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Identification of a Segment of the *Escherichia coli* Tsx Protein That Functions as a Bacteriophage Receptor Area

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The *Escherichia coli* outer membrane protein Tsx functions as a nucleoside-specific channel and serves as the receptor for colicin K and a number of T-even-type bacteriophages, including phage T6. To identify those segments of the Tsx protein that are important for its phage receptor function, we devised a selection and screening procedure which allowed us to isolate phage-resistant strains synthesizing normal amounts of Tsx. Three different Tsx-specific phages (T6, Ox1, and H3) were employed for the selection of phage-resistant derivatives of a strain expressing a *tsx*$^+$-*lacZ*$^+$ operon fusion, and 28 *tsx* mutants with impaired phage receptor function were characterized. Regardless of the Tsx-specific phage used for the initial mutant selection, cross-resistance against a set of six different Tsx phages invariably occurred. With one exception, these mutant Tsx proteins could still serve as a colicin K receptor. DNA sequence analysis of 10 mutant *tsx* genes revealed the presence of four distinct *tsx* alleles: two point mutations, an 18-bp deletion, and a 27-bp tandem duplication. In three isolates, Asn-249 was replaced by a Lys residue (*tsx-504*), and in four others, residue Asn-254 was replaced by Lys (*tsx-505*). The deletion (*tsx-506*; one isolate) removed six amino acids (residue 239 to residue 244) from the 272-residue Tsx polypeptide chain, and the DNA duplication (*tsx-507*; two isolates) resulted in the addition of nine extra amino acids (residue 229 to residue 237) to the Tsx protein. In contrast to the wild-type Tsx protein and the other mutant Tsx proteins, the Tsx-507 protein was cleaved by trypsin when intact cells were treated with this protease. The Tsx proteins encoded by the four *tsx* alleles still functioned in deoxyadenosine uptake in vivo, demonstrating that their nucleoside-specific channel activity was not affected by the alterations that caused the loss of their phage receptor function. The changes in the Tsx polypeptide that confer resistance against the Tsx-specific phages are clustered in a small region near the carboxy terminus of Tsx. Our results are discussed in terms of a model for the topological organization of the carboxy-terminal end of the Tsx protein within the outer membrane.

Escherichia coli can efficiently use exogenously provided nucleosides and deoxynucleosides as carbon and nitrogen sources and as precursors for the synthesis of nucleic acids. The structural genes encoding the proteins responsible for the uptake and metabolism of nucleosides are genetically organized in a regulon, which is negatively controlled by the DeoR and CytR repressors and positively affected by the cyclic AMP (cAMP)-cAMP receptor protein activator complex (33). The *tsx* gene is part of this regulon (8, 9, 23). It encodes a 272-amino-acid outer membrane protein, Tsx, that permits the efficient permeation of nucleosides and deoxynucleosides across this membrane when these substrates are present in submicromolar concentrations (9, 18, 23, 34). Tsx is a channel-forming protein whose nucleoside specificity results from the presence of a substrate-binding site within the Tsx channel (5, 25). Segments of the Tsx protein are exposed at the cell surface and serve as the receptor for colicin K, bacteriophage T6, and a number of other T-even-type phages (17, 27). Little is known about those regions of Tsx that are involved in its bacteriophage and colicin K receptor functions. On the basis of proteolytic digestion and chemical modification of cell envelope proteins, Weltzien and Jesaitis (47) have provided circumstantial evidence that the segments important for colicin K and phage T6 binding differ from each other. Only a single *tsx* missense mutant (*tsx-206*) with defective phage receptor function has been isolated (27), and its loss of phage receptor activity is due to the substitution of Asn-254 by a Tyr residue (26). This finding implies that Asn-254 is critical for the recognition of the Tsx receptor protein by the various Tsx-specific phages and that this residue is located in a segment of the Tsx protein exposed at the cell surface.

The aim of this study was to define more closely those segments of the Tsx polypeptide that are involved in its bacteriophage receptor function. It is clear from the study of Manning and Reeves (27) that direct selection for strains resistant against phage T6 yields predominantly *tsx* mutants that either lack Tsx entirely or synthesize it in greatly reduced amounts. We developed a genetic selection and immunological screening procedure that allowed us to isolate phage-resistant *tsx* mutants that synthesized normal amounts of the Tsx protein. The alterations resulting in phage resistance are all clustered near the COOH-terminal end of the Tsx polypeptide, thus identifying a segment of Tsx that is involved in its phage receptor function.

MATERIALS AND METHODS

Media, growth conditions, genetic procedures, bacteriophages, and construction of bacterial strains. Bacteria were grown aerobically at 37°C in rich medium (LB or DYT) or

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TABLE 1. Bacterial strains, bacteriophages, and plasmids

Strain, phage, or plasmid	Description[a]	Source or reference
Strains		
MC4100	F⁻ Δ(*argF-lac*)*U169 araD139 rpsL150 deoC1 relA1 ptsF25 flbB5501 rbsR*	11
BRE2050	F⁻ *metB ilv rpsL* Δ(*argF-lac*)*U169 cytR9 deoR8*	8
BRE2070	BRE2050 *tsx*	9
HS20	BRE2070 (λp1048)	This study
HF1	BRE2070 *ilv*⁺ *metB*⁺ Val^r (λp1048)	H. Fsihi
HF19	MC4100 *tsx*::Tn*10*(*kan*)	This study
CAG18413	*tsx*::Tn*10*(*kan*)	43
TG1	Δ(*lac-pro*) *supE thi hsdD5* F′ *traD36 proA*⁺*B*⁺ *lacI*^{q} *lacZM15*	40
BM630	MC4100 *cdd* Φ(*tsx*⁺-*lacZ*⁺)*630-2* (λp*lac*Mu55)	B. Mygind
BZB2116	pColK-K235; colicin K producer	39
Bacteriophages		
λp1048	Φ(*tyrT*′-*lacY*⁺)1048	6
λ630-2	Φ(*tsx*⁺-*lacZ*⁺); Lac⁺ transducing phage isolated from strain BM630	This study
Plasmids		
pGP15	*′lacZ lacY′* Tc^r	P. Gerlach
pHS11	pGP15 Φ(*tsx*⁺-*lacZ*⁺) Tc^r (Fig. 1A)	This study
pTX9	*lacPO-tsx* Δ(*Bst*EII) Ap^r (Fig. 1A)	26

