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Single Amino Acid Substitutions Affecting the Substrate Specificity of the *Escherichia coli* K-12 Nucleoside-specific Tsx Channel*

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The Tsx protein from the *Escherichia coli* outer membrane is a channel-forming protein containing a nucleoside-specific binding site. The antibiotic albicidin enters the cell via this substrate-specific channel. Because albicidin is toxic for *E. coli* at a very low external substrate concentration, the Tsx channel is likely to contain a binding site for this antibiotic. To identify residues involved in the Tsx substrate-specific channel activity, we devised a selection scheme to isolate albicidin-resistant *tsx* mutants synthesizing Tsx proteins with defects in their nucleoside uptake function. We recovered seven distinct albicidin-resistant *tsx* alleles, six point mutations and a 39-base pair duplication. The mutants with a duplication of residues 21–33 of Tsx or with single amino acid substitutions of residue Gly^{28} (to Arg) and Ser^{217} (to Arg) are completely deficient in nucleoside uptake at a low substrate concentration. Substitutions of Phe^{27} to Leu, Gly^{28} to Glu, Gly^{239} to Asp, and Gly^{240} to Asp result in a Tsx protein partially defective in nucleoside transport. These mutant proteins still permit nonspecific diffusion of serine indicating that the mutations do not result in a block of the Tsx channel. Our results are discussed in terms of a model for the topological organization of the Tsx protein within the outer membrane of *E. coli*.

The outer membrane of *Escherichia coli* protects the cells against noxious agents and serves as a molecular filter for hydrophilic substances. It contains a group of proteins that form open water-filled channels and allow the flux of nutrients and ions into the periplasm (Nikaido and Vaara, 1985; Benz and Bauer, 1988). One can distinguish between nonspecific porins such as OmpC, OmpF, and PhoE and substrate-specific channels such as LamB and Tsx. Nonspecific pores function as molecular sieves and permit the passive diffusion of a great variety of hydrophilic molecules across the outer membrane. The rate of penetration of a given substrate through a nonspecific porin depends essentially on the concentration gradient of the substrate across the outer membrane, its hydrophobicity, and its size. The flux of molecules with a molecular mass higher than a certain threshold value (>600 daltons) through general porins is severely restricted by the small pore diameter of these porins (Weiss *et al.*, 1990; Cowan *et al.*, 1992; Nikaido and Saier, 1992). In contrast, the substrate-specific channels contain a saturable substrate-binding site that permits the efficient flux of substrate across the channel at an exceedingly low substrate concentration, but the rate of solute diffusion saturates at high substrate concentrations (Freundlieb *et al.*, 1988; Trias *et al.*, 1989). Hence, these substrate-specific channels are of physiological importance for the bacterial cell in an environment with a low nutrient content where the flux of substrate through general porins is inefficient (Nikaido, 1992).

Work performed in our laboratory has focused on the *E. coli* Tsx protein, a nucleoside-specific channel. The 272-residue Tsx protein (Bremer *et al.*, 1990) is a minor component of the outer membrane and serves as a receptor for colicin K, bacteriophage T6, and a number of other lytic T-even-type phages (Hancock and Reeves, 1975; Mannings and Reeves, 1978). Tsx has an essential function for the uptake of deoxynucleosides and nucleosides at substrate concentrations below 1 μM (Hantke, 1976; Krieger-Brauer and Braun, 1980; Munch-Petersen *et al.*, 1979). Reconstitution of the purified Tsx protein into black lipid bilayers has proven that Tsx is a channel-forming protein whose *in vivo* substrate specificity is a consequence of the presence of a nucleoside-specific binding side inside the Tsx channel (Maier *et al.*, 1988; Benz *et al.*, 1988). This substrate binding site can discriminate between compounds closely related in structure. There is a stronger binding for deoxynucleosides than nucleosides, but curiously the channel shows no specificity for cytidine and deoxycytidine. Tsx plays no role in the uptake of the free bases or the phosphorylated derivatives of deoxynucleosides (Van Alphen *et al.*, 1978; Benz *et al.*, 1988). A comparison of the Tsx-dependent *in vivo* transport of adenosine and adenine arabinoside has indicated that the Tsx protein does not strongly differentiate between nucleosides with different pentose moieties (Krieger-Brauer and Braun, 1980). Like the maltose-specific LamB protein, the Tsx channel permits the passive diffusion of a number of small molecules (*e.g.* serine) with structures unrelated to nucleosides (Luckey and Nikaido, 1980; Heuzenroeder and Reeves, 1981).

The Tsx channel is also used by the antibiotic albicidin for its penetration through the outer membrane (Birch *et al.*, 1990). This antibiotic, produced by a strain of *Xanthomonas albilineans*, can specifically block DNA replication in intact *E. coli* cells within a few minutes when supplied at an external substrate concentration of 0.1 μM (Birch and Patil, 1985; Birch *et al.*, 1990). Selection for *E. coli* strains resistant to albicidin yielded exclusively *tsx* mutants, demonstrating that at a low substrate concentration the antibiotic uses the Tsx channel for its permeation across the outer membrane. These

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Alb^r[1] strains were also resistant against the Tsx-specific phage T6 and showed a defect in nucleoside uptake (Birch *et al.*, 1990). Such phenotypes are typical for *tsx* mutants that either lack Tsx entirely or synthesize it in a greatly reduced amount (Manning and Reeves, 1978). The structure of albicidin is still unknown. Partial characterization of the antibiotic by proton and ^{13}C-NMR spectroscopy suggests that it has several aromatic rings, 38 carbon atoms, and a molecular mass of 842 Da (Birch and Patil, 1985). It is notable that a molecule with a mass 3–4 times that of a common nucleoside can apparently readily permeate through the Tsx channel.

