INFECTION AND IMMUNITY, Oct. 2005, p. 6210–6219 Vol. 73, No. 10
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.10.6210–6219.2005

The *Salmonella enterica* Serovar Typhi *tsx* Gene, Encoding a Nucleoside-Specific Porin, Is Essential for Prototrophic Growth in the Absence of Nucleosides

Sergio A. Bucarey,[1] Nicolás A. Villagra,[2] Mara P. Martinic,[1] A. Nicole Trombert,[2] Carlos A. Santiviago,[1] Nancy P. Maulén,[2]† Philip Youderian,[3] and Guido C. Mora[2]*

Departamento de Genética Molecular y Microbiología, Pontificia Universidad Católica de Chile,[1] and Departamento de Ciencias Biológicas, Universidad Andrés Bello,[2] Santiago, Chile, and Department of Biology, Texas A&M University, College Station, Texas[3]

Received 5 January 2005/Returned for modification 23 March 2005/Accepted 2 June 2005

The *Salmonella enterica* serovar Typhi *tsx* gene encodes a porin that facilitates the import of nucleosides. When serovar Typhi is grown under anaerobic conditions, Tsx is among the outer membrane proteins whose expression increases dramatically. This increase in expression is due, at least in part, to increased transcription and is dependent on Fnr but not on ArcA. A mutant derivative of serovar Typhi strain STH2370 with a deletion of the *tsx* gene is an auxotroph that requires either adenosine or thymidine for growth on minimal medium. In contrast, an otherwise isogenic *nupG nupC* double mutant, defective in the inner membrane nucleoside permeases, is a prototroph. Because anaerobic growth enhances the virulence of serovar Typhi in vitro, we assessed the role that the *tsx* gene plays in pathogenicity and found that the serovar Typhi STH2370 Δ*tsx* mutant is defective in survival within human macrophage-like U937 cells. To understand why the Δ*tsx* mutant is an auxotroph, we selected for insertions of minitransposon T-POP in the Δ*tsx* genetic background that restored prototrophy. One T-POP insertion that suppressed the Δ*tsx* mutation in the presence of the inducer tetracycline was located upstream of the *pyrD* gene. The results of reverse transcription-PCR analysis showed that addition of the inducer decreased the rate of *pyrD* transcription. These results suggest that the Tsx porin and the balance of products of the *tsx* and *pyrD* genes play critical roles in membrane assembly and integrity and thus in the virulence of serovar Typhi.

Salmonella enterica serovar Typhi is a facultative intracellular pathogen that causes typhoid fever in humans. *Salmonella* serovar Typhi infections are responsible for significant morbidity and mortality worldwide, and there are an estimated 21.5 million cases per year, most of which occur in developing countries (14). Systemic infection of mice by the closely related bacterium *Salmonella enterica* serovar Typhimurium has been studied as a model of the systemic infection caused by serovar Typhi in humans, its only known host and reservoir.

To establish a successful infection, serovar Typhi must be able to adapt to changing environmental conditions encountered within the human host and must respond to these conditions by altering the expression of various genes (24, 27, 48, 64). *Salmonella* serovar Typhi virulence is enhanced in the presence of low oxygen, high osmolarity, and alkaline pH, conditions that apparently reflect the conditions encountered in the host (2, 4, 15, 25, 39, 40).

The first step in the infection process involves contact between serovar Typhi and host cells. This step likely involves initial interactions between the bacterial O antigen and specific host cell surface receptors and subsequent interactions between the bacterial outer membrane proteins and the host cell. The most abundant outer membrane proteins made by serovar Typhi are the porins. These include OmpC and OmpF, which have more general substrate specificities (6, 33, 54), and PhoE, MalL (LamB), and Tsx, which facilitate the uptake of phosphates, maltodextrins, and nucleosides, respectively (16, 45, 71). Like OmpC and OmpF, the specialized porins are water-filled channels and function without an energy requirement; however, they contain saturable substrate-binding sites that may limit transport at low substrate concentrations (8, 43, 53).

Once inside the host, intracellular pathogens like serovar Typhi encounter an environment with a scarcity of nutrients, including purines, pyrimidines, and aromatic amino acids (9, 20, 21, 28, 42, 47, 56). These pathogens can import nucleosides to serve as precursors for nucleic acid synthesis (1, 30, 52); this involves passive transport of the molecules across the outer membrane into the periplasm, mediated by the Tsx porin, and their active transport across the inner membrane into the cytoplasm, mediated by the permeases NupC and NupG (13, 50, 55, 73).

In this paper, we show that expression of the serovar Typhi Tsx porin is induced upon anaerobic growth, environmental conditions encountered by *Salmonella* inside the host (11, 25, 37). To understand the role that Tsx plays in the physiology and pathogenicity of serovar Typhi, we constructed a mutant of a clinical isolate of serovar Typhi with a deletion of the *tsx* gene. To our surprise, we found that this deletion conferred an

* Corresponding author. Mailing address: Laboratorio de Microbiología, Departamento de Ciencias Biológicas, Universidad Andrés Bello, República 217, Santiago, Chile. Phone: 56-2-6618374. Fax: 56-2-6618390. E-mail: gmora@unab.cl.

† Present address: Facultad de Medicina, Universidad Católica de la Santísima Concepción, Concepción, Chile.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid(s)	Genotype and/or phenotype	Source
Salmonella serovar Typhi strains		
STH2370	Wild type	Hospital Lucio Córdova
TYT2053	STH2370 *tsx*::*cam*	This study
TYT2075	STH2370 Δ*tsx*	This study
TYT2080	STH2370 Δ*tsx*/pSU19::*tsx*	This study
TYT2220	STH2370 Δ*nupC*	This study
TYT2214	STH2370 Δ*nupG*	This study
TYT2230	STH2370 Δ*nupG* Δ*nupC*	This study
TYT3039	STH2370 *pyrD*::Tn*10*	This study
TYT2152	STH2370 *ompC*::Tn*10*	This study
TYT2160	STH2370 *ompF*::Mu*dJ*	This study
TYT3033	STH2370 Δ*ompA*	This study
TYT3040	STH2370 *tsx*::*lacZY*	This study
TYT3055	STH2370 *tsx*::*lacZY fnr*-2::Tn*10*	This study
TYT4567	STH2370 *tsx*::*lacZY arcA*::Tn*10*	This study
TYT2090	STH2370 Δ*tsx STY2025*::T-POP	This study
TYT2095	STH2370 Δ*tsx STY0014*::T-POP	This study
TYT2010	STH2370 Δ*tsx STY1399*::T-POP	This study
STH795	Wild type	Hospital Lucio Córdova
TYT3020	STH795 Δ*tsx*	This study
Ty2	Laboratory strain	S. Maloy
TYT4050	Ty2 Δ*tsx*	This study
Salmonella serovar Typhimurium strains		
14028s	Wild type	S. Maloy
SC113	14028s Δ*tsx*	This study
LT2	Laboratory strain	
MST2970	*fnr*-2::Tn*10*	S. Maloy
MST2345	*arcA*::Tn*10*	S. Maloy
MST2947	*ompC*::Tn*10*	S. Maloy
MST1062	*ompF*::Mu*dJ*	S. Maloy
MST248	*pyrD*2260::Tn*10*	S. Maloy
MST1168	LT2/pNK972	S. Maloy
MST4208	F′::T-POP	S. Maloy
Plasmids		
pSU19	Cam^r, medium-copy-number Cloning vector	S. Maloy
pKD46	Amp^r, Red recombinase expression plasmid	K. Datsenko and B. Wanner
pCP20	Amp^r, FLP recombinase expression plasmid	K. Datsenko and B. Wanner
pKD4, pKD3	Cam^r and Kan^r template plasmids	K. Datsenko and B. Wanner
pCE36	Kan^r, FRT::*lacZY* plasmid	J. Slauch

auxotrophic requirement for nucleosides and reduced the ability of serovar Typhi to survive within human U937 cells.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The serovar Typhi and serovar Typhimurium strains used in this study are derivatives of wild-type strains STH2370 and 14028s, respectively, and are listed in Table 1. Bacteria were grown routinely at 37°C with aeration by vigorous shaking. Anaerobiosis was achieved by use of GasPack anaerobic jars. The rich medium used for growth was Luria-Bertani (LB) medium (Bacto tryptone, 10 g liter^{-1}; Bacto yeast extract, 5 g liter^{-1}; NaCl, 5 g liter^{-1}). The defined medium was M9-glucose medium (NaH_2PO_4, 6 mg ml^{-1}; K_2HPO_4, 3 mg ml^{-1}; NH_4Cl, 1 mg ml^{-1}; NaCl, 0.5 mg ml^{-1}; $MgSO_4$, 0.12 mg ml^{-1}; $CaCl_2$, 0.015 mg ml^{-1}; glucose, 2 mg ml^{-1}) supplemented with cysteine and tryptophan (50 mg liter^{-1}); this defined medium is referred to below as "minimal medium." When required, LB medium was supplemented with ampicillin (100 mg liter^{-1}), tetracycline (10 mg liter^{-1}), chlortetracycline (0.5 or 10 mg liter^{-1}), oxytetracycline (5 mg liter^{-1}), chloramphenicol (25 mg liter^{-1}), kanamycin (50 mg liter^{-1}), arabinose (2 mg ml^{-1}), and/or glucose (2 mg ml^{-1}). Solid media included 1.5% agar (Agar-agar ultra pure; Merck). Phosphate-buffered saline (PBS) contained $NaH_2PO_4 \cdot 7H_2O$ (55 mg ml^{-1}), K_2HPO_4 (15 mg ml^{-1}), and NaCl (4.25 mg ml^{-1}). Other reagents and chemicals were obtained from Sigma. *Escherichia coli* strain DH5α {*endA1 hsdR17* ($r^- m^+$) *supE44 thi-1 recA1 gyrA*(Nal^r) *relA1* Δ(*lacZYA-argF*)*U169 deoR* [Φ80Δ(*lacZ*)M15]} was used as the host for selection and preparation of plasmids.

