Analysis of the *tsx* gene, which encodes a nucleoside-specific channel-forming protein (Tsx) in the outer membrane of *Escherichia coli*

(Nucleoside uptake; recombinant DNA; bacteriophage receptor; colicin K receptor; promoters; gene regulation)

Erhard Bremer*, Anke Middendorf*, Jan Martinussen b and Poul Valentin-Hansen b

*Department of Biology, University of Konstanz, D-7750 Konstanz, (F.R.G.) and b Department of Molecular Biology, Odense University, DK-5230 Odense M (Denmark) Tel. (45)66-158600; Fax (45)66-158428

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SUMMARY

The *tsx* gene of *Escherichia coli* encodes an outer membrane protein, Tsx, which constitutes the receptor for colicin K and bacteriophage T6, and functions as a substrate-specific channel for nucleosides and deoxynucleosides. The mini-Mu element pEG5005 was used to prepare a gene bank in vivo, and this bank was used to identify T6-sensitive strains carrying the cloned *tsx* gene. Subcloning of the *tsx* gene into the multicopy plasmid, pBR322, resulted in a strong overproduction of Tsx. The sequence of a 1477-bp DNA segment containing *tsx* and its flanking regions was determined. An open reading frame (ORF) was found which was followed by a pair of repetitive extragenic palindromic sequences. This ORF translated into a protein of 294 amino acids (aa), the first 22 aa of which showed the characteristic features of a bacterial signal sequence peptide. The putative mature form of Tsx is composed of 272 aa and has an even distribution of charged residues (52 aa) and lacks extensive hydrophobic stretches. No significant homologies of Tsx to the channel-forming proteins OmpC, OmpF, PhoE and LamB from the *E. coli* outer membrane were detected. Using nuclease S1, we identified two transcription start points for the *tsx* mRNA which were separated by approx. 150 bp. Genetic data suggest that the synthesis of the larger mRNA species is directed by a weak promoter (P1) that is controlled by the DeoR repressor, whereas the smaller mRNA species is directed by the main promoter P2, which is negatively controlled by the CytR repressor and positively affected by the cyclic AMP/catabolite activator protein complex.

INTRODUCTION

The structural genes encoding the systems for nucleoside uptake and metabolism in *E. coli* are organized in a regulon that is controlled positively by the cAMP/CRP activator complex and negatively by the DeoR and CytR repressors. The *tsx* gene, located at 9.4 min on the *E. coli* linkage map, is part of this regulon (Krieger-Brauer and Braun, 1980; Bremer et al., 1988). It encodes a minor outer membrane protein, Tsx, which constitutes the receptor for colicin K and bacteriophage T6 (Manning and Reeves, 1976) and plays an important role in the permeation of nucleosides and deoxynucleosides across the outer membrane when these substrates are present at submicromolar extracellular concentration.

Abbreviations: aa, amino acid(s); bp, base pair(s); cAMP, cyclic AMP; CRP, catabolite activator protein; CytR, gene encoding CytR; CytR and DeoR repressor proteins; Δ, deletion; deoR, gene encoding DeoR; kb, kilobase(s) or 1000 bp; LB, Luria-Bertani (medium); nt, nucleotide(s); ORF, open reading frame; P, promoter; PAGE, polyacrylamide-gel electrophoresis; R, resistance/resistant; REP, repetitive extragenic palindromic sequence(s); S, sensitive/sensitivity; SDS, sodium dodecyl sulfate; Tn, transposon; tsp, transcription start point(s); *tsx*, gene encoding Tsx; Tsx, outer membrane protein; XP, 5-bromo-4-chloro-3-indolyl-phosphate; [], denotes plasmid-carrier state; :, novel joint (fusion).
concentrations (Hantke, 1976). In addition, the antibiotic albacidin, a potent inhibitor of DNA replication, uses Tsx as its primary entry pathway into E. coli (Birch et al., 1990). Reconstitution of purified Tsx into artificial membranes has shown that Tsx is a channel-forming protein with a nucleoside-specific binding site that can discriminate between compounds closely related in structure (Maier et al., 1988; Benz et al., 1988). Thus, it is functionally comparable to the outer membrane protein LamB, a channel-forming protein with a maltose- and maltodextrin-specific binding site (Nikaido and Vara, 1985). The substrate-specific binding sites enable Tsx and LamB to mediate the efficient permeation of their specific substrates across the outer membrane permeability barrier in a nutritionally impoverished environment. In contrast, the pores formed by the closely related E. coli proteins OmpC, OmpF, and PhoE (Mizuno et al., 1983) contain no substrate-specific binding sites. Hence, these pores mediate the diffusion of many hydrophilic substances across the outer membrane but function inefficiently at low substrate concentration (Nikaido and Vara, 1985). To aid further genetic and biochemical analysis of the tsx gene and its gene product, we decided to clone and sequence the tsx structural gene, analyze the tsx regulatory region and overproduce Tsx.

