DOI: 10.1534/genetics.105.054700

The Cotranscribed *Salmonella enterica* sv. Typhi *tsx* and *impX* Genes Encode Opposing Nucleoside-Specific Import and Export Proteins

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Manuscript received December 14, 2005
Accepted for publication February 16, 2006

ABSTRACT

The *Salmonella enterica tsx* gene encodes a nucleoside-specific outer membrane channel. The Tsx porin is essential for the prototrophic growth of *S. enterica* sv. Typhi in the absence of nucleosides. RT–PCR analysis shows that the *tsx* gene is cotranscribed with an open reading frame unique to *S. enterica*, *impX* (STY0450), which encodes an inner membrane protein 108 amino acids in length, which is predicted to have only two transmembrane α-helices. Fusions of the *lacZ* gene to both *tsx* and *impX* reveal that the transcription of both genes is induced in the presence of adenosine. A null mutation in the *S.* Typhi *impX* gene suppresses the induced auxotrophy for adenosine or thymidine resulting from a *tsx* mutation and confers sensitivity to high concentrations of adenosine or thymidine. The ImpX protein, when tagged with a 3xFLAG epitope, is functional and associates with the inner membrane; *impX* mutants are defective in the export of ^{3}H-radiolabeled thymidine. Taken together, these and other results suggest that the *S.* Typhi Tsx porin and ImpX inner membrane protein facilitate competing mechanisms of thymidine influx and efflux, respectively, to maintain the steady-state levels of internal nucleoside pools.

THE outer membrane of gram-negative bacteria forms a protective permeability barrier around the cells and serves as a molecular sieve. For this reason, channels are present in the outer membrane to mediate the transport of nutrients and ions across the outer membrane into the periplasm. These channels can be divided into three classes: the general porins, the substrate-specific porins, and the active transporters (Nikaido 2003). The general porins, including OmpC and OmpF, form trimeric, water-filled pores in the outer membrane through which relatively small (<600 Da), abundant solutes diffuse, a process driven by their concentration gradients (Benson and Decloux 1985; Nikaido 1994). For nutrients that are present at low (<1 mm) concentrations in the extracellular environment, passive diffusion is not efficient and transport occurs via substrate-specific and active transporters. The substrate-specific porins contain medium-affinity (micromolars to millimolars) substrate-binding sites that can be saturated, yet allow the efficient diffusion of substrates with shallow concentration gradients (Hantke 1976; McKeown *et al.* 1976; Nikaido 1994). The substrate-specific porins include LamB (maltose and maltodextrins; Luckey and Nikaido 1980), ScrY (sucrose; Hardesty *et al.* 1991), OprB (glucose; Trias *et al.* 1988), OprD (basic amino acids; Trias and Nikaido 1990), and Tsx (nucleosides; Hantke 1976; McKeown *et al.* 1976).

Both eukaryotes and prokaryotes salvage nucleosides for acting as precursors for nucleic acid synthesis and, in several cases, to serve as carbon and nitrogen sources (Hantke 1976; McKeown *et al.* 1976; Acimovic and Coe 2002). In gram-negative enteric bacteria, the first step in this salvage process is the transport of nucleosides across the outer membrane into the periplasm, mediated by the Tsx family of porins. After traversing the periplasmic space, nucleosides are transported across the inner membrane into the cytoplasm by the transporters NupC and NupG (Westh Hansen *et al.* 1987; Munch-Petersen and Jensen 1990; Craig *et al.* 1994; Norholm and Dandanell 2001).

In previous studies of the *Salmonella enterica* sv. Typhi (*S.* Typhi) Tsx porin, we found that Tsx is among the proteins in outer membrane preparations whose expression increases dramatically in response to anaerobiosis, a condition that favors the virulence of this pathogen. Surprisingly, we found that Tsx is essential for the prototrophic growth of *S.* Typhi in the absence of nucleosides. On the basis of these and other results, we have proposed that the Tsx porin plays a critical role in the assembly and integrity of the *S.* Typhi membrane, and thus in the virulence of *S.* Typhi (Bucarey *et al.* 2005). In this article, we show that a short open reading frame (ORF) adjacent to the *S.* Typhi *tsx* gene, *impX* (STY0450), encodes an additional product that mediates nucleoside transport across the *S.* Typhi membrane.

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TABLE 1

Bacterial strains and plasmids used in this study

Strain	Genotype	Source
S. Typhi		
STH2370	Wild type	Hospital Lucio Córdova
TYT2075	STH2370 Δ*tsx*	Bucarey *et al.* (2005)
TYT2080	STH2370 Δ*tsx*/pSU19::*tsx*	Bucarey *et al.* (2005)
TYT1014	STH2370 Δ*impX*	This work
TYT1026	STH2370 Δ*tsx* Δ*impX*	This work
TYT1033	STH2370 Δ*impX* /pET::*impX*	This work
TYT1044	STH2370 Δ*tsx* Δ*impX*/pGEM::*tsx-impX*	This work
TYT3020	STH2370 Δ*jajI*	This work
TYT3040	STH2370 *tsx*::*lacZY*	Bucarey *et al.* (2005)
TYT3052	STH2370 *impX*::*lacZY*	This work
TYT2014	STH2370 *impX*::3xFLAG	This work

Plasmids	Genotype	Source
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 and pKD3	Cam^R and Kan^R template plasmids	K. Datsenko and B. Wanner
pSU11	3xFLAG template plasmid	L. Bossi
pCE36	Kan^R, FRT::*lacZY* plasmid	J. Slauch

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions: The *S.* Typhi strains used in this study are derivatives of the wild-type strain STH2370 and are listed in Table 1. Bacteria were grown routinely at 37° and aerated by vigorous shaking. Rich medium used for growth was LB medium; defined medium was M9-glucose medium supplemented with cysteine and tryptophan 50 mg liter^{-1}, hereafter referred to as "minimal medium." When required, LB medium was supplemented with ampicillin (Amp; 100 mg liter^{-1}), chloramphenicol (Cam; 25 mg liter^{-1}), kanamycin (Kan; 50 mg liter^{-1}), arabinose (2 mg ml^{-1}), and/or glucose (2 mg ml^{-1}). Solid media included 15 g liter^{-1} agar (Agar–agar, Merck). PBS buffer is NaH2PO4·7H2O 55 mg ml^{-1}; K_2HPO_4, 15 mg ml^{-1}; NaCl, 4.25 mg ml^{-1}. Nucleosides and other reagents and chemicals were from Sigma (St. Louis). *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 the selection and preparation of plasmids.