Mutant construction. Mutant derivatives of serovar Typhi STH2370 with insertions of Tn*10* or Mu*dJ* in the *ompC*, *ompF*, *fnr*, *arcA*, or *pyrD* gene were constructed from derivatives of serovar Typhimurium LT2 (MST2947, MST1082 [19], MST2970, MST2345, and MST248, respectively) by generalized transduction carried out at a low multiplicity of infection using the high-frequency transducing phage P22 HT105/1 *int*-201 (46, 61). Mutant strains with deletions of the *tsx*, *nupC*, *nupG*, and *ompA* genes and concomitant insertions of kanamycin or chloramphenicol cassettes were constructed using the method of Datsenko and Wanner (17). Sixty-base PCR primers overlapping the start and stop codons of each gene (boldface type) were synthesized with 40-base 5′ ends corresponding to the ends of the desired substitutions. The primers used for the genes were *tsx*(*H1*+*P1*) (CAGTGGCATACAT**ATG**AAAAAAACTTTACTCGCAGTCAG CTGTAGGCTGGAGCTGCTTCG), tsx(H2+P2) (CTTTTTTGCAGGTTTAG AAGTTGTAACCCACGACCAGGTACATATGAATATCCTCCTTAG), *nupG*(H1+P1) (GGAAATTAAC**ATG**AATCTTAAGCTGCAGCTTAAAATACT CTGTAGGCTGGAGCTGCTTCG), *nupG*(H2+P2) (TATCCGGCCTGCGA AACAGGGCAAGGA**TTA**ATGTGCAACGCATATGAATATCCTCCTT AG), *nupC*(H1+P1) (ATTTGGAGCAAATATGGACCGCGTCCTTCATTTT GTCCTGTGTAGGCTGGAGCTGCTTCG), *nupC*(H2+P2) (ATTAAACGC AGAC**TTA**CAGTACCAGCGCCGCGATAGAGGCCATATGAATATCCTC CTTAG), *ompA*(H1+P1) (GGCGCATGCTGCC**ATG**CGGCGGCGAGTCGC GCCCACTTCATGTAGGCTGGAGCTGCTTCG), and *ompA*(H2+P2) (GAG

CAGAGTAGAA**TCA**GCGGTAAGCCGGGGCAACCCGGTGCATATGAA TATCCTCCTTAG).

The 3′ 20 bases of each primer (underlined) were annealed to the 5′ and 3′ ends of a chloramphenicol or kanamycin cassette flanked by the FRT sites present in template plasmids pKD3 and pKD4 (17), respectively. PCRs using *Taq* DNA polymerase were performed according to the manufacturer's instructions (Life Technologies). STH2370 carrying plasmid pKD46 (encoding the lambda Red recombinase functions) was grown at 30°C in LB medium containing ampicillin and 1 mM arabinose, made electrocompetent, and electroporated with approximately 500 ng of each PCR product. Electroporated cells were plated on LB medium with chloramphenicol or kanamycin at 37°C. Substitution mutations in recombinant strains resulting from this procedure were moved into a clean wild-type background by electroporation with linear chromosomal DNA. The presence of each substitution mutation was confirmed by PCR amplification, using primers complementary to the serovar Typhi genome flanking the sites of substitution. After backcrossing, the resistance gene was eliminated using pCP20, an ampicillin-resistant (Amp^r) plasmid that is temperature sensitive for replication and induction of the FLP recombinase. Serovar Typhi Cam^r or Kan^r mutants were transformed with pCP20, Amp^r recombinants were selected at 30°C, and isolated colonies were purified at 37°C before tests for loss of antibiotic resistance were performed.

Cloning and complementation of the serovar Typhi *tsx* gene. To clone the *tsx* gene, we amplified a 1.6-kb fragment of serovar Typhi STH2370 DNA that included the *tsx* coding sequence and its upstream promoter with primers TAG AATTCTACGGGCAAATTCAGGGCACTA and CCGGATCCGGAAAGAG AAAACCCCCGCACA. The PCR fragment was purified, digested with endonucleases BamHI and KpnI, and ligated to the same sites of the Cam^r, medium-copy-number plasmid vector pSU19 (69). The ligation mixture was electroporated into *E. coli* host strain DH5α, and recombinants that formed white colonies on LB medium plates containing chloramphenicol with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 40 μg/ml 5-bromo-4chloro-3-indolyl-β-D-galactopyranoside (X-Gal) at 37°C were screened. Plasmid DNA purified from one clone yielded an insert of the predicted size after digestion with BamHI and KpnI. This plasmid, pSU19::*tsx*, was electroporated into serovar Typhi Δ*tsx*; Cam^r recombinants were shown to have the Tsx protein in their outer membrane fractions (unpublished results).

Phenotypic analysis of the serovar Typhi Δ*tsx* mutant. We used two different assays to determine the phenotypes of the serovar Typhi Δ*tsx* mutant and its derivatives. Initially, we used a modification of the disk diffusion assay (5) to determine the auxotrophic requirements of these strains. The strains were grown overnight in LB medium with appropriate antibiotics, pelleted, washed twice with PBS, and diluted 1:100 in PBS. Aliquots (200 μl) containing approximately 2×10^6 cells were spread on M9-glucose medium plates and allowed to dry. Twenty-microliter portions of solutions of different nucleoside stocks (100 mM) were spotted on filter paper disks, the disks were placed in the centers of the plates, and the plates were incubated for 48 h at 37°C. The diameters of the zones of growth were calculated by measuring along two axes passing through the center of each disk.

To determine efficiencies of plating in the presence of nucleosides, overnight cultures of wild-type and mutant cells grown in LB medium were pelleted, washed twice with PBS, and diluted 10^6-fold in PBS. Aliquots (200 μl) were spread onto M9-glucose medium plates supplemented with cysteine and tryptophan and with adenosine, thymidine, guanosine, cytosine, inosine, or uridine at final concentrations of 0.5 mM. The plates were incubated at 37°C for 48 h, and the numbers of CFU were determined. Efficiencies of plating are expressed below as the ratios of survival on media with nucleosides to survival on media without nucleosides, divided by the titer of the wild-type strain without nucleosides.

SDS-polyacrylamide gel analysis and N-terminal sequencing of proteins. Outer membrane fractions were prepared as described by Lobos and Mora (44) based on a modification of the method of Schnaitman (62), and proteins in these fractions were separated in 12.5% polyacrylamide gels. Bacteria were grown to the mid-exponential phase (optical density at 600 nm [OD_{600}], 0.2), chilled on ice, pelleted by centrifugation at $3,000 \times g$ for 15 min at 4°C, resuspended in lysis buffer (10 mM Tris-HCl, pH 8, 10 mM $MgCl_2$), and sonicated, and the preparations were supplemented with 2 mM phenylmethylsulfonyl fluoride. Whole cells and debris were removed by low-speed centrifugation ($3,000 \times g$, 10 min), and total membrane fractions were obtained after 45 min of centrifugation at $13,000 \times g$ at 4°C. Cytoplasmic membranes were solubilized with 2% Triton X-100, and the outer membrane fraction was pelleted by centrifugation at $13,000 \times g$ and solubilized in 50 μl of 100 mM Tris-HCl (pH 8 buffer), 1% sodium dodecyl sulfate (SDS). For two-dimensional electrophoresis, outer membrane fractions were resolved in the first dimension by SDS-polyacrylamide gel electrophoresis (PAGE) and in the second dimension by isoelectric focusing by the method of Hochstrasser et al. (35). Proteins in gels were stained with Coomassie brilliant blue R-250. The protein content of each fraction was determined by the bicinchoninic acid method (Pierce). Proteins in gels were electroblotted onto polyvinylidene fluoride (Immobilon) membranes and detected by staining with 0.05% Coomassie brilliant blue R-250 in 50% methanol (high-performance liquid chromatography grade). N-terminal amino acid sequences were determined by sequential Edman degradation.

Assays for β-galactosidase activity. *Salmonella* serovar Typhi mutant strains with *lacZY* fusions were grown to an OD_{600} of 0.2 and then chilled to 4°C. β-Galactosidase activities were measured by a modification of the method of Miller (49). Fifty microliters of each bacterial culture was suspended in 950 μl of Z buffer (0.6 M Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol; pH 7.0). The bacteria were permeabilized with 10 μl chloroform, 10 μl 0.1% SDS, vortexed for 10 s, and incubated at 30°C for 10 min, and 200 μl of *o*-nitrophenyl-β-D-galactopyranoside (4 mg/ml) was added. Reactions were stopped by addition of 500 μl 1 M Na_2CO_3. β-Galactosidase activities are expressed below in Miller units, $10^3 \times (A_{420} - 1.75 \times A_{550})\ ml^{-1}\ min^{-1}\ A_{600}{}^{-1}$.

