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Exchange of Colicin Receptor Capacity Between Strains of *Escherichia coli* Sensitive or Resistant to Colicin K-K235

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Abstract. Phage and colicin-resistant mutants were derived from *Escherichia coli* K-12 P678. Two classes of phage T6 and colicin K-resistant mutants (genotype *tsx*) were isolated. Tsx-2 mutants, which demonstrated mucoid growth and increased sensitivities to many antibiotics, became sensitive to colicin K when pretreated with ethylenediaminetetraacetate (EDTA), whereas Tsx-1 mutants did not. Reassociation of EDTA-released material partially restored resistance to colicin K for Tsx-2 mutants. When EDTA-released material from strain P678 was associated with either class of K-resistant mutant, an increase in colicin K sensitivity resulted. Observations suggest that colicin K can act on its target site once it penetrates the cell surface. In addition, results suggest that functional colicin K receptors can be transferred from sensitive to resistant strains, thus conferring colicin sensitivity.

Key words: Colicin K – Phage T6 – *tsx* mutants – Outer membrane – Reconstitution – EDTA treatment – *Escherichia coli*

In the first stage of the interaction of a colicin with sensitive bacteria the colicin binds rapidly and tightly to a specific surface receptor (Hedges 1966). In all cases when a bacterial component able to adsorb a colicin has been identified it has proven to be a protein of the outer membrane. Mutants which are not killed by a given colicin because they do not adsorb it are by convention called resistant. Many such mutants have been shown not to make the outer membrane protein which in sensitive bacteria adsorbs the colicin, or to make an altered protein not apt for its adsorption (Braun and Wolff 1973; Manning and Reeves 1976; Sabet and Schnaitman 1973). Mutants which adsorb a colicin but are not killed by it are called colicin tolerant.

Molecules of colicin, or of active fragments of colicin, which have penetrated the outer membrane, presumably next interact with the outer face of the cytoplasmic membrane. Absence of the outer membrane, or its partial removal, might be expected to alter colicin sensitivity in either of two ways. In the case of a colicin-resistant (non-adsorbing) mutant, absence of the outer membrane or treatment making it permeable might restore sensitivity, by allowing direct access of the colicin to the cytoplasmic membrane. Indeed, Tilby et al. (1978) showed that colicin E3, inactive on *btuB* (formerly *bfe*) mutants which lack the outer membrane protein that adsorbs E colicins (and phage BF23), was active on *btuB* cells after osmotic shock, a treatment that is known to alter the functional integrity of the outer membrane. Foulds and Chai (1978) showed that tolerance to colicin L in *ompA* mutants was overcome by a variety of treatments that affect the outer membrane and that proline uptake by membrane vesicles from L-tolerant cells is sensitive to colicin L. On the other hand, bacteria of a colicin-sensitive strain, if treated in such a way as to remove all or most of the colicin receptor from the outer membrane, might be made nonsusceptible to colicin killing, by failure to adsorb.

In earlier reports (Brunner et al. 1977; Graham et al. 1979), it was shown that outer membrane material could be removed from cells by ethylenediaminetetraacetate (EDTA) treatment, and restored by allowing reassociation of the released material with EDTA-treated cells in the presence of Mg^{+2}. We now report the effect of such treatments on sensitivity of wild-type and colicin-resistant mutants to colicin K. Both colicin K and phage T6 bind to an outer membrane protein specified by gene *tsx*, at 9 min on the genetic map (Bachmann and Low 1980; Manning and Reeves 1976). Resistant mutants, obtained by selection with either colicin K or phage T6, have been shown to make no, or altered, *tsx* protein (Manning and Reeves 1976). Our results indicate that *tsx* mutants may be made susceptible to colicin K by EDTA treatment, and that the ability to adsorb (and be killed by) colicin K can be transferred from wild-type cells to K-resistant (*tsx*) cells by allowing EDTA-treated *tsx* mutants to reassociate EDTA-extracted outer membrane material from wild-type cells.

