P strains have similar proportions of OmpD in their outer membranes under conditions of moderate (+ NaCl) and low (− NaCl) osmolarity. Outer membrane proteins were prepared from cells grown in rich (LB) medium with and without NaCl. Only the portions of 12.5% polyacrylamide gels with bands corresponding to the major porins are shown. At the bottom, the levels of β-galactosidase activity made by otherwise isogenic *ompD1*::Mu*d*-J (SC7) (open bars) and *ompD1*::Mu*d*-J *envZ1005*::Mu*d*-P (SC9) (shaded bars) strains are compared. Cultures were grown to an optical density at 600 nm of 0.2 and permeabilized with chloroform and 0.1% SDS, and β-galactosidase activity was measured by the method of Miller (14). Activities are expressed in Miller units, $10^3(A_{420} - [1.75 \times A_{550}])/(\text{ml} \times \text{min} \times A_{600}^{-1})$, and represent the averages of at least three independent determinations.

ate thesis of C.A.S. as part of the requirements for the Master of Science degree in biochemistry from the University of Chile.)

Osmolarity does not affect OmpD regulation. To measure the relative abundance of OmpD in the serovar Typhimurium outer membrane, we prepared outer membrane fractions from cells grown at 37°C in Luria-Bertani (LB) medium as described by Schnaitman (24) and modified by Lobos and Mora (11), resolved the proteins in these fractions by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in 12.5% slab gels, and revealed these proteins by staining with Coomassie blue. Figure 1 shows that when wild-type strain LT2 is grown aerobically in LB medium at 37°C, it produces four abundant porins, OmpC, OmpF, OmpD, and OmpA, among which OmpD is the most abundant. For serovar Typhimurium, as for *E. coli* K-12, the abundance of OmpC relative to that of OmpF in the outer membrane decreases in response to decreasing osmolarity. The production of both OmpC and OmpF by serovar Typhimurium is dependent on *envZ*. A mutant derivative of LT2, SC2, with an *envZ1005*::Mu*d*-P insertion makes neither OmpC nor OmpF, as reported previously (10). In contrast, the relative abundance of OmpD in the outer membrane does not change in response to changes in osmolarity, nor is it dependent on *envZ* function. This is also the case when cells are grown in LB medium at very high osmolarity (10% sucrose or 0.3 M NaCl; data not shown).

The abundance of OmpD in the outer membrane increases in response to anaerobiosis. During the infection of its murine host, serovar Typhimurium must survive environments low in oxygen and pH. Because the outer membrane provides the first line of defense against unfavorable extracellular environments, the ability to change the composition of the outer membrane rapidly in response to environmental changes may be critical for virulence. Previously, we have found that the expression of a variety of serovar Typhimurium outer membrane proteins is induced by anaerobiosis (unpublished results). As shown in Fig. 2, in response to anaerobiosis, the relative abundance of OmpD in the outer membrane of wild-type cells roughly doubles with respect to other outer membrane proteins. Because OmpD is the major porin present in about 10^5 copies/cell under aerobic conditions, this twofold increase in OmpD corresponds to a dramatic change in the protein composition of the outer membrane. This result suggests that transcription of the *ompD* gene may be induced when oxygen is limiting.

In the absence of oxygen, serovar Typhimurium responds by utilizing alternative terminal electron acceptors or by activating fermentation pathways. Many of these responses to anaerobiosis are dependent on the global transcriptional regulator encoded by *fnr* (8, 25). Figure 2 shows that, in contrast with its wild-type parent, strain SC3 (*fnr2*::Tn*10*) does not have a