[a] The gene symbols are according to Bachmann (4). Genes marked with a prime are incomplete. The λp*lac*Mu55 prophage carries a kanamycin resistance gene.

minimal medium with 0.2% glycerol as the carbon source (29, 42). Agar plates spread with 0.1 ml of a 10-mg/ml solution of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) in dimethylformamide were used to distinguish between LacZ⁻ and LacZ⁺ phenotypes. Lactose-MacConkey agar plates were prepared as described previously (29, 42). Ampicillin, tetracycline, and kanamycin were added to liquid and solid media at 50, 5, and 50 μg/ml, respectively. The bacteria, phages, and plasmids used in this study are described in Table 1. All strains were *E. coli* K-12 derivatives. Standard techniques were used for the propagation of bacteria and bacteriophages, for general transduction with phage P1*vir*, and for the lysogenization of λ specialized transducing phages (29, 42). A P1*vir* lysate was prepared in strain CAG18413 [*tsx*::Tn*10*(*kan*)] and used to transduce strain MC4100 to kanamycin resistance. Loss of the Tsx protein in one of these transductants, strain HF19, was tested by cross-streaking against phage T6 and verified on sodium dodecyl sulfate (SDS)-polyacrylamide gels. A crude preparation of colicin K was prepared from strain BZB2116 after mitomycin C induction as described previously (23). Colicin K sensitivity of bacterial strains was tested by spotting 30-μl aliquots of a series of twofold dilutions of the crude colicin K preparation onto bacterial lawns plated in LB soft agar. The OmpA- and LamB-specific phages (TuII* and λ*vir*) and the Tsx-specific phages (T6, T6h3.1, Ox1, H1, H3, H8, and K18) have been previously described (13, 17, 26, 42) and were all propagated on strain MC4100.

Construction and characterization of the *tsx*⁺-*lacZ*⁺ operon fusion plasmid pHS11. During a search for CytR-regulated chromosomal genes from *E. coli*, a strain (BM630) was isolated that carried a Lac⁺ insertion of the *lacZ* operon fusion-generating phage λp*lac*Mu55 (10) tightly linked to the CytR-controlled *tsx* gene (35). This fusion strain was fully sensitive to the Tsx-specific phage T6 and produced normal amounts of the Tsx protein. A Lac⁺ λ specialized transducing phage (λ630-2) was isolated after UV irradiation of strain BM630 (42). DNA from phage λ630-2 was prepared and digested with *Eco*RI and *Sst*I; the resulting restriction fragments were cloned into the low-copy-number *lacZ* fusion vector pGP15. One of the resulting LacZ⁺ plasmids, pHS11 (Fig. 1A), was chosen for further analysis. When plasmid pHS11 was transformed into a strain carrying chromosomal *tsx* and Δ*lacZ* mutations, the transformants became LacZ⁺, sensitive against phage T6, and synthesized the Tsx protein (Fig. 2; lane 4). Restriction analysis of plasmid pHS11 showed that the λp*lac*Mu55 insertion had occurred close to the 3′ end of the *tsx* coding region (Fig. 1A). To determine the precise DNA sequence at the fusion junction, a 542-bp *Tha*I restriction fragment (Fig. 1A) was cloned into the *Hin*cII site of phage M13mp18 (37). DNA sequence analysis of the recombinant M13 phage revealed that the Mu*S* sequence (49) from the transposable λp*lac*Mu55 phage (10) was joined to the *tsx* sequence after position 1355 (bp) (9), 15 bp downstream of the *tsx* stop codon (Fig. 1B).

Isolation of phage-resistant Tsx mutants. The *tsx*⁺-*lacZ*⁺ fusion plasmid pHS11 does not contain an intact *lacY* gene (Fig. 1). In order to use lactose-MacConkey agar indicator plates for monitoring *lacZ* expression, we used strain HS20 as the host for plasmid pHS11. This strain carries a λ prophage, λp1048, integrated at *attB* in the *E. coli* chromosome and expresses the *lacY*⁺ gene constitutively under the control of the *tyrT* promoter (6). For the selection of strains resistant against Tsx-specific phages, cultures of strain HS20 (pHS11) were grown overnight in LB medium at 37°C; 100 μl of the undiluted culture and 100 μl from a 10^{-1} dilution were then separately spread onto lactose-MacConkey agar plates onto which 0.2 ml of a high-titered phage lysate of a Tsx-specific phage (either T6, Ox1, or H3) had been plated. The plates were then incubated overnight at 37°C. From each independent culture, two nonmucoid phage-resistant Lac⁺ colonies were picked, purified, and tested for sensitivity against the Tsx-specific phage used in the original selection. Whole-cell extracts from 1-ml cultures of the various phage-resistant mutants were prepared (42), the proteins were electrophoretically separated on a SDS–12% polyacrylamide gel, and the presence of the Tsx protein was then tested in a Western blot (immunoblot) experiment with a rabbit antiserum raised against denatured Tsx protein. Plasmid DNA was extracted from those colonies that showed a