Materials and Methods

Bacterial Strains and Phages. The bacterial strains and phages used in this study are listed in Table 1. Phage and colicin resistant mutants were isolated from strain P678 as described below.

Culture Media. TYS medium contained 1% tryptone, 0.5% yeast extract and 0.5% NaCl. M9 salts (Miller 1972) contained 1.0 g NH_4Cl, 0.13 g $MgSO_4$, 3.0 g KH_2PO_4, and 6.0 g Na_2HPO_4 per liter deionized water (final pH 7.2). Soft agar consisted of TYS medium supplemented with 0.75% agar. Antibiotic sensitivities were tested on DST agar (CM261, Oxoid Ltd). In all experiments involving quantitation of viable cells, 0.85% KCl was employed as the diluent prior to plating on trypticase soy agar (TSA, BBL).

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Non-standard Abbreviations: SDS = sodium dodecyl sulfate

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Table 1
Bacterial strains and phages

Strain	Genotype or relevant characteristics[a]	Source and/or reference
Escherichia coli K-12		
P678	*thr-1, leu-6, thi-1, lacY1, malT1, xyl-7, ara-13, mtl-2, tonA2, gal-6, rpsL135*	(Wollman et al. 1956)
K235	ColK-K235	A. Pugsley (Kunugita and Matsuhashi 1970)
X342	*proC29, metB1, relA1,* λ^-	B. J. Bachmann, CGSC[b]
KG16	*tsx-276* of P678 (= Tsx-2)	This study
KG19	*tsx-279* of P678 (= Tsx-2)	This study
GG24	*tsx-277* of P678 (= Tsx-1)	This study
GG26	*tsx-278* of P678 (= Tsx-1)	This study
Phages		
T6		B. A. D. Stocker (Manning and Reeves 1976)
P1::Tn*9*, c_{ts}		K. Smith (Miller 1972)

[a] Genetic symbols are as described by Bachmann and Low (1980)
[b] CGSC, *Escherichia coli* Genetic Stock Center, Yale University

Preparation and Titration of Colicin K. Colicin K was prepared as described by Kunugita and Matsuhashi (1970) through the ammonium sulfate precipitation step. Colicin activity was determined by serially diluting the solution to be tested with TYS broth and spotting a small drop on TYS soft agar containing approximately 5×10^7 freshly grown P678 cells. After incubation at 37° C for 12 h the plates were scored. The number of colicin units per milliliter (U/ml) was defined as the reciprocal of the highest dilution which gave a clear zone of inhibition of growth of strain P678.

Isolation and Characterization of Colicin-Resistant Mutants. Mutants resistant to colicin K were isolated by selection with colicin K as described by Takagaki et al. (1973). Phage sensitivities were determined either by cross-streaking on nutrient agar plates or by spotting 10 µl drops of lysate (titer 10^6 to 10^7 plaque forming units/ml) with a multiloop applicator onto nutrient agar plates inoculated by flooding from broth cultures and then allowing them to dry. Colicin sensitivity was determined either by using soft agar inoculated with the test strain to overlay a master plate containing macrocolonies (sterilized by chloroform vapors) of colicin-producing strains or by the cross-streaking technique of Davies and Reeves (1975). Parental strain P678 and all mutant derivatives were tested for sensitivities to the following colicins: L, K, A, S1, S4, X, E1, E2, E3, D, Ia, Ib, M, V, G, H, and N. Antibiotic sensitivities were determined using antibiotic Sensi-Discs (BBL) and the method of Bauer et al. (1959). Survival curves following ultraviolet irradiation were determined as described by Mortelmans and Stocker (1976).