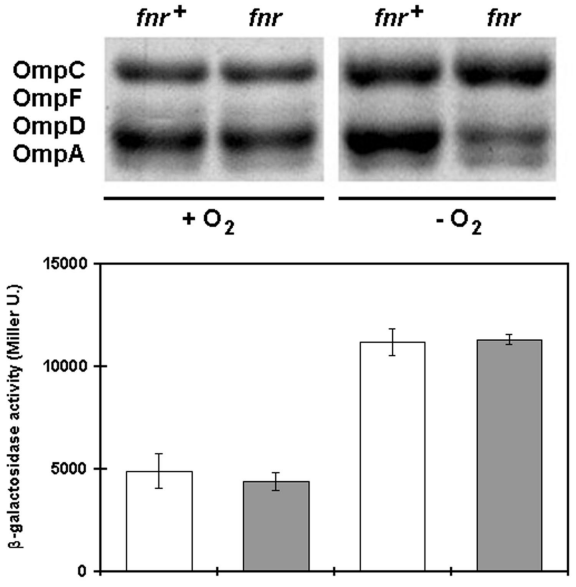

FIG. 2. The abundance of OmpD in the outer membrane of serovar Typhimurium doubles during anaerobic growth, dependent on *fnr* function. At the top are shown the outer membrane proteins prepared from otherwise isogenic *fnr*$^+$ and *fnr2*::Tn*10* cells grown in rich medium with (+ O_2) and without (− O_2) oxygen. At the bottom, the levels of transcription of the *ompD1*::Mu*d*-J operon fusion in the same genetic backgrounds (*fnr*$^+$ [open bars] and *fnr2*::Tn*10* [shaded bars]) are represented as β-galactosidase activities, as in the legend to Fig. 1. Note that although the level of transcription of the operon fusion doubles in both wild-type and *fnr* mutant genetic backgrounds (bottom), the abundance of OmpD in the outer membrane doubles only in the wild-type genetic background (top right) under anaerobic conditions.

higher percentage of OmpD in its outer membrane under anaerobic growth conditions. This result suggests that the transcription of the *ompD* gene is dependent on *fnr* function.

The relative abundance of OmpD that we observe in the outer membrane is the end product of a series of events dependent on the rates of *ompD* transcription and OmpD translation and the efficiency of insertion of OmpD in the outer membrane (as well as the stability of intermediates in this process). Because gene expression is regulated primarily at the level of transcription initiation, we tested whether the rate of transcription of the *ompD* gene is dependent on *fnr.* To do this, we constructed an operon fusion in which the *ompD* promoter is fused to the *lacZY* structural genes, by isolating a mutant with an *ompD::*Mu*d*-J insertion.

The increase in abundance of OmpD, but not in the rate of *ompD* transcription, in response to anaerobiosis is dependent on Fnr. To make a serovar Typhimurium *ompD*::Mu*d*-J mutant, we used the method of transitory *cis* complementation to generate a pool of mutants of serovar Typhimurium with Mu*d*-J insertions (7). The donor serovar Typhimurium strain TT10288 (*hisD*9953::Mu*d*-J *hisD*9941::Mu*d*-1) was grown in LB medium with ampicillin (30 μg/ml) and kanamycin sulfate (40 μg/ml) to exponential density and infected with phage P22 HT105/1 *int201*. Ten pools of each of 1,600 Kan^r transductants of recipient strain LT2 colonies were made on LB plates (1.5% agar), and each pool was infected with P22 to make a series of secondary transducing lysates. These secondary donor lysates were used to transduce the Tet^r recipient strain SC1 (*ompD159*::Tn*10*), and Tet^s Kan^r recombinants that form blue colonies on plates with fusaric acid and chlortetracycline to counterselect resistance to tetracycline (12) and 40-μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside were selected. Overnight cultures were prepared from four Lac^+ Kan^r Tet^s colonies and the profiles of their outer membrane proteins were analyzed by SDS-polyacrylamide gel electrophoresis, and two were found to be missing OmpD in their outer membrane. The *ompD1*::Mu*d*-J mutation in one of these strains was backcrossed into a wild-type LT2 recipient to yield strain SC7. To confirm that the Mu*d*-J insertion in SC7 is in *ompD*, the junctions of the Mu*d*-J insertion with the chromosome were amplified using the PCR with primer pairs MUR (5′-TTCGCAT TTATCGTGAAACGCTTCC) and OMPD1 (GACAAAGAC AAAACCCGTT) as well as MUL (TTCGTACTTCAAGT GAAT) and OMPD2 (CGTCCAGCAGGTTGATTTT). The sizes of the products of these amplifications, as well as the results of Southern analysis, show that the Mu*d*-J insertion is in the center of *ompD*.