Transposon mutagenesis. T-POP (58) mutagenesis was carried out as described by Hidalgo et al. (32), using STH2370 Δ*tsx* (pNK972) as the recipient. The recipient was constructed by electroporation of plasmid pNK972 DNA isolated from strain MST1168, followed by selection for Amp^r recombinants at 30°C. Equal volumes of an overnight culture of Amp^r recipient cells and a generalized transducing lysate grown on donor strain MST4208 were mixed, incubated for 2 h at 37°C, and then spread onto M9 medium plates containing tetracycline. Colonies recovered after 48 h were used to grow overnight cultures in M9 medium containing tetracycline. Chromosomal DNA purified from these cultures was used to electroporate the serovar Typhi parental strain, STH2370 Δ*tsx*, as described by Toro et al. (72), and Tet^r recombinants were selected on M9 medium plates containing tetracycline.

Cloning and sequencing of T-POP insertions. Chromosomal DNA from backcrossed mutants with T-POP insertions was digested with PstI, which did not cleave within the T-POP element, and ligated to the PstI site of plasmid pSU19. Ligation mixtures were electroporated into *E. coli* DH5α, and transformants were selected on LB medium plates containing oxytetracycline. Unlike tetracycline, oxytetracycline does not induce transcription of the *tetRA* genes and can be used to select for medium- or high-copy-number plasmids with subcloned T-POP inserts. Plasmid DNAs were purified using QIAGEN columns and were used as templates in PCRs with the T-POP-EX1 primer (CCTTTTTCCGTGATGGTA), internal to the T-POP element and extending outward from *tetR*, to define the serovar Typhi Δ*tsx zxx*::T-POP insertion join points. This primer corresponded to bp 2191 to 2207 in the GenBank accession no. AY150213 sequence. Mutant alleles were designated by numbers corresponding to the coordinates in the genome sequence of serovar Typhi strain CT18 (57) for the 9-bp direct repeat generated by T-POP insertion. For the STY0014, STY2025, and STY1399 genes, these coordinates are bp 15655 to 15663, 1898126 to 1898134, and 1351895 to 1351903, respectively.

RNA isolation and RT-PCR. To isolate RNA, cells were grown overnight in LB medium at 37°C. Total RNA was extracted and purified using Trizol and was treated with RNase-free DNase I (amplification grade; Gibco-BRL). Reverse transcription (RT)-PCR was performed with 200 ng of DNase-treated RNA using a single-tube RT-PCR kit (Gibco-BRL). Amplification was performed for 25 to 30 cycles (94°C for 30 s, 55°C for 30 s, and 72°C for 2 min, followed by a 7-min extension at 72°C). Primers GGGGAGTGCATCGACGCATT and TTT ACTGCCACCGGCACAT, corresponding to internal region of the serovar Typhi *pyrD* gene, were designed based on the CT18 genome sequence (57); universal primers AGAGTTTGATCC TGGCTCAG and AAGGAGGTGATC CAGCCGCA were used to amplify16S rRNA. Genomic DNA served as a positive control, and DNase-treated RNA that had not been reverse transcribed was used as a negative control. Twenty-microliter aliquots removed after 25, 30, and 35 cycles of each PCR were electrophoresed on 1% agarose gels with ethidium bromide, and the gels were analyzed using a Digital Science 120 system (Kodak). The amounts of PCR products were normalized to the amounts of 16S rRNA amplified in the cDNA samples.

Assays for the survival of serovar Typhi within human macrophage-derived U937 cells. Infection of U937 cells was carried out as described by Baker et al. (3), with the following modifications. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum, seeded into 24-well tissue culture plates at a concentration of 10^5 cells per well, and activated with 100 nM phorbol myristate acetate. The cells were incubated for 2 days at 37°C in 5% CO_2 and air, until a confluent monolayer was formed, and then they were centrifuged and washed three times with PBS. Approximately 2×10^6 to 5×10^6 CFU of exponential-phase (OD_{600}, 0.15 to 0.20) anaerobically

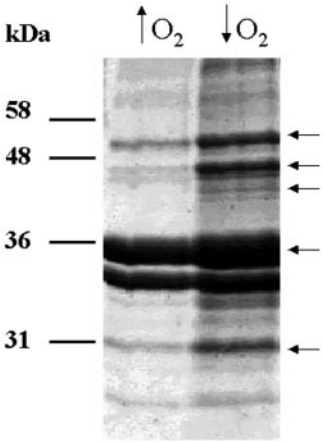

FIG. 1. Profiles of proteins present in outer membrane fractions extracted from serovar Typhi grown under aerobic and anaerobic conditions are different. Proteins present in outer membrane fractions of serovar Typhi were resolved by SDS-PAGE as described in Materials and Methods. The steady-state levels of at least five proteins appear to be higher when serovar Typhi is grown under anaerobic conditions.

grown bacteria was pelleted, washed twice with PBS, and resuspended in 1 ml of minimal essential medium without nucleosides. Aliquots (100 μl) of bacteria were added to U937 cells at a multiplicity of infection of 20:1 to 50:1. After 1 h of infection, cells were centrifuged and washed three times with PBS, and the medium was replaced with Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum containing gentamicin (200 μg/ml). After additional incubation for 2, 24, and 48 h, U937 cells were washed three times with PBS and lysed with 0.5% deoxycholate, and the titers of intracellular bacteria were determined by serial dilution of cell lysates on agar plates.

RESULTS

Expression of serovar Typhi Tsx and other porins is induced in response to anaerobiosis. Previously, we found that the ability of serovar Typhi to invade and proliferate within epithelial and macrophage-like human cell lines is stimulated by prior anaerobic growth (11). Because the proteins made by serovar Typhi that first come in contact with host cells are presented on the outer membrane, we reasoned that a subset of outer membrane proteins made under anaerobic conditions might contribute to the virulence of serovar Typhi.

To test this hypothesis, we identified a subset of the proteins present in outer membrane fractions of serovar Typhi that are expressed at higher steady-state levels under anaerobic conditions, and we are attempting to assess the roles of these proteins in virulence. A comparison of the proteins resolved by one-dimensional SDS-polyacrylamide gel electrophoresis present in outer membrane fractions of serovar Typhi grown aerobically and anaerobically showed that expression of at least five proteins found in the outer membrane fractions of serovar Typhi cells is induced upon the shift to anaerobiosis (Fig. 1). These proteins have mobilities corresponding to apparent molecular masses of 50, 46, 44, 36, and 31 kDa (Table 2).

Previously, we have shown that the protein with an apparent molecular mass of 36 kDa corresponds to the porin OmpC (12, 44). Bands corresponding to the other induced proteins were isolated, and the N-terminal sequences of the proteins present in these bands were determined by sequential Edman degradation and are the sequences of serovar Typhi FliC (flagellin) and the MalL, PhoE, and Tsx porins (Table 2).

Transcription of the serovar Typhi *tsx* gene increases in response to anaerobiosis and is dependent on Fnr. Because the majority of the regulation of prokaryotic gene expression occurs at the level of transcription initiation, it is likely that the increase in the level of Tsx porin in response to anaerobiosis is due to the increased transcription of the *tsx* gene. To show that transcription of the *tsx* gene is induced during anaerobiosis, we constructed a transcriptional fusion of the *tsx* and *lacZY* genes in single copy on the serovar Typhi genome, using FLP-mediated site-specific recombination. Plasmid pCE36 was integrated at the site of the "FLP scar" in the serovar Typhi Δ*tsx* strain (22). As shown in Fig. 2, the β-galactosidase activity produced by the recombinant serovar Typhi *tsx*::*lacZY* strain during anaerobic growth was threefold higher than the activity during aerobic growth. This result suggests that the increased steady-state level of the Tsx protein observed under anaerobic growth conditions is likely due to increased transcription of the *tsx* gene.

Because Fnr regulates a global response to oxygen availability at the level of transcription (36, 67, 68), we constructed an *fnr*::Tn*10* *tsx*::*lacZY* double mutant and measured the levels of β-galactosidase activity of the double mutant during aerobic and anaerobic growth. As shown in Fig. 2, the presence of the *fnr*::Tn*10* allele abolished induction of β-galactosidase activity from the *tsx*::*lacZY* fusion under anaerobic conditions. This result suggests that Fnr regulates the transcription of *tsx*. Because the two-component ArcAB system regulates the transition between aerobic and anaerobic respiratory control (67), we also constructed an *arcA*::Tn*10* *tsx*::*lacZY* double mutant and measured the levels of β-galactosidase activity of the double mutant during aerobic and anaerobic growth. As shown in

TABLE 2. Amino-terminal sequences of proteins in the outer membrane fraction of *Salmonella* serovar Typhi induced by anaerobiosis

Protein[a]	Locus tag	N-terminal sequence	M_r (10^3)	
			Predicted	Observed
FliC	STY2167	AQVINTNSLSLLTQNNLNKSQXALGTAI	53.3	50
LamB	STY4427	VDFHGYARSGIGWTG	50.1	46
PhoE	STY0365	AEIYNKDGNKLDVIG	38.7	44
OmpC	STY2493	Identified genetically	41.2	36
Tsx	STY0451	AENDQPQYL	32.8	31

[a] The N-terminal sequences of the proteins shown in Fig. 1 with apparent molecular masses of 50, 46, 44, and 31 kDa were determined and found to match those predicted from the *Salmonella* serovar Typhi flagellin and porin gene sequences. To confirm the identity of Tsx, we also sequenced the internal peptide, TFDWGNGNDK. The identity of the OmpC porin was determined by comparison of the outer membrane protein profiles of wild-type and mutant strains (12, 44).