EDTA Treatment. Cells were grown in TYS broth to a concentration of 5×10^8 cells/ml and collected by centrifugation at 25° C. The cells were washed twice and resuspended at 5×10^9 cells/ml in 0.12 M Tris buffer (pH 8.0). Treatment was performed essentially as described by Leive and Shovlin (1968) by exposure to EDTA (1 µmol/10^{10} cells) for 2 min, with shaking, at 37° C. For preparation of EDTA-released material the cells were resuspended at 5×10^{11} cells/ml and treated with EDTA. The suspension was centrifuged at 8,000 × *g* for 15 min at 25° C, and the supernatant fraction filtered through a membrane filter (Millipore, 0.45 µm) to separate bacterial cells from their released material. Total protein in the released material was determined according to the method of Lowry et al. (1951).

Effect of EDTA on Colicin Sensitivity. Cells were treated with EDTA as described above and the cells were diluted to 5×10^7 cells/ml in M9 salts. Untreated (washed and suspended to 5×10^7 cells/ml in M9 salts) and EDTA-treated cells were tested for colicin sensitivity as described by Foulds and Chai (1978).

Colicin Neutralization by EDTA-Released Material. $MgCl_2$ was added to EDTA-released material to a final concentration of 50 mM. A 0.9 ml amount of EDTA-released material was mixed with 0.1 ml M9 salts containing various concentrations of colicin. After a 30 min incubation at 37° C, 0.1 ml of freshly grown P678 cells (washed and suspended at 5×10^8 cells/ml in M9 salts) was added. After a further 15 min incubation the number of survivors was quantitated.

Reconstitution Protocol. Cells to be reconstituted were treated with EDTA as described, washed twice, and resuspended at 5×10^7 cells/ml in M9 salts. 8.0 ml of cells were then pelleted by centrifugation and resuspended in 8.0 ml EDTA-released material from a sensitive (P678) or resistant strain. Reassociation of EDTA-released material was achieved by the addition of $MgCl_2$ to a final concentration of 50 mM and incubation at 37° C for 20 min (Brunner et al. 1977; Graham et al. 1979). The reconstituted cells were then pelleted by centrifugation and washed with M9 salts to remove unbound EDTA-released material. 0.1 ml of various concentrations of colicin in M9 salts were added to 0.9 ml of untreated, EDTA-treated or reconstituted cells (suspended at 5×10^7 cells/ml in M9 salts). Viable cells were quantitated after 20 min incubation at 37° C.

Adsorption of Colicin. Strains resistant to phage T6 and colicin K (*tsx*) were reconstituted as described above and tested for adsorption of colicin K (Foulds and Chai 1978). 0.9 ml (5×10^8 cells/ml) of reconstituted washed cells was mixed with 0.1 ml of various concentrations of colicin K in M9 salts. After a 15 min incubation at 37° C, the colicin K

adsorbed by the cells was removed by centrifugation. The supernatant fluid was then filtered using a 0.45 μm filter to remove any remaining cells. 0.1 ml of a freshly grown culture of P678 (washed and suspended at 5×10^8 cells/ml in M9 salts) was added to 0.9 ml of the supernatant fluid. After a further 15 min incubation the number of survivors was determined.

Polyacrylamide Gel Analysis of Outer Membrane Proteins. Cells used for isolation of outer membrane proteins were grown to approximately 1×10^9 cells/ml in TYS broth, collected by centrifugation, washed, and resuspended in 0.01 M sodium phosphate buffer (pH 7.4). The cells were disrupted by sonication (Sonifer Cell Disruptor, model W-185-E) at a power setting of 100 W for five 30 s pulses in an ice bath. The mixture was then centrifuged ($8{,}000 \times g$ for 10 min at 4° C) to pellet unbroken cells. The cell envelopes were pelleted by ultracentrifugation ($100{,}000 \times g$ for 60 min at 4° C) and washed twice in 10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) buffer (pH 7.4). Outer membrane proteins were prepared using the method of Schnaitman (1974) to obtain the Triton X-100 insoluble component of the cell envelope.

