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Double Negative and Positive Control of *tsx* Expression in *Escherichia coli*

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The *Escherichia coli tsx* gene encodes an outer membrane protein that is involved in nucleoside uptake and serves as the receptor protein for colicin K and several bacteriophages. Regulation of its expression was studied by using *tsx-lacZ* protein and operon fusion strains carrying mutations in *deoR*, *cytR*, and *crp*. The *cytR*-encoded repressor had a stronger influence on *tsx* transcription than the DeoR repressor did, and the level of *tsx* expression in a *deoR cytR* double mutant was approximately the sum of those found in the single *deoR* and *cytR* strains. This double negative control of Tsx synthesis was superceded by a positive control mechanism mediated by the cyclic AMP-catabolite activator protein (cAMP-CAP) complex. Our results suggest that *tsx* expression is controlled at two separate and differently regulated promoters: the weaker promoter (P_1) is repressible by DeoR, while the stronger promoter (P_2) is subject to negative and positive control by the CytR repressor and the cAMP-CAP complex, respectively. A mutant was isolated that showed unaltered *tsx* regulation by DeoR and the cAMP-CAP complex but strongly reduced repression by CytR. This *tsx* operator mutant was used to obtain a suppressor mutation located on a plasmid carrying the cloned *cytR* gene that restored CytR control of *tsx* expression. The direction of *tsx* transcription was determined and found to be counterclockwise on the *E. coli* chromosome.

The Tsx protein is a minor component of the outer membrane of *Escherichia coli*, with an apparent molecular weight of 28,000 (22). It acts as the receptor protein for colicin K and several bacteriophages, including phage T6 (23, 38). The Tsx protein is involved in the permeation of nucleosides across the outer membrane. This was first demonstrated by Hantke (16), who found that strains lacking Tsx were impaired in nucleoside uptake. Further analysis of this phenomenon revealed a remarkable specificity in the Tsx-mediated permeation of nucleosides across the outer membrane (19, 20, 27). The rate of uptake of adenosine and thymidine is strongly reduced in the absence of Tsx, whereas that of cytidine remains unchanged. The importance of the Tsx protein for nucleoside uptake becomes apparent only at low (<1 μM) substrate concentration or under conditions of fast cell growth (19, 27). At higher concentration of substrate, the Tsx protein becomes dispensable and the nucleosides permeate the outer membrane by diffusion through the nonspecific porins OmpC and OmpF (28, 37, 39). These and other studies (18) led to the suggestion that Tsx is a pore-forming protein with some specificity for nucleosides and deoxynucleosides. Recently, we reconstituted purified Tsx protein in vitro into black lipid membranes (C. Maier, E. Bremer, A. Schmid, and R. Benz, J. Biol. Chem., in press). These experiments demonstrated that Tsx can form a pore in vitro. They also provided strong evidence that this protein contains a nucleoside-specific binding site that has a high affinity for adenosine and a low affinity for cytidine. Thus, the Tsx protein appears to be functionally comparable to another pore-forming outer membrane protein, LamB, which specifically facilitates the permeation of maltose and maltodextrins and contains a substrate-binding site (5, 30).

The involvement of Tsx in nucleoside uptake is reflected in the regulation of its structural gene, *tsx*, which is located at 9 min on the *E. coli* linkage map (2). As first demonstrated by Krieger-Brauer and Braun (19), expression of the *tsx* gene is under double negative control of the DeoR and CytR proteins, which are known to regulate the synthesis of two nucleoside transport systems (NupC and NupG) and several nucleoside-catabolizing enzymes (for reviews, see references 14 and 26). In addition, the cyclic AMP-catabolite activator protein (cAMP-CAP) complex (11) has been shown to positively affect the transcription of the *tsx* gene (1, 20, 24). However, the interrelationship of the double negative (DeoR; CytR) and positive (cAMP-CAP) control in *tsx* expression has not been analyzed to any great extent. Furthermore, it has been difficult to quantitate the effects of these regulatory proteins on Tsx synthesis.

To gain a deeper insight into the complex regulation of the *tsx* gene, we have studied Tsx synthesis in an isogenic set of strains with mutations in *deoR*, *cytR*, and *crp*. We have monitored and quantitated *tsx* expression by using *tsx-lacZ* protein and operon fusions and have isolated a strain with a mutation in the *tsx* regulatory region that reduces the binding of the CytR repressor.

MATERIALS AND METHODS

Media and chemicals. Bacteria were grown aerobically in a shaking incubator (28 or 37°C, as indicated) in LB medium (10 g of tryptone [Difco Laboratories] per liter, 5 g of yeast extract [Difco] per liter, 5 g of NaCl per liter) or in minimal medium A (MMA; 10.5 g of K_2HPO_4 per liter, 4.5 g of KH_2PO_4 per liter, 1 g of $(NH_4)SO_4$ per liter, 0.5 g of hydrated sodium citrate per liter, 0.1 g of $MgSO_4 \cdot 7H_2O$ per liter) supplemented with a carbon source (glucose or glycerol) at 0.4%. The medium was supplemented with a solution of methionine, valine, and isoleucine to a final concentration of 0.02% as required. MacConkey and tetrazolium media were prepared as previously described (25, 29). Agar plates spread with 0.1 ml of a 10-mg/ml solution of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) in dimethylformamide were used to distinguish between $LacZ^-$ and $LacZ^+$ phenotypes. The indicator dye 5-bromo-4-chloro-3-indolyl-

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phosphate (XP) was used in a similar manner to detect colonies synthesizing high levels of alkaline phosphatase.

Strains, phages, and genetic procedures. The bacteria and phages used are described in Table 1. All strains were *E. coli* K-12 derivatives. Standard techniques were used for the growth and titration of bacteria and bacteriophages and for generalized transduction with phage P1 (25, 29). Lysates of phage Mu were obtained by heat induction of a Mu *c*Its62 lysogen (29), and immunity to superinfecting Mu phages was tested as described previously (7). The procedure for the induction of a λ p*lac*Mu prophage by UV irradiation has been reported previously (29).