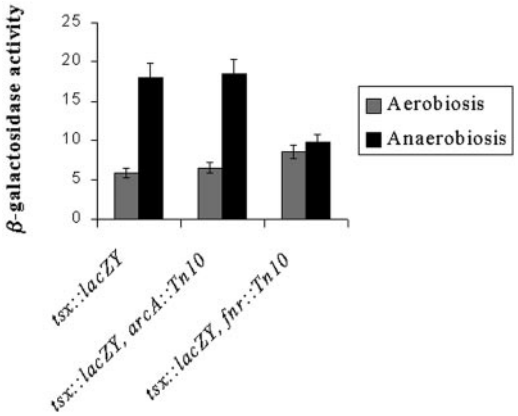

FIG. 2. Induced expression of β-galactosidase activity from a *tsx*::*lacZY* fusion operon is dependent on Fnr. The data are the values from one representative experiment performed in triplicate; similar results were obtained in three additional, independent experiments. Each point represents the arithmetic mean of three determinations of β-galactosidase activity expressed in Miller units (49).

Fig. 2, the induction of the serovar Typhi *tsx* gene in response to anaerobiosis is not dependent on the ArcAB two-component system.

Deletion of the serovar Typhi *tsx* gene confers an auxotrophic requirement for nucleosides. To characterize the role that the serovar Typhi *tsx* gene may play in the physiology and virulence of serovar Typhi, we took a genetic approach. We constructed a derivative of serovar Typhi with a deletion of the *tsx* gene (Δ*tsx*) and cloned the *tsx* gene into a plasmid vector to complement the loss-of-function mutation. First, we constructed a mutant of serovar Typhi with a Cam^r cassette substituted for the *tsx* gene. Subsequently, the Cam^r cassette was eliminated by FLP recombinase-mediated site-specific recombination (17) to generate a serovar Typhi Δ*tsx* strain. Figure 3 shows that the Tsx porin was not present in outer membrane fractions isolated from this mutant.

In a subset of enteric gram-negative pathogens, Tsx has been shown to be a nucleoside-specific porin that is dispensable for growth in minimal media (52). However, we found that the serovar Typhi Δ*tsx* mutant was unable to grow in M9-glucose or E-glucose minimal medium, and the efficiency of plating was 5×10^{-7} compared to the wild-type parent on M9-glucose medium plates. In contrast, a serovar Typhimurium Δ*tsx* mutant was not an auxotroph and grew well in minimal media (data not shown).

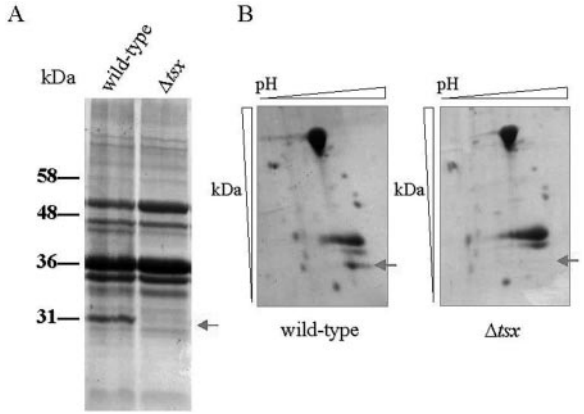

FIG. 3. SDS-PAGE and two-dimensional profiles of serovar Typhi wild-type and serovar Typhi Δ*tsx* outer membrane fractions. Proteins present in outer membrane fractions of serovar Typhi were resolved by SDS-PAGE as described in Materials and Methods. (A) Results after electrophoresis in one dimension. (B) Results after electrophoresis in two dimensions. The arrow indicates the position of the Tsx porin.

TABLE 3. Efficiencies of plating[a]

Strain	Efficiency of plating on M9-Glc (%)
Salmonella serovar Typhi wild-type	1
Salmonella serovar Typhi Δ*tsx*	5×10^{-7}
Salmonella serovar Typhi Δ*tsx*/pSU19::*tsx*	1
Salmonella serovar Typhi Δ*nupC*	1
Salmonella serovar Typhi Δ*nupG*	1
Salmonella serovar Typhi Δ*nupC* Δ*nupG*	1

[a] Overnight cultures of each strain grown aerobically in LB medium at 37°C were pelleted, washed twice with PBS, diluted serially in PBS, and spread on M9-glucose medium (M9-Glc) plates. Numbers of CFU were determined after incubation for 48 h at 37°C. Efficiencies of plating are expressed as the titer of each strain divided by the titer of the wild type.

To confirm that this auxotrophy was due to loss of function of the *tsx* gene, we amplified the serovar Typhi *tsx* gene using a PCR and subcloned this gene into the medium-copy-number plasmid pSU19. Plasmid pSU19::*tsx* was transformed into the serovar Typhi Δ*tsx* mutant, and we found that the serovar Typhi Δ*tsx*/pSU19::*tsx* recombinants could grow in minimal medium (Table 3).

Because Tsx has been shown to be a porin involved in the transport of nucleosides, we asked whether the addition of nucleosides could mask the auxotrophy of the serovar Typhi Δ*tsx* mutant. We supplemented minimal medium with different concentrations of nucleosides and nucleotide bases and found that either adenosine or thymidine could correct the auxotrophy of the serovar Typhi Δ*tsx* mutant (Table 4).

We also found that when the serovar Typhi Δ*tsx* mutant was grown on a minimal medium plate and a filter paper disk with adenosine was placed in the center of the plate, a growth halo formed around the disk. However, when serovar Typhi

TABLE 4. *Salmonella* serovar Typhi Δ*tsx* mutant is an auxotroph[a]

Medium	Growth of:		
	Salmonella serovar Typhi wild type	*Salmonella* serovar Typhi Δ*tsx*	*Salmonella* serovar Typhi Δ*tsx*/pSU19::*tsx*
M9-Glc	++++	−	++++
M9-Glc + guanosine	+++	−	++++
M9-Glc + inosine	+++	+	+++
M9-Glc + adenosine	+++	++++	+++
M9-Glc + uridine	+++	++	+++
M9-Glc + cytidine	+++	−	++++
M9-Glc + thymidine	+++	+++	+++

[a] Overnight cultures of each strain grown aerobically in LB medium at 37°C were pelleted, washed twice with PBS, diluted serially in PBS, and spread on M9-glucose medium (M9-Glc) plates supplemented with guanosine, inosine, adenosine, uridine, cytidine, or thymidine at a final concentration of 0.5 mM. ++++, excellent growth after incubation for 48 h at 37°C; +++, good growth; ++, poor growth; +, barely visible growth; −, no growth.

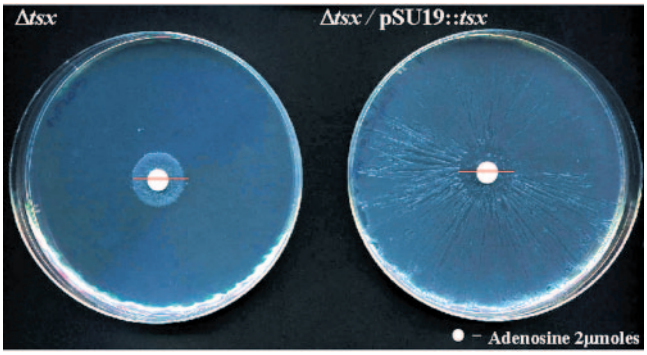

FIG. 4. Adenosine masks the auxotrophy of the mutant serovar Typhi Δ*tsx* strain. Overnight cultures of the serovar Typhi Δ*tsx* and serovar Typhi Δ*tsx*/pSU19::*tsx* strains were grown aerobically in LB medium at 37°C, pelleted, washed twice with PBS, and diluted 100-fold in PBS. Two hundred microliters of each dilution was spread on an M9-glucose agar plate. A sterile paper disk with 20 μl of 100 mM adenosine was placed in the center each plate, and the plates were incubated at 37°C for 48 h. The red lines indicate the diameters of the growth halo of the serovar Typhi Δ*tsx* strain and the inhibition halo of the serovar Typhi Δ*tsx*/pSU19::*tsx* strain.

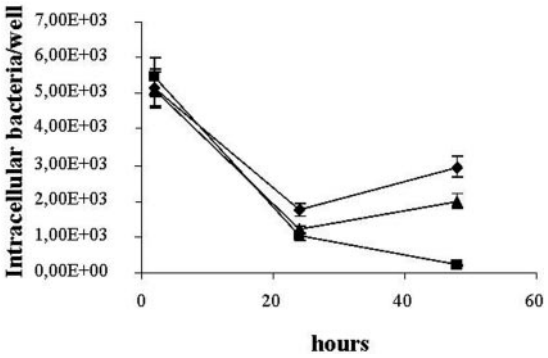

FIG. 5. Survival of the serovar Typhi wild-type (◆), serovar Typhi Δ*tsx* (■), and serovar Typhi Δ*tsx*/pSU19::*tsx* (▲) strains in human U937 cells. *Salmonella* serovar Typhi cells were placed in direct contact with U937 cells by brief centrifugation of tissue culture plates after mixing. After 1 h of incubation at 37°C, extracellular bacteria were removed by washing with PBS, and the remaining extracellular bacteria were killed by addition of gentamicin to the growth medium. Intracellular bacterial survival was determined by serial dilution of cell lysates, followed by growth of serial dilutions on LB medium plates at 37°C for 48 h. The correlation between time and intracellular survival was established in four independent experiments performed in triplicate. Each point represents the arithmetic mean of three determinations, expressed as number of CFU/well.