Polyacrylamide gel electrophoresis (PAGE) was carried out in slabs 1.5 mm thick and 25 cm long. The electrophoresis buffer system employed was the Tris-glycine system of Laemmli (1970). The separating gel contained 15.0% acrylamide, 0.21% bisacrylamide, 0.1% sodium dodecyl sulfate (SDS), 0.05% ammonium persulfate, 0.05% N,N,N′,N′-tetramethylethylenediamine (TEMED), and was prepared in 0.375 M Tris (pH 8.8). The stacking gel consisted of 6.0% acrylamide, 0.12% bisacrylamide, 0.1% SDS, 0.05% ammonium persulfate, 0.05% TEMED, and was prepared in 0.125 M Tris (pH 6.8). Samples were solubilized by boiling for 5 min in the sample buffer which contained 0.0625 M Tris (pH 6.8), 2.0% SDS, 12.5% glycerol, and 5% 2-mercaptoethanol. Molecular weight determinations were calculated using a low molecular weight calibration kit (Pharmacia). Electrophoresis was carried out at 120 V for 8 h. Gels were run at room temperature. Gels were stained at 37° C for 2 h with 0.25% coomassie brilliant blue dissolved in 7.5% acetic acid and 50% methanol. Gels were destained in 7.5% acetic acid and 30% methanol for 24 h.

Genetic Techniques. Transductions were performed as described by Miller (1972) using a derivative of phage P1 (Table 1). Transductants were screened for unselected colicin or phage resistances as described above.

Results

Mutants Resistant to Phage T6 and Colicin K. Using the method of Takagaki et al. (1973), two distinct classes of colicin K-resistant mutants were isolated. All colicin K-resistant mutants were screened for resistance to phage T6. Independently isolated phage T6 and colicin K-resistant class 2 mutants (Tsx-2) represented less than 10% (3/39) of the total resistant mutants. Class 2 mutants were insensitive to colicin L, in addition to colicin K, but were identical to P678 for all other colicins tested. Class 1 mutants (Tsx-1) remained sensitive to colicin L, as well as to all other colicins examined, and were slightly sensitive to colicin K only when the colicin was partially purified from broth and used at high concentrations, but not by either the soft-agar overlay or cross-streak methods. The slight sensitivity may be due to either non-specific or secondary colicin binding or the presence of trace amounts of a second colicin, other than K, produced by strain K235 (i.e., colicin V). Phage P1::Tn*9*, c_{ts} lysates of two mutants from each resistance class were used to transduce strain X432 (*proC*) to Pro^+. Examination of 80 Pro^+ transductants from each class showed 49% cotransduction of phage T6 and colicin K resistance for class 2 mutants (KG16 and KG19) and 48% for class 1 mutants (GG24 and GG26), in agreement with previous data for the cotransduction of *tsx* and *proC* (Manning et al. 1977).

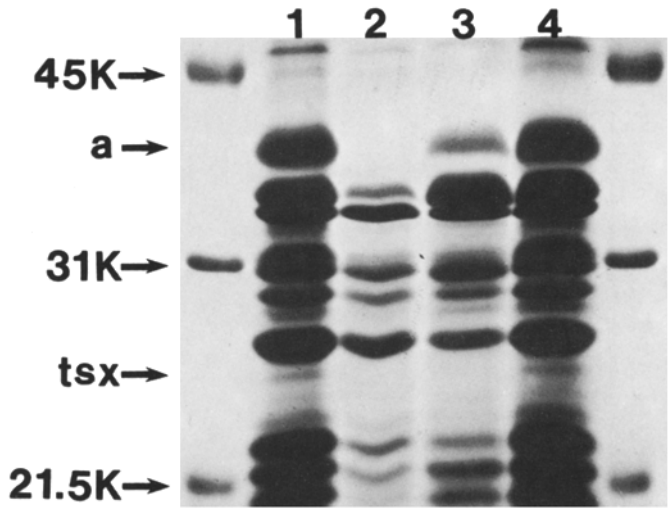

Fig. 1. Pattern of outer membrane proteins present in strains P678, KG16 (Tsx-2) and GG24 (Tsx-1). Outer membrane proteins were purified and subjected to SDS-polyacrylamide gel electrophoresis as described. Only the region of the gel between molecular weights of 21,000 and 45,000 daltons is shown. Lanes 1 and 4, P678; lane 2, KG16 (Tsx-2); lane 3, GG24 (Tsx-1). Indicated on the left are the positions of the *tsx* protein, protein a, and molecular weight standards (21,500, 31,000, and 45,000 daltons)