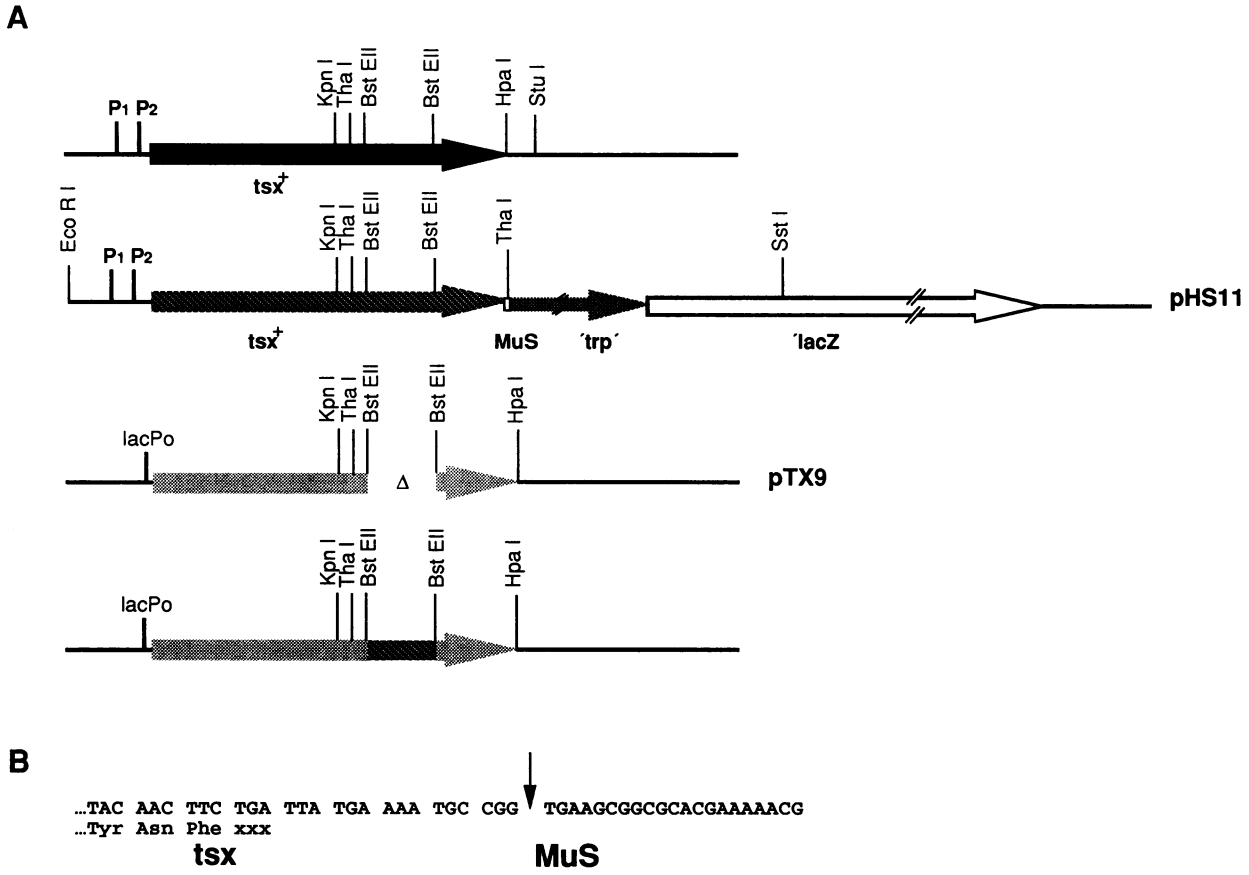

FIG. 1. (A) Physical structure and genetic organization of the plasmids used in this study. The positions of restriction sites relevant only for this work are indicated, and the regions outside the *tsx* coding region are not drawn to scale. (B) DNA sequence of the fusion junction between the *tsx* gene and the λ*plac*Mu55-derived Mu*S* sequence.

positive immunological reaction with the Tsx antiserum and used to transform strain HF1 (*tsx* Δ*lacZ lacY*$^+$; Table 1). The Lac$^+$ phenotype of the transformants was tested on lactose-MacConkey agar indicator plates, and the presence of the Tsx protein in outer membrane preparations was verified by SDS-polyacrylamide gel electrophoresis (PAGE). Phage sensitivity or resistance of the *tsx* mutants was determined by spotting or plating phage dilutions onto LB plates that had been overlaid with LB soft agar containing the bacteria. Tsx-specific phages (T6, T6h3.1, Ox1, H1, H3, H8, and K18), an OmpA-specific phage (TuII*), and a LamB-specific phage (λ*vir*) were used for these tests.

Mapping and DNA sequence analysis of the mutant *tsx* alleles. To avoid the resequencing of the entire *tsx* gene encoding the mutant Tsx proteins, we first mapped the approximate position of the mutations conferring phage resistance. A 305-bp *Bst*EII restriction fragment carrying part of the *tsx* gene was cut out of the mutant pHS11 derivatives and cloned into the unique *Bst*EII site of plasmid pTX9 (Fig. 1A). Plasmid pTX9 carries a partially deleted *tsx* gene (Δ*Bst*EII-*Bst*EII) under *lacPO* control, and strains carrying pTX9 do not synthesize a functional Tsx protein that can be detected in outer membrane preparations. Cloning of the 305-bp *Bst*EII fragment into pTX9 in the correct orientation restores the coding sequence of *tsx* and allows synthesis and insertion of Tsx into the outer membrane (26). The resulting recombinant plasmids were transformed into a *tsx* host strain, and the sensitivity of the transformants was