RESULTS AND DISCUSSION

(a) Cloning the tsx gene

Since a selection for Tsx + strains was not available, we took advantage of the close physical linkage of the tsx and phoR genes. Mutations in phoR result in the constitutive synthesis of alkaline phosphatase, and such strains form dark-blue colonies when plated on media containing the alkaline phosphatase indicator dye XP. A gene bank was grown in the presence of Km and at 28 ° C, frequent PhoR- strains were isolated. To examine plasmid-directed Tsx synthesis, we analyzed the cell envelope proteins from strain BRE2070[pTX3] by SDS-PAGE and compared them with those from the control strain BRE2070[pBR322] (Fig. 2A). In the strain carrying the

(b) Subcloning of the tsx gene and overproduction of Tsx

Although the PhoR + Tsx + strain BRE2116[pTX2] was grown in the presence of Km and at 28 ° C, frequent PhoR- T6R segregants were found. These colonies are probably the result of secondary transpositions of pTX2 (Groisman and Casadaban, 1986). To obtain a more stable tsx plasmid, we digested the tsx + plasmid pTX2 with ClaI, ligated the resulting restriction fragments into the unique ClaI site of plasmid pBR322 (Bolivar et al., 1977), transformed the ligation mixture into the tsx strain BRE2070, and screened the resulting transformants for sensitivity to phage T6. Plasmids from several T6R strains were analysed and found to contain a 4.2-kb ClaI fragment in addition to the 4.36-kb pBR322 vector segment. A restriction map of one of these plasmids, pTX3, is shown in Fig. 1. We then deleted restriction fragments from pTX3 or subcloned defined restriction fragments into pBR322 and were able to locate the tsx gene on a 1.17-kb ClaI-SstI restriction fragment (Fig. 1). The direction of transcription of the cloned tsx gene was established by comparing the restriction maps of the tsx plasmids pTX3 and pTX5 with that of plasmid pMLB705(DPstI) carrying a tsx-lacZ operon fusion (Fig. 1). In a d30R+ cytR+ strain, Tsx is only a minor component of the E. coli outer membrane, but in a d30R cytR mutant (e.g., strain BRE2050) Tsx is overproduced (Krieger-Brauer and Braun, 1980; Bremer et al., 1988). To examine plasmid-directed Tsx synthesis, we analyzed the cell envelope proteins from strain BRE2070[pTX3] by SDS-PAGE and compared them with those from the control strain BRE2070[pBR322] (Fig. 2A). In the strain carrying the plasmid Tsx