Essentially nothing is known about the determinants of the Tsx protein that are important for its nucleoside-specific channel activity. One approach to better understand this substrate specificity is the isolation of *tsx* missense mutants that synthesize Tsx but exhibit altered channel characteristics. Such a genetic approach has been fruitfully used to characterize the maltose/maltodextrine-specific LamB channel (Charbit *et al.*, 1988; Dargent *et al.*, 1988) and the general porins OmpC and OmpF (Misra and Benson, 1988; Benson *et al.*, 1988). The information gained through the genetic and physiological analysis of this type of *E. coli* mutants has greatly aided the understanding of the structure and function relationship of the three-dimensional structure of the OmpF and PhoE porins (Cowan *et al.*, 1992). The isolation of mutants with functionally altered Tsx proteins is difficult since no positive selection for Tsx$^+$ strains is available. We have reported the use of a *tsx*$^+$-*lacZ*$^+$ operon fusion in a mutant isolation scheme to detect strains synthesizing Tsx proteins with defects in their phage receptor function (Schneider *et al.*, 1993). We have adopted this strategy for the isolation of *tsx* mutants with altered channel characteristics by exploiting the use of the nucleoside-specific Tsx channel by the antibiotic albicidin. The molecular mass of albicidin and its toxicity for *E. coli* at a very low substrate concentration (Birch *et al.*, 1990) suggests the presence of a binding site for albicidin inside the Tsx channel. We consider it likely that this binding site is identical to or at least overlapping with the nucleoside binding site inside the Tsx channel. Thus, *tsx* mutations that confer albicidin resistance but allow Tsx synthesis are apt to be informative about those determinants of the Tsx channel that contribute to its substrate specificity. In this paper, we describe the characterization of albicidin-resistant *tsx* mutants with single amino acid substitutions and show that the altered Tsx proteins exhibit a defect in the *in vivo* uptake of deoxynucleosides.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The *E. coli* K-12 strain BRE2050 (F$^-$ *metB ilv rpsL cytR9 deoR8* Δ (*argF-lac*)*U169*) and its *tsx* derivative, strain BRE2070, have been previously described (Bremer *et al.*, 1988, 1990). Strain HF1, used for most of the experiments, was constructed as follows. First, the *metB* and *ilv* mutations present in strain BRE2070 were successively removed by P1*vir*-mediated transduction by selecting for Ilv$^+$ and Met$^+$ colonies on glucose minimal plates using a P1*vir* lysate grown on the *metB*$^+$ *ilv*$^+$ strain MC4100 (Casadaban, 1976). A valine-resistant derivative of the resulting Ilv$^+$ Met$^+$ transductant was then selected on glucose minimal plates containing 40 μg/ml L-valine as described by Miller (1972). One of the resulting strains, BKM6, was subsequently lysogenized with the specialized transducing phage λp1048 carrying a *lacY*$^+$ gene expressed constitutively under the control of the *tyrT* promoter (Berman and Jackson, 1984), yielding strain HF1. This strain construction was done to avoid the killing of *E. coli* K-12 strains by the excess of valine synthesized by the albicidin-producer *X. albilineans* LS155 (Birch and Patil, 1985) and to allow the detection of Lac$^+$ colonies on lactose MacConkey plates when strain HF1 carried the *tsx*$^+$-*lacZ*$^+$ operon fusion plasmid pHS11 (Fig. 1*A*). A derivative of strain MC4100 lacking the Tsx, OmpC, and OmpF proteins was constructed by first transducing with phage P1*vir* the *tsx*::Tn*10*(*kan*) insertion from strain CAG18413 (Singer *et al.*, 1989) into MC4100. Into the resulting Tsx$^-$ strain HF19 we then transduced the ø(*ompF-lacZ*) operon fusion from strain PLB3260 (Benson and Decloux, 1985) by selecting for Lac$^+$ colonies on lactose minimal plates. The Tsx$^-$ OmpF$^-$ strain HF23 was subsequently used as a recipient for a P1*vir*-mediated transduction using a lysate grown on strain RAM191, which carries a Tn*10* insertion tightly linked to a deletion in the *ompC* gene (Δ(*ompC*::Tn*10*) (Misra and Benson, 1988). Loss of the OmpC, OmpF, and Tsx proteins in the resulting strain HF24 was tested by cross-streaking against the OmpC-, OmpF-, and Tsx-specific phages TuIb, TuIa, and T6, respectively (Datta *et al.*, 1977; Manning and Reeves, 1978) and verified by electrophoresis of the outer membrane protein preparations on SDS-polyacrylamide gels. Strain BZB2116 was used to obtain a colicin K preparation (Krieger-Brauer and Braun, 1980; Pugsley, 1985). The antibiotic albicidin was isolated from cultures of *X. albilineans* strain LS155. For control experiments cultures of the *X. albilineans* strain LS156, an Alb$^-$ derivative of LS155 that carries a Tn*5* in the albicidin biosynthetic genes, was used (Birch *et al.*, 1990).

The construction and characterization of the low copy number plasmid *tsx*$^+$-*lacZ*$^+$ operon fusion plasmid pHS11 (Fig. 1*A*) have been described by Schneider *et al.* (1993). The vector used for the construction of pHS11 is the low copy number *lacZ* protein fusion plasmid pGP15, which carries a tetracycline resistance gene.[2] A 4.5-kilobase *Stu*I-*Hin*dIII restriction fragment carrying the *tsx* gene was isolated from plasmid pHS11 (tetracycline resistant) (Fig. 1*A*) and ligated into the *Sma*I and *Hin*dIII sites in the polylinker of the low copy number plasmid pPD1 (chloramphenicol resistant);[3] the resulting *tsx*$^+$ plasmid was called pHF1. The same cloning strategy was used for each of the seven pHS11-derived Alb^r *tsx* alleles to transfer the mutant *tsx* genes into the chloramphenicol resistant vector pPD1. The plasmids obtained were pHF2 (*tsx-511*), pHF3 (*tsx-510*), pHF4 (*tsx-509*), pHF5 (*tsx-512*), pHF6 (*tsx-514*), pHF7 (*tsx-513*), and pHF8 (*tsx-508*). The recloning of the wild-type and mutant Alb^r *tsx* genes from the tetracycline-resistant *tsx*$^+$-*lacZ*$^+$ operon fusion plasmid pHS11 into the chloramphenicol-resistant vector pPD1 was necessary because strain HF24 ((*tsx*::Tn*10*(*kan*) ø(*ompF-lacZ*) Δ(*ompC*::Tn*10*)) used as the host strain for serine uptake experiments (see Fig. 4) is tetracycline-resistant.

Media and Growth Conditions—*E. coli* strains were grown aerobically at 37 °C in rich media (LB, DYT, or NB) or minimal medium A (MMA) with 0.2% glycerol as the carbon source as described (Miller, 1972; Silhavy *et al.*, 1984). LB and lactose MacConkey plates were prepared as previously described (Miller, 1972). Tetracycline, kanamycin, and chloramphenicol were added to liquid and solid media at 5, 30, and 30 μg/ml, respectively. *X. albilineans* was grown at 28 °C in SP medium (20 g of sucrose, 5 g of peptone, 0.5 g of K_2HPO_4, 0.25 g of $MgSO_4 \times 7H_2O$/liter, pH 7.0) according to Birch and Patil (1985).

Albicidin Production—The *X. albilineans* strain LS155 excretes in stationary phase the antibiotic albicidin into the growth medium. The supernatant of such cultures contains enough albicidin to kill bacteria susceptible to this antibiotic (Birch and Patil, 1985). One-liter cultures of strain LS155 were grown in SP medium for 96 h at 28 °C (Birch and Patil, 1985). Bacterial cells were removed by centrifugation (8,000 × *g* for 20 min), and the supernatant was used to determine the minimal amount of culture fluid necessary to kill a Tsx$^+$ strain without impairing the growth of a Tsx$^-$ mutant. Various amounts of the supernatant were added to the lactose MacConkey medium used for the selection plates. Usually, 8–9 ml of culture fluid/agar plate (30 ml) was required to differentiate between Tsx$^+$ and Tsx$^-$ strains. To ensure that the toxicity of the supernatant from cultures of *X. albilineans* strain LS155 for Tsx$^+$ *E. coli* strains was due to albicidin production, lactose MacConkey plates containing the supernatant of the Alb$^-$ derivative (*X. albilineans* LS156) of strain *X. albilineans* strain LS155 were used to monitor the growth of Tsx$^+$ and Tsx$^-$ *E. coli* strains. The minimum inhibitory concentration of the crude albicidin preparation for Tsx$^+$ strains was determined for each new culture of *X. albilineans* LS155. The supernatant from the albicidin producer was stored at −70 °C until use. For quantitative antibiotic sensitivity assays, albicidin was partially purified from the supernatant of *X. albilineans* LS155 by adsorption to Amberlit XAD-

[1] The abbreviations used are: Alb^r, resistance against the antibiotic albicidin; bp, base pair; MMA, minimal medium A.

[2] P. Gerlach and E. Bremer, unpublished results.