Construction of bacterial strains. The Δ(*argF-lac*) *U169* mutation was transduced with phage P1 into strains SO656 (*deoR*$^+$ *cytR*$^+$), SO664 (*deoR*$^+$ *cytR9*), SO665 (*deoR8* *cytR*$^+$), and SO774 (*deoR8* *cytR9*) by using a phage lysate prepared on strain ECB457 [*proC*::Tn*10* Δ (*argF-lac*)*U169*]. One tetracycline-resistant LacZ$^-$ colony from each group of transductants was purified on LB agar containing tetracycline (20 μg/ml), and the *proC*::Tn*10* insertion was subsequently removed from these strains by P1 transduction with a P1 lysate grown on the *proC*$^+$ Δ(*argF-lac*)*U169* strain MC4100. Strains SO656, SO664, SO665, and SO744 (15) were found to be resistant to λ *vir*. This phenotype must result from a *lamB* mutation, because we found that these strains are Mal$^+$. To remove this *lamB* mutation from the Δ*lac* derivatives, the *malF*::Tn*10* insertion from strain TST7 (*lamB*$^+$) was first introduced with phage P1. Then the *malF*::Tn*10* insertion was removed from λ *vir*^{s} (*lamB*$^+$) colonies by transduction to Mal$^+$ with a P1 phage lysate prepared on strain MC4100. These steps yielded strains BRE2047 (*deoR*$^+$ *cytR*$^+$), BRE2048 (*deoR*$^+$ *cytR9*), BRE 2049 (*deoR8* *cytR*$^+$), and BRE2050 (*deoR8* *cytR9*). The presence of the *deoR* and *cytR* mutations was verified by streaking these strains on minimal plates with inosine or uridine as the sole carbon source (14). The Δ(*crp*)*96* mutation present in strain BD18 (Table 1) was transduced with phage P1 into various strains by selecting for the closely linked *zhd-732*::Tn*10* insertion and then testing the tetracycline-resistant transductants for a Mal$^-$ phenotype by streaking on maltose MacConkey plates.

Isolation of the *tsx-lacZ* fusion. We used the transposable λ p*lac*Mu phages (8) to construct *tsx-lacZ* protein and operon fusions. Strains were infected with λ p*lac*Mu9 (*lacZ* protein fusions) and λ p*lac*Mu55 (*lacZ* operon fusions) in the presence of the λ pMu507 helper phage, and kanamycin-resistant colonies were selected as previously described (8). Approximately 10,000 colonies each were pooled, washed three times with 40 ml of LB medium containing 40 mM sodium citrate, and suspended in 5ml of LB medium. To select *lacZ* fusions to the *tsx* gene, we prepared serial dilutions from the pooled kanamycin-resistant cells (10^{-1} to 10^{-8}) in LB medium and plated them onto lawns of phage T6 (0.2 ml of a 10^{11}-PFU/ml lysate) on LB agar containing kanamycin and X-Gal. Nonmucoid LacZ$^+$ colonies were purified twice and retested for their resistance to phage T6. To confirm that the LacZ$^+$ and T6^r phenotypes were due to a λ p*lac*Mu insertion into the *tsx* gene, we prepared a P1 lysate on such strains. These lysates were used to transduce strain BRE2047 to kanamycin resistance, and the resulting Lac$^+$ transductants were tested for T6 resistance. We retained one *tsx-lacZ* protein and one *tsx-lacZ* operon fusion strain. The λ p*lac* Mu9 and λ p*lac*Mu55 insertions were originally isolated in strains MC4100 and BRE2047, respectively.

β-Galactosidase assay. Specific β-galactosidase activity was assayed as described previously (25) and expressed as micromoles of substrate cleaved per minute per milligram of protein. Protein concentration was estimated from the optical density at 600 nm of the culture, assuming that an optical density at 600 nm of 1.4 corresponds to approximately 150 μg of protein per ml of culture (25).

TABLE 1. Bacterial strains, bacteriophages, and plasmids

Strain, phage, or plasmid	Relevant genotype[a]	Source or reference
Strains		
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169* *rpsL150* *relA* *deoC1* *ptsF25* *rbsR* *flbB5301*	10
ECB457	MC4100 *proC*::Tn*10*	S. Benson
TST7	MC4100 *malF*::Tn*10*	T. J. Silhavy
K797	*phoR*::Tn*10*	M. Villarejo
RM1036	F′ *traD36* *proAB* *lacI*^{q}Δ*lac* *M15*/Δ(*lac-pro*) *thi* *rpsL* *supE* *endA* *sbcB* r$^-$ m$^-$ *mutD5* *zaf-13*::Tn*10*	R. Maurer through P. Gött
BD18	HfrH *rpsL* *thi* Δ*cya-851* Δ*crp-96* *zhd-732*::Tn*10*	4
SO656	F$^-$ *metB* *ilv* *rpsL* *lamB*	15
SO664	F$^-$ *metB* *ilv* *rpsL* *lamB* *cytR9*	15
SO665	F$^-$ *metB* *ilv* *rpsL* *lamB* *deoR8*	15
SO744	F$^-$ *metB* *ilv* *rpsL* *lamB* *cytR9* *deoR8*	15
BRE2047	SO656 *lamB*$^+$ Δ(*argF-lac*)*U169*	This study
BRE2048	SO664 *lamB*$^+$ Δ(*argF-lac*)*U169*	This study
BRE2049	SO665 *lamB*$^+$ Δ(*argF-lac*)*U169*	This study
BRE2050	SO744 *lamB*$^+$ Δ(*argF-lac*)*U169*	This study
BRE2055	BRE2047 Δ(*crp*)*96* *zhd-732*::Tn*10*	This study
BRE2056	BRE2048 Δ(*crp*)*96* *zhd-732*::Tn*10*	This study
BRE2057	BRE2049 Δ(*crp*)*96* *zhd-732*::Tn*10*	This study
BRE2058	BRE2050 Δ(*crp*)*96* *zhd-732*::Tn*10*	This study
BRE2101	BRE2047 *phoR*::Tn*10*	This study
Bacteriophages		
λ p*lac*Mu9	Mu *c*Its62 *ner*$^+$ *A′* *′ara′* Mu *S′* *′lacZ* *lacY*$^+$ *lacA′* *kan* *imm* λ	8
λ p*lac*Mu55[b]	Mu *c*Its62 *ner*$^+$ *A′* *am1093* *′uvrD′* Mu *S′* *′trp′* *lacZ*$^+$ *lacY*$^+$ *lacA′* *kan* *imm* λ	E. Bremer
λ pMu507	Mu cIts62 *ner*$^+$ *A*$^+$ *B*$^+$ *d*ts857 *S*am7	8
Plasmids		
pBR322		6
pCB008	*cytR*$^+$	3
pGP 1[c]	Mutated derivative of pCB008	This study

[a] Genes or sequences marked with a prime are incomplete. The genetic nomenclature is that of to Bachmann (2).