Tsx-2 mutants gave mucoid growth at 30° C and 37° C on nutrient agar. In addition, Tsx-2 mutants showed increased sensitivities to chloramphenicol, neomycin, kanamycin, ampicillin, nalidixic acid, carbenicillin and novobiocin, when compared to either strain P678 or Tsx-1 mutants (data not shown). Dose-survival curves for ultraviolet irradiated Tsx-2 mutants indicated that the mucoid growth was not a result of a mutation at *lon* (*capR*) as the mutants were as UV-resistant as strain P678. Rather, it is thought that the mucoid phenotype demonstrated by Tsx-2 mutants is due to the loss of protein a (= 3b) in their outer membranes (Fig. 1). Protein a has been implicated to be involved in the regulation of capsular polysaccharide synthesis in *Escherichia coli* K-12 (Gayda et al. 1979). In addition to the loss of protein a in the outer membranes of Tsx-2 strains, several other proteins were either reduced in concentration or missing (Fig. 1). Tsx-1 mutants also demonstrated an altered outer membrane protein profile, compared to parental strain P678, however, protein a remained present and these mutants were all non-mucoid. Both Tsx-1 and Tsx-2 strains lack the *tsx* protein (25,000 daltons) in their outer membranes (Fig. 1), as was genetically characterized by cotransduction frequencies for *tsx* and $proC^+$.

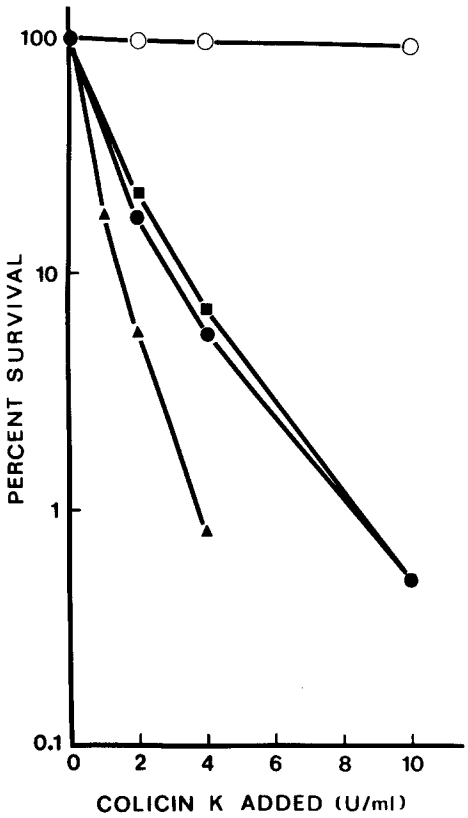

Fig. 2. Neutralization of colicin K by EDTA-released material. Colicin neutralization assays were performed as described. EDTA-released material was extracted from equivalent concentrations of cells and each yielded approximately 160 μg protein/ml. P678 was the test strain. Symbols: ▲ No EDTA-released material; ○ P678 EDTA-released material; ■ GG26 (Tsx-1) EDTA-released material; ● KG16 (Tsx-2) EDTA-released material