Δ*tsx*/pSU19::*tsx* was grown under the same conditions, a discrete inhibition halo formed around the disk (Fig. 4). This result suggests that whereas a high concentration of adenosine is essential to support the growth of the serovar Typhi Δ*tsx* mutant, adenosine is toxic to otherwise isogenic cells expressing the *tsx* gene from a intermediate-copy-number plasmid. Under the same conditions, the growth of wild-type serovar Typhi was not affected by adenosine (data not shown).

We constructed Δ*tsx* mutant derivatives of two additional serovar Typhi strains, Ty2, whose genome sequence has been determined (18), and another clinical isolate, STH795. Like the STH2370 mutant, mutant derivatives of these strains were auxotrophs, and their auxotrophies could be masked by addition of either adenosine or thymidine.

Survival of the serovar Typhi Δ*tsx* mutant in human macrophage-like U937 cells. U937 is a human monocytic suspension cell line (70), and the cells express many of the normal macrophage characteristics when they are activated (31). Survival of *Salmonella* within macrophages is essential for virulence (23) and involves a dynamic equilibrium between bacterial growth and death (10). To determine whether the serovar Typhi Δ*tsx* mutant has an altered interaction with human macrophages, we compared the survival of wild-type serovar Typhi with the survival of the otherwise isogenic Δ*tsx* mutant strain in activated U937 cells. As shown in Fig. 5, the number of wild-type bacteria recovered from infected U937 cells decreased during the first 24 h after infection and then increased slightly during the next 24 h. In contrast, the titers of serovar Typhi Δ*tsx* mutant bacteria recovered from U937 cells decreased throughout the first 48 h of infection. Complementation of the Δ*tsx* mutant with the plasmid carrying the serovar Typhi *tsx* gene resulted in an intermediate phenotype (Fig. 5). This finding is consistent with the finding that complementation of auxotrophy by this plasmid also did not result in a wild-type phenotype; the complemented strain, unlike the wild type, did not grow in the presence of high concentrations of adenosine.

Insertion of transposon T-POP upstream of the serovar Typhi *pyrD* gene suppresses the auxotrophy of the Δ*tsx* mutant. The auxotrophy resulting from the Δ*tsx* mutation is surprising for four reasons. First, deletion of no other porin-encoding gene in gram-negative bacteria closely related to serovar Typhi, including other serovars of *S. enterica* and *E. coli*, results in auxotrophy. *Salmonella* serovar Typhi strains with mutations in the porin-encoding *ompC*, *ompF*, and *ompA* genes grow on minimal medium (unpublished results). Second, the Δ*tsx* mutation, unlike mutations in the essential purine and pyrimidine biosynthetic pathways of *S. enterica*, causes auxotrophy that can be masked by the addition of nucleosides but not by the addition of their nucleotide counterparts. Third, the Δ*tsx* mutation causes auxotrophy that can be masked by the addition of either purine or pyrimidine nucleosides (adenosine or thymidine). Fourth, the Δ*tsx* mutation causes auxotrophy that can be masked by the addition of specific purine and pyrimidine nucleosides. Adenosine and thymidine work, but why don't guanosine and uridine work? And why do these particular nucleosides reflect the substrate specificity of the Tsx porin? This phenotype is clearly novel among auxotrophs of *S. enterica*.

To address this puzzle, we took a genetic approach. We reasoned that a study of reversion mutations that suppress this phenotype might allow us to understand why a serovar Typhi Δ*tsx* mutant is an auxotroph. Because deletion of no other porin-encoding gene in gram-negative enteric bacteria closely related to serovar Typhi results in auxotrophy, including deletion of the serovar Typhimurium *tsx* gene (unpublished results), we reasoned that serovar Typhi may have an additional gene function missing in serovar Typhimurium that makes *tsx* an essential gene. To test this hypothesis, we looked for loss-of-function mutations in these genes that suppressed the auxotrophic defects of a Δ*tsx* mutation. Success in our efforts would have supported our rejection of the alternative hypothesis that serovar Typhi is missing a gene function present in serovar Typhimurium, the absence of which makes *tsx* essential.

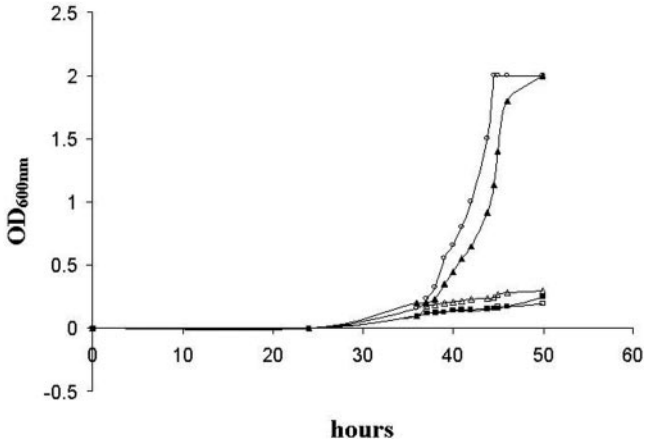

FIG. 6. Growth of the serovar Typhi Δ*tsx* STY2025::T-POP double mutant is dependent on the presence of inducer. Overnight cultures of the serovar Typhi wild-type (○), serovar Typhi Δ*tsx* (□), and serovar Typhi Δ*tsx* STY2025::T-POP (△) strains were grown aerobically at 37°C in LB medium, pelleted, washed twice with PBS, and diluted 100-fold in PBS. Two hundred microliters of each dilution was subcultured in 30 ml of M9-glucose medium. ▲ and ■, growth of the serovar Typhi Δ*tsx* STY2025::T-POP mutant in M9-glucose medium with 0.5 and 2 μg ml^{-1} of tetracycline, respectively. Results of a representative experiment are shown; similar results were observed in three independent experiments and experiments in which the nontoxic inducer chlortetracycline was added.

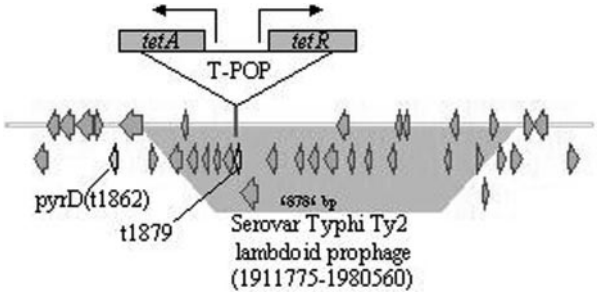

FIG. 7. Insertion of T-POP in the serovar Typhi STY2025 locus, upstream of *pyrD*, suppresses the auxotrophy resulting from the Δ*tsx* mutation. The join point of the T-POP insertion in the STY2025 locus was determined using a primer internal to the T-POP element and extending outward from *tetR*. The diagram shows that this gene is part of a 69-kb lambdoid prophage integrated at a site adjacent to the *pyrD* gene. This region of the serovar Typhi STH2370 genome is conserved with the corresponding region of strain Ty2. In this region of the sequenced Ty2 genome, the prophage t1879 locus (bp 1939277 to 1938267), identical to STY2025 in strain CT18, lies 14 kb upstream of the *pyrD* gene (t1862; bp 1924812 to 1923802) (18). The T-POP insertion places *pyrD* gene expression under control of the *tetA* promoter.

To look for mutations in the gene(s) that makes *tsx* essential in serovar Typhi but not in serovar Typhimurium, we looked for transposon insertions that inactivated genes, whose loss of function suppressed the Δ*tsx* defect. Recently, we have shown that insertion of the transposon T-POP can be used to identify essential serovar Typhi genes by placing their expression under the control of the tetracycline-inducible *tetA* promoter (32). The T-POP element carries the *tetR* (repressor) and *tetA* (resistance) genes flanked by active IS*10* inverted repeats required in *cis* for transposition. Tetracycline induces divergent transcription of *tetR* and *tetA*, which can extend outside the ends of the transposon into adjacent genes (58).

We isolated derivatives of the serovar Typhi Δ*tsx* mutant strain with insertions of the T-POP element and selected among these derivatives for tetracycline-resistant prototrophs. Sequence analysis of the T-POP insertion present in one such revertant showed that the insertion was within the STY2025 gene, which is predicted to encode a tail protein of a lambdoid prophage.

To demonstrate that this insertion is responsible for the suppression of the auxotrophic phenotype of the serovar Typhi Δ*tsx* mutant, the insertion was backcrossed into the mutant strain by electroporation with linear DNA. As shown in Fig. 6, the STH2370 Δ*tsx* STY2025::T-POP double mutant grew at the same rate as the wild type on M9-glucose medium with tetracycline at a concentration of 0.5 μg ml^{-1}. This strain did not grow in the absence of tetracycline, suggesting that expression of a nearby chromosomal gene from the divergent *tetRA* promoters in this strain is required for suppression. Surprisingly, this strain also did not grow when the medium was supplemented with tetracycline at a concentration of 2 μg/ml^{-1} or more, suggesting that the overexpression of a nearby chromosomal gene from the divergent *tetRA* promoters in this strain is toxic to serovar Typhi.