Mutant constructions: Mutant derivatives of *S.* Typhi STH2370 with deletions of the *tsx*, *impX*, and *jajI* genes, and concomitant insertions of kanamycin (Kan^R)- or chloramphenicol (Cam^R)-resistance cassettes were constructed using the method of Datsenko and Wanner (2000). PCR primers 60 bases long overlapping the start and stop codons of each gene were synthesized with 40 base 5′-ends corresponding to the ends of the desired substitutions. The primers used for each gene were (start and stop codons are in boldface type): *tsx*(H1 + P1) 5′-CAGTGGCATACAT**ATG**AAAAAAACTTTAC TCGCAGTCAGCTGTAGGCTGGAGC TGCTTCG, *tsx*(H2 + P2) CTTTTTTGCAGGT**TTA**GAAGTTGTAACCCACGACCAGGT ACATATGAATATCCTCCTTAG, *impX* (H1 + P1) GGCGC**ATG** CTGCCATGCGGCGGCGAGTCGCGCCCACTTCATGTAGGC TGGAGC TGCTTCG, *impX*(H2 + P2) 5′-GAGCAGAGTAGAA **TCA**GCGGTAAGCCGGGGCAACCCGGTGCATATGAATATCC TCCTTAG, *jajI*(H1 + P1) GGAAATGATCGAC**ATG**ACAAGACG TTACCTAAGAATTCTCTGTAGGCTGGAGCTGCTTCG, and *jajI* (H2 + P2) TCCGCCAGAACGT**TTA**TTGATTAACAGGCT GAATATCATGCATATGAATATCCT CCTTAG.

The 3′ 20 bases of each primer (underlined) were annealed to the 5′- or 3′-ends of Cam or Kan^R cassettes flanked by FRT sites in template plasmids pKD3 and pKD4, respectively (Datsenko and Wanner 2000). PCR reactions using Taq DNA polymerase were made according to the manufacturer's instructions (Life Technologies). STH2370 carrying plasmid pKD46 (encoding the λ-Red recombinase functions) was grown at 30° in LB medium with Amp and 1 mm arabinose, made electrocompetent, and electroporated with ~500 ng of each PCR product. Electroporated cells were plated on LB medium with Cam or Kan at 37°. Substitution mutations in recombinant strains resulting from this procedure were moved into a clean wild-type background by electroporation with linear chromosomal DNA as described by Toro *et al.* (1998). The presence of each substitution mutation was confirmed by PCR amplification, using primers complementary to the *S.* Typhi genome flanking the sites of substitution. After backcrosses, resistance determinants were eliminated to avoid polar effects using plasmid pCP20, an Amp^R plasmid that is temperature sensitive for replication and the production of FLP recombinase (Datsenko and Wanner 2000). *S.* Typhi Cam^R or Kan^R mutants were transformed with pCP20, Amp^R recombinants were selected at 30°, and isolated colonies were purified at 37° before testing for loss of antibiotic resistance. After the elimination of resistance cassettes, we obtained nonpolar mutations, as described previously by Datsenko and Wanner (2000).

Cloning and complementation of the *S.* Typhi *tsx-impX* operon: To clone the *tsx-impX* operon, we amplified a 1.9-kb fragment of *S.* Typhi STH2370 DNA, including the *tsx* and *impX* coding sequences and their upstream promoter with primers TAGAATTCTACGGGCAAATTCAGGGCACTA and TTGAATGTTAGGTTACGCTTC. The PCR fragment was purified and ligated to Amp^R plasmid vector pGEM-T (Promega, Madison, WI). The ligation mix was electroporated into *E. coli* host strain DH5α, and recombinants that form white colonies on LB Amp plates with 0.5 mm IPTG and 40 μg/ml X-gal at 37° were screened. Plasmid DNA purified from one clone yielded an insert of the predicted size after digestion with *Eco*RI. This

plasmid, pGEM::*tsx-impX*, was electroporated into *S.* Typhi Δ*tsx* Δ*impX*; Amp^R recombinants were found to have a wild-type phenotype (our unpublished results).

Phenotypic analysis of *S.* Typhi mutants: A modification of the disk diffusion assay (Bauer *et al.* 1966) was used to determine the auxotrophic requirements of mutant strains, as well as their sensitivities to nucleosides. Assays to measure the efficiencies of plating of wild-type and mutant strains in the presence of nucleosides were performed as described previously (Bucarey *et al.* 2005) and in the text. Efficiencies of plating are expressed as the titers of colony-forming units on media with or without nucleosides, divided by the titer of the wild-type strain on media without nucleosides.

Subcellular fractionation of inner and outer membrane proteins: Membrane fractions were prepared as described by Lobos and Mora (1991) on the basis of the modification of the method of Schnaitman (1971). Bacteria were grown to midexponential phase, chilled on ice, pelleted by centrifugation at 3000 × *g* for 15 min at 4°, resuspended in lysis buffer (Tris–HCl 10 mm pH 8, 10 mm MgCl2), sonicated, and then supplemented to 2 mm phenylmethylsulfonyl fluoride. Whole cells and debris were removed by low-speed centrifugation (3000 × *g*, 10 min), and total membrane fractions were obtained after 45 min of centrifugation at 13,000 × *g* at 4°. Inner membrane fractions were solubilized with 2% Triton X-100, and the outer membrane fraction was pelleted by centrifugation at 13,000 × *g* and solubilized in 50 μl of Tris–HCl 100 mm, pH 8 buffer, 1% SDS. Supernatants containing proteins associated with the inner membrane were precipitated by the addition of trichloroacetic acid to 10%, washed twice with acetone, dried, and resuspended in 50 μl of Tris–HCl 100 mm, pH 8 buffer, 1% SDS. Proteins were separated in 12% SDS–polyacrylamide gels.