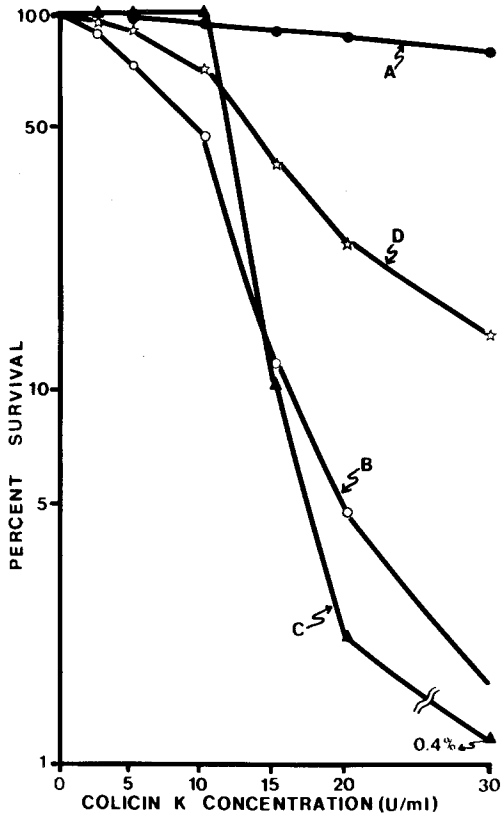

Fig. 3A—D. Colicin K sensitivity of reconstituted Tsx-2 mutants. Reconstitution of strain KG16 (Tsx-2 mutant) was performed as described. (**A**) untreated cells; (**B**) EDTA-treated cells; (**C**) KG16 reconstituted with P678 EDTA-released material; (**D**) KG16 reconstituted with KG16 EDTA-released material

Neutralization of Colicin by EDTA-Released Material. Neutralization tests were performed to determine whether the colicin K receptors were released by EDTA treatment. A previous report indicated that isolated lipopolysaccharide (LPS) was unable to bind colicin K (Weltzien and Jesaitis 1971). This is not to assume that the native LPS does not play a role in making the protein receptor functionally active in the outer membrane.

EDTA-released material obtained from both mutant classes neutralized significantly less colicin K than EDTA-released material from parental strain P678 (Fig. 2) in agreement with expectations for *tsx* mutants. The addition of $MgCl_2$ (50 mM final concentration) to the EDTA-released material was of critical importance, as little detectable colicin K neutralization was detected using P678 EDTA-released material without the addition of $MgCl_2$.

Reconstitution of tsx Mutants. Pretreatment of Tsx-2 mutants with EDTA increased sensitivity to colicin K (Fig. 3). Undoubtedly, the release of outer membrane material results in exposure of colicin K target sites. The response of Tsx-1 mutants to EDTA treatment and subsequent challenge with colicin K was different from that observed with Tsx-2 mutants. EDTA treated Tsx-1 mutants were less sensitive to colicin K than untreated cells (Fig. 4). Presumably this decreased sensitivity following EDTA exposure may result from removal of outer membrane material containing the colicin V receptor; colicin V is also produced by strain K235 in trace amounts. In fact, the slightly greater native sensitivity demonstrated by Tsx-1 strains compared with Tsx-2 strains at high colicin doses may be attributed to this trace contaminating colicin and critical architectural differences in the outer membranes of these two mutant classes. The nature of these differences is not known.

Reconstitution of either Tsx class with EDTA-released material from parental strain P678 allowed for increased colicin K sensitivities (Figs. 3 and 4) when compared to either EDTA-treated cells or cells reconstituted with their own EDTA-released material (presumed to be receptor deficient). The difference in sensitivity for Tsx-2 mutants reconstituted with parent strain released material was only evident at the higher concentrations of colicin tested (Fig. 3), as might be expected for reassociation of exogenous material containing the colicin K receptor.

In previous studies it was shown that Mg^{+2} alone was not sufficient to repair EDTA-induced sensitivity to actinomycin D (Brunner et al. 1977; Graham et al. 1979). In order to repair the permeability barrier, exogenous EDTA-released material had to be added during the reconstitution procedure. Similar control experiments performed with Tsx-1 and Tsx-2 mutants demonstrated the absolute requirement for the addition of exogenous EDTA-released material and not only Mg^{+2}.