[b] This phage is a derivative of λ p*lac*Mu53 (8). It carries the Mu *A*am1093 mutation, which prevents secondary transposition.

[c] This plasmid is a derivative of pCB008 obtained after *mutD* mutagenesis.

Preparation of proteins samples, gel electrophoresis, and immunological detection of a Tsx–β-galactosidase hybrid protein. Cell envelopes were prepared from 40-ml overnight cultures grown in LB medium as described by Henning et al. (17). The membrane proteins were solubilized in sample buffer containing 2% sodium dodecyl sulfate (SDS) by boiling for 10 min, and portions were electrophoresed on an SDS-polyacrylamide gel (12% polyacrylamide) by the method of Lugtenberg et al. (21). Protein bands were visualized by staining with Coomassie brilliant blue. To detect the hybrid protein synthesized in the Φ(*tsx-lacZ*)*1*(Hyb)

fusion strain GP4, we prepared total cellular proteins (29) from a 5-ml overnight culture grown in LB medium. These were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (7% acrylamide) and stained with Coomassie brilliant blue. A high-molecular-weight calibration kit (Sigma Chemical Co.) was used to estimate the molecular weight of the hybrid protein. For the immunological detection of the Tsx–β-galactosidase hybrid protein, we transferred the separated cell proteins electrophoretically onto a sheet of nitrocellulose and probed the bound proteins with a rabbit antibody raised against β-galactosidase. The formed antigen-antibody complex was visualized with a second goat anti-rabbit immunoglobulin G peroxidase coupled antibody (33).

RESULTS

Negative and positive regulation of *tsx* expression. To study the expression of the *tsx* gene of *E. coli*, we used an isogenic set of strains (kindly provided by A. Munch-Petersen) carrying mutations in the *deoR* and *cytR* regulatory genes. These strains were made *lamB*$^+$ and Δ*lac* (see Materials and Methods) (Table 1), so that they could also be used for isolating *tsx-lacZ* protein and operon fusions with the λ *plac*Mu system (8). Figure 1 shows the pattern of cell envelope proteins of this set of strains after electrophoretic separation on an SDS-polyacrylamide gel (12% polyacrylamide). The DeoR protein exerts only a weak negative effect on Tsx synthesis, whereas CytR represses *tsx* expression more strongly. The amount of Tsx was greatest in the *deoR-cytR* double mutant. Our results thus support an earlier report by Krieger-Brauer and Braun (19), who concluded that synthesis of the Tsx protein increased in the following sequence: wild-type strain < *deoR* < *cytR* < *deoR cytR* mutants.

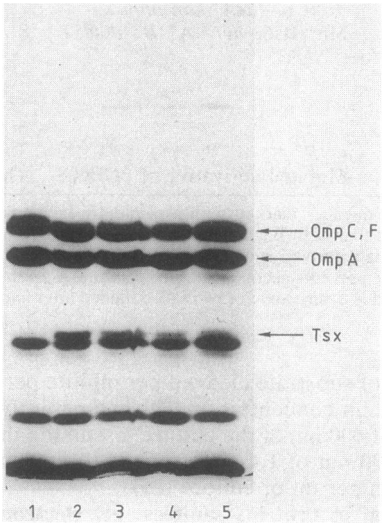

FIG. 1. Synthesis of Tsx protein in response to *deoR* and *cytR* mutations. Cell envelope proteins of LB medium overnight cultures of strains BRE2054 (*deoR cytR tsx*::Tn*10*) (lane 1), BRE2050 (*deoR cytR*) (lane 2), BRE2048 (*cytR*) (lane 3), BRE2049 (*deoR*) (lane 4), and BRE2047 (*deoR*$^+$ *cytR*$^+$) (lane 5) were separated by SDS-PAGE (12% acrylamide). The positions of the outer membrane proteins OmpC, OmpF, OmpA, and Tsx are indicated by arrows. Note that the Tsx protein migrates above the heavy band that represents several unresolved polypeptides (see Fig. 2). The gel system used does not resolve the OmpC and OmpF proteins; the gel was stained with Coomassie blue.

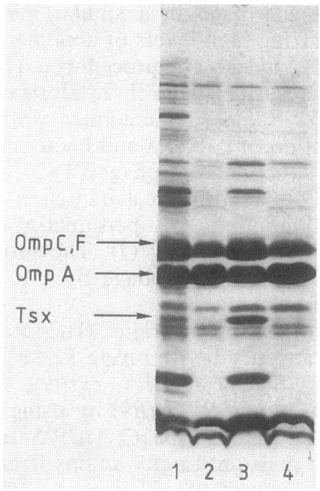

FIG. 2. Effect of the Δ(*crp*)*96* mutation on Tsx synthesis. Cell envelope proteins of LB medium overnight cultures of strains BRE2047 (*deoR*$^+$ *cytR*$^+$ *crp*$^+$) (lane 1), BRE2055 [*deoR*$^+$ *cytR*$^+$ Δ(*crp*)*96*] (lane 2), BRE2050 (*deoR cytR crp*$^+$) (lane 3), and BRE2058 [*deoR cytR* Δ(*crp*)*96*] (lane 4) were separated by SDS-PAGE (12% acrylamide), and the gel was stained with Coomassie blue.

It is well established that *tsx* expression is subject to catabolite repression (1, 20, 24), but it is unknown how this positive regulatory mechanism interacts with DeoR- and CytR-mediated regulation. To investigate such interaction, we introduced with phage P1 the Δ(*crp*)*96* mutation into the four strains described above and analyzed the profile of cell envelope proteins by SDS-PAGE. No Tsx protein could be clearly detected in wild-type or *deoR-cytR* double-mutant strains carrying the Δ(*crp*)*96* mutation (Fig. 2, lanes 2 and 4); the same result was obtained when this mutation was present in either the *deoR* or *cytR* single mutant (data not shown). Thus, the positive regulation of *tsx* expression mediated by the cAMP-CAP complex has a stronger effect than the negative regulation by the DeoR and CytR proteins. Although the Tsx protein was not detected in the above experiment, *tsx* expression was not completely abolished in the Δ(*crp*)*96* mutants, since these strains were still sensitive to the Tsx-specific bacteriophage T6. From these data we conclude that Tsx synthesis is subject to double negative control by the DeoR and CytR repressors, but is most strongly affected by positive regulation through the cAMP-CAP complex.