[3] P. Dersch, H. Fsihi, and E. Bremer, manuscript in preparation.

7 resin (Sigma) and by elution with methanol (Birch and Patil, 1985). 500 ml of culture supernatant was passed through a column (10 × 2.5 cm) containing 50 g of Amberlit XAD-7 resin previously equilibrated with SP medium. The column was washed with 100 ml of SP medium, and the antibiotic was then eluted from the Amberlit XAD-7 resin with 50 ml of 95% methanol. Two-ml fractions were collected, and the presence of albicidin was assayed by placing 30-μl aliquots into wells (0.5 mm) cut into LB agar plates overlaid with soft agar containing cells of either a Tsx^+ or a Tsx^- strain. The zone of growth inhibition around the wells was recorded after overnight incubation of the plates at 37 °C.

Isolation of Plasmid pHS11-encoded Alb^r tsx Mutants—Aliquots (0.1 ml of undiluted and 10- and 100-fold diluted) of overnight LB-grown cultures of strain HF1 (pHS11) were spread on lactose MacConkey agar plates containing an inhibitory concentration of albicidin for the growth of a Tsx^+ strain and 5 μg/ml tetracycline. The selection plates were incubated for 16 h at 37 °C. Between four and six Lac^+ Alb^r colonies from each independent culture were picked and purified by restreaking on the same plates. In this way, we isolated approximately 400 Lac^+ Alb^r mutants, which originate from 25 *N*-methyl-N′-nitro-*N*-nitrosoguanidine-treated, 20 2-aminopurine-treated, and 30 unmutagenized cultures of strain HF1 (pHS11). The 400 Lac^+ Alb^r mutants were cross-streaked against the Tsx-specific T6 phage, and 60 were found to be sensitive against this bacteriophage. Thirty-eight colonies of these $T6^s$ strains were of independent origin and were further analyzed by Western immunoblotting of whole cell extracts using a Tsx-specific antiserum. Each of the 38 independent Lac^+ Alb^r $T6^s$ strains gave a positive reaction with the antiserum. We tested 40 of the Lac^+ Alb^r mutants that were resistant against phage T6 in Western immunoblotting experiments using whole cell protein extracts. Thirty-seven of these strains did not synthesize the Tsx protein. Three of the Lac^+ Alb^r $T6^r$ strains reacted with the antiserum but produced unstable Tsx proteins.

Isolation and Electrophoresis of Outer Membrane Proteins and Immunoblotting—Cell envelopes from 40-ml cultures grown overnight in DYT medium containing 5 μg/ml tetracycline were prepared as described by Henning *et al.* (1978). Proteins were separated by electrophoresis on 12% SDS-polyacrylamide gels by the method of Lugtenberg *et al.* (1975). For the immunological detection of the Tsx protein, whole cell extracts were prepared from 5-ml cultures of strain HF1 (pHS11) according to Silhavy *et al.* (1984). Western immunoblotting experiments were carried out as described by Sambrook *et al.* (1989).

DNA Manipulations—Routine manipulations of plasmid DNA were all carried out by standard techniques (Sambrook *et al.*, 1989). Chemical mutagenesis of cultures of strain HF1 containing plasmid pHS11 with *N*-methyl-N′-nitro-*N*-nitrosoguanidine or 2-aminopurine (Sigma) was performed as described by Silhavy *et al.* (1984) and Miller (1972). Plasmid DNA used for sequencing was purified on Quiagen columns (Diagen) according to the manufacturer's instructions. Sequencing of double-stranded plasmid DNA was performed according to the method of Sanger *et al.* (1977) using the Sequenase 2.0 kit (U. S. Biochemical Corp.) and the conditions recommended by the suppliers. Sequencing reactions of alkaline-denatured plasmid DNA were primed with a number of synthetic oligonucleotide primers spaced along the *tsx* coding region (Bremer *et al.*, 1990). The following primers were used: oligonucleotide 1, 5′-TTTCACTCCCGCAAGGG-3′ (417–433 bp); oligonucleotide 2, 5′-CTGGTGGCACCAGAGCG-3′ (554–570 bp); oligonucleotide 3, 5′-GGCGGTAACTCCGATGC-3′ (702–718 bp); oligonucleotide 4, 5′-GTCGCCAGAGCACCTGG-3′ (874–890 bp); oligonucleotide 5, 5′-ACCGATCTGTGGGGCGG-3′ (1041–1057 bp); oligonucleotide 6, 5′-GAACTACGATCACTGGC-3′ (1187–1203 bp). To confirm the presence of a single mutation in the Alb^r *tsx* mutants we sequenced the entire *tsx* gene for one representative from each class of *tsx* alleles (Table I). Sequencing reactions for the corresponding DNA sequence from the *tsx* wild-type gene were always run in parallel to facilitate the unambiguous identification of the alteration in the mutant *tsx* genes. For the remaining Alb^r *tsx* mutants (Table I) the region between 585 and 1270 bp of the *tsx* gene was sequenced using oligonucleotides 2, 3, 4, and 5 to prime the sequencing reactions.

Uptake Assays—For the measurements of [^{14}C]deoxyadenosine (46.6 mCi/mmol; Du Pont-New England Nuclear) and [^{14}C]thymidine (59 mCi/mmol; Du Pont-New England Nuclear) uptake, bacterial cultures of strain HF1 harboring plasmid pHS11 and its mutant Alb^r *tsx* derivatives were freshly grown in MMA with 0.2% glycerol as a carbon source and 5 μg/ml tetracycline to prevent loss of the plasmids. At an optical density (A_{578}) of 0.2, the cells were harvested by centrifugation and washed twice with MMA. To a 2-ml cell suspension, ^{14}C-radiolabeled deoxyadenosine and thymidine were added to a final concentration of 0.55 and 0.85 μM, respectively. Samples (300 μl) were removed at various time intervals, filtered through MMA-prewashed membrane filters (ME 25, 0.45 μm; Schleicher & Schuell), and washed twice with MMA. The radioactivity retained on the membrane filters was determined in a scintillation counter. Each uptake experiment was repeated at least four times. Uptake of [^{14}C] serine (180.5 mCi/mmol; Du Pont-New England Nuclear) was measured as described by Heuzenroeder and Reeves (1981) except that the plasmid-bearing cells were grown to an A_{625} of 0.5 in double-strength nutrient broth medium supplemented with chloramphenicol (30 μg/ml). The final substrate concentration of serine in the uptake assays was 2.5 μM. These serine uptake experiments were repeated three times.

RESULTS

Isolation of Albicidin-resistant tsx Mutants Synthesizing Full Amounts of the Tsx Protein—The Alb^r *tsx* mutants characterized by Birch *et al.* (1990) show phenotypes typical for *E. coli* strains that either lack the Tsx protein entirely or synthesize it in greatly reduced amounts (Manning and Reeves, 1978). To detect Alb^r *tsx* mutants synthesizing the full amount of Tsx we used strain HF1 (*tsx*) carrying the low copy number *tsx*$^+$-*lacZ*$^+$ operon fusion plasmid pHS11. In this operon fusion an intact *tsx* gene is expressed from its natural promoters (P_1 and P_2) (Bremer *et al.* 1988, 1990) and is followed by a promoterless *lacZ* gene (Fig. 1*A*). Since the expression of the *lacZ* indicator gene in the *tsx*$^+$-*lacZ*$^+$ operon fusion is dependant on the *tsx* transcriptional initiation signals, mutations that reduce *tsx* expression can be recognized by their weak Lac phenotype. We therefore search for the Alb^r mutants with a strong Lac^+ phenotype on lactose MacConkey plates containing a concentration of a crude albicidin preparation that prevented the growth of Tsx^+ strains but not that of Tsx^- mutants. In this way we isolated from 75 cultures of strain HF1 (pHS11) 38 independent Alb^r strains, which synthesized full amounts of Tsx and were sensitive against the Tsx-specific phage T6. We found that the mutations conferring albicidin resistance were in each case plasmid-encoded since retransformation of the plasmid DNA prepared from the original Lac^+ Alb^r mutants into strain HF1 resulted again in a Lac^+ Alb^r phenotype and the concomitant synthesis of Tsx.