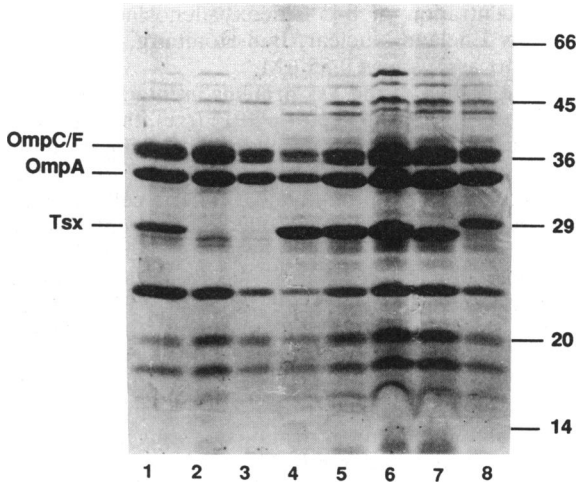

FIG. 2. Synthesis of the Tsx proteins in phage-resistant mutants. Cell envelope proteins of strains BRE2050 (*deoR cytR*) (lane 1), BRE2070 (*deoR cytR tsx*) (lane 2), and HS20 (*deoR cytR tsx*) carrying the vector pGP15 (lane 3) and the *tsx*$^+$ plasmid pHS11 (lane 4) and the pHS11 mutant derivatives, *tsx-505* (lane 5), and *tsx-504* (lane 6), *tsx-506* (lane 7), and *tsx-507* (lane 8), were separated by SDS-PAGE. The gel system used does not resolve the OmpC and OmpF proteins; the gel was stained with Coomassie brilliant blue. Molecular size markers (in thousands) are indicated on the right.

then tested against Tsx-specific phages and colicin K. In this way, we could prove that the mutation conferring phage resistance in isolates T6 5-1, T6 15-1, T6 85-1, Ox1 4-2, Ox1 10-2, Ox1 14-1, Ox1 28-2, H3 5-2, H3 7-2, and H3 8-1 was located in the 305-bp *Bst*EII restriction fragment of the *tsx* gene. Two strategies were used to determine the DNA sequence alterations in the mutant *tsx* genes. For four mutants (isolates T6 85-1, Ox1 4-2, Ox1 14-1, and H3 5-2), a 542-bp *Tha*I fragment (Fig. 1A) was isolated from the pHS11 derivatives and inserted into the *Hin*cII site of phage M13mp18 (37). For the remaining isolates, a 478-bp *Kpn*I-*Hpa*I restriction fragment (Fig. 1A) was cloned into phages M13mp18 and M13mp19. Recombinant M13 phages were propagated on strain TG1 as described previously (37, 40). The DNA sequence of the cloned restriction fragment was determined by using the method of Sanger et al. (41) and the Sequenase 2.0 kit under the conditions recommended by the supplier (United States Biochemical, Bad-Homburg, Germany). Routine DNA manipulations were carried out as described previously (40, 42).

Preparation of protein samples, gel electrophoresis, immunological detection of the Tsx protein, and nucleoside uptake assays. Cell envelopes were prepared from 40-ml overnight cultures grown in DYT medium as described previously (8). The membrane proteins were solubilized in sample buffer containing 2% SDS by boiling for 5 min, and portions were then electrophoresed on an SDS–12% polyacrylamide gel by the method of Lugtenberg et al. (24). Protein bands were visualized by staining with Coomassie brilliant blue. For the immunological detection of the Tsx protein, electrophoretically separated proteins were transferred to a nylon membrane (Immobilon; pore size, 0.45 μm; Millipore). The bound proteins were then probed with antiserum, and the antigen-antibody complex formed was visualized with a second goat anti-rabbit immunoglobulin G–alkaline phosphatase-coupled antibody (Sigma, Deisenhoefen, Germany) (40). The initial uptake of deoxyadenosine was measured in cells grown in minimal medium with 0.2% glycerol as the carbon source as described previously (23). The final substrate concentration of 8-[^{14}C]deoxyadenosine (46.6 mCi/mmol; New England Nuclear, Bad-Homburg, Germany) in the transport assays was 0.55 μM.

Trypsin digestion of the Tsx proteins in intact cells. Strain HF1 harboring either the vector pGP15 (*tsx*), the *tsx*$^+$-*lacZ*$^+$ fusion plasmid pHS11, or its derivatives carrying the mutant *tsx* alleles was grown in DYT medium at 37°C to an optical density at 578 nm of approximately 0.7. The cells from 5-ml portions of the cultures were collected by centrifugation, washed with 6 ml of a solution containing 10 mM Tris-HCl (pH 8.0) and 10 mM $MgCl_2$, and resuspended in 6 ml of the same solution. One half was treated with 1 mg of trypsin (resuspended in 10 mM Tris-HCl [pH 8.0]–10 mM $MgCl_2$) per ml, and the other half was treated with the same solution containing no protease. The cells were incubated at 37°C for 6 h, after which trypsin activity was stopped by adding 2 mg of trypsin inhibitor from egg white (Boehringer Mannheim, Mannheim, Germany) per ml. The cell suspensions were further incubated for 15 min at 37°C. The cells were subsequently pelleted by centrifugation, washed twice with 10 ml of a solution of 10 mM Tris-HCl (pH 8.0)–10 mM $MgCl_2$, and finally resuspended in 100 μl of SDS loading buffer (42). The proteins were solubilized by boiling the samples for 5 min at 95°C, and 20-μl aliquots were then loaded onto an SDS–12% polyacrylamide gel. After SDS-PAGE, a Western blot was performed to visualize the Tsx protein and its trypsin digestion product.

RESULTS

Rationale for the isolation of phage-resistant *tsx* mutants. To detect phage-resistant *tsx* mutants producing normal amounts of the Tsx protein, we took advantage of a low-copy-number plasmid, pHS11, that carries a *tsx*$^+$-*lacZ*$^+$ operon fusion. In this operon, an intact *tsx*$^+$ gene is transcribed from its natural DeoR- and CytR-controlled promoters (*tsx*-p1 and *tsx*-p2, respectively) (9) and is followed by a promotorless *lacZ* indicator gene (Fig. 1A). This *tsx*$^+$-*lacZ*$^+$ fusion was fortuitously isolated during a search for CytR-controlled chromosomal genes in *E. coli* (35) by using the *lacZ* operon fusion-generating phage λp*lac*Mu55 (10). Cloning of the *tsx*$^+$-*lacZ*$^+$ operon fusion and DNA sequence analysis of the fusion junction revealed that the joining of *tsx* and the λp*lac*Mu55-derived Mu*S* sequence had occurred 15 bp downstream of the *tsx* stop codon (Fig. 1B). Mapping of the 3′ end of the *tsx* mRNA has shown that the *tsx* transcripts proceeds at least 116 bp downstream of the *tsx* translational termination signal (9). As a result, expression of the promoterless *lacZ* gene in the *tsx*$^+$-*lacZ*$^+$ operon is dependent on the *tsx* transcription initiation signals, and strains (*tsx* Δ*lacZ lacY*$^+$) carrying plasmid pHS11 are sensitive against Tsx-specific phages and show a strong Lac$^+$ phenotype on lactose-MacConkey agar indicator plates. Consequently, a weak Lac phenotype of colonies selected as being resistant against Tsx-specific phages can be taken as an indication for blocked or reduced expression of *tsx* in the mutant strains. Since phage-resistant Lac$^+$ mutants could also correspond to unstable Tsx proteins or to nonpolar nonsense or deletion mutations in *tsx*, we examined Tsx production by Western blot experiments. This two-step selection and screening procedure allowed us to identify *tsx* mutants displaying defects in the phage receptor activity of Tsx.