The prophage in which this suppressing T-POP insertion is located is integrated at two different sites in the two sequenced genomes of serovar Typhi, the genomes of strains CT18 and Ty2 (18, 57). The results of PCR amplifications across the prophage *attL* and *attR* sites using STH2370 chromosomal DNA as the template showed that the site of integration of this prophage in STH2370 is the same as that in strain Ty2 (unpublished results) and corresponds to the site of integration of the serovar Typhimurium lambdoid prophage Gifsy-2 (34). Examination of the region of the serovar Typhi genome with this T-POP insertion revealed that it contains only one gene known to be involved in nucleoside transport or metabolism, *pyrD*, which encodes dihydroorotate dehydrogenase, the only membrane-associated protein involved in nucleoside biosynthesis (Fig. 7). The site of prophage integration lies three genes upstream of *pyrD* (t1862 in the Ty2 genome sequence), which places the T-POP insertion in STY2025 (corresponding to the t1879 locus in the Ty2 genome sequence) about 14 kb upstream of *pyrD*. Therefore, we formed the hypothesis that overexpression of the *pyrD* gene from the *tetA* promoter in the STH2370 Δ*tsx* STY2025::T-POP double mutant may suppress the *tsx* defect, because it restored an essential balance of products of the *tsx* and *pyrD* genes.

To test this hypothesis, we asked whether expression of the *pyrD* gene is altered in the double mutant at different concentrations of inducer and used RT-PCR to measure the relative levels of the *pyrD* gene transcript in the presence or absence of inducer. Surprisingly, we found that transcripts corresponding to the *pyrD* gene were produced at higher levels in the absence of inducer than in its presence (Fig. 8). Thus, it appears that the induced transcription in the direction of the *pyrD* gene from the *tetA* promoter results in decreased, not increased, expression of *pyrD*. If this is the case, then the failure of the Δ*tsx* STY2025::T-POP double mutant to grow at high concentrations of inducer may be simply due to the loss of *pyrD*

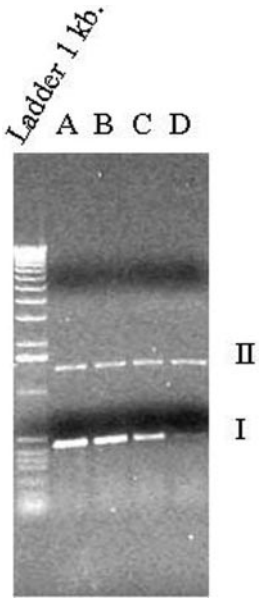

FIG. 8. Expression of *pyrD* in the Δ*tsx* STY2025::T-POP double mutant decreases in response to increasing concentrations of inducer. RNA was isolated from overnight cultures grown aerobically in LB medium at 37°C. RT-PCR was used to determine the relative amounts of *pyrD* mRNA (band I) and 16S rRNA (band II). Lane A, serovar Typhi wild type; lane B, serovar Typhi Δ*tsx* STY2025::T-POP; lane C, serovar Typhi Δ*tsx* STY2025::T-POP with 0.5 μg ml^{-1} chlortetracycline; lane D, serovar Typhi Δ*tsx* STY2025::T-POP with 10 μg ml^{-1} chlortetracycline. The relative amount of transcript corresponding to an internal portion of the *pyrD* gene decreased about twofold upon addition of low concentrations of the nontoxic inducer chlortetracycline and more than fivefold upon addition of high concentrations of chlortetracycline.

expression under these conditions, resulting in pyrimidine auxotrophy. Consistent with this idea, we found that growth of the Δ*tsx* STY2025::T-POP double mutant was restored in the presence of high concentrations of inducer by the addition of uridine, unlike what happens with its Δ*tsx* parental strain (Table 4). Uridine also satisfies the auxotrophic requirement of a serovar Typhi *pyrD*::Tn*10* mutant (unpublished results).

In addition, we isolated and characterized two additional T-POP insertions that suppress the phenotype of the Δ*tsx* mutation. These insertions lie within the STY0014 and STY1399 genes, which are predicted to encode a helix-turn-helix protein in the LysR family of transcription regulators and a protein with an unknown function, respectively.

DISCUSSION

The porin Tsx is among the serovar Typhi outer membrane proteins that are induced in response to anaerobiosis. We found that expression of the serovar Typhi *tsx* gene is regulated by Fnr but not by the ArcAB system. This is consistent with results that show that although the subsets of *E. coli* genes controlled by Fnr and ArcAB overlap extensively, the Fnr regulon includes a set of genes expressed under strictly anaerobic conditions that are not controlled by ArcAB (60). In *E. coli*, expression of *tsx* is controlled by two differentially regulated promoters. One promoter is subject to negative regulation by both the DeoR and CytR repressors, and the other is positively regulated by the cyclic AMP/cyclic AMP receptor protein complex (7, 8, 29). These elements are conserved in the serovar Typhi *tsx* promoter sequence. In addition, the sequence of the serovar Typhi *tsx* promoter also contains two inverted repeats corresponding to Fnr consensus binding sites (TTGATNNNNATCAA) (63), 77 bp upstream of its DeoR operator.

Surprisingly, we found that deletion of the serovar Typhi *tsx* gene confers an auxotrophic requirement for adenosine or thymidine. Prototrophy can be restored by complementation of a Δ*tsx* mutant with an intermediate-copy-number plasmid carrying the *tsx* gene, but the complemented strain acquires sensitivity to the nucleosides, presumably as a consequence of overexpression of *tsx*. It is not surprising that the serovar Typhi Δ*tsx* mutant is unable to survive as well as its wild-type parent in human cells. Many of the genes required for the pathogenicity of serovars of *S. enterica* encode proteins involved in normal housekeeping functions of the bacillus, including genes involved in purine and amino acid biosynthesis, as well as genes involved in nutrient uptake. Mutations in these housekeeping genes can attenuate the virulence of *Salmonella* and at the same time allow the immune system to clear infecting organisms more rapidly. Mutants of serovar Typhimurium with auxotrophic requirements for nucleosides are attenuated in vivo, most likely because serovar Typhimurium encounters a scarcity of nucleosides and nucleotide bases within macrophages (23, 41, 47, 56).

Why is the serovar Typhi *tsx* mutant an auxotroph for these nucleosides? In both *E. coli* K-12 (52) and serovar Typhimurium (unpublished results), deletions of the *tsx* gene do not confer auxotrophy, and the importance of the Tsx protein for nucleoside uptake becomes apparent only at low substrate concentrations (<1 μM) (26). This auxotrophy does appear to reflect the substrate specificity of the Tsx porin. In *E. coli*, the rate of uptake of adenosine and thymidine is strongly reduced in the absence of the Tsx porin, whereas the rate of uptake of cytidine remains unchanged (51).

Clearly, the Δ*tsx* mutation must result in a defect in the transport of nucleosides across the outer membrane. However, the auxotrophy resulting from the Δ*tsx* mutation is not an auxotrophy in the classical sense, for the four reasons described above, and the growth defect caused by the Δ*tsx* mutation is unlikely to be a consequence of its function in nucleoside transport per se. In support of this idea, we have shown that serovar Typhi *nupC* and *nupG* mutants, as well as a *nupC nupG* double mutant defective in the two major inner membrane nucleoside permeases conserved among the gram-negative enteric bacteria, retain the ability to grow in minimal media.

Rather, our results suggest that the *tsx* gene product must play a critical role in the assembly or integrity of the serovar Typhi membrane. This hypothesis is consistent with the finding that overexpression of Tsx is detrimental to growth in the presence of its preferred substrates. Recent studies of the mechanisms of colicin transport across the outer membrane suggest that porins such as Tsx may participate in assembly of translocon complexes involving other outer membrane proteins (74) and other periplasmic and inner membrane proteins (59). Thus, Tsx may be required for assembly of an essential translocon complex, and perhaps the conformational changes that an alternative porin, such as OmpF or OmpC, undergo

during nucleoside transport may allow it to substitute for the essential role of Tsx. Alternatively, the absence of the Tsx protein may result in the formation of a complex detrimental to cell growth, by facilitating the interaction between two membrane proteins whose abnormal association can be disrupted by the restoration of nucleoside transport. Tsx may play an important role in cell structure, because in *E. coli* K-12, the Tsx protein is enriched in minicells (38) and may play a role in maintaining the integrity of the cell poles.

The analysis of second-site revertants of the Δ*tsx* mutant provides an initial clue about the nature of the complex in which Tsx participates; PyrD may be a part of this complex. Decreased expression of the *pyrD* gene from the *tetA* promoter in the presence of intermediate concentrations of tetracycline suppresses the defect caused by the Δ*tsx* mutation. Expression of the serovar Typhimurium *pyrD* gene is regulated in response to the ratio of intracellular CTP to intracellular GTP. Under repressing conditions, when this ratio is high, the *pyrD* transcript initiates upstream of its preferred starting point under inducing conditions, when the ratio is low. The longer transcript begins with a stem-loop structure (attenuator) that may sequester the *pyrD* start codon, thereby preventing efficient translation of the *pyrD* gene product (65, 66); this attenuator is also present upstream of the serovar Typhi *pyrD* gene. Our results suggest that the regulation of *pyrD* transcription is more complicated and that transcripts initiating from the *tetA* promoter extending into the *pyrD* attenuator either may terminate prematurely or may be degraded more rapidly, because our RT-PCR analyses showed that less transcript corresponding to an internal region of the *pyrD* gene is made when expression of *pyrD* from the upstream *tetA* promoter is induced.