![Fig. 1. Physical maps of the plasmids used. Restriction maps of plasmids carrying the tsx region or a tsx-lacZ operon fusion are shown; only restriction sites relevant for this study are indicated. The chromosomal DNA is indicated by the open boxes, the vector DNA is represented by solid bars and the position of the tsx coding region is indicated by arrows. Hatched bar represents the 'lacZ' gene; shaded bars represent lacY' and a segment of the E. coli trp operon plus some material from the S end of phage Mu in plasmid pMLB705[PstI] which carries a tsx-lacZ operon fusion. Plasmid pMLB705[PstI] is a derivative of plasmid pMLB705 (M. Berman, unpublished data), which was made smaller by partial digestion with PstI and subsequent religation. The tsx gene carried by plasmids pTX3 and pTX5 lacks its minor promoter P1, since it is located upstream from the ClaI site used to construct these plasmids. The origin of the 750-bp EcoRV-PvuII fragment used as a probe for the S1 mapping of the trp for tsx is indicated. C, ClaI; E, EcoRV; H, HindIII; K, KpnI; P, PstI; Pvu, PvuII; R, EcoRI; S, StuI. The restriction sites shown in parentheses were destroyed during the blunt-end ligation of restriction fragments.]
cloned tsx gene, the quantity of Tsx is comparable to that of the major outer membrane protein OmpA (Fig. 2A) and greatly exceeds the amount of Tsx present in strain BRE2050, which maximally expresses the chromosomally encoded tsx gene due to mutations in the deoR and cytR repressor genes (Bremer et al., 1988).

e) Nucleotide sequence of tsx

The nt sequence of the tsx gene and its flanking regions, for which we have determined the nt sequence on both strands, is shown in Fig. 3. This chromosomal DNA segment contains an ORF of 882 bp that begins with an ATG start codon at bp positions 456–458 and ends with a TGA stop codon at bp positions 1338–1340. The ATG codon is preceded by a putative ribosome-binding site complementary to the 3' end of the 16S RNA with a typical spacing of 7 nt to the ATG start codon. A potential stem-loop structure is found downstream from the tsx stop codon (Figs. 3 and 4D), but this region is not followed by the stretch of T's typical of factor-independent transcription terminators. An nt sequence downstream from this region is highly homologous to the consensus REP sequence (Higgins et al., 1988).
Fig. 4. S1 nuclease mapping of the in vivo tsx mRNAs. (A) Structure of the DNA fragment used as a probe to map the 3' end of the tsx mRNA. The region which shows dyad symmetry and the REP sequence are indicated by paired arrows. (B) S1 nuclease mapping of the tsp for tsx-specific mRNAs. A 750-bp EcoRV-PvuII restriction fragment (32P-labelled at the PvuII end), which encompasses the nt sequence from bp 1–522 (Figs. 1 and 3), was used as a hybridization probe; this DNA fragment was isolated from plasmid pMLB705(zlPstI). Approx. 50 μg of RNA isolated from strain SO744 (tsx+ cytR− deoR−; Hammer-Jespersen and Nygaard, 1976) was annealed to the DNA probe (0.1 μg) and treated with 100 units of S1 nuclease for 10 min at 36°C. Lanes: 1 the purine-specific cleavage of the 5'-end-labelled hybridization probe; 2 DNA fragments protected by tsx-specific mRNAs. The bp are numbered as in Fig. 3. (C) S1 nuclease mapping of the tsx mRNAs 3' ends. Approx. 50 μg of total RNA isolated from strain BRE2070[pTX3] was hybridized to 0.2 μg of the BstEII-NheI DNA fragment (uniquely 3'-end-labelled at the BstEII site); this DNA fragment was isolated from plasmid pTX5 (see part A). The S1 digestion was carried out for 15 min at 36°C with 50 units of S1 nuclease. Lane 1 shows that two kinds of transcripts with different 3' ends are found in vivo: a 'short' mRNA having its 3' ends within the sequence AACA (marked by arrows in D) just after the REP sequence and a 'longer' transcript covering the region between the tsx stop codon and the junction of the chromosomal and vector DNA. Lane 2, sequencing ladder of the G + A reaction of the hybridization probe. The nt are numbered as in Fig. 3. DNA fragments used as probes for S1 mapping were radiolabelled either at the 5' end of strands by polynucleotide kinase or at the 3' end of strands by Pollk. Isolation of RNA, purification of labelled DNA fragments and S1 mapping experiments were performed as described previously (Valentin-Hansen et al., 1984). (D) Stem-loop structures at the end of the tsx coding region; the position of the tsx stop codon is indicated by three asterisks and the position of the 3' ends of the detected tsx mRNA are indicated by arrows.
(d) Mapping of the in vivo \textit{tsx} mRNA