Serovar Typhimurium does not have the *lac* operon. This fact enables us to use *lacZ* as a reporter for transcription in both wild-type and mutant serovar Typhimurium strains by introducing Mu*d*-J insertions. For example, the otherwise isogenic *envZ*$^+$ and *envZ1005*::Mu*d*-P strains whose outer membrane proteins are shown in Fig. 1 show no detectable β-galactosidase activity. When the *ompD1*::Mu*d*-J insertion is introduced into these genetic backgrounds, the recombinant *ompD1*::Mu*d*-J and *ompD1*::Mu*d*-J *envZ1005*::Mu*d*-P strains are found to produce similar levels of β-galactosidase activity under conditions of both low and high osmolarity. This result supports the idea that the transcription of *ompD* does not change in response to changes in osmolarity.

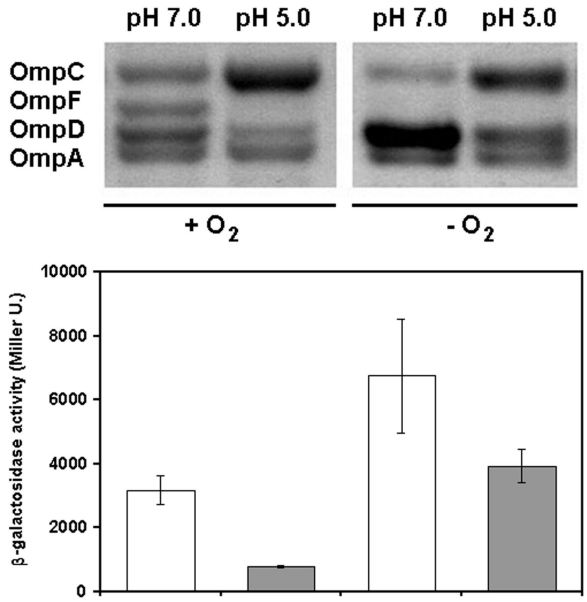

FIG. 3. The abundance of OmpD in the outer membrane of serovar Typhimurium decreases in response to lower pH. At the top are shown the profiles of outer membrane proteins prepared from cells grown in LB medium at pH 7.0 or at pH 5.0, aerobically (+ O_2) or anaerobically (− O_2). At the bottom, β-galactosidase activities made by the *ompD1*::Mu*d*-J strain grown under the same conditions (pH 7.0 [open bars] and 5.0 [shaded bars]) are presented as in Fig. 1 and 2.

As shown in Fig. 2, we compared the levels of β-galactosidase activity made by otherwise isogenic *ompD1*::Mu*d*-J *fnr*$^+$ (SC7) and *ompD1*::Mu*d*-J *fnr*2::Tn*10* (SC8) strains under aerobic and anaerobic growth conditions. Under anaerobic growth conditions, the β-galactosidase activity shown by the mutant *ompD1*::Mu*d*-J strain is slightly more than twice that shown by the same strain under aerobic conditions, a result that reflects what we observe when we assay the abundance of OmpD protein in the outer membrane of the wild-type parent of this strain under anaerobic versus aerobic growth conditions. In contrast, when we compare the levels of β-galactosidase activity shown by the *ompD1*::Mu*d*-J *fnr2*::Tn*10* strain under aerobic and anaerobic growth conditions, we find a different result. The *fnr* mutation has no effect on the expression of β-galactosidase activity from the operon fusion under anaerobic conditions compared to expression under aerobic conditions. These results show that *fnr* function does not play a direct role in the regulation of *ompD* transcription. Rather, the increase in the abundance of OmpD in the outer membrane in response to anaerobiosis must be due to an Fnr-dependent posttranscriptional regulatory mechanism. One possible step at which this *fnr*-dependent posttranscriptional control may occur is at the level of control of mRNA stability. The turnover of the transcripts of many genes is slowed under anaerobic conditions (4), and the extended half-life of mRNA species observed under anaerobic conditions may depend on an *fnr*-regulated function. Alternatively, the rate of degradation of OmpD may depend on an *fnr*-regulated function.