The finding that the Δ*tsx* STY2025::T-POP double mutant displays different growth phenotypes at three different concentrations of inducer shows that the induced expression of genes from the *tetA* promoter is not an all-or-nothing phenomenon but rather is rheostatic. Only intermediate levels of *pyrD* expression from the *tetA* promoter suppress the Δ*tsx* defect, indicating that the balance of products of the *tsx* and *pyrD* genes is critical for the growth of serovar Typhi. Therefore, it is not surprising that one of the additional second-site suppressors of the Δ*tsx* mutation is an insertion of T-POP into the STY0014 locus, which is predicted to encode a regulator of transcription. Presumably, this regulatory mutation affects the balance of the *tsx* and *pyrD* gene products. Unfortunately, the two genes in which these additional T-POP insertions map are unique to the genomes of serovars Typhi, Paratyphi A, and Typhimurium, and we cannot obtain clues about their functions from comparative genomics. We must conduct additional genetic studies to identify their roles in nucleoside transport and biosynthesis.

Our hypothesis that the growth of serovar Typhi is dependent on a balance of products involved in nucleoside transport and biosynthesis is supported by the fact that this is not the only reported case in which an imbalance of such products results in a growth defect. When the NupG inner membrane transporter is expressed from a high-copy-number plasmid in *E. coli*, high concentrations of adenosine inhibit cell growth (55). Finally, if PyrD, a protein associated with the inner membrane, forms part of a translocon complex with Tsx, an outer membrane porin, then there likely is an inner membrane protein that can bridge these two components. Preliminary results from additional genetic experiments have allowed us to identify a candidate for such a third, integral inner membrane component.

ACKNOWLEDGMENTS

We thank Cecilia Tapia, Juan Fuentes, Daniela Castro, and Jorge Lévican for critical reading of the manuscript.

This work was supported by FONDECYT (Chile) grant 1020485 to G.C.M. S.B. was supported by fellowships from MECESUP and DI-PUC.

REFERENCES

1. **Acimovic, Y., and I. R. Coe.** 2002. Molecular evolution of the equilibrative nucleoside transporter family: identification of novel family members in prokaryotes and eukaryotes. Mol. Biol. Evol. **19:**2199–2210.
2. **Arricau, N., D. Hermant, H. Waxin, C. Ecobichon, P. S. Duffey, and M. Y. Popoff.** 1998. The RcsB-RcsC regulatory system of *Salmonella typhi* differentially modulates the expression of invasion proteins, flagellin and Vi antigen in response to osmolarity. Mol. Microbiol. **29:**835–850.
3. **Baker, S. J., J. S. Gunn, and R. Morona.** 1999. The *Salmonella typhi* melittin resistance gene *pqaB* affects intracellular growth in PMA-differentiated U937 cells, polymyxin B resistance and lipopolysaccharide. Microbiology **145:**367–378.
4. **Basso, H., F. Rharbaoui, L. H. Staendner, E. Medina, F. Garcia-Del Portillo, and C. A. Guzman.** 2002. Characterization of a novel intracellularly activated gene from *Salmonella enterica* serovar Typhi. Infect. Immun. **70:**5404–5411.
5. **Bauer, A. W., W. M. M. Kirby, J. C. Sherris, and M. Turck.** 1966. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. **45:**493–496.
6. **Benson, S. A., and A. Decloux.** 1985. Isolation and characterization of outer membrane permeability mutants in *Escherichia coli* K-12. J. Bacteriol. **161:** 361–367.
7. **Bremer, E., P. Gerlach, and A. Middendorf.** 1988. Double negative and positive control of *tsx* expression in *Escherichia coli.* J. Bacteriol. **170:**108–116.
8. **Bremer, E., A. Middendorf, J. Martinussen, and P. Valentin-Hansen.** 1990. Analysis of the *tsx* gene, which encodes a nucleoside-specific channel-forming protein (Tsx) in the outer membrane of *Escherichia coli.* Gene **96:**59–65.
9. **Brown, R. F., and B. A. Stocker.** 1987. *Salmonella typhi* 205aTy, a strain with two attenuating auxotrophic characters, for use in laboratory teaching. Infect. Immun. **55:**892–898.
10. **Buchmeier, N. A., and S. J. Libby.** 1997. Dynamics of growth and death within a *Salmonella typhimurium* population during infection of macrophages. Can. J. Microbiol. **43:**29–34.
11. **Contreras, I., C. S. Toro, G. Troncoso, and G. C. Mora.** 1997. *Salmonella typhi* mutants defective in anaerobic respiration are impaired in their ability to replicate within epithelial cells. Microbiology **43:**2665–2672.
12. **Contreras, I., L. Munoz, C. S. Toro, and G. C. Mora.** 1995. Heterologous expression of *Escherichia coli* porin genes in *Salmonella typhi* Ty2: regulation by medium osmolarity, temperature and oxygen availability. FEMS Microbiol. Lett. **133:**105–111.
13. **Craig, J. E., Y. Zhang, and M. P. Gallagher.** 1994. Cloning of the *nupC* gene of *Escherichia coli* encoding a nucleoside transport system, and identification of an adjacent insertion element, IS 186. Mol. Microbiol. **11:**1159–1168.
14. **Crump, J. A., S. P. Luby, and E. D. Mintz.** 2004. The global burden of typhoid fever. Bull. W. H. O. **82:**346–353.
15. **Daigle, F., J. E. Graham, and R. Curtiss.** 2001. Identification of *Salmonella typhi* genes expressed within macrophages by selective capture of transcribed sequences (SCOTS). Mol. Microbiol. **41:**1211–1222.
16. **Dargent, B., J. Rosenbusch, and F. Pattus.** 1987. Selectivity for maltose and maltodextrins of maltoporin, a pore-forming protein of *E. coli* outer membrane. FEBS Lett. **220:**136–142.
17. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA **97:**6640–6645.
18. **Deng, W., S. R. Liou, G. Plunkett 3rd, G. F. Mayhew, D. J. Rose, V. Burland, V. Kodoyianni, D. C. Schwartz, and F. R. Blattner.** 2003. Comparative genomics of *Salmonella enterica* serovar Typhi strains Ty2 and CT18. J. Bacteriol. **185:**2330–2337.
19. **Dorman, C. J., S. Chatfield, C. F. Higgins, C. Hayward, and G. Dougan.** 1989. Characterization of porin and *ompR* mutants of a virulent strain of *Salmonella typhimurium*: *ompR* mutants are attenuated in vivo. Infect. Immun. **57:**2136–2140.
20. **Dougan, G., D. Maskell, D. Pickard, and C. Hormaeche.** 1987. Isolation of stable *aroA* mutants of *Salmonella typhi* Ty2: properties and preliminary characterisation in mice. Mol. Gen. Genet. **207:**402–405.
21. **Edwards, M. F., and B. A. Stocker.** 1988. Construction of Δ*aroA his* Δ*pur* strains of *Salmonella typhi.* J. Bacteriol. **170:**3991–3995.
22. **Ellermeier, C. D., A. Janakiraman, and J. M. Slauch.** 2002. Construction of targeted single copy *lac* fusions using lambda Red and FLP-mediated site-specific recombination in bacteria. Gene **290:**153–161.