Selection and characterization of mutants resistant against Tsx-specific phages. For the selection of phage-resistant Tsx mutants, we chose three different Tsx-specific phages: T6, Ox1, and H3. These phages were originally isolated in the United States, England, and Australia, respectively (17). From 119 cultures of strain HS20(pHS11), we identified 28 independent phage-resistant strains that still synthesized normal amounts of the Tsx protein. In each case, the mutation conferring resistance against the Tsx-specific phages was plasmid encoded. These strains were fully sensitive to the OmpA- and LamB-specific phages TuII* and λ *vir* (data not shown), demonstrating that the pHS11-encoded mutations did not cause a general pattern of resistance against phages that do not use Tsx as their receptor. Analysis of the outer membrane protein profile of the mutant strains by SDS-PAGE demonstrated that the mutationally altered Tsx proteins were synthesized in amounts similar to that of the pHS11-encoded Tsx protein (Fig. 2). In two cases (isolates T6 15-1 and Ox1 28-2), the Tsx protein showed a slower electrophoretic mobility than the wild-type protein (Fig. 2, lane 8).

We tested the sensitivity of the 28 *tsx* missense mutants selected as being resistant towards either phage T6, Ox1, or H3 against seven Tsx-specific phages and colicin K (Table 2). All mutant strains were fully resistant against phages Ox1, H1, H3, H8, and K18. With one exception (isolate H3 7-2), phage T6 was still partially able to infect the strains synthesizing the mutant Tsx proteins. This was also the case for those mutants that were originally isolated as being T6 resistant (Table 2). The plating efficiency of phage T6 on all mutant strains was reduced at least 10-fold, and the phage

TABLE 2. Characteristics of the phage-resistant Tsx mutants[a]

Gene	Isolate	Number of nonsequenced isolates with identical phenotypes	Characteristic with:							
			T6	T6h3.1	Ox1	H1	H3	H8	K18	Colicin K
tsx$^+$			S	S	S	S	S	S	S	S
tsx			R	R	R	R	R	R	R	R
tsx-504	T6 85-1	1	R/S	R	R	R	R	R	R	S
	Ox1 4-2	2	R/S	R	R	R	R	R	R	S
	H3 8-1	7	R/S	R	R	R	R	R	R	S
tsx-505	T6 5-1	3	R/S	S	R	R	R	R	R	S
	Ox1 10-2	5	R/S	S	R	R	R	R	R	S
	Ox1 14-1		R/S	S	R	R	R	R	R	S
	H3 5-2	0	R/S	S	R	R	R	R	R	S
tsx-506	H3 7-2	0	R	R	R	R	R	R	R	R
tsx-507	T6 15-1	0	R/S*	R	R	R	R	R	R	S
	Ox1 28-2	0	R/S*	R	R	R	R	R	R	S

[a] All *tsx* alleles are plasmid encoded and are derivatives of plasmid pHS11 carrying the *tsx*$^+$ gene. Plasmid pGP15 was the vector used for the construction of pHS11 and served as the *tsx* control. All plasmids were present in strain HF1 (*tsx*). S, sensitivity; R, resistance; R/S, reduced plating efficiency and turbid plaque morphology; R/S*, the plaques formed were rapidly overgrown by the bacterial cells.

plaques were very small and turbid. Isolates T6 15-1 and Ox1 28-2 (Table 2), which synthesize Tsx proteins with slower electrophoretic mobilities (Fig. 2, lane 8), showed particularly impaired T6 receptor function, since the cell lawns rapidly overgrew the very turbid plaques formed by phage T6. Strains both resistant against and sensitive to phage T6h3.1 are present in the collection of the *tsx* mutant strains (Table 2). This host-range derivative of T6 can recognize both the wild-type Tsx protein and the mutant Tsx-206 protein (Asn-254 to Tyr substitution), which is impaired in its phage receptor function (26). The colicin K receptor activity of the Tsx protein (27) was not affected by most mutations, except in isolate H3 7-2, which is also the only strain that is fully resistant against phage T6 (Table 2). Regardless of the bacteriophage (T6, Ox1, or H3) used for the initial selection of *tsx* mutants, cross-resistance against the other Tsx-specific phages was observed (Table 2). This suggests that the various Tsx-specific phages require identical or similar determinants for their recognition of Tsx.

T-even-type phages recognize their cell surface receptors with the tips of the long tail fibers, and in a second step, the short tail fibers bind to lipopolysaccharide (20). We tested the mutants carrying various *tsx* alleles (see below) that confer phage resistance for their ability to inactivate the Tsx-specific phages T6, H3, and K18. The three phages were inactivated by a strain synthesizing the wild-type Tsx protein, but there was no significant irreversible binding of the phages to any of the mutant Tsx proteins (Table 3). Phage T6 can still plate, albeit with reduced efficiency, on most of the *tsx* mutant strains (Table 2). However, there was no strong irreversible binding of T6 to the cells synthesizing the mutant Tsx proteins, indicating that this phage still recognizes the mutant receptor proteins but cannot interact with them in an effective manner.