Genetic experiments have previously indicated that \textit{tsx} expression is mediated by two differently regulated promoters: a minor promoter, \textit{P1}, which is negatively controlled by DeoR and a main promoter, \textit{P2}, which is repressible by CytR and activated by the cAMP/CRP complex (Bremer et al., 1988). To identify the position of these promoters within the \textit{tsx} regulatory region we mapped the 5' ends of the \textit{tsx} mRNAs by the S1 nuclease mapping technique. RNA was isolated from the \textit{tsx} regulatory mutant strain SO744 and hybridized to a radio-labelled DNA probe from plasmid pMLB705 (Ap*stI) (Fig. 1), which spans the \textit{tsx} regulatory region. The results of the S1 analysis (Fig. 4B) clearly show that \textit{tsx} transcription initiates at two positions approx. 150 bp apart. A minor transcript starts near bp position 224 (P1) and a major transcript near bp position 378 (P2). Each mRNA tsp is preceded by a Pribouw-box-like sequence (Fig. 3), and a putative \textit{deoR} operator (Fig. 5) overlaps the -10 region of the \textit{tsx} \textit{P1} promoter. No such sequences are present in the \textit{tsx} \textit{P2} promoter region. However, in analogy with other CytR-regulated promoters (Valentin-Hansen et al., 1989) the \textit{tsx} \textit{P2} promoter contains tandem putative binding sites for the cAMP/CRP activator complex (Fig. 3). Taken together, the results of the S1 mapping experiment and the regulatory features of \textit{tsx} gene expression (Krieger-Brauer and Braun, 1980; Bremer et al., 1988) strongly suggest that transcription initiating from the weak \textit{tsx} \textit{P1} promoter is repressible by DeoR, whereas the activity of the main \textit{tsx} promoter, \textit{P2}, is stimulated by the cAMP/CRP complex and negatively controlled by the CytR repressor.

To pinpoint the 3' end of the \textit{tsx} mRNA we likewise used high-resolution S1 mapping to analyse the in vivo transcripts entering the nt sequence 3' to the translational stop codon of \textit{tsx}. Fig. 4C shows the results of such an experiment using mRNA isolated from a strain harbouring pTX3 and a radio-labelled probe (Fig. 4A) isolated from plasmid pTX5 and carrying the junction between the cloned chromosomal DNA and pBR322 sequences. Two types of transcripts are found in vivo: a read-through transcript which has its 3' end close to the junction between the chromosomal and the vector sequences and a second transcript with its 3' end within the sequence AACA after the REP sequence (Fig. 4D). Since REP sequences are commonly believed to act as a degradation barrier against 3'-to 5'-exonuclease activity (Valentin-Hansen et al., 1984; Higgins et al., 1988), it seems likely that the actual \textit{tsx} terminator lies 3' to the nt sequence which we have determined.