Colicin K neutralization assays employing reconstituted mutants (Fig. 5) demonstrated that both classes of *tsx*

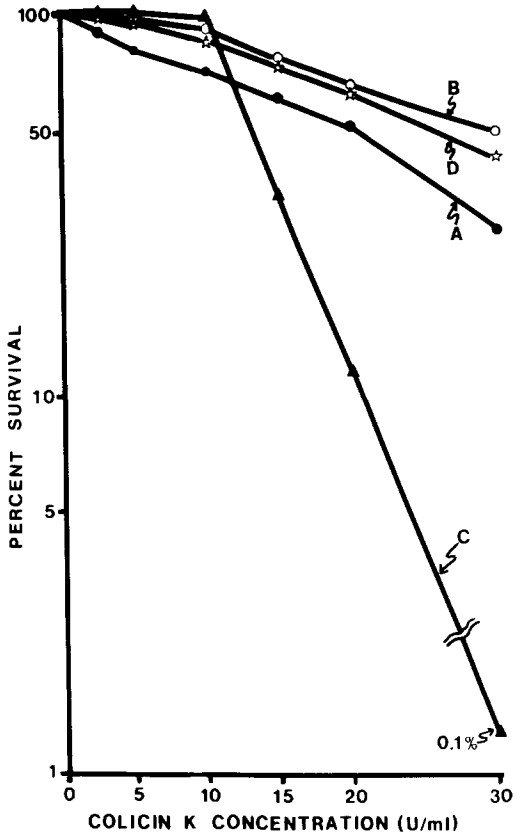

Fig. 4A—D. Colicin K sensitivity of reconstituted Tsx-1 mutants. Reconstitution of strain GG26 (Tsx-1 mutant) was performed as described. (**A**) untreated cells; (**B**) EDTA-treated cells; (**C**) GG26 reconstituted with P678 EDTA-released material; (**D**) GG26 reconstituted with GG26 EDTA-released material

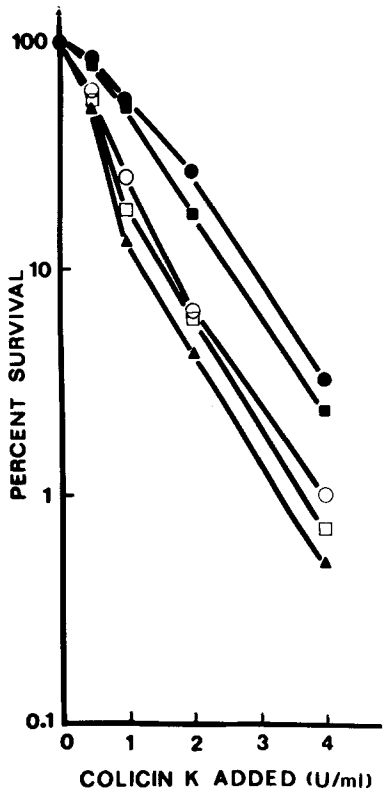

Fig. 5. Adsorption of colicin K by *tsx* mutants reconstituted with EDTA-released material from colicin-sensitive and colicin-resistant strains. Reconstitution and colicin adsorption were performed as described. P678 was the test strain. Symbols: ▲ No cells; ■ GG26 (Tsx-1) + P678 EDTA-released material; □ GG26 (Tsx-1) + GG26 (Tsx-1) EDTA-released material; ● KG16 (Tsx-2) + P678 EDTA-released material; ○ KG16 (Tsx-2) + KG16 (Tsx-2) EDTA-released material

mutants would reassociate P678 EDTA-released material and express colicin K binding activity. When these mutants were reconstituted with EDTA-released material from themselves, neutralization of less colicin K was observed, consistent with genetic evidence indicating both classes have a mutation at *tsx* and lack the primary colicin K receptor.

Discussion

Conclusions based upon genetic and phenotypic data indicate that the phage T6 and colicin K-resistant mutants isolated here can be divided into two distinct classes (Tsx-1 and Tsx-2), both of which have the *tsx* mutation, known to be responsible for resistance to phage T6 and colicin K (Hantke 1976; Manning and Reeves 1976). In addition, Tsx-2 mutants demonstrate increased sensitivities to many antibiotics, colicin L resistance, and mucoid growth on nutrient agar.