Isolation of *tsx-lacZ* protein and operon fusions. In a wild-type strain, the Tsx protein is a relatively minor component of the outer membrane proteins. Additionally, on SDS-polyacrylamide gels it migrates to an area densely populated with other proteins (Fig. 1 and 2), making it difficult to quantitate changes in Tsx synthesis. To obtain a more sensitive tool to monitor *tsx* expression, we isolated *tsx-lacZ* protein and operon fusions. Such fusions were selected from pools of kanamycin-resistant insertions of λ *plac*Mu9 (*lacZ* protein fusion) and λ *plac*Mu55 (*lacZ* operon fusion) on the basis of their resistance to phage T6. One *tsx-lacZ* protein fusion strain and one *tsx-lacZ* operon fusion strain were subjected to the following tests to confirm the fusion of the *tsx* and *lacZ* genes. (i) The λ *plac*Mu insertions were transduced with phage P1 into strain BRE2047 by selecting for kanamycin-resistant colonies in the presence of X-Gal. LacZ$^+$ Kan^r transductants were then tested by cross-

streaking against phage T6 and λ *vir*. All transductants tested were resistant to phage T6 but sensitive to λ *vir*. (ii) A *tsx*::Tn*10* insertion was transduced with phage P1 into both fusion strains by selecting for tetracycline resistance in the presence of X-Gal. All transductants examined were LacZ$^-$ Kan^s. (iii) Cell envelope proteins from both fusion strains were analyzed by SDS-PAGE, and no Tsx protein was detected. This is shown in Fig. 3A (lane 2) for the Φ(*tsx-lacZ*)*1* (Hyb) fusion strain GP4 (*deoR cytR*) in comparison with its parent strain BRE2050 and strain BRE2054, a *tsx*::Tn*10* control. Taken together, these tests provide proof that *tsx-lacZ* protein and operon fusion strains had been isolated.

The Φ(*tsx-lacZ*)*1*(Hyb) protein fusion present in strain GP4 should encode a hybrid protein whose size depends on the insertion point of the λ *plac*Mu9 phage into *tsx*. This hybrid protein was detected in total cell extracts electrophoretically separated on an SDS-polyacrylamide gel (7% polyacrylamide) (data not shown) and reacted with an antibody directed against β-galactosidase (Fig. 3B, lane 1). It is slightly larger than β-galactosidase (Fig. 3B, lane 3), and we estimate its size to be approximately 120,000 daltons. Since the portion of such hybrid proteins encoded by the incomplete *lacZ* gene and the 117 base pairs from the *S* end of Mu is constant (approximately 117,000 daltons) (8), the fusion joint probably lies early in the *tsx* gene. It should be noted, however, that for large proteins, molecular size estimates based on SDS-PAGE are not precise.

Regulation of *tsx-lacZ* expression by DeoR, CytR, and the cAMP-CAP complex. To quantitate the influence of the various regulatory proteins on *tsx* expression, we transduced with phage P1 the Φ(*tsx-lacZ*)*1*(Hyb) protein fusion and Φ(*tsx-lacZ*)*2* operon fusion into an isogenic set of strains, carrying, in various combinations, mutations in the *deoR*, *cytR* and *crp* genes (Table 2). When these strains were grown in minimal medium with glycerol as the carbon source, both the *tsx-lacZ* protein fusion and operon fusion showed the same regulatory pattern, as determined by β-galactosidase activity. Expression of the fused genes increased in the following order: wild-type < *deoR* < *cytR* < *deoR cytR* double mutant, where the absence of the DeoR repressor increased *tsx-lacZ* expression about 1.5- to 2-fold and the absence of CytR resulted in 6-fold-enhanced β-galactosidase activity. The level of β-galactosidase activity found in the *deoR cytR* strains GP4 and BRE2094 is approximately the sum of that observed in the single *deoR* (GP3 and BRE2093) and *cytR* (GP2 and BRE2092) mutants. Thus, the extent of the DeoR- and CytR-mediated repression of *tsx* expression appears to be additive.

To analyze the influence of the cAMP-CAP complex on *tsx* expression, we grew the various fusion strains in MMA containing glucose or glycerol and compared the β-galactosidase activities (Table 2). The *deoR* and *cytR* strains differed in their response to glucose in the growth medium. A strong reduction of *tsx-lacZ* expression in glucose-grown cultures could be measured only in strains carrying a *cytR* mutation, whereas the wild-type and *deoR* mutant strains showed only slightly reduced β-galactosidase activity. Hence, the high levels of β-galactosidase activity found in glycerol-grown *cytR* strains must be due to the activation of *tsx* transcription by the cAMP-CAP complex, which is prevented when the CytR repressor is present. In contrast, the DeoR-mediated regulation of *tsx* appears to be independent of the cAMP-CAP activator complex, since in *deoR*

TABLE 2. Effects of mutations in *deoR*, *cytR*, and *crp* on *tsx-lacZ* expression

Strain[a]	Relevant genotype[b]			β-Galactosidase sp act (μmol/min per mg of protein) after growth in[c]:	
	deoR	*cytR*	*crp*	MMA with glycerol	MMA with glucose
GP1	+	+	+	0.8	0.8
GP2	+	−	+	5.4	1.1
GP3	−	+	+	1.3	1.1
GP4	−	−	+	6.6	1.6
GP5	+	+	−	−[d]	0.2
GP6	+	−	−	−	0.2
GP7	−	+	−	−	0.9
GP8	−	−	−	−	0.6
BRE2091	+	+	+	0.9	0.7
BRE2092	+	−	+	6.1	1.7
BRE2093	−	+	+	2.0	1.6
BRE2094	−	−	+	10.5	3.3
BRE2095	+	+	−	−	0.5
BRE2096	+	−	−	−	0.2
BRE2097	−	+	−	−	0.9
BRE2098	−	−	−	−	0.5

[a] Strains GP1 through GP8 carry the Φ(*tsx-lacZ*)*1*(Hyb) protein fusion as a single copy in the chromosome. Strains BRE2091 through BRE2098 carry the Φ(*tsx-lacZ*$^+$)*2* operon fusion as a single copy in the chromosome. The symbol Φ indicates the presence of a *lacZ* fusion, and the abbreviation Hyb indicates that the fusion encodes a hybrid protein. The symbols *lacZ*$^+$ and *lacZ* denote *lacZ* genes with or without translational initiation signals, respectively.