(e) Features of the deduced aa sequence of Tsx

The ORF that we identified can encode a polypeptide of 294 aa with a calculated \( M_r \) of 33 594. The first 22 aa of the deduced aa sequence (Fig. 3) show the characteristic features of a bacterial signal sequence peptide, suggesting that Tsx, like most outer membrane proteins from \textit{E. coli}, is initially synthesized as a precursor molecule (Michaelis and Beckwith, 1982). Indeed, in minicells that show reduced processing of such precursors, we detected a protein that reacted specifically with a Tsx antiserum and that migrated slower than the mature Tsx protein in the SDS gel (Fig. 2B). This polypeptide is likely to be the Tsx precursor because it was not found in whole cells, which rapidly process such precursor molecules. The aa sequence Asn\(^{\text{21}}\)-Ala\(^{\text{22}}\)-Ala\(^{\text{23}}\)-Glu\(^{\text{2}}\) is identical to the aa sequence around the known cleavage sites of the signal peptides of the major outer membrane proteins OmpC and OmpF (Mizuno et al., 1983). In the absence of N-terminal sequence data for Tsx we presume that the cleavage of the Tsx precursor molecule occurs between the two Ala residues. The mature Tsx would then contain 272 aa residues and have a calculated \( M_r \) of 31 418. This is in close agreement with the estimates for the Tsx protein derived from its electrophoretic mobility on a 0.1% SDS–12% polyacrylamide gel (28 kDa; Maier et al., 1988) and after sedimentation equilibrium ultracentrifugation in the presence of a detergent (29 ± 2 kDa; Yawato and Hinz, 1982). Of the 272 aa in Tsx, there are 52 charged residues; 32 are acidic and 20 are basic, resulting in an excess of twelve negative charges. This is very similar to the net charges of the channel-forming proteins OmpC, OmpF, PhoE and LamB, i.e., -14, -11, -9 and -19, respectively (Clement and Hofnung, 1981; Mizuno et al., 1983). Positively and negatively charged residues are scattered throughout the Tsx sequence. Like the OmpC, OmpF and PhoE porins
(Mizuno et al., 1983), the Tsx protein has no Cys residues. The OmpC, OmpF and PhoE proteins are strongly homologous to each other but differ from LamB. A pairwise comparison of these channel-forming proteins to Tsx revealed no significant homologies, and a search of the NBRF database (release July 1989), revealed no other proteins closely related to Tsx. We did find limited aa sequence homology between Tsx and FhuA (Coulton et al., 1986), an outer membrane protein of E. coli involved in the uptake of ferrichrome-iron: there was a consecutive string of eight identical aa (S-V-N-V-V-S-G-S-~) in Tsx at aa positions 16–23 and in FhuA at aa positions 581–588. Whether this region of homology is of functional importance or is merely coincidental remains to be seen. The dual function of nucleoside-specific channel and receptor protein for colicin K and phage Tö indicate that Tsx spans the outer membrane. The hydrophobicity profile of the Tsx precursor shows, however, that Tsx is not appreciably nonpolar in its composition (Fig. 6). There are no long hydrophobic segments (with the exception of the signal sequence peptide) that would be obvious candidates for membrane-spanning segments in Tsx, but this is a typical feature of many proteins from the E. coli outer membrane. Secondary structure predictions made for Tsx using the method of Garnier et al. (1978) indicate similar percentages of α-helical (11.3%), β-extended (30.1%), random-coil (32.4%) and β-turn (32.4%) regions. We consider it likely that the Tsx polypeptide transverses the outer membrane several times but find it premature to speculate about its arrangement within the membrane. However, the characterization of tsx missense mutants which impair the function of Tsx as a phage and colicin K receptor should aid the analysis of Tsx. We have recently characterized in detail an Asn^254 to Tyr mutant which acquires resistance to Tö and other Tsx-specific phages, without altering the colicin K receptor or nucleoside-specific channel functions (Maier et al., 1990). These findings imply that Asn^254 lies in a segment that is exposed to the cell surface.

(f) Conclusions

The results of our studies show unambiguously that we have cloned the tsx gene from E. coli. Expression of tsx from a multicopy plasmid resulted in strong overproduction of Tsx. The ability to obtain large amounts of Tsx should significantly facilitate the purification of this protein for further biochemical and biophysical studies, since current purification schemes still rely on strains synthesizing Tsx from the chromosomally encoded tsx gene (Yamato and Hinz, 1982; Maier et al., 1988). Sequence analysis of tsx and the mapping of the initiation sites for the tsx mRNA allowed us to deduce the transcriptional and translational organization of this gene and to predict the primary structure of Tsx. Two separate promoters, which were previously inferred from genetic experiments, were accurately mapped within the tsx regulatory region. The aa sequence predicted from the tsx mRNA indicates that Tsx is initially synthesized as precursor molecule. Like other proteins from the E. coli outer membrane, Tsx is not strongly nonpolar in its overall composition, contains many charged residues and lacks longer segments containing exclusively hydrophobic or uncharged residues. It does not share extensive homology with other channel-forming proteins from the E. coli outer membrane. Knowledge of the tsx sequence is a necessary base for detailed investigation of both the tsx regulatory region and the structural and functional organization of Tsx.

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