DNA Sequence Analysis of the tsx Mutations—To establish the nature of the alterations in *tsx* that result in an Alb^r phenotype but still permit Tsx production, we characterized the 38 independently isolated *tsx* mutants by DNA sequence analysis. Seven different *tsx* alleles were detected and three types of mutations were represented, transversions, transitions, and a duplication of a 39-bp DNA segment. All of the point mutations were isolated more than once (Table I). The entire *tsx* coding region was sequenced from a representative isolate of each mutant Alb^r *tsx* allele along with the wild-type *tsx* gene to ensure the unambiguous identification of the mutations conferring albicidin resistance. We found that each *tsx* allele contained only a single change and the position of the mutations with respect to the *tsx* coding region are summarized in Fig. 1*B*.

Point mutations that alter residues Gly^{28} and Ser^{217} are the major class (31/38) of *tsx* mutants with an Alb^r phenotype (Table I). In 20 of the 38 *tsx* mutants, the codon for residue Gly^{28} of the Tsx protein was changed, resulting in the substitution of the neutral Gly residue by either a positively charged Arg residue (14 isolates) (*tsx-509*) or by a negatively charged Glu residue (6 isolates) (*tsx-510*). In eleven mutants, Ser^{217} was replaced by Arg (*tsx-511*); this class of *tsx* mutants comprise all spontaneously isolated Alb^r strains (nine isolates)

(Table I). Strikingly, the same C to A transversion in codon 217 has occurred in each of the eleven recovered *tsx-511* alleles, although three other single base pair changes can convert the AGC (Ser^{217}) codon into a codon directing the insertion of an Arg residue into the growing Tsx polypeptide chain. This transversion alters the most frequently used Ser codon into an infrequently used Arg (AGA) codon (Wada *et al.*, 1992). Single amino acid substitutions of Phe^{27} (*tsx-508*), Gly^{239} (*tsx-512*), and Gly^{240} (*tsx-513*) represent a minor class of *tsx* mutations that confer resistance against albicidin (Table I). The Gly residues at position 239 and 240 in the Tsx protein have each been replaced with a negatively charged Asp residue in the Tsx-512 and Tsx-513 proteins (Fig. 1*B*). In contrast to the *tsx* alleles described above, the point mutation present in the *tsx-508* gene does not cause the incorporation of an additional charge into the Tsx protein. A substitution of the hydrophobic Phe^{27} residue by a hydrophobic Leu residue (Fig. 1*B*) is the cause of the Alb^r phenotype exhibited by strains synthesizing the Tsx-508 protein. In the *tsx-514* mutant, a 39-bp insertion has occurred, resulting in the duplication of the region between residues 21 and 33 of the Tsx polypeptide. We note that the duplicated protein segment comprises residues Phe^{27} and Gly^{28}, sites at which single amino acid substitutions can confer an Alb^r phenotype (Fig. 1*B*).

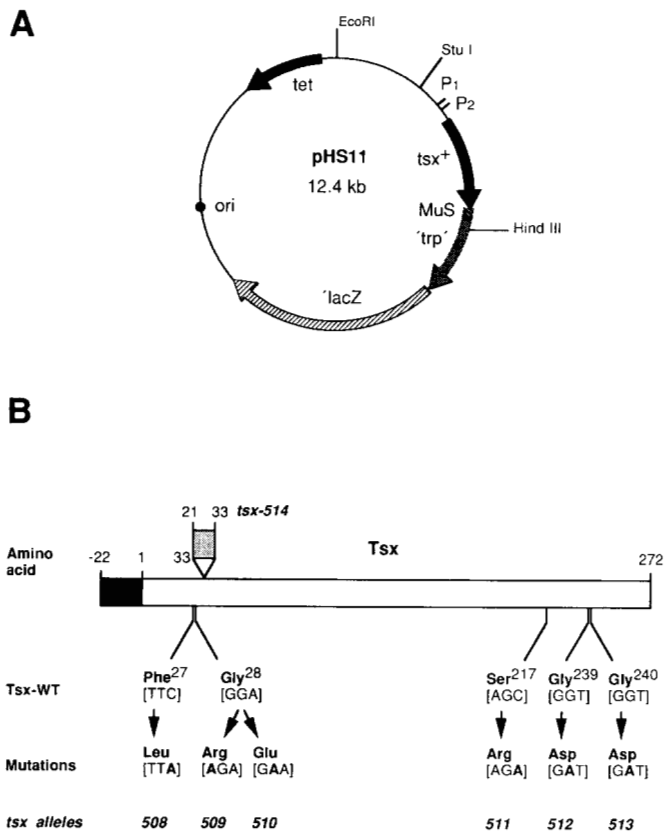

FIG. 1. **Genetic structure of plasmid pHS11 and location of Alb^r *tsx* mutations.** *A*, schematic representation of the tsx^+ plasmid pHS11 used for the selection of the Alb^r *tsx* mutants. This low copy number plasmid carries a tsx^+-$lacZ^+$ operon fusion in which the intact *tsx* gene is expressed from its natural promoters (P_1 and P_2). The Mu*S* and "*trp*" DNA sequences are derived from the transposable λp*lac*Mu55 bacteriophage originally used to construct the chromosomal tsx^+-$lacZ^+$ operon fusion. The fusion junction between the *tsx* gene and the Mu*S* sequences is located 15 bp downstream of the stop codon (Schneider *et al.*, 1993). The position of relevant restriction sites in pHS11 is indicated. *B*, position of the *tsx* mutations conferring albicidin resistance. The mature Tsx protein is composed of 272 amino acid residues and is initially synthesized with a 22-amino acid residue signal sequence (Bremer *et al.*, 1990).

The Mutant Tsx Proteins Are Routed into the Outer Membrane—To test whether the mutant Tsx proteins were inserted into the *E. coli* outer membrane, we prepared cell envelope fractions from strain HF1 expressing the seven Alb^r *tsx* alleles recovered and analyzed the outer membrane protein profile by SDS-polyacrylamide gel electrophoresis. Each of the mutant Tsx proteins was present in the outer membrane and was synthesized in amounts similar to that of the Tsx wild-type protein (Fig. 2). The Tsx-514 protein exhibited a slightly slower electrophoretic mobility on the SDS-polyacrylamide gel in comparison to the Tsx wild-type protein (Fig. 2, *lanes 2* and *5*). Such an increase in the apparent molecular weight of the Tsx-514 protein is expected since it carries an additional 13 amino acids (Fig. 1*B*). Thus, none of the alterations present in the mutant proteins negatively affect the amount of Tsx protein produced or the export of the mutationally altered Tsx proteins in the *E. coli* outer membrane.