OmpD expression decreases in response to low pH. As shown in Fig. 3, when serovar Typhimurium is grown in LB medium at pH 5.0 versus pH 7.0, there is an increase in the

abundance of OmpC relative to that of OmpF at this lower pH, consistent with previously published results (3). Also, we find that OmpD is present in the outer membrane in significantly lower abundance at the lower pH. This down-regulation of the abundance of OmpD in response to low pH is most likely due to decreased transcription of the *ompD* gene. At the lower pH, the strain with the *ompD1*::Mu*d*-J reporter fusion shows about one-half the β-galactosidase activity as at the higher pH. Thus, the relative change in abundance of OmpD in response to pH is regulated at the level of transcription initiation. When cells are grown anaerobically at pH 5.0, however, the abundance of OmpD remains about the same as in cells grown aerobically at pH 7.0 (Fig. 3). This result shows that the mechanism of *fnr*-dependent posttranscriptional control of OmpD in response to anaerobiosis apparently acts independently of the mechanism of transcriptional control that results in a decrease in its abundance in response to low pH.

Transcription from the *ompD* promoter is activated by the cAMP/Crp complex. Catabolite repression, or the glucose effect, is a global regulatory system that coordinates the balanced expression of genes involved in the catabolism of alternative carbon sources. Because the OmpD porin, like the OmpC and OmpF porins, may be involved in the influx of nutrients including saccharides, we also tested whether *ompD* expression is regulated by catabolite repression. When the mutant *ompD1*::Mu*d*-J strain is grown in defined, minimal medium (E medium) (0.02 g of $MgSO_4 \cdot 7H_2O$/liter, 2 g of citric acid $\cdot H_2O$/liter, 13.1 g of $Na_2HPO_4 \cdot 3H2O$/liter, 3.3 g of $NaNH_4HPO_4 \cdot 4H_2O$/liter) supplemented with glucose (2 g/liter) or glycerol (2 g/liter), the levels of β-galactosidase activity made by the mutant under the two different growth conditions differ by about twofold (4,200 ± 700 versus 7,700 ± 1,500 Miller units, respectively), suggesting that the *ompD* promoter is sensitive to catabolite repression.

Catabolite repression in enteric gram-negative bacteria is dependent on the interaction of cyclic AMP (cAMP) with cAMP receptor protein (Crp) to form an active DNA-binding complex that activates transcription from some promoters and represses transcription at others. Assembly of this transcription factor requires the products of the *cya* and *crp* genes, encoding adenylyl cyclase and cAMP receptor protein, respectively (20). We constructed two double mutants of serovar Typhimurium carrying the *ompD1*::Mu*d*-J mutation paired with either the *cya961*::Tn*10* or *crp773*::Tn*10* mutation. When we grow the *ompD1*::Mu*d*-J *cya961*::Tn*10* double mutant (SC12) aerobically in minimal glucose medium, we find that this mutant shows extremely low levels of β-galactosidase activity, less than 1/10 the level of activity made by the otherwise isogenic wild-type parent. This effect can be reversed by the addition of 1 mM cAMP to the medium. Similarly, the *ompD*::Mu*d*-J *crp773*::Tn*10* double mutant (SC13) also shows extremely low levels of β-galactosidase activity when grown in minimal glucose medium, a phenotype that cannot be masked by the addition of cAMP (Table 1). Thus, the transcription of the *ompD* gene is dependent on positive activation by the cAMP/Crp complex. Direct measurements of the abundance of OmpD in outer membrane preparations of the same cells are consistent with this interpretation (data not shown). The sequence of the region upstream of the serovar Typhimurium *ompD* gene (bp 1655173 to 1656261 in GenBank NC_003197) includes a near-consensus cAMP/Crp binding site located 160 to 139 bp upstream of the ATG start codon of *ompD*; this may be the site required for the positive regulation of the *ompD* gene by Crp.