DNA sequence analysis of the mutant *tsx* genes. We chose for DNA sequence analysis 10 *tsx* mutants representative of the phage resistance and sensitivity patterns that we observed (Table 2). We first mapped the approximate positions of the *tsx* mutations by cloning a 305-bp *Bst*EII restriction fragment from the mutant pHS11 derivatives into a nonfunctional *tsx* gene (Δ*Bst*EII-*Bst*EII) carried by plasmid pTX9 (Fig. 1A). We chose this mapping strategy because we have previously shown that the *tsx-206* mutation, which impairs the phage receptor function of Tsx, is located on this *Bst*EII restriction fragment (26). In the 10 *tsx* alleles analyzed, the mutations were all associated with this restriction fragment (data not shown). The precise alterations present in the mutants were subsequently determined by DNA sequencing, and four different *tsx* alleles were detected. In *tsx-504* (three isolates), a C-to-A transversion in codon 249 (AAC) results in the replacement of Asn-249 by a Lys residue. In *tsx-505* (four isolates), a C-to-A change affected codon 254 (AAC), causing the substitution of Asn-254 by a Lys residue. The *tsx-506* mutation (one isolate) is a deletion of 18 bp that affects codons 239 to 244, and it is the only allele that confers complete resistance to all Tsx-specific phages and to colicin K (Table 2). A direct repeat of 6 bp is present in the vicinity of the deleted DNA segment (Fig. 3A). The *tsx-507* allele (two isolates) has a 27-bp duplication that results in the addition of nine extra amino acids (residue 229 to residue 237) to the Tsx polypeptide chain. Surrounding the duplication in the *tsx-507* gene is an 8-bp directly repeated DNA sequence (Fig. 3B).

We found that the pattern of sensitivity to phage T6 and its host-range derivative, T6h3.1, in the *tsx* mutants that we analyzed at the DNA level was predictive of their specific mutation. The *tsx-504* and *tsx-505* alleles confer T6$^{r/s}$ T6h3.1^r and T6$^{r/s}$ T6h3.1^s phenotypes, respectively. The same phenotypes are exhibited by the 18 unsequenced *tsx* mutants (Table 2), and it is therefore highly likely that these strains are repeated isolates that synthesize the Tsx-504 and Tsx-505 mutant proteins.

TABLE 3. Adsorption of Tsx-specific phages to the mutant Tsx proteins[a]

Gene	Alteration in Tsx	No. of phage		
		T6	H3	K18
tsx$^+$		80	5	23
tsx		340	1,600	105
tsx-504	Asn-249-Lys	300	1,400	96
tsx-505	Asn-254-Lys	337	1,680	90
tsx-506	Δ(Gly-239-Asp-244)	300	1,680	107
tsx-507	Duplication of (Tyr-229-His-237)	284	1,763	104

[a] The Tsx-specific phages were incubated with cells of strain HF19 carrying either the vector plasmid pGP15 (*tsx*), pHS11 (*tsx*$^+$), or its mutant derivatives expressing the various Tsx proteins. After incubation for 20 min at 37°C, phages adsorbed to the bacteria were pelleted by centrifugation and the number of phages in the supernatant was determined by plating on the *tsx*$^+$ strain MC4100.

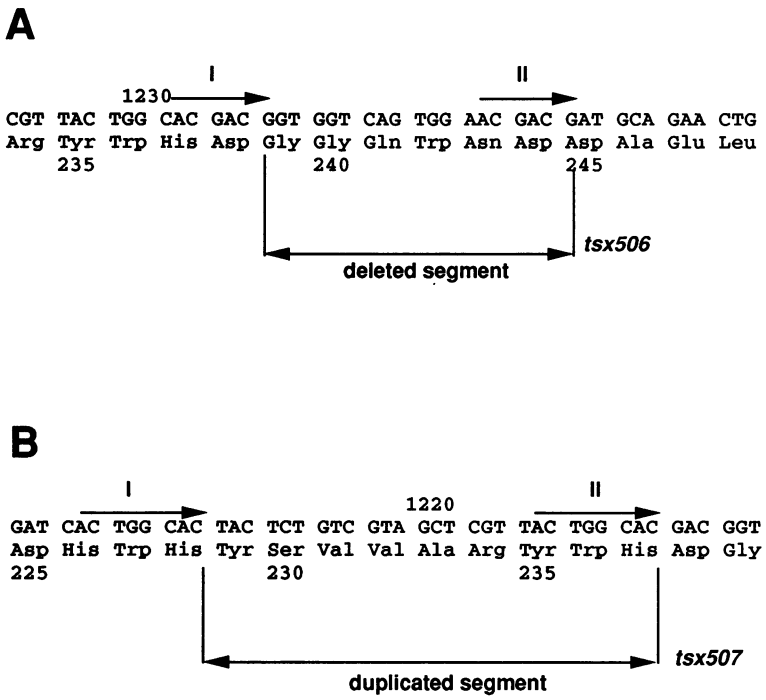

FIG. 3. DNA sequences of the *tsx-506* (A) and the *tsx-507* (B) mutations.