The Mutant Tsx Proteins Function as Receptors for Tsx-specific Phages and for Colicin K—That the mutant Tsx proteins were indeed present in the *E. coli* outer membrane was also evident from the fact that they still could function as receptors for colicin K and Tsx-specific bacteriophages. Each of the seven Alb^r *tsx* mutants was fully sensitive to colicin K (Table II). Likewise, the mutant Tsx proteins with single amino acid substitutions were fully proficient as receptors for a panel of six Tsx-specific bacteriophages (Table II). In contrast, the Tsx-514 protein carrying an additional 13 amino acids (Fig. 1*B*) was entirely resistant against the Tsx-specific phages H3, H8, and Ox1, and the plaques formed by the other bacteriophages were small and turbid (Table II). Thus, with the exception of Tsx-514, each of the mutant Tsx proteins shows only a limited functional defect.

Different Levels of Resistance against Albicidin Are Conferred by the Mutant Tsx Proteins—We used in the course of our study different crude preparations of albicidin to prepare the plates for the selection of Alb^r derivatives of strains HF1

TABLE I
Summary of the isolated Alb^r tsx mutants

tsx-allele	Mutation	Alteration in Tsx[a]	Number of isolates	Mutagen[b]
tsx-508	C-A transversion	Phe^{27}-Leu	2	2-AP
tsx-509	G-A transition	Gly^{28}-Arg	14	2-AP (10), NG (4)
tsx-510	G-A transition	Gly^{28}-Glu	6	NG
tsx-511	C-A transversion	Ser^{217}-Arg	11	2-AP (2), None (9)
tsx-512	G-A transition	Gly^{239}-Asp	2	NG
tsx-513	G-A transition	Gly^{240}-Asp	2	NG
tsx-514	39-bp duplication	From Gly^{21} to Asn^{33}	1	2-AP

[a] The numbering of the alterations in Tsx is according to Bremer *et al.* (1990).
[b] 2-AP, 2-aminopurine; NG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

(pHS11). Since the amount of albicidin might vary from one preparation to the other, it was possible that the various Alb^r *tsx* mutations confer different levels of resistance against albicidin. We therefore partially purified the antibiotic and performed a more quantitative assay by monitoring the zone of growth inhibition of the Alb^r strains around wells in LB agar plates containing various dilutions of the same albicidin preparation. As expected from the data reported by Birch *et al.* (1990), the growth of a Tsx$^+$ strain was strongly inhibited by albicidin even when present at a low concentration (Table III). Under such conditions, a Tsx$^-$ strain was entirely resistant against the partially purified albicidin (Table III), attesting to the importance of the Tsx channel at a low external substrate concentration for the permeation of albicidin across the outer membrane. However, at a high albicidin concentration, growth inhibition was evident for the Tsx$^-$ strain as well (Table III), indicating that in addition to Tsx, alternative pathways can be used by albicidin to cross the *E. coli* outer membrane permeability barrier.

We found that there are three classes of *tsx* mutants among the albicidin-resistant strains (Table III). The first group comprises the *tsx-508*, *tsx-509*, *tsx-511*, and *tsx-514* alleles; strains synthesizing these mutant Tsx proteins show a level of resistance very similar to an *E. coli* strain lacking the Tsx channel entirely. Mutants expressing the *tsx-510* and *tsx-512* genes form the second class and are more sensitive to the antibiotic than the first group of Alb^r *tsx* mutants. Growth of these strains is inhibited up to a dilution of 1:32 of the albicidin preparation, whereas a Tsx$^+$ strain is still sensitive to a 1:128 dilution of the antibiotic. The third class is represented by the *tsx-513* allele, which exhibits only a very weak albicidin resistance phenotype (Table III). Thus, the seven *tsx* mutants can be differentiated with respect to their level of resistance against albicidin. This finding indicates that the various *tsx* mutations affect the ability of the Tsx protein to function as an albicidin-specific channel in an allele-specific fashion.

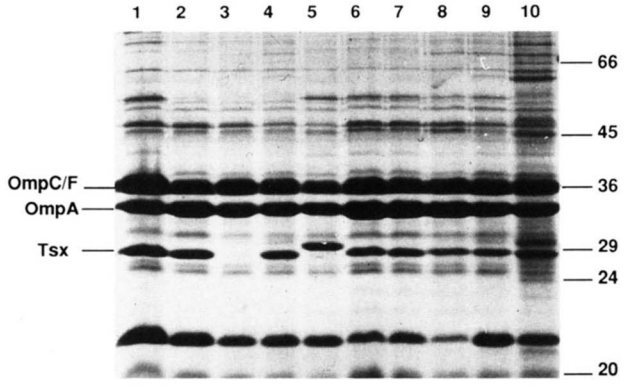

FIG. 2. **SDS-polyacrylamide gel electrophoresis of outer membrane proteins.** Cell envelope proteins of strain BRE2050 (*tsx*$^+$, *lane 1*), strain HF1 (*tsx*) harboring the *tsx*$^+$ plasmid pHS11 (*lane 2*), the vector pGP15 (*lane 3*), and the mutant derivatives of pHS11 with the Alb^r *tsx* alleles *tsx-508* (*lane 4*), *tsx-514* (*lane 5*), *tsx-509* (*lane 6*), *tsx-510* (*lane 7*), *tsx-511* (*lane 8*), *tsx-512* (*lane 9*), and *tsx-513* (*lane 10*) were electrophoretically separated on a 12% SDS-polyacrylamide gel. The position of the Tsx, OmpA, OmpC, and OmpF outer membrane proteins is indicated; the gel system used does not resolve the OmpC and OmpF proteins. The molecular mass (in kDa) of marker proteins is indicated on the *right*. The gel was stained with Coomassie Brilliant Blue.

The Mutant Tsx Proteins Are Impaired in Their Nucleoside-specific Channel Activity—We speculated at the beginning of our study that the determinants of the Tsx protein that govern the efficient permeation of the antibiotic albicidin through the Tsx channel are identical, or at least overlapping, with those that determine its nucleoside specificity. To test whether the mutations in the *tsx* gene causing increased resistance to albicidin also cause defects in the nucleoside-specific channel function of Tsx, we measured *in vivo* the initial transport activity of the *tsx* mutants proteins for both a purine and a pyrimidine deoxynucleoside at submicromolar substrate concentrations. At such a low external deoxynucleoside level, the uptake of [^{14}C]deoxyadenosine (0.55 μM) and [^{14}C]thymidine (0.85 μM) is entirely dependent on the Tsx protein and each of the Alb^r *tsx* mutants showed a defect in deoxynucleoside transport (Fig. 3, *A* and *B*). Strains synthesizing the Tsx-509 (Gly^{28} to Arg), Tsx-511 (Ser^{217} to Arg), and Tsx-514 (13-amino acid duplication) mutant proteins were entirely deficient for both deoxyadenosine and thymidine uptake. Their deoxynucleoside transport activity was indistinguishable from a strain lacking the Tsx protein entirely. The Gly^{28} to Glu and Gly^{239} to Asp substitutions present in the Tsx-510 and Tsx-513 proteins, respectively, caused a drop in the initial deoxynucleoside uptake activity to approximately 50% of that measured in the Tsx$^+$ wild-type strain (Fig. 3, *A* and *B*). A similar reduction in the transport activity (to approximately 40% of the wild-type level) was caused by the Phe^{27} to Leu alteration present in the Tsx-508 mutant protein (Fig. 3, *A* and *B*). In contrast, the substitution of Gly^{240} by an Asp residue in the Tsx-513 protein had practically no influence on [^{14}C]deoxyadenosine uptake (Fig. 3*A*) and reduced the transport of [^{14}C]thymidine only slightly (Fig. 3*B*). The weak level of resistance against albicidin conferred by the *tsx-513* allele (Table III) is thus reflected by only a weak impair-