[b] Symbols: +, intact chromosomally encoded *deoR*, *cytR*, and *crp* genes; −, mutated chromosomally encoded *deoR*, *cytR*, and *crp* genes.

[c] The strains were grown overnight in MMA with 0.4% glycerol or 0.4% glucose as the carbon source. Samples were withdrawn, and the specific β-galactosidase activity was determined (25). A parent strain (BRE2047) was always included as a control. The data shown are the mean values from two to three independent experiments.

[d] −, Strains with a mutation in *crp* do not grow on glycerol as the sole carbon source.

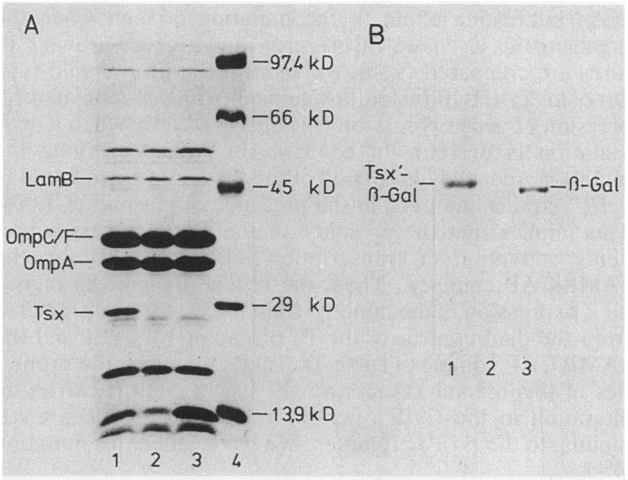

FIG. 3. Absence of Tsx protein in the Φ(*tsx-lacZ*)*1*(Hyb) fusion strain GP4 and immunological detection of the hybrid protein. (A) Cell envelope proteins of LB medium overnight cultures of strains BRE2050 (*deoR cytR*) (lane 1), GP4 [*deoR cytR* Φ(*tsx-lacZ*)*1*(Hyb)] (lane 2), and BRE2054 (*deoR cytR tsx*::Tn*10*) (lane 3) were separated by SDS-PAGE (12% acrylamide). Lane 4 shows a molecular weight standard; the gel was stained with Coomassie blue. (B) Total cellular proteins from strains GP4 (lane 1), BRE2050 (lane 2), and a high-molecular-weight calibration kit, containing β-galactosidase, were separated by SDS-PAGE (7% acrylamide). The proteins were transferred onto a sheet of nitrocellulose, and polypeptides able to react with the antibody raised against β-galactosidase were visualized as described in Materials and Methods.

strains, *tsx-lacZ* expression is increased to about the same extent regardless of the growth medium (Table 2). In agreement with the strongly reduced amount of Tsx present in strains with the Δ(*crp*)*96* mutation (Fig. 2), we found a further reduction of *tsx-lacZ* expression in glucose-grown cells when the Δ(*crp*)*96* mutation was present (Table 2). From these data, we conclude that Tsx synthesis is controlled from two differently regulated promoters. The weaker promoter (P_1) is repressible by DeoR, while the main promoter (P_2) is subject to negative and positive control by CytR and the cAMP-CAP complex, respectively.

A CytR operator mutant in the hybrid *tsx-lacZ* gene. Plasmid pCB008 carrying the *cytR*$^+$ gene (3) strongly repressed the expression of the Φ(*tsx-lacZ*)*1*(Hyb) protein fusion regardless of the presence of the chromosomally encoded DeoR and CytR repressors (Table 3). Consequently, strain GP4 (*deoR8 cytR9*) harboring pCB008 formed only small colonies on lactose minimal plates. We took advantage of this weak *tsx-lacZ* expression to obtain mutants with an altered response to CytR. Strain GP4(pCB008) (Table 3) was streaked on lactose minimal plates with ampicillin, and fast-growing colonies were isolated. Increased *tsx-lacZ* expression in these strains could result from mutations in the cloned *cytR*$^+$ gene that reduced or abolished CytR synthesis. Alternatively, this strong Lac$^+$ phenotype could be due to a mutation in the *tsx* regulatory region that prevents repression by CytR. To distinguish between these possibilities, we streaked the isolated mutant strains on minimal plates containing uridine as the sole carbon source. Efficient growth on uridine requires the expression of the CytR-controlled *udp* gene and the *deo* operon (14). Consequently, the isolated Lac$^+$ mutants will form normal-size colonies on uridine minimal plates only if they carry mutations altering the expression of the cloned *cytR*$^+$ gene or the function of the gene product. In contrast, strains with mutations in the *tsx* regulatory region will show weak growth on these plates. Most (18 of 19) of the independently isolated Lac$^+$ mutants grew well on uridine plates. The remaining strain (GP28) formed only very small colonies, indicating that it contained a mutation affecting the repression of the Φ(*tsx-lacZ*)*1*(Hyb) fusion by CytR.

TABLE 3. Repression of *tsx* expression by the cloned *cytR*$^+$ gene and altered response of a *tsx-lacZ* mutant (*O*^{c}-1) to the CytR repressor

Strain[a]	*cytR*$^+$ plasmid[b]	Chromosomal repressor[c]		β-Galactosidase sp act (μmol/min per mg of protein) after growth in[d]:	
		deoR	*cytR*	MMA with glycerol	MMA with glucose
GP1	−	+	+	0.7	0.9
GP1	+	+	+	0.1	0.2
GP2	−	+	−	4.8	2.2
GP2	+	+	−	0.3	0.3
GP3	−	−	+	1.0	0.9
GP3	+	−	+	0.09	0.1
GP4	−	−	−	6.3	1.7
GP4	+	−	−	0.4	0.4
GP25	−	+	+	4.2	1.4
GP25	+	+	+	2.3	1.1
GP26	−	+	−	5.5	1.1
GP26	+	+	−	2.1	1.2
GP27	−	−	+	5.2	1.0
GP27	+	−	+	4.0	1.9
GP28	−	−	−	7.0	2.6
GP28	+	−	−	4.7	2.0

[a] Strains GP1 through GP4 carry the wild-type Φ(*tsx-lacZ*)*1*(Hyb) fusion as a single copy in the chromsome. Strains GP25 through GP28 carry the Φ(*tsx-lacZ*)*1*(Hyb) fusion with mutation *O*^{c}-1 as a single copy in the chromosome.