The mutant Tsx-507 is accessible in whole cells to trypsin. A potential cleavage site for trypsin (after Arg-234 [Fig. 3B]) exists in the extra nine amino acids present in the Tsx-507 protein. Whole cells synthesizing either the wild-type Tsx or Tsx-507 protein were treated with the protease, and the Tsx proteins and their possible proteolytic digestion products were then visualized by immunoblotting. The wild-type Tsx protein was completely resistant against trypsin (Fig. 4, lanes 1 and 2), as were the mutant Tsx-504, Tsx-505, and Tsx-506 proteins (data not shown). The Tsx-507 protein, however, exhibited sensitivity towards trypsin (Fig. 4, lanes 5 and 6). The wild-type Tsx protein migrates on SDS-polyacrylamide gels, with an apparent molecular weight of 28,000 (Fig. 2). The nine-amino-acid duplication present in the Tsx-507 protein increases the apparent molecular weight by approximately 1,500, and trypsin digestion of the Tsx-507 protein in intact cells yielded a stable cleavage product that migrated on the SDS-polyacrylamide gel as a 27,000 polypeptide species (Fig. 4, lane 6). No other stable degradation products of the Tsx-507 protein were detected in the immunoblot (data not shown). Thus, the protease sensitivity of the Tsx-507 protein towards trypsin indicates that the duplicated segment is exposed at the cell surface. Furthermore, the size of the Tsx-507 proteolytic degradation product is consistent with the idea that a small carboxy-terminal segment is removed from the Tsx polypeptide, whereas the main part of the Tsx-507 protein remains unaccessible to the protease. We note that an *ompA* mutant (OmpA-105) with characteristics similar to those of the Tsx-507 protein has been recovered during the search for phage-resistant OmpA strains (12, 32).

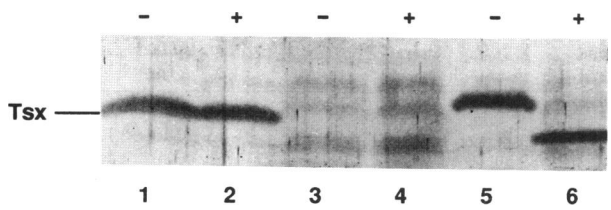

FIG. 4. Sensitivity of the Tsx-507 protein against trypsin. Intact cells were incubated in the absence (−) or presence (+) of trypsin, and the Tsx protein was immunologically detected after the electrophoretic separation of whole-cell proteins. Proteins from strain HF1 (*tsx*) harboring plasmid pHS11 (*tsx*$^+$) (lanes 1 and 2), pGP15 (*tsx*) (lanes 3 and 4), or pHS11 (*tsx-507*) (lanes 5 and 6) were used for the assay. The position of the wild-type Tsx protein is indicated.

Nucleoside uptake is not affected by the alterations in Tsx that result in phage resistance. To test whether the *tsx* mutants that confer phage resistance affect the channel-forming activity of the Tsx protein, we measured the initial uptake of [^{14}C]deoxyadenosine at a low substrate concentration (0.55 μM). Strains HF1(pHS11; *tsx*$^+$) and HF1(pGP15; vector plasmid) showed the expected (18, 23, 34) strong dependence on the Tsx protein for deoxyadenosine uptake. Deoxyadenosine uptake was not affected by any of the *tsx* mutations conferring phage resistance (data not shown).

DISCUSSION

Studies of missense mutants that confer phage resistance have yielded valuable insight into the functional and topological organization of a number of integral outer membrane proteins (1, 12, 16, 19, 20, 22, 28, 30–32, 46). Direct selection for phage resistance usually yields mutants that lack the receptor protein entirely or synthesize it in greatly reduced amounts. Our two-step selection with a *tsx*$^+$-*lacZ*$^+$ operon fusion yielded *tsx* mutants that synthesize the normal amount of Tsx (28 of 119 phage-resistant strains isolated). Although not highly efficient, the procedure is a significant improvement over previously used direct selections for Tsx

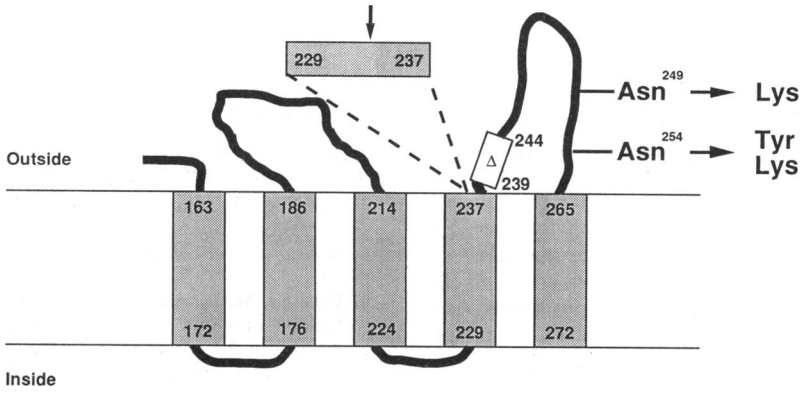

FIG. 5. Model for the topological arrangement of the carboxy-terminal end of the Tsx protein in the outer membrane. The positions of the alterations in Tsx that confer phage resistance are indicated, and a potential cleavage site for trypsin in the duplicated segment is marked by an arrow. Tsx-206, Asn-254→Tyr; Tsx-504, Asn-249→Lys; Tsx-505, Asn-254→Lys; Tsx-506, Δ(amino acids 239 to 244); Tsx-507, duplication of amino acids 229 to 237.

phage receptor mutants (27). The number of strains producing Tsx was fairly high among the phage-resistant mutants selected against phage H3 (10 of 18 cultures) and phage Ox1 (11 of 31 cultures) but was very low (7 of 70 cultures) among those selected against phage T6. We detected residual sensitivity against phage T6 in essentially all *tsx* phage receptor mutants (Table 2), and this phenotype could hamper the recognition of the desired types of *tsx* mutants when the colonies were picked from the original selection plates and retested for their resistance against T6 by cross-streaking against a high-titered phage lysate.

A model for the topological organization of the carboxy-terminal end of the Tsx protein within the outer membrane is shown in Fig. 5. This model is based on the structure prediction rules developed for the bacterial porin superfamily (21). The carboxy terminus of Tsx is shown facing the periplasm, as predicted from its homology to a consensus sequence of the carboxy-terminal ends of many outer membrane proteins (44). The regions of Tsx drawn in the model as cell-surface-exposed loops contain many charged residues, whereas those segments of Tsx drawn as membrane-spanning segments contain only a single charged residue (9). The regions representing the membrane-spanning segments are strongly conserved in the Tsx proteins from *Salmonella typhimurium*, *Enterobacter aerogenes*, and *Klebsiella pneumoniae*, whereas the two cell-surface-exposed regions are highly variable (36). Similar variation of externally exposed domains of integral outer membrane proteins has been found for the OmpA, PhoE, and LamB proteins as well (7, 14, 45).