TABLE II

Sensitivity of the Alb^r tsx mutant toward albicidin, colicin K, and Tsx-specific phages

tsx allele[a]	Alteration in Tsx	Resistance/sensitivity[b] to							
		Alb	col K	T6	H1	H3	H8	Ox1	K18
tsx$^-$		R	R	R	R	R	R	R	R
tsx$^+$		S	S	S	S	S	S	S	S
tsx-508	Phe^{27}-Leu	R	S	S	S	S	S	S	S
tsx-509	Gly^{28}-Arg	R	S	S	S	S	S	S	S
tsx-510	Gly^{28}-Glu	R	S	S	S	S	S	S	S
tsx-511	Ser^{217}-Arg	R	S	S	S	S	S	S	S
tsx-512	Gly^{239}-Asp	R	S	S	S	S	S	S	S
tsx-513	Gly^{240}-Asp	S/R	S	S	S	S	S	S	S
tsx-514	Duplication from Gly^{21} to Asn^{33}	R	S	S/R	S/R	R	R	R	S/R

[a] The *tsx* wild-type and its Alb^r derivatives are pHS11-encoded and are present in strain HF1 (*tsx*). The vector plasmid pGP15 was used as a Tsx$^-$ control.

[b] S, sensitivity; R, resistance; S/R, reduced sensitivity.

ment of the *in vivo* nucleoside-specific channel activity of the mutant Tsx-513 protein. Taken together, these data show that the alterations in Tsx conferring albicidin resistance result simultaneously in a decrease of deoxynucleoside permeation through the Tsx channel at a low external substrate concentration.

The Mutant Tsx Proteins Still Function as Nonspecific Pores—Strains synthesizing the mutant Tsx-509, Tsx-511, and Tsx-514 proteins show a strong defect in deoxyadenosine and thymidine uptake (Fig. 3). This defect in the function of Tsx might have the rather trivial explanation that the alterations present in the mutant proteins result in the collapse of the Tsx channel. To address this question, we probed the *in vivo* pore function of the seven mutant Alb^r Tsx proteins. In addition to its primary function as a nucleoside-specific channel (Maier *et al.*, 1988; Benz *et al.*, 1988), the Tsx protein can also serve as a nonspecific pore for some solutes unrelated to nucleosides (Heuzenroeder and Reeves, 1981). This nonspecific element of the Tsx channel can be monitored *in vivo* by measuring the uptake of radiolabeled serine in strains carrying mutations in the *ompB* operon whose gene product controls the synthesis of the major general diffusion porins OmpC and OmpF (Heuzenroeder and Reeves, 1981). Since mutations in the *ompB* operon generally strongly reduce but do not completely abolish production of the OmpC and OmpF proteins we constructed a derivative of strain HF19 (*tsx*::Tn*10* (*kan*)) that lacked the OmpC and OmpF porins entirely due to mutations in the *ompC* and *ompF* structural genes. The resulting strain, HF24, showed a severe defect in its ability to accumulate [^{14}C]serine at a substrate concentration of 2.5 μM in comparison with its OmpC$^+$ and OmpF$^+$ parent strain HF19 (Fig. 4*A*). When the *tsx*$^+$ plasmid pHF1 was introduced into strain HF24, [^{14}C]serine uptake was significantly increased in comparison with strain HF24 carrying the vector plasmid (pPD1) used to construct plasmid pHF1 (Fig. 4*B*). Thus, the Tsx channel partially compensates for the loss of the OmpC and OmpF general diffusion porins in strain HF24 with respect to serine permeation across the outer membrane. We tested the ability of each of the seven mutant Tsx proteins to serve as a nonspecific porin. All of the mutant Tsx-channels were fully proficient in [^{14}C]serine uptake in comparison with the wild-type Tsx protein, and this is documented in Fig. 4*B* for strains synthesizing the mutant Tsx-508, Tsx-509, and Tsx-511 proteins. These data show that neither the single amino acid substitutions nor the duplication present in the mutant Tsx proteins simply lead to a collapse of the Tsx channel. Rather, they strongly indicate that these alterations, perhaps with the exception of the Tsx-514 protein, exert quite specific effects on the ability of the Tsx protein to function as a nucleoside- and albicidin-specific channel.

TABLE III

Inhibitory activity of albicidin toward E. coli strains carrying mutant tsx genes

tsx allele[a]	Albicidin dilution[b]				
	1:8	1:16	1:32	1:64	1:128
tsx$^-$	8	2	R	R	R
tsx$^+$	8	6	4	2	1
tsx-508	8	3	R	R	R
tsx-509	6	2	R	R	R
tsx-510	8	6	2	R	R
tsx-511	8	4	R	R	R
tsx-512	6	5	1	R	R
tsx-513	8	6	4	1	R
tsx-514	8	4	R	R	R

[a] The *tsx* wild-type and its Alb^r derivatives are pHS11-encoded and are present in strain HF1 (*tsx*). The vector plasmid pGP15 was used as a Tsx$^-$ control.

[b] Sensitivity of the *E. coli* strains towards albicidin is expressed as the zone of growth inhibition (in mm) around wells in LB agar plates containing dilutions of the antibiotic.

DISCUSSION

In the present study we have used a genetic approach to characterize mutant Tsx proteins with altered channel char-