Epitope tagging: A fusion of the sequence encoding the 3xFLAG epitope with the *impX* gene was constructed using the method of Datsenko and Wanner (2000) as modified by Uzzau *et al.* (2001). Primers were designed with 36- to 40-base 5′ extensions corresponding to the 3′-end of the *impX* coding sequence and to the region immediately downstream of *impX* to amplify plasmid pSUB11. The sequences of the primers used are: FLAG-impX1 CGCAACATTGCGCCTCACCGGGTT GCCCCGGCTTACCGCGACTACAAAGACCATGACGG and FLAG-impX2 CTGACATTTTTTCAGCCCCGGAGTTTGTCG CCAGCCCCAACATATGAATATCCT CCTTAG.

The PCR product was used to transform strain STH2370 carrying plasmid pKD46. The presence of the genetic fusion was confirmed by PCR amplification, using primers complementary to the *S.* Typhi genome flanking the fusion.

Immunoblot analysis: The 3xFLAG fusion protein was detected in immunoblots by the use of anti-FLAG M2 monoclonal antibody from Sigma. Strains carrying the epitope-tagged gene were grown in cultures (2 ml) to stationary phase and centrifuged. Bacterial pellets were resuspended in 50 μl of 100 mm Tris–HCl (pH 8), mixed with 50 μl of Laemmli lysis buffer, and incubated at 100° for 10 min. The resulting lysates were centrifuged to remove cell debris and resolved by SDS–PAGE. Total bacterial lysates and inner and outer membrane fractions were resolved by SDS–PAGE, transferred to polyvinylidenedifluoride (PDVF) membranes, and probed with mouse antiflag Ab M2 (1:10,000) and then with alkaline phosphatase-conjugated goat anti-mouse IgG [1:5000 (Sigma)]. Alkaline phosphatase activity was revealed by using the nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate dye system.

Assays for β-galactosidase activity: *S.* Typhi mutant strains with *lacZY* fusions were grown to an OD_{600nm} of 0.2 and then chilled to 4°. β-Galactosidase activities were assayed as described and are expressed in Miller units, $10^3 \times ((OD_{420nm} - 1.75 \times OD_{550nm}) \times OD^{-1}_{600nm})$ ml^{-1} min^{-1} (Miller 1972).

RNA isolation and reverse transcriptase–PCR: To isolate RNA, cells were grown overnight in LB medium at 37°. Total RNA was extracted and purified using TRIzol and treated with RNase-free DNase I (amplification grade; GIBCO-BRL, Gaithersburg, MD). Reverse transcription (RT)–PCR was performed on 200 ng of DNase-treated RNA using Superscript II RT (Invitrogen, San Diego). Amplification was for 30 cycles (94° for 30 sec, 55° for 30 sec, and 72° for 2 min, followed by a 7-min extension at 72°). Primers jajI TAGAATTCTACGGG CAAATTCAGGGCACTA, impX CCGGATCCGGAAAGAGAA AACCCCCGCACA, tsx2 CCGGATCCAATCCAATCGCCGTCG CCCCAGTTCAG, and tsx1 CCGGTACCCAAAGCCAGCCGC CAGAGCACA, corresponding to internal regions of the *S.* Typhi *jajI*, *impX*, and *tsx* genes, were designed on the basis of the *S.* Typhi strain CT18 genome sequence (Parkhill *et al.* 2001). 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 30 cycles of amplification were resolved in 1% agarose gels with ethidium bromide, and gels were analyzed using a Digital Science 120 system (Kodak).

Uptake and efflux assays: For the measurements of [^{3}H]thymidine (85 Ci/mmol, Perkin-Elmer, Norwalk, CT) uptake and efflux, bacterial cultures of strain *S.* Typhi STH2370 and its mutant derivatives were grown in LB medium to an OD_{600nm} of 0.2 or to overnight density, and cells were harvested by centrifugation and washed twice with M9 glycerol medium or 20 mm HEPES buffer (pH 7), respectively. To measure uptake, a mix of 1 μCi ^{3}H-radiolabeled thymidine (0.01 nmol) and cold thymidine (0.415 nmol) was added to a 0.5-ml cell suspension to yield a final concentration of 0.85 μm. Samples (100 μl) were removed at various time intervals, filtered through membrane filters (ME 25, 0.45 pm; Schleicher & Schuell, Keene, NH), prewashed with M9 medium, and washed twice with 1 ml M9 medium. The radioactivity retained on the membrane filters was determined by scintillation counting. To measure efflux, cells were resuspended in 0.5 ml of 20 mm HEPES buffer (pH 7) and a mix of 1 μCi ^{3}H-radiolabeled thymidine (0.01 nmol) and cold thymidine (0.415 nmol) was added to a final concentration of 0.85 μm. Carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) was added to a final concentration of 20 μm to dissipate the membrane potential, and cells were incubated for 15 min at 37° and aerated by shaking. Cells were then washed twice and resuspended in a 900-μl buffer at 4°. Efflux was initiated by the addition of sodium succinate to 0.1 m. Samples (100 μl) were removed at various time intervals and filtered. Radioactivity present in the supernatants was determined by scintillation counting. Efflux was expressed as the counts per minute released to the supernatant, divided by the counts per minute retained on the membrane filters.

RESULTS

The loss of *impX* function suppresses the induced auxotrophy caused by a *tsx* mutation: We and others have found that the nucleoside-specific porin, Tsx, made by a subset of enteric gram-negative pathogens including *S.* Typhimurium and *E. coli*, is dispensable for growth in minimal media (Nieweg and Bremer 1997). In contrast, in three independent isolates of *S.* Typhi, a Δ*tsx* nonpolar mutation confers an auxotrophic requirement for the nucleosides adenosine or thymidine (Bucarey *et al.* 2005).

TABLE 2

A Δ*impX* mutation confers sensitivity to high concentrations of specific nucleosides and suppresses the auxotrophy resulting from a Δ*tsx* mutation

Genotype	Adenosine	Thymidine	Inosine	Uridine	Guanosine	Cytocine	None
Wild type	1	1	1	1	1	1	1
Δ*impX*	2×10^{-4}	3×10^{-4}	2×10^{-4}	1	1	1	1
Δ*tsx* Δ*impX*	4×10^{-5}	3×10^{-5}	4×10^{-5}	1	1	1	1
Δ*tsx*	1	1	5×10^{-7}	5×10^{-7}	5×10^{-7}	5×10^{-7}	5×10^{-7}

Overnight cultures of each strain grown aerobically in LB medium at 37° were pelleted, washed twice with PBS, diluted serially in PBS, and spread on M9-glucose plates supplemented with adenosine, thymidine, guanosine, cytosine, inosine, or uridine to final concentrations of 0.5 mM. Colony-forming units were determined after incubation for 48 hr at 37°. Efficiencies of plating were expressed as the titers of each strain divided by the titer of the wild type.

