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 Gly28 (to Arg) and Ser217 (to Arg) are completely deficient in nucleoside uptake at a low substrate concentration. Substitutions of Phe27 to Leu, Gly28 to Gln, Gly30 to Asp, and Gly240 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, below the rate of substrate 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 deoxyribonucleosides and nucleosides at substrate concentrations below 1 μM (Hantke, 1976; Krieger-Brauer and Braun, 1980; Munch-Peterson *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 site inside the Tsx channel (Maier *et al.*, 1988; Benz *et al.*, 1988). The Tsx substrate binding site can discriminate between compounds closely related in structure. There is a stronger binding for deoxyribonucleosides 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 deoxyribonucleosides (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...
Alb\textsuperscript{β} 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 \textsuperscript{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/maltodextrin-specific LamB channel (Charbit et al., 1988; Dargent et al., 1988) and the outer membrane 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 \textit{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\textsuperscript{+} strains is available. We have constructed a scheme to detect strains synthesizing Tsx proteins that exhibit altered channel characteristics. Such a genetic approach has been fruitfully used to characterize the maltose/maltodextrin-specific LamB channel (Charbit et al., 1988; Dargent et al., 1988) and the outer membrane 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 \textit{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).

EXPERIMENTAL PROCEDURES

**Bacterial Strains and Plasmids**—The \textit{E. coli} K-12 strain BRE2050 (\textit{F} \textsuperscript{-} metB ilo rpsL cytB9 deoR8 Δ (argF-lac)U169) and its derivative, strain BRE2070, have been previously described (Bremer et al., 1988, 1990). Strain HFI, used for most of the experiments, was constructed as follows. First, the \textit{metB} and \textit{ilo} mutations present in strain BRE2070 were successively removed by P1vir-mediated transduction from selecting for Ilv\textsuperscript{+} and Met\textsuperscript{+} colonies on glucose minimal plates using P1vir lysate grown on the \textit{metB} \textit{ilv} strain MC1400 (Casadaban, 1976). A viable-resistant derivative of the resulting \textit{metB} \textit{ilv} strain MC1400 was selected on glucose minimal plates as described by Miller (1972). One of the resulting strains, BK6M, was subsequently lysogenized with the specialized transducing phage A1048 carrying a lac\textsuperscript{Y} gene expressed constitutively under the control of the \textit{tlyT} promoter (Berman and Jackson, 1984), yielding strain HFI. This strain construction was done to avoid the killing of \textit{E. coli} K-12 strains by the excess of vaccine synthesized by the albicidin-producer \textit{X. albilineans} LS155 (Birch and Patil, 1985) and to allow the detection of Lac\textsuperscript{+} colonies on lactose MacConkey plates when strain HFI carried the \textit{tsx}-lacZ\textsuperscript{α} operon fusion plasmid pH11 (Fig. 1A). A derivative of strain MC1400 lacking the Tsx, OmpC, and OmpF proteins was constructed by first transducing with phage P1vir the tsx::Tn10(kan) insertion from strain CAG18143 (Singh et al., 1989) into MC1400. Into the resulting strain HF19 we then introduced the Tn10 lacZ\textsuperscript{α} insertion tightly linked to a deletion in the \textit{ompC} gene (ΔompC::Tn10) (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 TlB, Tula, and T6, respectively (Datta et al., 1977; Manning and Reeves, 1978) and verified by electrophoresis of the outer membrane protein preparations on SDS-polyacylamide gels. Strain RZB2116 was used to obtain a colicin K preparation (Krieger-Brauer and Braun, 1980; Pugsley, 1985). The antibiotic albicidin was isolated from cultures of \textit{X. albilineans} strain LS156, an Alb\textsuperscript{α} derivative of LS155 that carries a\textit{tsr} in the albicidin biosynthetic genes, was used (Birch et al., 1990).