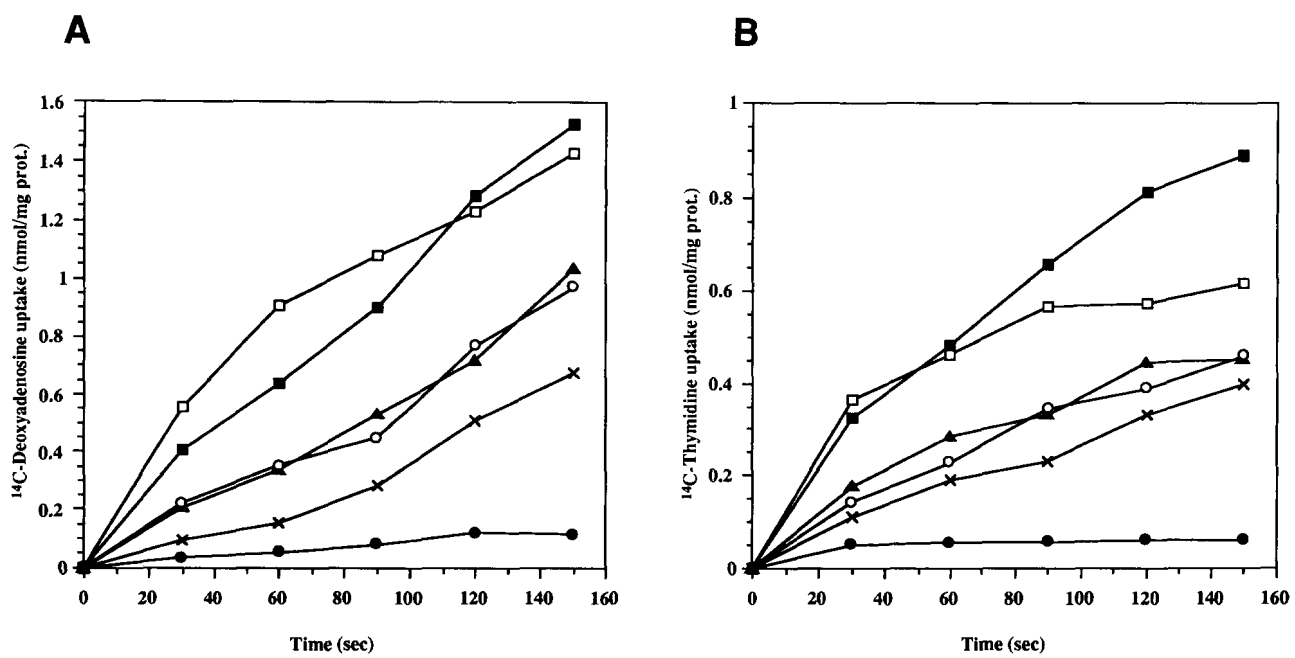

FIG. 3. **Uptake of ^{14}C-radiolabeled deoxyadenosine and thymidine.** The initial transport of [^{14}C]deoxyadenosine (*A*) and [^{14}C] thymidine (*B*) in *E. coli* cells synthesizing either the Tsx wild-type or the mutant Alb^r Tsx proteins was measured. The final substrate concentration of deoxyadenosine and thymidine in the uptake assays was 0.55 and 0.85 μM, respectively. The uptake of nucleosides was compared in strain HF1 (*tsx*) containing the vector plasmid pGP15 (●), the *tsx*$^+$ plasmid pHS11 (■), and its derived Alb^r alleles *tsx-508* (×), *tsx-509* (●), *tsx-510* (▲), *tsx-511* (●), *tsx-512* (○), *tsx-513* (□), and *tsx-514* (●), respectively.

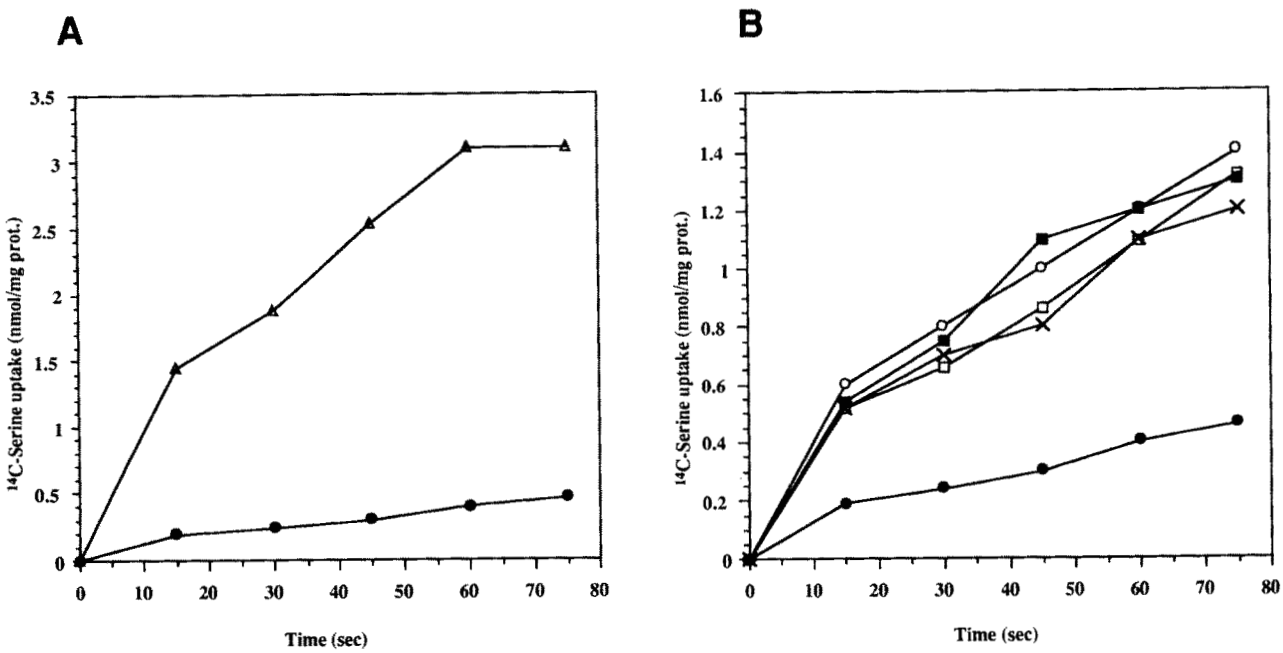

FIG. 4. **Uptake of [^{14}C]serine.** The initial transport of [^{14}C]serine at a final substrate concentration of 2.5 μM was measured (*A*) in strain HF19 (Tsx$^-$ OmpC$^+$ OmpF$^+$) (△) and strain HF24 (Tsx$^-$ OmpC$^-$ OmpF$^-$) (●) and (*B*) in strain HF24 carrying either the vector plasmid pPD1 (●), the *tsx*$^+$ plasmid pHF1 (□) or its Alb^r *tsx* derivatives, plasmids pHF2 (*tsx-511*, ×), pHF4 (*tsx-509*; ○), and pHF8 (*tsx-508*; ■), respectively.

acteristics. We took advantage of the permeation of the antibiotic albicidin through the Tsx channel (Birch *et al.*, 1990) to devise a genetic selection and immunological screening procedure identifying Alb^r mutants producing full amounts of Tsx. It is known that some antibiotics use substrate-specific channels to enter the cell. The antibiotic imipenem, for example, mimics the structure of basic amino acids, one of the natural substrates of the OprD channel, to diffuse efficiently through the outer membrane of *Pseudomonas aeruginosa* (Trias *et al.*, 1989). Despite the apparent differences in the structure of albicidin and nucleosides (Birch and Patil, 1985), a binding site appears to exist for the antibiotic inside the Tsx channel since this relatively large molecule (M_r 842) can rapidly cross the outer membrane even at very low (0.1 μM) substrate concentration (Birch *et al.*, 1990). Because it is unlikely that *E. coli* has specifically evolved a substrate-binding site for a toxic substance, albicidin probably uses the nucleoside binding site of the Tsx channel. Indeed, our data (Table III and Fig. 3) reveal a correlation between the efficiency of nucleoside uptake and sensitivity toward albicidin.