TABLE 1. Transcription of the *ompD* gene is regulated by catabolite repression[a]

Genotype	β-Galactosidase activity	
	+ glucose	+ glucose, cAMP
ompD1::Mu*d*-J	4,200 ± 700	ND
ompD1::Mu*d*-J *cya961*::Tn*10*	170 ± 50	3,800 ± 400
ompD1::Mu*d*-J *crp773*::Tn*10*	240 ± 50	270 ± 90

[a] Serovar Typhimurium strains carrying the *ompD1*::Mu*d*-J fusion in wild-type and mutant *cya* or *crp* genetic backgrounds were grown to exponential density at 37°C in minimal medium supplemented with 0.2% glucose or 0.2% glucose plus 1 mM cAMP, and β-galactosidase activities were determined as described in the text. Values (β-galactosidase activities in Miller units) represent the averages and standard deviations of at least three determinations. ND, not determined.

The serovar Typhimurium *ompD* gene is regulated similarly in serovar Typhi. Prior to the determination of the complete and draft genome sequences of many serovars of *S. enterica*, we found that the *ompD* gene is present in all serovars of *S. enterica* with the exception of serovar Typhi (22), results consistent with all currently available genome sequence data. As a complementary approach to understand the function of *ompD*, we constructed a serovar Typhi hybrid carrying the serovar Typhimurium *ompD* gene. This serovar Typhi hybrid expresses the OmpD porin in its outer membrane and has a gain-of-function phenotype, increased resistance to methyl viologen (21, 22).

To determine how the *ompD* gene is regulated in the serovar Typhi genetic background, we constructed eight derivatives of serovar Typhi with the genotypes *ompD*$^+$, *ompD159*::Tn*10*, *ompD*$^+$ *fnr2*::Tn*10*, *ompD*$^+$ *envZ1005*::Mu*d*-P, *ompD1*::Mu*d*-J, *ompD1*::Mu*d*-J *fnr2*::Tn*10*, *ompD1*::Mu*d*-J *envZ1005*::Mu*d*-P, *ompD1*::Mu*d*-J *cya961*::Tn*10*, and *ompD1*::Mu*d*-J *crp773*::Tn*10*, by transferring alleles from serovar Typhimurium donor strains to serovar Typhi recipients using P22-mediated generalized transduction. We repeated each of the experiments described above for serovar Typhimurium with these serovar Typhi strains and find that *ompD* is regulated in serovar Typhi in a manner identical to its regulation in serovar Typhimurium (data not shown).

In summary, we have found that the relative abundance of the OmpD porin in the outer membrane of serovar Typhimurium is regulated by a multiplicity of environmental factors, including anaerobiosis, lower pH, and catabolite repression. Unlike the porins OmpC and OmpF, the abundance of OmpD does not change under different conditions of osmolarity and does not depend on the *envZ-ompR* two-component regulatory system.

What is the physiological function of OmpD? We have shown that the *ompD* gene is necessary for resistance to the toxic compound methyl viologen (21). More recently, we have shown that at least two other porins, OmpA and OmpW, also are critical for resistance to toxic compounds (unpublished results). The regulation of the *ompD* gene may provide us with some clues to the function of OmpD. Because the transcription of *ompD* is dependent on catabolite repression and increases in response to anaerobiosis, we suspect that OmpD may be in-

volved in the efflux of toxic compounds made during the catabolism of alternative, poorer carbon sources.

We are indebted to Stanley Maloy for generous gifts of bacterial strains.

This work was funded by FONDECYT (Chile) grant 1020485 (to G.C.M.) and NIH Fogarty Center Senior International Fellowship TW05645 (to P.Y.). C.A.S. was supported by fellowships from CONICYT and DIPUC.

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