Five Tsx proteins with altered phage receptor activities have been characterized in detail (26; this study). The underlying mutations are all clustered in a small region close to the 3′ end of the *tsx* coding region. If one projects the alterations present in the mutant Tsx proteins onto our topological model, four mutations (*tsx-206*, *tsx-504*, *tsx-505*, and *tsx-506*) affect residues located in the cell-surface-exposed loop found next to the carboxy terminus of Tsx. The fifth mutation (*tsx-507*) results in a duplication of one of the membrane-spanning segments that flank this loop (Fig. 5) and renders the Tsx-507 protein sensitive to trypsin in whole cells. These mutant Tsx proteins show only limited functional defects since their nucleoside-specific channel activity has been maintained, and with the exception of the Tsx-506 polypeptide, they can also serve as colicin K receptor proteins. The characteristics of the *tsx* phage receptor mutants and the protease sensitivity of the Tsx-507 protein thus provide experimental support for the cell surface exposure of the region between residues 237 and 265 of Tsx.

Amino acid substitutions at residues Asn-249 and Asn-254 (Fig. 5) strongly impaired the phage receptor function of Tsx but did not affect colicin K sensitivity or nucleoside uptake, strongly suggesting that these residues are directly involved in the interaction of Tsx with the various bacteriophages. Residue Asn-249 is conserved in the Tsx proteins from *E. aerogenes* and *K. pneumoniae*, which also function as receptors for Tsx-specific phages (36). Likewise, substitutions of Asn-254 by Asp in the *E. aerogenes* Tsx protein and by a Pro residue in the *K. pneumoniae* Tsx protein do not abolish phage receptor activity (36). Since the uncharged polar Asn-254 residue in the *E. coli* Tsx protein can be replaced by a negatively charged Asp or a hydrophobic Pro residue, it appears that it is the loss of the original Asn-254 residue that causes phage resistance and not the gain of the positively charged Lys (*tsx-505*) and aromatic Tyr (*tsx-206*) residues (Fig. 5). The nine-amino-acid duplication present in the Tsx-507 protein could have an indirect effect on the phage receptor function by changing the secondary structure of the cell-surface-exposed loop and thus obscuring the bacteriophage-binding area. Such changes must, however, be locally confined since the Tsx-507 protein has retained its colicin K receptor function and its property as a nucleoside-specific channel. Indirect effects on secondary structure might also account for the phage resistance phenotype conferred by the deleted six-amino-acid segment in the Tsx-506 protein. Alternatively, one could speculate that some of the deleted residues might be directly involved in bacteriophage recognition. Mutant analysis of the phage receptor areas of OmpA, LamB, and PhoE have shown that in each protein, several cell-surface-exposed regions are involved in the phage receptor function (1, 12, 16, 19, 28, 32, 46). It is therefore somewhat surprising that we found the mutations conferring phage resistance to be clustered in a single region. However, our data do not prove that the phage receptor area is confined to this region, since spontaneous mutations may not reveal the full spectrum of alterations affecting the phage receptor function of Tsx.

There is very limited information about those segments of the Tsx protein that are involved in its colicin K receptor activity. Only one of our phage-resistant mutants showed resistance to colicin K. Removal of six amino acids (residue

239 to residue 244) (Fig. 3A) in the Tsx-506 protein renders the cell entirely resistant against the colicin. We do not know whether the *tsx-506* mutation affects colicin K binding or its translocation from the cell surface to its target site, the cytoplasmic membrane (39). The influence of the *tsx-506* mutation on the colicin K receptor activity of Tsx could be indirect since the deletion might remove amino acids residues that are not directly involved in colicin K binding or translocation. The colicin K receptor activity of the Tsx proteins from *S. typhimurium* and *E. aerogenes* and those of mutant *E. coli* Tsx proteins support such a view. Replacement of Gly-239 or Gly-240 in the *E. coli* Tsx protein by Asp residues does not negatively affect colicin K or phage sensitivity (15). Likewise, the substitution of Asp-244 by an Asn residue in the *S. typhimurium* Tsx protein or the substitution of Asn-243 by Gln or Ala in the Tsx proteins from *S. typhimurium* and *E. aerogenes*, respectively, does not abolish colicin K receptor activity (36). Nevertheless, the colicin K resistance phenotype associated with the Tsx-506 protein suggests that the integrity of the last cell-surface-exposed region of the Tsx protein (Fig. 5) is important for the ability of Tsx to function as colicin K receptor.

Three of the mutant *tsx* genes characterized here contain small DNA rearrangements: an 18-bp deletion (*tsx-506*) and a 27-bp tandem duplication (*tsx-507*) that was recovered twice. Directly repeated DNA segments with lengths of 6 and 8 bp, respectively, are present in the DNA region flanking the deleted or duplicated sequence (Fig. 3). There is evidence for the involvement of such short repeated sequences in the formation of deletions and duplications (2, 38, 48). Misalignment of the repeated DNA sequences present in the *tsx* gene during DNA strand separation and subsequent DNA replication could readily explain the DNA rearrangements that we detected in the *tsx-506* and *tsx-507* alleles. Duplications and deletions are assumed to serve an important function for evolution (3). Both the duplication and the deletion demonstrate that spontaneous DNA rearrangements can confer beneficial properties to the bacterial cell, e.g., phage resistance, in response to selective pressure. The deletion is, in this respect, a particularly telling example. A single mutational event relieves simultaneously the detrimental effects on cell viability exerted by colicin K and the infection by lytic bacteriophages without affecting the physiological relevant function of Tsx, its nucleoside-specific channel activity.

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