The construction and characterization of the low copy number plasmid \textit{tsx}-lacZ\textsuperscript{α} operon fusion plasmid pH11 (Fig. 1A) have been described by Schneider et al. (1989). The vector used for the construction of strain PH11 was the low copy number \textit{lacZ} operon plasmid pGP15, which carries a tetracycline resistance gene. A 4.5-kilobase \textit{Stul}-\textit{Hind}III restriction fragment carrying the \textit{tsr} gene was isolated from plasmid pH11 (tetracycline resistant) (Fig. 1A) and ligated into the \textit{Smal} and \textit{Hind}III sites in the polylinker of the low copy number plasmid pPDI (chloramphenicol resistant). The resulting \textit{tsx} plasmid was called pHF1. The same cloning strategy was used for each of the seven \textit{phl}11-derived Alb\textsuperscript{β} plasmids to transfer the mutant \textit{tsx} genes into the chloramphenicol resistant vector pPDI. 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 resulting wild-type and mutant Alb\textsuperscript{β} tsx genes from the tetracycline-resistant \textit{tsx}-lacZ\textsuperscript{α} operon fusion plasmid pH11 into the chloramphenicol-resistant vector pPDI was necessary because strain HF24 (tsx::Tn10(kan) Δ(\textit{ompC::Tn10})) used as the host strain for serine uptake experiments (see Fig. 4) is tetracycline-resistant.

**Media and Growth Conditions**—\textit{E. coli} strains were grown aerobically at 37 °C in rich media (LB, DYT, or NB) or minimal medium (MMA) (Miller, 1972) with 0.2% glycerol as the carbon source as described (Miller, 1972; Silhavy et al., 1984). LB and lactose MacConkey plates were used as the host strain for serine uptake experiments (see Fig. 4) is tetracycline-resistant. The construction and characterization of the low copy number plasmid \textit{tsx}-lacZ\textsuperscript{α} operon fusion plasmid pH11 (Fig. 1A) have been described by Schneider et al. (1989). The vector used for the construction of strain PH11 was the low copy number \textit{lacZ} operon plasmid pGP15, which carries a tetracycline resistance gene. A 4.5-kilobase \textit{Stul}-\textit{Hind}III restriction fragment carrying the \textit{tsr} gene was isolated from plasmid pH11 (tetracycline resistant) (Fig. 1A) and ligated into the \textit{Smal} and \textit{Hind}III sites in the polylinker of the low copy number plasmid pPDI (chloramphenicol resistant). The resulting \textit{tsx} plasmid was called pHF1. The same cloning strategy was used for each of the seven \textit{phl}11-derived Alb\textsuperscript{β} plasmids to transfer the mutant \textit{tsx} genes into the chloramphenicol resistant vector pPDI. 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 resulting wild-type and mutant Alb\textsuperscript{β} tsx genes from the tetracycline-resistant \textit{tsx}-lacZ\textsuperscript{α} operon fusion plasmid pH11 into the chloramphenicol-resistant vector pPDI was necessary because strain HF24 (tsx::Tn10(kan) Δ(\textit{ompC::Tn10})) used as the host strain for serine uptake experiments (see Fig. 4) is tetracycline-resistant.
7 resin (Sigma) and by elution with methanol (Birch and Patil, 1985). 500 ml of culture supernatant was passed through a column (10 x 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 antibiotic was assayed by absorbance at 305 nm. The fractions were pooled and evaporated to dryness. The final antibiotic preparation was dissolved in a small volume of water

Isolation of Plasmid pHs11-encoded Alb' tsx Mutants—Aliquots (0.1 ml of undiluted and 10- and 100-fold diluted) of overnight LB-grown cultures of strain HFI (pHS11) were spread on lactose MacConkey agar plates containing an inhibitory concentration of albicidin for the growth of a Tsx' strain and 5 ug/ml tetracycline. The selection plates were incubated for 16 h at 37 °C. Between four and six Lac' Alb' colonies from each independent culture were picked and purified by restreaking on the same plates. In this way, we isolated approximately 400 Lac' Alb' mutants, which originate from 25 N-methyl-N'-nitro-N-nitrosoguanidine-treated, 20 2-aminopurine-treated, and 30 unmutagenized cultures of strain HFI (pHS11). The 40 Lac' Alb' mutants were assayed streaked against the Tsx-specific T6 phage, and 60 were found to be sensitive against this bacteriophage. Thirty-eight colonies of these T6' 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' T6' strains gave a positive reaction with the antiserum. We tested 40 of the Lac' Alb' 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' T6' 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 ug/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 Loganberg et al. (1975). For the immunological detection of the Tsx protein, whole cell extracts were prepared from 5-ml cultures of strain HFI (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 HFI containing plasmid pHs11 with N-methyl-N'-nitro-N-nitrosoguanidine or 2-aminopurine (Sigma) was performed as described by Silhavy et al. (1984) and Mitsuhashi (1987). Plasmid DNA used for sequencing was purified on Qiagen 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 using 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'-TTCACCTCCCGCGAACGGG-3' (417-433 bp); oligonucleotide 2, 5'-CTGGTGGCACCGAGCCG-3' (554-570 bp); oligonucleotide 3, 5'-GGCGGCTACTCCGATGC-3' (702-718 bp); oligonucleotide 4, 5'-GTGCGCGACACCTGG-3' (874-890 bp); oligonucleotide 5, 5'-ACCGATCCTGTCGCGG-3' (1041-1057 bp); oligonucleotide 6, 5'-GAATACGATCCTGCG-3' (1187-1203 bp). To confirm the presence of a single mutation in the Alb' tsx mutants we sequenced the entire tsx gene for one representative strain or colony of each mutant Alb' 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. 1B.