The auxotrophy resulting from the Δ*tsx* mutation is unusual. Unlike mutations affecting the essential purine and pyrimidine biosynthetic pathways of *Salmonella enterica*, the Δ*tsx* mutation causes an auxotrophy that can be masked by the addition of nucleosides, but not their nucleotide counterparts, and that can be masked by the addition of either purine or pyrimidine nucleosides (adenosine or thymidine) and only by these specific nucleosides. To understand this unusual phenotype, we have taken a genetic approach, and have attempted to isolate second-site suppressors of a Δ*tsx* mutation that restore prototrophic growth.

During this search, we noted a small ORF (STY0450) immediately downstream and adjacent to the *S.* Typhi *tsx* gene (STY0451). The gene corresponding to ORF STY0450, hereafter referred to as *impX*, is predicted to encode a protein product 108 amino acids in length with a molecular mass of 12 kDa. This gene is unique to serovars of *S. enterica*. To determine the function of the *S.* Typhi *impX* gene, we constructed a mutant derivative of *S.* Typhi strain STH2370 with a Δ*impX* mutation, using the method of Datsenko and Wanner (2000), and determined its phenotype. As shown in Table 2, the Δ*impX* mutant is a prototroph; however, the deletion of the *S.* Typhi *impX* gene also results in an inability to grow in the presence of higher concentrations of adenosine (or inosine) and thymidine. For comparison, a deletion of the gene upstream of *tsx* (*jajI*) confers a wild-type phenotype (our unpublished results).

Moreover, unlike a Δ*tsx* mutant, which is an auxotroph that requires either adenosine or thymidine for growth in minimal medium, a Δ*impX* Δ*tsx* double mutant is a prototroph whose growth is sensitive to adenosine or thymidine. Clearly, the Δ*tsx* mutation does not have a polar effect on the expression of the *impX* gene, because the phenotype of the double mutants is different from that of the Δ*tsx* single mutant. Rather, the Δ*impX* mutation is epistatic to the Δ*tsx* mutation and suppresses the auxotrophy caused by the Δ*tsx* mutation (Figure 1). Formally, these genetic results suggest that the products of the *impX* and *tsx* genes participate in the same pathway in which the activity of ImpX precedes that of Tsx; however, we show below that this is not the case.

Previously, we have shown that complementation of an *S.* Typhi Δ*tsx* mutant with a derivative of the moderate-copy-number plasmid pSU19 carrying the cloned *tsx* gene yields a strain that is prototrophic, yet cannot grow in the presence of high concentrations of adenoside or thymidine, suggesting that overproduction of the Tsx porin results in an imbalance of membrane proteins that renders *S.* Typhi sensitive to adenosine or thymidine (Bucarey *et al.* 2005). This is the same phenotype exhibited by the *S.* Typhi Δ*impX* mutant. In addition, expression of *impX* from a high-copy-number plasmid complements the adenosine or thymidine sensitivity of a Δ*impX* mutant only partially. In contrast, we find that the high-copy-number plasmid vector pGEM-T carrying an insert of the entire *tsx impX* operon with its promoter complements a Δ*tsx* Δ*impX* double mutant completely and restores both prototrophy and the ability to grow in the presence of high concentrations of adenosine or thymidine (our unpublished results). Taken together, these results suggest that the balance of Tsx and ImpX products is implicated in both the prototrophic growth of *S.* Typhi in the absence of adenosine or thymidine and the growth of *S.* Typhi in the presence of high concentrations of adenosine or thymidine.

The *S.* Typhi *tsx* and *impX* genes are cotranscribed: As illustrated in Figure 2, the *tsx* and *impX* genes are transcribed in the same direction and are separated by 53 bp of noncoding sequence. These genes lie 299 bp downstream of an open reading frame designated *jajI* (STY0452), which is also transcribed in the same direction and is separated by 252 bp from the potential ORF STY0449, which is divergently transcribed.

To determine whether the *tsx* and *impX* genes are part of the same transcription unit, we isolated RNA from wild-type *S.* Typhi cells and prepared cDNA templates using the primers *tsx2* and *impX1*, internal to the *tsx* and *impX* genes, respectively. These templates were amplified with pairs of primers corresponding to the *jajI*, *tsx*, and *impX* coding sequences. As shown in Figure 2,

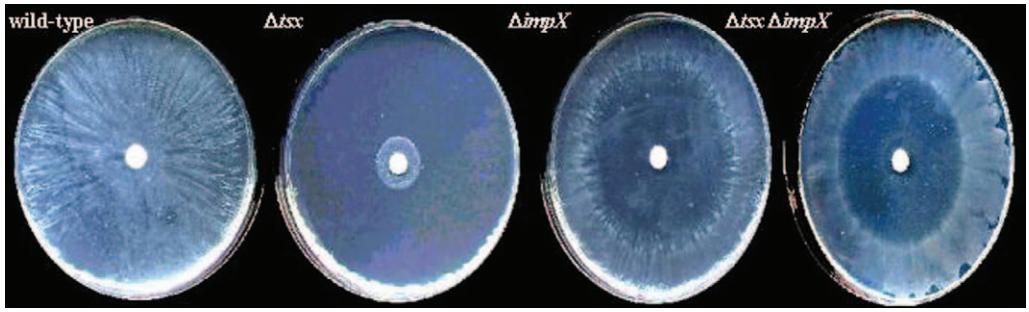

FIGURE 1.—An *S.* Typhi Δ*impX* mutation is epistatic to a Δ*tsx* mutation. *S.* Typhi strains STH2370 and their otherwise isogenic Δ*impX*, Δ*tsx*, and Δ*impX* Δ*tsx* mutant derivatives were grown to overnight density in LB medium and diluted 1:100 in PBS. Aliquots (200 μl) of the dilutions were spread on minimal plates, and sterile filter paper disks with 20 μl of 0.1 M adenosine were placed in the center of each plate. Plates were incubated to 37° for 48 hr. The figure shows that, whereas a Δ*tsx* mutant requires adenosine for growth, a Δ*impX* Δ*tsx* mutant does not and has a phenotype more similar to that of a Δ*impX* mutant.