23. **Fields, P. I., R. V. Swanson, C. G. Haidaris, and F. Heffron.** 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proc. Natl. Acad. Sci. USA **83:**5189–5193.
24. **Finlay, B. B., and S. Falkow.** 1989. Common themes in microbial pathogenicity. Microbiol. Rev. **53:**210–230.
25. **Francis, C. L., M. N. Starnbach, and S. Falkow.** 1992. Morphological and cytoskeletal changes in epithelial cells occur immediately upon interaction with *Salmonella typhimurium* grown under low-oxygen conditions. Mol. Microbiol. **6:**3077–3087.
26. **Fsihi, H., B. Kottwitz, and E. Bremer.** 1993. Single amino acid substitutions affecting the substrate specificity of the *Escherichia coli* K-12 nucleoside-specific Tsx channel. J. Biol. Chem. **268:**17495–17503.
27. **Galan, J. E.** 1996. Molecular genetic bases of *Salmonella* entry into host cells. Mol. Microbiol. **20:**263–271.
28. **Garcia del Portillo, F.** 2001. *Salmonella* intracellular proliferation: where, when and how? Microbes Infect. **3:**1305–1311.
29. **Gerlach, P., P. Valentin-Hansen, and E. Bremer.** 1990. Transcriptional regulation of the *cytR* repressor gene of *Escherichia coli*: autoregulation and positive control by the cAMP/CAP complex. Mol. Microbiol. **4:**479–488.
30. **Hantke, K.** 1976. Phage T6-colicin K receptor and nucleoside transport in *Escherichia coli.* FEBS Lett. **70:**109–112.
31. **Harris, P., and P. Ralph.** 1985. Human leukemic models of myelomonocytic development: a review of the HL-60 and U937 cell lines. J. Leukoc. Biol. **37:**407–422.
32. **Hidalgo, A. A., A. N. Trombert, J. C. Castro-Alonso, C. A. Santiviago, B. R. Tesser, P. Youderian, and G. C. Mora.** 2004. Insertions of mini-Tn10 transposon T-POP in *Salmonella enterica* sv. Typhi. Genetics **167:**1069–1077.
33. **Hikita, C., Y. Satake, H. Yamada, T. Mizuno, and S. Mizushima.** 1989. Structural and functional characterization of the OmpF and OmpC porins of the *Escherichia coli* outer membrane: studies involving chimeric proteins. Res. Microbiol. **140:**177–190.
34. **Ho, T. D., N. Figueroa-Bossi, M. Wang, S. Uzzau, L. Bossi, and J. M. Slauch.** 2002. Identification of GtgE, a novel virulence factor encoded on the Gifsy-2 bacteriophage of *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **184:**5234–5239.
35. **Hochstrasser, D. F., M. G. Harrington, A. C. Hochstrasser, M. J. Miller, and C. R. Merril.** 1988. Methods for increasing the resolution of two-dimensional protein electrophoresis. Anal. Biochem. **173:**424–435.
36. **Jamieson, D. J., and C. F. Higgins.** 1986. Two genetically distinct pathways for transcriptional regulation of anaerobic gene expression in *Salmonella typhimurium.* J. Bacteriol. **168:**389–397.
37. **Kapoor, S., R. D. Singh, P. C. Sharma, and M. Khullar.** 2002. Anaerobiosis induced virulence of *Salmonella typhi.* Indian J. Med. Res. **115:**184–188.
38. **Lai, E. M., U. Nair, N. D. Phadke, and J. R. Maddock.** 2004. Proteomic screening and identification of differentially distributed membrane proteins in *Escherichia coli.* Mol. Microbiol. **52:**1029–1044.
39. **Leclerc, G. J., C. Tartera, and E. S. Metcalf.** 1998. Environmental regulation of *Salmonella typhi* invasion-defective mutants. Infect. Immun. **66:**682–691.
40. **Lee, C. A., and S. Falkow.** 1990. The ability of *Salmonella* to enter mammalian cells is affected by bacterial growth state. Proc. Natl. Acad. Sci. USA **87:**4304–4308.
41. **Leung, K. Y., and B. B. Finlay.** 1991. Intracellular replication is essential for the virulence of *Salmonella typhimurium.* Proc. Natl. Acad. Sci. USA **88:** 11470–11474.
42. **Linde, K.** 1983. Stable, highly immunogenic mutants of "*Salmonella*" with two independent, attenuating markers as potential live vaccine and their validity for "*Shigella*" and other bacteria. Dev. Biol. Stand. **53:**15–28.
43. **Liu, X., and T. Ferenci.** 1998. Regulation of porin-mediated outer membrane permeability by nutrient limitation in *Escherichia coli.* J. Bacteriol. **180:**3917–3922.
44. **Lobos, S. R., and G. C. Mora.** 1991. Alteration in the electrophoretic mobility of OmpC due to variations in the ammonium persulfate concentration in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Electrophoresis **12:**448–450.
45. **Maier, C., E. Bremer, A. Schmid, and R. Benz.** 1988. Pore-forming activity of the Tsx protein from the outer membrane of *Escherichia coli.* Demonstration of a nucleoside-specific binding site. J. Biol. Chem. **263:**2493–2499.
46. **Maloy, S. R.** 1990. Experimental techniques in bacterial genetics. Jones & Bartlett Publishers, Boston, MA.
47. **McFarland, W. C., and B. A. Stocker.** 1987. Effect of different purine auxotrophic mutations on mouse-virulence of a Vi-positive strain of *Salmonella dublin* and of two strains of *Salmonella typhimurium.* Microb. Pathog. **3:**129–141.
48. **Mekalanos, J. J.** 1992. Environmental signals controlling expression of virulence determinants in bacteria. J. Bacteriol. **174:**1–7.
49. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
50. **Munch-Petersen, A., and N. Jensen.** 1990. Analysis of the regulatory region of the *Escherichia coli nupG* gene, encoding a nucleoside-transport protein. Eur. J. Biochem. **190:**547–551.
51. **Munch-Petersen, A., B. Mygind, A. Nicolaisen, and N. J. Pihl.** 1979. Nucleoside transport in cells and membrane vesicles from *Escherichia coli* K12. J. Biol. Chem. **254:**3730–3737.
52. **Nieweg, A., and E. Bremer.** 1997. The nucleoside-specific Tsx channel from the outer membrane of *Salmonella typhimurium*, *Klebsiella pneumoniae* and *Enterobacter aerogenes*: functional characterization and DNA sequence analysis of the *tsx* genes. Microbiology **143:**603–615.
53. **Nikaido, H.** 1994. Porins and specific diffusion channels in bacterial outer membranes. J. Biol. Chem. **269:**3905–3908.
54. **Nikaido, H., E. Y. Rosenberg, and J. Foulds.** 1983. Porin channels in *Escherichia coli*: studies with beta-lactams in intact cell. J. Bacteriol. **153:**232–240.
55. **Norholm, M. H., and G. Dandanell.** 2001. Specificity and topology of the *Escherichia coli* xanthosine permease, a representative of the NHS subfamily of the major facilitator superfamily. J. Bacteriol. **183:**4900–4904.
56. **O'Callaghan, D., D. Maskell., J. Tite, and G. Dougan.** 1990. Immune responses in BALB/c mice following immunization with aromatic compound or purine-dependent *Salmonella typhimurium* strains. Immunology **69:**184–189.
57. **Parkhill, J., G. Dougan, K. D. James, N. R. Thomson, D. Pickard, J. Wain, C. Churcher, K. L. Mungall, S. D. Bentley, M. T. Holden, M. Sebaihia, S. Baker, D. Basham, K. Brooks, T. Chillingworth, P. Connerton, A. Cronin, P. Davis, R. M. Davies, L. Dowd, N. White, J. Farrar, T. Feltwell, N. Hamlin, A. Haque, T. T. Hien, S. Holroyd, K. Jagels, A. Krogh, T. S. Larsen, S. Leather, S. Moule, P. O'Gaora, C. Parry, M. Quail, K. Rutherford, M. Simmonds, J. Skelton, K. Stevens, S. Whitehead, and B. G. Barrell.** 2001. Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. Nature **413:**848–852.
58. **Rappleye, C. A., and J. R. Roth.** 1997. A Tn*10* derivative (T-POP) for isolation of insertions with conditional (tetracycline-dependent) phenotypes. J. Bacteriol. **179:**5827–5834.
59. **Rigal, A., E. Bouveret, R. Lloubes, C. Lazdunski, and H. Benedetti.** 1997. The TolB protein interacts with the porins of *Escherichia coli.* J. Bacteriol. **179:**7274–7279.
60. **Salmon, K. A., S. P. Hung, N. R. Steffen, R. Krupp, P. Baldi, G. W. Hatfield, and R. P. Gunsalus.** 2005. Global gene expression profiling in *Escherichia coli* K12: effects of oxygen availability and ArcA. J. Biol. Chem. **280:**15084–15096.
61. **Schmieger, H.** 1972. Phage P22-mutants with increased or decreased transduction abilities. Mol. Gen. Genet. **119:**75–88.
62. **Schnaitman, C. A.** 1971. Solubilization of the cytoplasmic membrane of *Escherichia coli* by Triton X-100. J. Bacteriol. **108:**545–552.
63. **Scott, C., J. D. Partridge, J. R. Stephenson, and J. Green.** 2003. DNA target sequence and FNR-dependent gene expression. FEBS Lett. **541:**97–101.
64. **Slauch, J. M., M. J. Mahan, and J. J. Mekalanos.** 1994. In vivo expression technology for selection of bacterial genes specifically induced in host tissues. Methods Enzymol. **235:**481–492.
65. **Sorensen, K. I.** 1994. Conformational heterogeneity in the *Salmonella typhimurium pyrC* and *pyrD* leader mRNAs produced in vivo. Nucleic Acids Res. **22:**625–631.
66. **Sorensen, K. I., K. E. Baker, R. A. Kelln, and J. Neuhard.** 1993. Nucleotide pool-sensitive selection of the transcriptional start site in vivo at the *Salmonella typhimurium pyrC* and *pyrD* promoters. J. Bacteriol. **175:**4137–4144.
67. **Spiro, S., and J. R. Guest.** 1991. Adaptive responses to oxygen limitation in *Escherichia coli.* Trends Biochem. Sci. **16:**310–314.
68. **Strauch, K. L., J. B. Lenk, B. L. Gamble, and C. G. Miller.** 1985. Oxygen regulation in *Salmonella typhimurium.* J. Bacteriol. **161:**673–680.
69. **Suh, S. J., and J. C. Escalante-Semerena.** 1993. Cloning, sequencing and overexpression of *cobA* which encodes ATP:corrinoid adenosyltransferase in *Salmonella typhimurium.* Gene **129:**93–97.
70. **Sundstrom, C., and K. Nilsson.** 1976. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int. J. Cancer **17:**565–577.
71. **Tommassen, J., P. Overduin, B. Lugtenberg, and H. Bergmans.** 1982. Cloning of *phoE*, the structural gene for the *Escherichia coli* phosphate limitation-inducible outer membrane pore protein. J. Bacteriol. **149:**668–672.
72. **Toro, C. S., G. C. Mora, and N. Figueroa-Bossi.** 1998. Gene transfer between related bacteria by electrotransformation: mapping *Salmonella typhi* genes in *Salmonella typhimurium.* J. Bacteriol. **180:**4750–4752.
73. **Westh Hansen, S. E., N. Jensen, and A. Munch-Petersen.** 1987. Studies on the sequence and structure of the *Escherichia coli* K-12 *nupG* gene, encoding a nucleoside-transport system. Eur. J. Biochem. **168:**385–391.
74. **Zakharov, S. D., and W. A. Cramer.** 2004. On the mechanism and pathway of colicin import across the *E. coli* outer membrane. Front. Biosci. **9:**1311–1317.

Editor: A. D. O'Brien