[b] The *cytR*$^+$ plasmid used was pCB008 (3); the other strains contained plasmid pBR322 (6) as a control. Symbols: +, presence of pCB008; −, absence of pCB008.

[c] Symbols: +; presence of intact chromosomally encoded *deoR* and *cytR* genes; −, presence of mutated chromosomally encoded *deoR* and *cytR* genes.

[d] The strains were grown overnight with 0.4% glycerol or 0.4% glucose as the carbon source. Samples were withdrawn, and the specific β-galactosidase activity was determined (25). The values shown are the mean values from two independent experiments.

The mutation in strain GP28 was shown by P1 transduction to be closely linked to the Φ(*tsx-lacZ*)*1*(Hyb) fusion. When kanamycin-resistant transductants in strain BRE2047 were (*deoR*$^+$ *cytR*$^+$) selected, all 10 transductants tested were still resistant to phage T6. They showed a much higher expression of the gene fusion on lactose MacConkey indicator plates than did the isogenic strain GP1, which carries the wild-type Φ(*tsx-lacZ*)*1*(Hyb) fusion. To analyze the regulation of the mutated fusion by the chromosomally encoded DeoR and CytR repressors, we transduced this fusion into *deoR* and *cytR* strains and subsequently measured β-galactosidase activity in glycerol- and glucose-grown cultures (Table 3). In glycerol minimal medium, the expression of the mutated Φ(*tsx-lacZ*)*1*(Hyb) fusion in GP25 (*deoR*$^+$ *cytR*$^+$) was about fivefold stronger than in the control strain, GP1 (Table 3). In a *deoR* strain, expression of the mutated and the wild-type fusion increased to the same extent: 1.2-fold and 1.3-fold, respectively. This indicated that the mutation did not affect DeoR-mediated repression of *tsx*. However, the response to CytR was different in the mutant: a *cytR* mutation resulted only in a 1.3-fold increase in the expression of the fusion, whereas the corresponding value for the wild-type fusion was 6.6-fold (Table 3). This shows that in the isolated mutant the repression of *tsx-lacZ* expression by CytR was strongly reduced but not completely abolished.

An interesting effect of the mutation is seen when the β-galactosidase values of glycerol- and glucose-grown cultures are compared (Table 3). In strains with the wild-type Φ(*tsx-lacZ*)*1*(Hyb) fusion, a significant glucose-mediated repression of gene expression was observed only when a *cytR* mutation is present. In contrast, in strains carrying the mutation, reduced expression of the fusion was also found in *cytR*$^+$ strains and even in the presence of plasmid pCB008. This implies that the presence of the CytR repressor prevents activation of transcription initiation at P_2 by the cAMP-CAP complex. Thus, the regulatory pattern of *tsx-lacZ* expression in the mutant strain is exactly that predicted from the dual control of the P_2 promoter by CytR and the cAMP-CAP complex (Table 2). Taken together, the properties of this mutant strain strongly suggest that it carries an alteration in the CytR operator region that reduces CytR binding to the *tsx* P_2 promoter. We have called this mutation *O*^{c}-1.

Suppression of the operator mutation by a mutated *cytR* gene. Plasmid pCB008 was mutagenized in vivo by transformation into the *mutD5* strain RM1036; transformants were grown overnight in LB medium (29). Isolated plasmid DNA was then retransformed into strain GP28 (*deoR cytR*) carrying the Φ(*tsx-lacZ*)*1*(Hyb) fusion with the *O*^{c}-1 operator mutation. We looked for suppressors of this mutation by selecting the transformants on lactose tetrazolium plates containing ampicillin and screening the transformants for a Lac$^-$ phenotype. Such Lac$^-$ colonies can result from either a reversion of the *O*^{c}-1 mutation or an alteration in the *cytR*$^+$ gene that suppresses the *O*^{c}-1 Lac$^+$ phenotype. Several

Lac$^-$ colonies were found in this experiment, and one of these carried a mutated pCB008 plasmid. This was ascertained by retransforming this plasmid (pGP1) into strain GP28. This plasmid most probably encodes an altered *cytR* gene (*cytR**), since the presence of pGP1 strongly reduced the expression of the fusion in strain GP28 (Table 4). The *cytR** mutation is not allele specific, since the expression of the wild-type *tsx-lacZ* fusion in strain GP4 is also repressible by pGP1 (Table 4). Both pCB008 and pGP1 showed the same restriction pattern after digestion with *Cla*I, *Ava*I, and *Pvu*II, indicating that no gross rearrangement had occurred in pGP1. We did not notice an increase in the amount of plasmid DNA isolated from strain GP28(pGP1) in comparison with strain GP28(pCB008), suggesting that the suppressor phenotype was not due to a mutation drastically increasing the copy number of pGP1 and thereby the amount of the CytR protein.

Direction of *tsx* transcription in the *E. coli* chromosome. The tightly linked *phoB*, *phoR*, and *tsx* genes are located at 9 min on the *E. coli* linkage map (2), with about 15 kilobases separating *tsx* from the *phoB-phoR* operon (31). The *tsx* gene is distal to the *phoB-phoR* operon, and *phoB* is known to be transcribed in a clockwise direction (2). These data enabled us to use the Φ(*tsx-lacZ*)*1*(Hyb) protein fusion to determine the direction of *tsx* transcription in the *E. coli* chromosome. The λ p*lac*Mu9 prophage inserted into the *tsx* gene is genetically marked at both ends: at one end there is the Φ(*tsx-lacZ*)*1*(Hyb) protein fusion, which confers a Lac$^+$ phenotype, and the other end carries the Mu *c*Its62 gene, whose gene product confers a temperature-sensitive immunity to superinfecting Mu phages (7). Depending on the direction of *tsx* transcription, the λ p*lac*Mu9 prophage is inserted in one of two possible orientations relative to *phoR* (Fig. 4a and c). Upon UV irradiation, the λ p*lac*Mu prophage can excise from the chromosome with variable endpoints, giving rise to specialized lambda transducing phages that can carry bacterial genes located near the original λ p*lac*Mu insertion (8). We reasoned that it should be possible to isolate a specialized lambda transducing phage that carries the *phoR*$^+$ gene and only one of the genetic markers flanking the original λ p*lac*Mu9 insertion in *tsx*. The phenotypes of a lysogen harboring such a *phoR*$^+$ transducing phage would then unambiguously indicate the direction of *tsx* transcription. If transcription is clockwise (Fig. 4a), the lysogen will be Lac$^+$ and not immune to phage Mu (Fig. 4b); if transcription is counterclockwise (Fig. 4c), the lysogen will be Lac$^-$ and immune to phage Mu (Fig. 4d).