primer pairs internal to the *jajI* and *tsx* genes or the *jajI* and *impX* genes do not yield PCR products with either cDNA template, suggesting that the *jajI* transcript does not include the *tsx* or *impX* genes. In contrast, both cDNA templates yield products when amplified with a primer pair internal to the *tsx* gene. In addition, the cDNA template made by reverse transcription with the primer internal to *impX* yields a product when amplified with a pair of primers internal to *tsx* and *impX*. These results show that the *tsx* and *impX* genes are cotranscribed.

Transcription of the *tsx* and *impX* genes is induced in the presence of adenosine: To support the conclusion that the *tsx* and *impX* genes are cotranscribed, we made two additional experiments. First, we asked whether transcription of the *tsx* and *impX* genes is subject to a common mechanism of regulatory control by intracellular nucleosides. Expression of the *E. coli tsx* gene is controlled by two differentially regulated promoters. One promoter is regulated negatively by the DeoR repressor, and the other is regulated negatively by the CytR repressor and positively by the cAMP/CRP complex (BREMER *et al.* 1988; GERLACH *et al.* 1990). Repression by DeoR and CytR is relieved by adenosine or cytidine (VALENTIN-HANSEN *et al.* 1978); these nucleosides are inducers of the *tsx* gene. The operators recognized by DeoR and CytR are present in the sequence of the *S.* Typhi *tsx* promoter, and the *deoR* and *cytR* genes are conserved in the *S.* Typhi genome sequence (PARKHILL *et al.* 2001). To ask whether transcription of the *tsx* and *impX* genes is regulated by adenosine in *S.* Typhi, we constructed transcriptional fusions of the *tsx* and *impX* genes to the *lacZY* genes in single copy on the *S.* Typhi genome, using FLP-mediated site-specific recombination. Plasmid pCE36 was integrated at the site of the FLP scar in Δ*tsx* and Δ*impX* strains of *S.* Typhi to generate these fusions (ELLERMEIER *et al.* 2002). As shown in Figure 3, the β-galactosidase activity produced by the recombinant *S.* Typhi *impX*::*lacZY* strain in presence of 2 mM adenosine is about twofold higher than that in the absence of adenosine. The β-galactosidase activity produced by a recombinant *S.* Typhi *tsx*::*lacZY* strain also increases by a similar amount in response to adenosine induction.

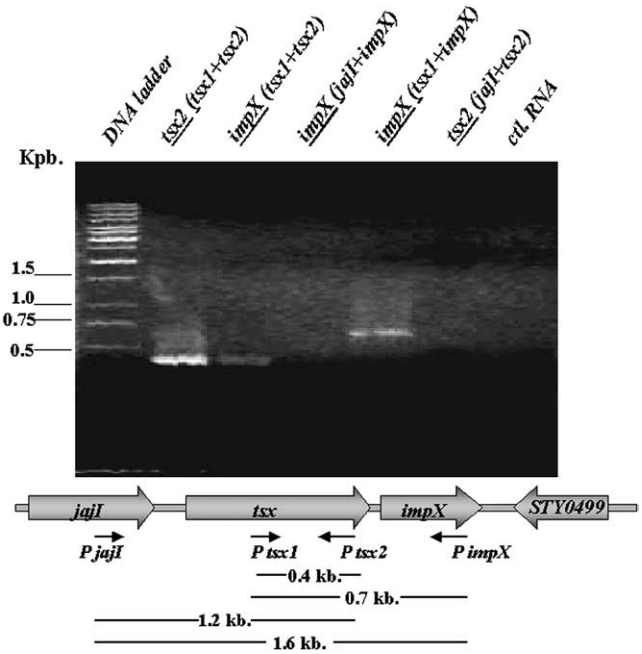

FIGURE 2.—The *tsx* and *impX* genes are cotranscribed. (Bottom) The locations of the primers used to prepare and amplify cDNA. Primers *impX* and *tsx2* (underlined) were used to prepare cDNA corresponding to a portion of the *tsx–impX* transcript prepared from total RNA. PCR products were subsequently amplified using primer pairs (*tsx1* + *tsx2*), (*jajI* + *impX*), (*tsx1* + *impX*), and (*jajI* + *tsx2*). The right-most lane shows the products of amplification of RNA prior to treatment with reverse transcriptase.

Second, we constructed a mutant derivative of *S.* Typhi carrying a fusion of the coding sequence for a threefold repeated FLAG epitope to the 3′-end of the *impX* coding sequence (UZZAU *et al.* 2001). This insertion mutant has a wild-type phenotype (our unpublished results), showing that the C terminus of the ImpX protein is not essential for function. To confirm the result that the expression of the *impX* gene is induced by the presence of adenosine, we grew the *impX*::3xFLAG mutant in the presence of various concentrations of adenosine, resuspended pelleted cells in sample buffer with SDS, and resolved the proteins in cells by SDS–PAGE. Proteins in gels were transferred to PDVF membranes,

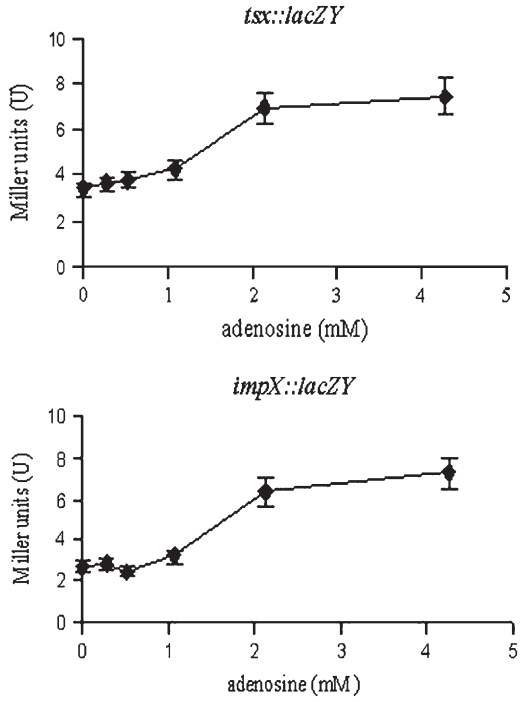

FIGURE 3.—The transcription of the *tsx* and *impX* genes is induced by the presence of adenosine. Data are the average values of β-galactosidase activities expressed in Miller units (MILLER 1972) obtained from one representative experiment performed in triplicate. Similar results were obtained from two additional, independent experiments.