The Alb^r *tsx* mutants characterized in this study comprise six *tsx* alleles with single-base pair changes and one with a 39-bp insertion that results in the duplication of 13 amino acids of Tsx. The point mutations do not alter the phage and colicin K receptor function of Tsx, indicating that they exert only local effects. Consistent with such a partial functional defect is our finding that the mutant proteins still mediate the nonspecific diffusion of serine. The increased resistance against albicidin and the decreased deoxynucleoside transport activity in these Alb^r *tsx* mutants are consequently not the result of a collapse of the Tsx channel. The phenotypes of these mutants strongly suggest that the single amino acid substitutions specifically impair the substrate-specific channel activity of Tsx. In contrast, the duplication present in the Tsx-514 protein (Fig. 1*B*) probably disturbs the proper folding of the Tsx polypeptide chain. The altered phage sensitivity pattern of the strain synthesizing the Tsx-514 protein is an

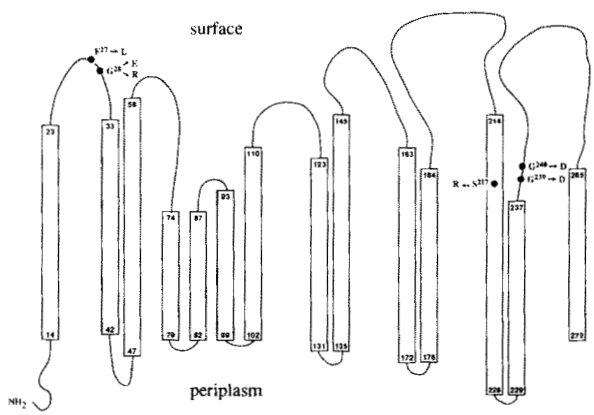

FIG. 5. **Model for the topological arrangement of the Tsx protein in the *E. coli* outer membrane.** The position of the single amino acid substitutions in Tsx conferring resistance against the antibiotic albicidin is indicated. Segments of the Tsx protein assumed to be transmembranous are *boxed*.

indication of possible long range effects of the duplication in the vicinity of the amino terminus since a major bacteriophage receptor area of Tsx is located near its carboxyl terminus (Maier *et al.*, 1990; Schneider *et al.*, 1993).

A model for the topological arrangement of the Tsx protein in the outer membrane is shown in Fig. 5. This model is based on the structure prediction rules developed for the bacterial porin family (Jeanteur *et al.*, 1991; Struve *et al.*, 1991), the analysis of *tsx* missense mutants affecting phage binding (Maier *et al.*, 1990; Schneider *et al.*, 1993) and a comparison of the amino acid sequence of the Tsx protein from *E. coli* (Bremer *et al.*, 1990) with those of *Salmonella typhimurium*, *Enterobacter aerogenes*, and *Klebsiella pneumoniae*.[4] We discuss below the properties of the single amino acid substitutions that confer albicidin resistance and impair deoxynucle-

[4] A. Nieweg and E. Bremer, manuscript in preparation.

oside uptake in the context of this topological model. Four of the 5 residues affected in the mutant Tsx proteins (Phe^{27}, Gly^{28}, Gly^{239}, and Gly^{240}) are located at the cell surface, whereas residue Ser^{217} is present in a transmembrane segment of Tsx (Fig. 5). These amino acids are all conserved in the Tsx proteins from *S. typhimurium*, *E. aerogenes*, and *K. pneumoniae* except Gly^{28}, which has been replaced in the *S. typhimurium* Tsx protein by a Ser residue.[4]

There is good experimental evidence that the region between residues 238 and 264 of Tsx is exposed at the cell surface. This segment comprises residues involved in the phage receptor function of Tsx (Maier *et al.*, 1990; Schneider *et al.*, 1993). In addition, the amino acid sequence of this area is highly variable in the Tsx proteins from *S. typhimurium*, *E. aerogenes*, and *K. pneumoniae*, whereas the sequence of the surrounding regions (residues 229–237 and residues 265–272) is strongly conserved.[4] Residues Gly^{239} and Gly^{240} are part of this external loop of the Tsx polypeptide and are replaced by an Asp residue in the mutant Tsx-512 and Tsx-513 proteins (Fig. 5). Although the nature of the substitution in the Tsx-512 and Tsx-513 is identical, an allele-specific phenotype is observed with respect to albicidin resistance and deoxynucleoside uptake (Table III and Fig. 3). The Tsx-513 mutant is only marginally affected in its deoxynucleoside transport activity, whereas the substitution in the Tsx-512 protein causes a significant decrease in deoxynucleoside uptake. Residues Gly^{239} and Gly^{240} might not be part of the substrate binding site in Tsx. These amino acids are surrounded by three negatively charged Asp residues (position 238, 244, and 245), and the introduction of an additional Asp residue in the Tsx-512 and Tsx-513 mutant proteins might therefore alter electrostatic interactions at the mouth of the pore and thus hinder the entrance of the substrates into the Tsx channel. Single amino acid substitutions that cause hindrance at the mouth of the maltose/maltodextrine-specific LamB channel have been previously characterized (Dargent *et al.*, 1988; Charbit *et al.*, 1988).

Substitutions of Gly^{28} by a positively charged Arg residue (Tsx-509) and by the negatively charged Glu residue (Tsx-510) were the most frequent of Alb^r *tsx* mutants recovered in our selection (Table I). The nature and charge of the residues of the replacements at position Gly^{28} are important for the ability of the Tsx channel to facilitate deoxynucleoside uptake. Deoxyadenosine and thymidine transport in the *tsx-509* mutant cannot be distinguished from that of a strain lacking the Tsx protein entirely, whereas deoxynucleoside uptake activity in the *tsx-510* mutant is reduced to approximately half of that measured in a Tsx^+ strain (Fig. 3). In contrast, the replacement of Gly^{28} by a neutral Ser residue in the *S. typhimurium* Tsx protein has no influence on the substrate specificity of the Tsx channel.[4] Two Arg residues (Arg^{26} and Arg^{32}) are present in the vicinity of Gly^{28}, and the introduction of an additional positive charge into the Tsx-509 protein might explain the drastic decrease of deoxyadenosine and thymidine transport observed in the Alb^r *tsx-509* mutant. The only mutation that does not introduce a charged amino acid into the Tsx polypeptide chain is the Phe^{27} to Leu substitution in the Tsx-508 protein; this mutation causes a partial defect in deoxynucleoside transport (Fig. 3). Both Phe^{27} and Gly^{28} are located on a cell surface-exposed segment of the Tsx protein (Fig. 5), and they are part of the 13-amino acid duplication that causes the defect in nucleoside uptake in the mutant Tsx-514 protein (Fig. 1*B*). Loss of deoxynucleoside transport function of the Tsx-514 protein (Fig. 3) is likely the consequence of alterations in the secondary structure of the amino-terminal end of Tsx. Thus, the integrity and spatial organization of this cell surface-exposed region comprising residues Phe^{27} and Gly^{28} are important determinants for the activity of the Tsx channel. Substitutions at these residues might negatively affect the entrance of the substrate into the nucleoside-specific Tsx channel.

Ser^{217} is located in a transmembrane segment of the Tsx protein (Fig. 5). Its substitution by Arg in the Tsx-511 protein causes strong resistance against albicidin and a severe defect in deoxynucleoside uptake (Table III and Fig. 3). The phenotypes conferred by the *tsx-511* allele and the position of Ser^{217} suggest that this residue might be part of the selective filter that determines the substrate specificity of the Tsx channel. Possibly, the OH group of the Ser side chain might interact through hydrogen bonding with nitrogen atoms present in the purine and pyrimidine rings of the nucleobases. Hydrogen bonding is also known to be of great functional importance for substrate recognition and binding in carbohydrate-binding proteins (Quiocho, 1986). Alternatively, the positive charge of the Arg residue and its bulky side chain could cause a steric block in the lumen of the pore. The genetic approach used in this study has helped define several residues of the Tsx protein that seem to be important for its nucleoside- and albicidin-specific channel function. The purification of the genetically modified Tsx proteins and their *in vitro* reconstitution into lipid bilayers should open the way for a characterization of the substrate specificity of the Tsx channel at the molecular level.

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