We isolated a collection of lambda specialized transducing phages by UV irradiation of strain GP1 [*phoR*$^+$ Φ(*tsx-lacZ*)*1*(Hyb)] and used this lysate to transduce strain

TABLE 4. Suppression of the operator mutation O^c-1 by a mutated *cytR* gene

Strain[a]	Plasmid present	β-Galactosidase sp act (μmol/min per mg of protein) after growth in[b]:	
		MMA with glycerol	MMA with glucose
GP28	pBR322	7.1	3.1
GP28	pCB008 (*cytR*$^+$)	4.7	2.0
GP28	pGP1 (*cytR**)[c]	0.5	0.5
GP4	pBR322	6.1	1.2
GP4	pCB008 (*cytR*$^+$)	0.2	0.1
GP4	pGP1 (*cytR**)[c]	0.1	0.07

[a] The chromosomal background of the strains was *deoR cytR*. Strain GP28 carries the wild-type (*tsx-lacZ*)*1*(Hyb) fusion with the mutation O^c-1 as a single copy in the chromosome; strain GP4 carries the Φ(*tsx-lacZ*)*1*(Hyb) fusion without the mutation.

[b] The strains were grown overnight with 0.4% glycerol or 0.4% glucose as the carbon source in the presence of ampicillin (50 μg/ml). Samples were withdrawn, and the specific β-galactosidase activity was determined (25). The data shown are the mean values from two independent experiments.

[c] Plasmid pGP1 was isolated after in vivo mutagenesis in the *mutD5* mutator strain RM1036.

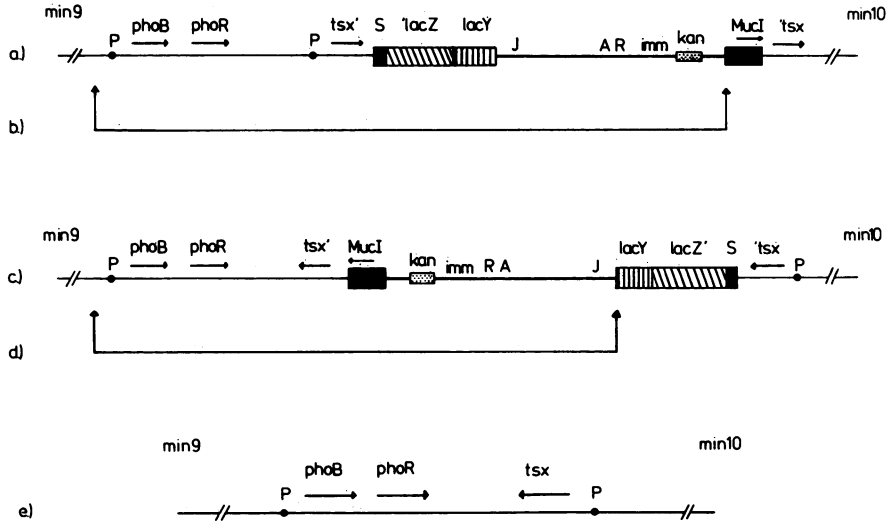

FIG. 4. Direction of the *tsx* transcription in the *E. coli* chromosome. The physical structure of the λ p*lac*Mu9 insertion in the *tsx* gene is shown in the two possible orientations (clockwise [line a] and counterclockwise [line c]) relative to the *phoB phoR* operon. Lines b and d depict two possible excision events of the lambda prophage after UV induction. Line e indicates the determined orientation of *tsx* transcription. Segments from phage Mu are represented by the black boxes and flank the λ prophage positioned next to the Φ(*tsx-lacZ*)*1*(Hyb) protein fusion. The right end Mu attachment site is designated S. The Mu *c*I gene of λ p*lac*Mu9 carries the *c*Its62 allele, which encodes a temperature-sensitive Mu repressor protein. The orientation of the λ prophage is shown by the position of the genes A, R, J, and the imm region. The direction of transcription of a particular gene is shown by an arrow, and its promoter region is designated (P). A prime indicates that a particular gene is not completely present or is interrupted by other DNA sequences. The figure is not drawn to scale.

BRE2101 (*phoR*::Tn*10*Δ*lac*) to kanamycin resistance in the presence of XP. Strains carrying mutations in *phoR* produce alkaline phosphatase constitutively (32) and therefore form dark-blue colonies when plated with the indicator dye XP, whereas *phoR*$^+$ strains form white colonies. We used this phenotype to identify lambda specialized transducing phages carrying the *phoR*$^+$ gene. Of approximately 1,200 transductants inspected, 2 PhoR$^+$ colonies were found. Both strains were lambda lysogens as judged by their immunity to phage λ *c*Ih80, they were Lac$^-$ when streaked on indicator plates containing X-Gal, and they were immune to superinfecting Mu phages at 32°C but not at 42°C. We therefore concluded that the direction of *tsx* transcription is counterclockwise (Fig. 4e). Since this conclusion is dependent on *phoR*$^+$ being carried by the transducing phage, we ruled out the possibility that the PhoR$^+$ phenotype in these lysogens resulted from fortuitous excision of the chromosomal *phoR*::Tn*10* insertions. We isolated and tested several spontaneous PhoR$^-$ segregants and found that they were still resistant to tetracycline but were no longer lambda lysogens, were kanamycin sensitive, and were not immune to phage Mu at 32°C. This demonstrates that the PhoR$^+$ phenotype of the above described lysogens was conferred by the lambda specialized transducing phage.

DISCUSSION

We have used *tsx-lacZ* protein and operon fusions to study the *cis*-acting transcriptional regulatory elements controlling synthesis of the *E. coli* outer membrane protein Tsx. Previous analyses have demonstrated that the cAMP-CAP complex positively affects *tsx* transcription (1, 20, 24), whereas the *deoR*- and *cytR*-encoded proteins act as repressors of *tsx* expression (19). These three regulatory proteins also control the expression of a number of genes involved in nucleoside uptake and metabolism (14, 26). This coregulation of *tsx* thus underlines the function of the Tsx protein in the permeation of nucleosides across the outer membrane (16, 19, 20, 27; Maier et al., in press).