and the presence of the fusion protein was detected by Western blot analysis, as described in MATERIALS AND METHODS. Immunoblot analysis shows that this mutant produces a protein with an apparent molecular mass of ~14 kDa that binds anti-FLAG monoclonal antibody. This analysis also shows that the steady-state level of the ImpX–3xFLAG protein increases in response to increasing concentrations of adenosine (Figure 4).

The ImpX protein is associated with the inner membrane: In our previous search for suppressors of the induced auxotrophy caused by a Δ*tsx* mutation, we encountered a second-site revertant with an insertion of the mini-Tn10 transposon derivative, T-POP (RAPPLEYE and ROTH 1997), upstream of the *S.* Typhi *pyrD* gene. The *pyrD* gene encodes a dehydrogenase, the only membrane-associated protein required for *de novo* nucleoside biosynthesis (HANSEN *et al.* 2004). This T-POP insertion suppresses the auxotrophy caused by a Δ*tsx* mutation presumably by lowering the amount of PyrD protein product relative to that of Tsx (BUCAREY *et al.* 2005).

PyrD is an unusual membrane protein, because, like ImpX, it is predicted to have only two transmembrane α-helices (HANSEN *et al.* 2004). Because our genetic evidence suggests that, like PyrD, ImpX may interact with Tsx, we determined the subcellular localization of the ImpX protein. As shown in Figure 5, when proteins made by the *impX*::3xFLAG mutant are fractionated, ImpX–3xFLAG protein is found associated with the inner membrane fraction.

A Δ*impX* mutation suppresses the defect in nucleoside uptake caused by a Δ*tsx* mutation and is defective in nucleoside export: A deletion of the *S.* Typhi *impX* gene suppresses the auxotrophy resulting from a Δ*tsx* mutation (Figure 2). In *E. coli*, a null mutation in the *tsx* gene results in a complete defect in the uptake of both adenosine and thymidine when these nucleosides are present at low (micromolars to millimolars) extracellular concentrations (FSIHI *et al.* 1993). To determine the effects of the Δ*tsx* and Δ*impX* mutations on nucleoside uptake, we measured the initial rates of [^{3}H]thymidine uptake by wild-type and mutant strains of *S.* Typhi. As shown in Figure 6, unlike the case of *E. coli* in which a Δ*tsx* mutation results in a complete defect in thymidine uptake under similar conditions, an *S.* Typhi Δ*tsx* mutation results in only a partial defect in the initial rate of thymidine uptake; mutant cells uptake thymidine at a rate approximately half that of the wild type. From this result, we can conclude that *S.* Typhi has other

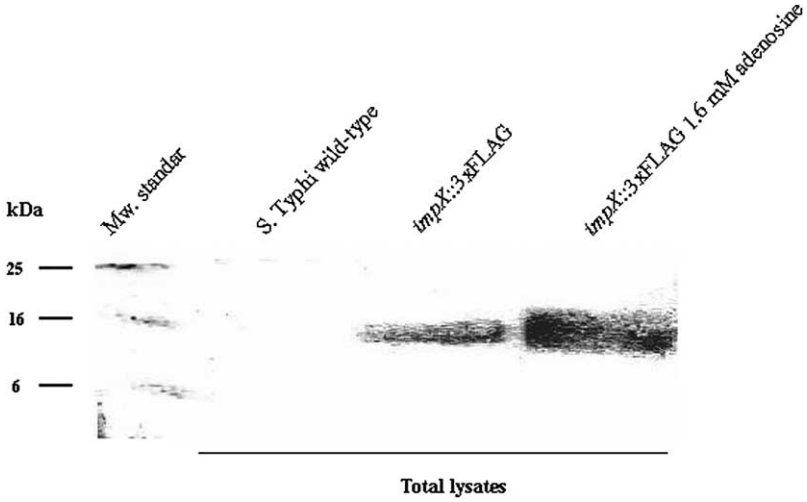

FIGURE 4.—Steady-state levels of the ImpX–3xFLAG hybrid protein increase in response to increasing concentrations of adenosine. The *S.* Typhi *impX*::3xFLAG mutant was grown to overnight density in LB medium in the absence and presence (1.6 mM) of adenosine, cells were concentrated by centrifugation and resuspended in sample buffer with SDS, and proteins were resolved by SDS–PAGE. Proteins in gels were transferred to PDVF membranes, and the presence of the fusion protein was detected by Western blot analysis, as described in MATERIALS AND METHODS.