DNA Sequence Analysis of the tsx Mutations—To establish the nature of the alterations in tsx that result in an Alb' 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, transversions, 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' tsx allele along with the wild-type tsx gene to ensure the unambiguous identification of the mutations conferring albicidin resistance. We found for 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. 1B.

Point mutations that alter residues Gly26 and Ser217 are the major class (31/38) of tsx mutants with an Alb' phenotype (Table I). In 20 of the 38 tsx mutants, the codon for residue Gly26 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, Ser217 was replaced by Arg (tsx-511); this class of tsx mutants comprise all spontaneously isolated Alb' strains (nine isolates)
Tsx Proteins with Altered Substrate Specificity

(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) 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 Arg (AGA) codon (Schneider et al., 1992). Single amino acid substitutions of Phe27 (tsx-508), Gly239 (tsx-512), and Gly240 (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. 1B). In contrast to the tsx alleles described above, the point mutations present in the tsx-508 gene do not cause the incorporation of an additional charge into the Tsx protein. A substitution of the hydrophobic Phe27 residue by a hydrophobic Leu residue (Fig. 1B) is the cause of the Alb' 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 comprises residues Phe27 and Gly239, sites at which single amino acid substitutions can confer an Alb' phenotype (Fig. 1B).

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' 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. 1B). 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' 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. 1B) 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' derivatives of strains HF1.

<table>
<thead>
<tr>
<th>tsx-allele</th>
<th>Mutation</th>
<th>Alteration in Tsx</th>
<th>Number of isolates</th>
<th>Mutagen</th>
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<tbody>
<tr>
<td>tsx-508</td>
<td>C-A transversion</td>
<td>Phe27-Leu</td>
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<td>2-AP</td>
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<tr>
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<td>Gly28-Arg</td>
<td>14</td>
<td>2-AP (10), NG (4)</td>
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<td>tsx-510</td>
<td>G-A transition</td>
<td>Gly239-Glu</td>
<td>6</td>
<td>NG</td>
</tr>
<tr>
<td>tsx-511</td>
<td>C-A transition</td>
<td>Ser217-Arg</td>
<td>11</td>
<td>2-AP (2), None (9)</td>
</tr>
<tr>
<td>tsx-512</td>
<td>C-A transition</td>
<td>Gly239-Asp</td>
<td>2</td>
<td>NG</td>
</tr>
<tr>
<td>tsx-513</td>
<td>G-A transition</td>
<td>Gly240-Asp</td>
<td>2</td>
<td>NG</td>
</tr>
<tr>
<td>tsx-514</td>
<td>39-bp duplication</td>
<td>From Gly21 to Asp24</td>
<td>1</td>
<td>2-AP</td>
</tr>
</tbody>
</table>

* The numbering of the alterations in Tsx is according to Bremer et al. (1990).

* 2-AP, 2-aminopurine; NG, N-methyl-N'-nitro-N-nitrosoguanidine.
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' 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.