The use of *tsx-lacZ* fusions has allowed us to study the interrelationship of the DeoR, CytR, and cAMP-CAP regulatory proteins and to quantitate their contribution to the regulation of *tsx*. These experiments revealed interesting new features of *tsx* expression. The DeoR and CytR regulatory proteins repress *tsx* transcription to different extents. Synthesis of Tsx is strongly reduced by CytR, whereas DeoR influences *tsx* expression only weakly. In the *cytR deoR* double mutant, the amount of Tsx protein is greatest and the level of *tsx* expression is approximately the sum of those found in the single mutants. Thus, *tsx* expression increases in the following sequence: wild-type < *deoR* < *cytR* < *deoR cytR*. This regulatory pattern has been established by using several different approaches: the adsorption rate of phage T6, the rate of permeation of nucleosides across the outer membrane, the amount of Tsx (19), and, as shown here, the use of *tsx-lacZ* fusions. In both *tsx-lacZ* protein and operon fusion strains, the same regulatory pattern was found, indicating that *tsx* regulation occurs at the transcriptional level. It is of interest to compare the regulatory pattern of *tsx* expression with those of other DeoR- and CytR-controlled genes. The best-characterized system is the *deoCABD* operon, which encodes four nucleoside-catabolizing enzymes (14, 26). This operon is expressed from two differently controlled promoters, P_1 and P_2, which are separated by 599 base pairs; the former is regulated by the DeoR repressor, and the latter is regulated by both the CytR and DeoR repressors (10, 34). In contrast to *tsx* regulation, the DeoR repressor exerts the main effect on the synthesis of the *deo*-encoded enzymes, whereas the CytR repressor influences the expression of the *deo* operon only weakly. Furthermore, *deo* repression by CytR and DeoR is cooperative rather than additive (14). This is exemplified by the levels of the *deoA*-encoded thymidine phosphorylase: in a *deoR cytR* double mutant, a 188-fold increase is observed in comparison with that in the wild type, whereas a single *deoR* or *cytR* mutation results in a 70- or 12-fold-higher enzyme level, respectively (34). Our data strongly indicate that such a cooperation between DeoR and CytR repressors does not play any role in *tsx* regulation. Therefore, the results presented in this study point to important differences in the regulation of *tsx* and the *deo* operon by CytR and DeoR.

Expression of *tsx* is most strongly affected by the cAMP-CAP complex. This positive regulation supercedes that of the two repressors: in a Δ(*crp*) *deoR cytR* mutant strain which lacks the CAP protein, Tsx protein was barely detectable. However, such strains are still sensitive to phage T6, indicating that some Tsx synthesis occurs independently of the activator complex. Indeed, a low β-galactosidase activity is found in *tsx-lacZ* fusion strains carrying the Δ(*crp*)*96* mutation (Table 2). The amount of the cAMP-CAP complex present in the cell is strongly influenced by the available carbon source (11). Expression of *tsx-lacZ* fusions in *deoR* and *cytR* mutants is differently affected in glucose- and glycerol-grown cells. Derepression of *tsx-lacZ* fusions in *deoR* strains occurs to the same extent in glucose- and glycerol-grown cells, demonstrating that the DeoR-regulated *tsx* expression is independent of cAMP-CAP activation. This is in contrast to the strong reduction of *tsx-lacZ* transcription seen in glucose-grown *cytR* strains. Therefore, it is apparent that the CytR-controlled *tsx* transcription is also subject to activation by the cAMP-CAP complex. This regulatory pattern follows that for other CytR- and DeoR-controlled genes. All known promoters repressible by CytR are activated by the cAMP-CAP complex, while transcription occurring from DeoR-controlled promoters is independent of this activator complex (14, 26).

Our results suggest a simple model for the transcriptional control of Tsx synthesis. Expression of *tsx* is controlled at two separate and differently regulated promoters. The weaker of these, P_1, is repressible by DeoR, whereas the main promoter, P_2, is activated by the cAMP-CAP complex and is negatively controlled by CytR. This model predicts that it should be possible to isolate *tsx* mutants that specifically alter the response to only one of the repressors. By taking advantage of the strong repression of *tsx-lacZ* expression in cells carrying the cloned *cytR*$^+$ gene, we have been able to isolate a mutant that shows strongly decreased repression by CytR but no change in regulation by DeoR. This clearly demonstrates that the DeoR and CytR control of Tsx synthesis can be genetically separated. The regulatory pattern of this *tsx* mutant most probably results from an alteration in the CytR operator. The positive regulation of *tsx* expression by the cAMP-CAP complex still occurs in this operator mutant. In contrast to the wild-type Φ(*tsx-lacZ*) *1*(Hyb) fusion strain, *cytR*$^+$ strains are sensitive to glucose repression. This effect is most probably due to the strongly reduced CytR binding, emphasizing the dual control of the *tsx* P_2 promoter by CytR and the cAMP-CAP complex. Our model suggests that Tsx synthesis should occur from two mRNAs that are different in length. This prediction can now

be experimentally tested, since the *tsx* gene has recently been cloned (13; E. Bremer, unpublished results).

Although the results presented here strongly suggest the existence of two *tsx* promoters, they do not provide any clues about the arrangement of these promoters at the 5′ end of *tsx*. Recently, *tsx* regulation has also been studied by Valentin-Hansen and co-workers. The data obtained in this independent investigation are in agreement with our results and provide conclusive evidence that the DeoR-controlled P_1 promoter precedes that of the CytR- and cAMP-CAP-regulated *tsx* P_2 promoter (P. Valentin-Hansen, personal communication).

We are interested in studying the interaction of the CytR and DeoR repressors with their respective *tsx* operator sequences. As a first step, we were able to isolate a suppressor mutation on a plasmid carrying the cloned $cytR^+$ gene that restored the negative control of the O^c-1 operator mutant by CytR. This suppressor mutation is not allele specific, because *tsx-lacZ* expression was reduced in both the wild type and the O^c-1 operator mutant. Two mutational events could account for the suppressor phenotype. It could result from increased synthesis of the wild-type CytR protein, since in the operator mutant CytR binding is not completely abolished. Alternatively, the mutation could result from an altered repressor protein able to interact with both the wild-type and the mutated *tsx* P_2 region. Clearly, it is necessary to isolate more operator mutants and correlate them with allele-specific suppressor mutations in *cytR* and *deoR* (35, 36) to identify the interactions of specific amino acid side chains of the repressor proteins with specific base pairs in the operator sequences (12). Such studies are currently in progress.

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