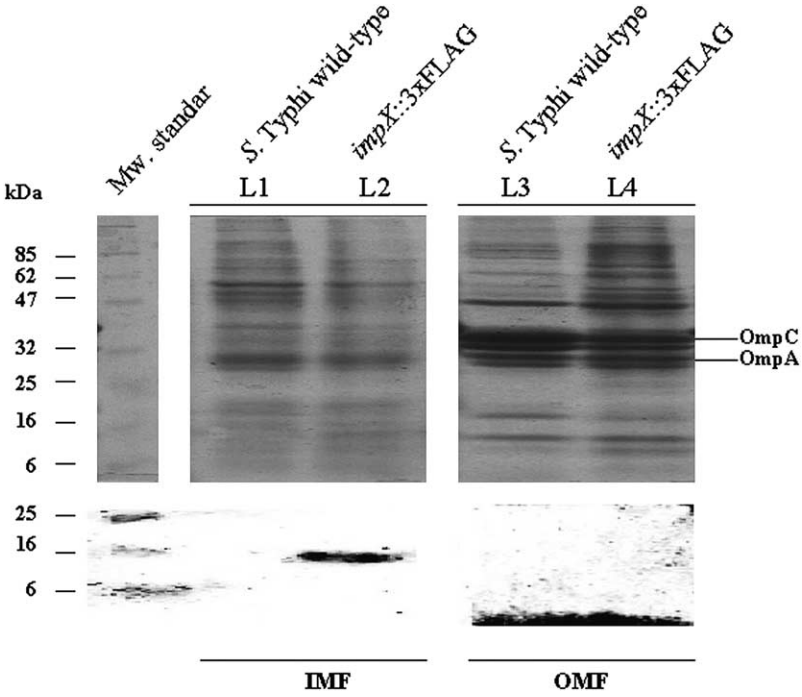

FIGURE 5.—The ImpX protein tagged with the 3xFLAG epitope is associated with the *S.* Typhi inner membrane. Subcellular fractions enriched for proteins in the inner membrane (IMF) and outer membrane (OMF) were separated by SDS–PAGE and transferred to PDVF membranes. (Bottom) The presence or absence of the fusion protein in inner (lanes 1 and 2) and outer (lanes 3 and 4) membrane fractions was detected by Western blot analysis as described in MATERIALS AND METHODS. (Top) The typical pattern of abundant outer membrane proteins made by *S.* Typhi (lanes 3 and 4) (LOBOS and MORA 1991) is not observed for the inner membrane fraction (lanes 1 and 2).

outer membrane proteins in addition to the Tsx porin that facilitates the import of thymidine across the outer membrane. Thus, unlike the case for *E. coli*, thymidine uptake in *S.* Typhi is mediated by multiple porins. Surprisingly, the Δ*impX* mutant uptakes thymidine at a greater initial rate than the wild type, as does the Δ*impX* Δ*tsx* double mutant. Again, we find that the Δ*impX* mutation is epistatic to the Δ*tsx* mutation with respect to thymidine uptake, reinforcing the idea that the Tsx channel is not the only mechanism by which thymidine can enter *S.* Typhi and arguing that ImpX either inhibits the uptake of thymidine or facilitates the competing export of thymidine.

To distinguish between these alternatives, we measured the initial rates of thymidine efflux from wild-type and mutant cells. Cells were loaded with [^{3}H]thymidine in the presence of the uncoupling agent, CCCP, and washed to remove extracellular thymidine. Export was reactivated by the addition of succinate to regenerate the proton motive force due to respiratory electron transport, and the initial rates of thymidine efflux were determined by measuring the rates of release of radioactive thymidine from the loaded cells into the supernatants of cell suspensions. As shown in Figure 6, wild-type cells have a mechanism that results in the efflux of thymidine that is dependent on the proton gradient. The Δ*tsx* mutant shows a somewhat higher rate of thymidine efflux than the wild type. This result is consistent with the idea that Tsx facilitates the directional transport of extracellular thymidine across the outer membrane and into the periplasm. In contrast, the Δ*impX* single mutant shows a lower rate of efflux than does the wild type, implicating the product of the *impX* gene directly in thymidine efflux. The Δ*impX* Δ*tsx* double mutant resembles the wild-type strain in its initial rate of thymidine efflux.

DISCUSSION

In *E. coli*, the Tsx porin is essential for the uptake of nucleosides and deoxynucleosides at low (less than millimolar) substrate concentrations (FSIHI *et al.* 1993). The rate of uptake of adenosine and thymidine is strongly reduced in the absence of the Tsx porin, whereas that of cytidine and guanosine remains unchanged (MUNCH-PETERSEN *et al.* 1979; BENZ *et al.* 1988; MAIER *et al.* 1988). Tsx does not play a role in the transport of free nucleobases or monophosphate nucleosides (MCKEOWN *et al.* 1976; VAN ALPHEN *et al.* 1978; BENZ *et al.* 1988). In addition to its role as a nucleoside-specific channel, the *E. coli* Tsx protein functions as a receptor for a number of bacteriophages and colicin K (HANTKE 1976; NIEWEG and BREMER 1997; NIKAIDO 2003). Tsx also transports albicidin, a potent inhibitor of prokaryotic DNA replication produced by *Xanthomonas albilineans* (BIRCH and PATIL 1985; BIRCH *et al.* 1990; NIEWEG and BREMER 1997). To understand the mechanism of Tsx-mediated transport across the bacterial outer membrane, crystal structures of Tsx have been determined alone and with different bound nucleosides. Tsx forms a monomeric β-barrel consisting of 12 β-strands with a long, narrow central pore. The structures of Tsx in a complex with nucleosides reveal several distinct substrate-binding sites and suggest a "sequential binding" mechanism for the transport of nucleosides across the outer membrane (YE and VAN DEN BERG 2004).

Previously, we found that a deletion of the *S.* Typhi *tsx* gene confers an auxotrophic requirement for