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 [14C]deoxyadenosine (0.55 μM) and [14C]thymidine (0.85 μM) is entirely dependent on the Tsx protein and each of the Alb' *tsx* mutants showed a defect in deoxynucleoside transport (Fig. 3, A and B). Strains synthesizing the Tsx-509 (Glym to Arg), Tsx-511 (Serm to Arg), and Tsx-514 (13-amino acid duplication) mutant proteins were entirely deficient for both deoxynucleosine and thymidine uptake. Their deoxynucleoside transport activity was indistinguishable from a strain lacking the Tsx protein entirely. The Glym to Glu and Glym 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 Phe27 to Leu alteration present in the Tsx-508 mutant protein (Fig. 3, A and B). In contrast, the substitution of Glym by an Asp residue in the Tsx-513 protein had practically no influence on [14C]deoxynucleosine uptake (Fig. 3A) and reduced the transport of [14C]thymidine only slightly (Fig. 3B). The weak level of resistance against albicidin conferred by the tsx-513 allele (Table III) is thus reflected by only a weak impair-

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**TABLE II**

<table>
<thead>
<tr>
<th>tsx allele*</th>
<th>Alteration in Tsx</th>
<th>Resistance/sensitivity to</th>
<th>Alb</th>
<th>col K</th>
<th>T6</th>
<th>H1</th>
<th>H3</th>
<th>H8</th>
<th>Otxl</th>
<th>K18</th>
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<td>tsx-514</td>
<td>Duplication from Glym to Asn</td>
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<td>S/R</td>
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</tbody>
</table>

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

* 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' 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::TnlO (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 \([^{14}\text{C}]\text{serine}\) at a substrate concentration of 2.5 \(\mu\text{M}\) in comparison with its OmpC' and OmpF' parent strain HF19 (Fig. 4A). When the tsx' plasmid pHFl was introduced into strain HF24, \([^{14}\text{C}]\text{serine}\) uptake was significantly increased in comparison with strain HF24 carrying the vector plasmid (pPD1) used to construct plasmid pHFl (Fig. 4B). 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 \([^{14}\text{C}]\text{serine}\) uptake in comparison with the wild-type Tsx protein, and this is documented in Fig. 4B 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

### Table III

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*The tsx wild-type and its Alb' derivatives are pHS11-encoded and are present in strain HF1 (tsx). The vector plasmid pGP15 was used as a tsx" control.

* 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-

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**Fig. 3. Uptake of \([^{14}\text{C}]\text{radiolabeled deoxyadenosine and thymidine}\).** The initial transport of \([^{14}\text{C}]\text{deoxyadenosine} (A) and \([^{14}\text{C}]\text{thymidine} (B) in E. coli cells synthesizing either the Tsx wild-type or the mutant Alb' Tsx proteins was measured. The final substrate concentration of deoxyadenosine and thymidine in the uptake assays was 0.55 and 0.85 \(\mu\text{M}\), respectively. The uptake of nucleosides was compared in strain HF1 (tsx) containing the vector plasmid pGP15 (.), the tsx' plasmid pHS11 (●), and its derived Alb' alleles tsx-508 (●), tsx-509 (●), tsx-510 (●), tsx-511 (●), tsx-512 (●), tsx-513 (●), and tsx-514 (●), respectively.
characteristics. 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' 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 (Mr 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' 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' tsx derivatives, plasmids pHF2 (tsx-511; ×), pHF4 (tsx-509; ○), and pHF8 (tsx-508; ■), respectively.

A model for the topological arrangement of the Tsx protein in the E. coli 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. A we discuss below the properties of the single amino acid substitutions that confer albicidin resistance and impair deoxynucleo-

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**Fig. 4.** Uptake of [14C]serine. The initial transport of [14C]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' tsx derivatives, plasmids pHF2 (tsx-511; ×), pHF4 (tsx-509; ○), and pHF8 (tsx-508; ■), respectively.

**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.
oside uptake in the context of this topological model. Four of the 5 residues affected in the mutant Tsx proteins (Phe27, Gly28, Gly228, and Gly240) are located at the cell surface, whereas residue Ser217 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 Gly28, 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 Gly228 and Gly240 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 Gly228 and Gly240 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/maltodextrin-specific LamB channel have been previously characterized (Dargent et al., 1988; Charbit et al., 1988).

Substitutions of Gly28 by a positively charged Arg residue (Tsx-509) and by the negatively charged Glu residue (Tsx-510) were the most frequent of Alb+ mutant Tsx-510), which have been previously characterized (Dargent et al., 1988; Charbit et al., 1988). The nature and charge of the residues of the replacements at position Gly28 are important for the activity of the Tsx channel, whereas the substitution in the Tsx-512 protein causes a partial defect in deoxynucleoside uptake (Table III and Fig. 3). The phenotype conferred by the tsx-511 allele and the location of Ser217 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 (Quirocho, 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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Tsx Proteins with Altered Substrate Specificity