The mucoid growth of Tsx-2 mutants is not the result of a *lon* (*capR*) mutation (also *proC* linked), since Tsx-2 mutants did not demonstrate increased sensitivity to UV irradiation, exhibited by *lon* mutants (Markovitz 1977). Mutations which alter the membrane structure could activate the functioning of colanic acid polymerases (Markovitz 1977). For example, colicin-tolerant mutants (*tolA* and *tolB*) demonstrate traits which are consistent with outer membrane alterations and form mucoid colonies (Bhattacharyya et al. 1970). Tsx-2 mutants also have altered outer membranes, as they are tolerant to colicin L (but remain sensitive to phages TuII* and K3), demonstrate either loss of or reductions in the quantity of several outer membrane proteins (Fig. 1), and have increased sensitivites to several antibiotics. It is unlikely that a mutation solely at *tsx* could account for the pleiotropic phenotype demonstrated by Tsx-2 mutants. Rather, the presence, in a portion of the parental P678 population, of pre-existing variants negatively regulated with respect to several outer membrane proteins and subsequent selection among this population for *tsx* mutants resulted in the two Tsx classes encountered. Joint regulation of the synthesis of several outer membrane proteins has recently been attributed to two alleles, *perA* and *tpo*, which are thought to be identical (Lundrigan and Earhart 1981; Wandersman et al. 1980; Wanner et al. 1979). As *tsx*, *proC*$^+$ cotransductants obtained from Tsx-2 donors were non-mucoid yet colicin K and phage T6 resistant, the Tsx-2 phenotype can be attributed to mutations at *tsx* and a second allele (perhaps *perA*).

EDTA treatment, as described in this report, causes a disruption of the outer membrane permeability barrier, but has little effect on protein synthesis, RNA synthesis or viability of the cells (Leive 1974). The results presented here indicate that pretreatment of one class of *tsx* mutants (Tsx-2) with EDTA increased sensitivity to colicin K. These results

support those of Foulds and Chai (1978) for *ompA* mutants and colicin L.

We previously reported the ability to reconstitute the cell's permeability barrier following EDTA treatment (Brunner et al. 1977; Graham et al. 1979). Data presented here support our previous studies by showing that the permeability barrier to colicin K could be reconstituted following EDTA treatment of colicin-resistant strains. Of greater significance are the data (Figs. 3 and 4) indicating that biologically active colicin K receptors, extracted from sensitive cells, could be transferred to resistant cells, thus conferring colicin K sensitivity. The apparent reduced efficacy of reconstitution of Tsx-2 strains (Fig. 3) when compared to Tsx-1 strains (Fig. 4) is presumably due to the EDTA sensitization of Tsx-2 mutants, thus masking the total effect of receptor addition. Comparing adsorption of colicin K (Fig. 5) and colicin K sensitivity of the reconstituted *tsx* mutants (Figs. 3 and 4) it is evident that both Tsx classes, reconstituted with EDTA-released material from the colicin-sensitive parent, bind relatively small amounts of colicin K, yet these reconstituted strains are significantly sensitized to colicin K, an effect never observed when reconstituted with receptor-deficient material.

In summary, these data support the conclusion that the colicin molecule, or a portion of it, must breach the outer membrane barrier to interact with the target (Bradbeer et al. 1976; Chai and Foulds 1974; Foulds and Chai 1978; Takagaki et al. 1973; Tilby et al. 1978). This suggests the role of the outer membrane receptor proteins in permitting access of the colicin, either directly or indirectly, to the underlying cytoplasmic membrane. Data concerning reconstitution of the colicin K receptor with colicin-resistant cells support this suggestion. Using purified outer membrane components and the reconstitution procedure described, more precise analysis of outer membrane receptor functions may be possible.

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