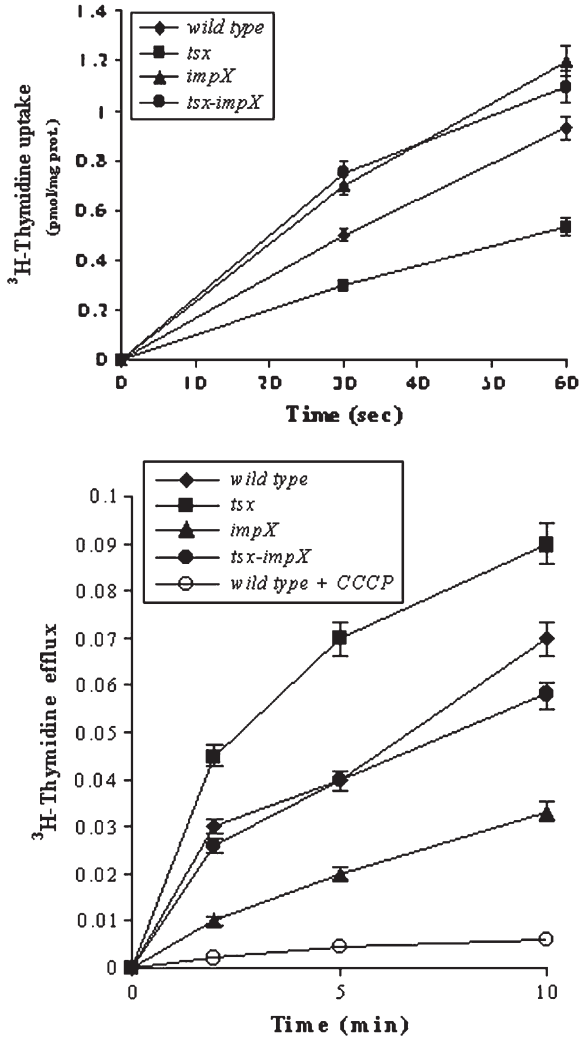

FIGURE 6.—Uptake and efflux of ^{3}H-radiolabeled thymidine by *S.* Typhi. (Top) The initial rates of uptake of [^{3}H]thymidine by *S.* Typhi wild-type or mutant cells was measured at a substrate concentration of 0.85 μM thymidine. Data shown represent the means of the values obtained from two separate experiments in which duplicate samples were taken at each time point. (Bottom) Efflux was initiated by the addition of 0.1 M sodium succinate to cells loaded with [^{3}H]thymidine in the presence of CCCP. Efflux was expressed as the counts per minute released to the supernatant at various times after initiation, divided by the counts per minute retained on the membrane filters. Data shown represent the means of the results from two separate experiments in which duplicate samples were taken at each time point.

adenosine or thymidine. Prototrophy can be restored by the complementation of a Δ*tsx* mutant with an intermediate-copy-number plasmid carrying the *tsx* gene, but the complemented strain acquires sensitivity to these nucleosides, presumably as a consequence of the overexpression of *tsx*. To understand these unusual phenotypes, we have isolated revertants of an *S.* Typhi Δ*tsx* mutant that can grow in minimal medium. One of these revertants carries an insertion that places the *pyrD* gene under the control of the tetracycline-inducible *tetA* promoter from transposon T-POP. This revertant grows in minimal medium only in the presence of intermediate concentrations of tetracycline, suggesting that the balance of products of the *tsx* and *pyrD* genes, an outer membrane porin and an inner-membrane-associated dehydrogenase, is essential for the growth of *S.* Typhi (BUCAREY *et al.* 2005). In this article, we have extended our search for suppressors of the induced auxotrophy resulting from a Δ*tsx* nonpolar mutation and have found that mutations in the cotranscribed and coregulated gene, immediately downstream of *tsx*, *impX*, can suppress this auxotrophy.

The *impX* gene, like *pyrD*, is predicted to encode an inner membrane protein with only two transmembrane α-helices, similar in size to that of the small multidrug resistance (SMR) proteins. This smallest family of membrane proteins has members that range in size from 100 to 120 aminoacyl residues and participate in the efflux of a wide variety of antibiotics (GRINIUS *et al.* 1992; GRINIUS and GOLDBERG 1994). These proteins function as homo- or hetero-oligomers, but unlike ImpX, have four transmembrane α-helices. Indeed, ImpX shares no sequence similarity with proteins of the SMR family, nor with larger multidrug resistance proteins or other membrane transporters, and represents the first member of a new class of inner membrane exporters.

Our genetic evidence argues strongly that the Tsx outer membrane protein and ImpX inner membrane protein act in opposition to maintain a balance of nucleoside transport. Not only does an Δ*impX* mutation suppress a Δ*tsx* mutation, but also it confers the same phenotype, sensitivity to a specific subset of nucleosides (the preferred substrates of Tsx), which results from the overexpression of the *tsx* gene. These results argue that the balance of products of the *tsx* and *impX* genes is critical for nucleoside transport in *S.* Typhi.

However, nucleoside import and export in *S.* Typhi must involve both outer and inner membrane protein in addition to Tsx and ImpX, respectively. The findings that the Δ*tsx* mutant is only partially defective in thymidine import reveals that *S.* Typhi has another porin involved in nucleoside import. Similarly, the Δ*impX* mutant is only partially defective in thymidine export. Because the inner membrane is impermeable to thymidine, this result reveals that there is another, *impX*-independent, energy-dependent thymidine efflux system. The simplest model to explain our result is that the Tsx channel and other porins facilitate the import of nucleosides to the periplasm, and additional inner membrane transporters, including those encoded by the *nupC*, *nupG*, and potentially other *S.* Typhi genes, complete the import process. In addition, there is a competing export pathway that involves the principal ImpX and another inner membrane transporter and potentially as-yet-unknown outer membrane proteins. Thus, both Δ*impX* and Δ*impX* Δ*tsx* mutants show a higher rate of nucleoside import, because the major

route of competing nucleoside export is blocked by the Δ*impX* mutation. The Δ*tsx* mutant likely has a higher rate of nucleoside efflux, because Tsx-mediated nucleoside import in this mutant cannot counter the effects of ImpX-mediated export. Both the Δ*impX* mutant and mutants that overproduce Tsx are sensitive to high concentrations of nucleosides, presumably because they accumulate levels of these metabolites sufficient to inhibit cell growth. The Δ*tsx* mutant is an auxotroph, presumably because the combination of the alternative uptake system and a functional ImpX-dependent efflux system in the absence of Tsx does not permit the accumulation of intracellular levels of nucleosides sufficient for growth.

Our results are of particular importance to our understanding of epistatic interactions. Normally, the result that one loss-of-function mutation (for example, Δ*impX*) is epistatic to another loss-of-function mutation (for example, Δ*tsx*) is taken as a strong indication that the product of the first gene acts before that of the second gene in the same pathway. In the case of the *impX* and *tsx* genes, it is clear that their products act in opposing pathways involving redundant functions to maintain a balance of the concentrations of their substrates. It is likely that other examples of this type of epistasis will be encountered upon the genetic analysis of other transport mechanisms, as well as of competing pathways involving multiple, redundant functions in general.

We thank Phil Youderian for his helpful assistance in the preparation of this manuscript. This work was supported by Fondo Nacional de Desarrollo Científico y Tecnológico (Chile) grant 1020485 to G.C.M. S.B. was supported by fellowships from Programa de Mejoramiento de la Equidad y Calidad de la Educación Superior and Dirección General de Postgrado e Investigación, Pontificia Universidad Católica de Chile.

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Communicating editor: A. L. Sonenshein