Site-Directed Mutagenesis of the Charged Residues near the Carboxy Terminus of the Colicin E1 Ion Channel†

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ABSTRACT: Colicin E1 was altered by oligonucleotide-directed mutagenesis at the site of three charged residues on the COOH side of the 35-residue hydrophobic segment in the channel-forming domain. Asp-509 is one of five conserved acidic residues in the channel domain of colicins A, B, E1, Ia, and Ib and is the first charged residue following the hydrophobic segment, followed by the basic residues Lys-510 and Lys-512. Asp-509 and Lys-512 were changed to *amber* and *ochre* stop codons, respectively, while Lys-510 was mutated to a Met codon. Proteins truncated after residue 508 or 511, and missing the last 14 or 11 residues, were obtained from a nonsuppressing cell strain harboring the mutant plasmid while full-length colicin molecules with single residue changes at Asp-509 to Leu, Ser, and Gln, and Lys-512 to Tyr, were obtained by using appropriate suppressor strains. The truncated colicins displayed (i) a low cytotoxicity, ~1% of intact wild-type colicin, (ii) 10-fold less in vitro channel activity with liposomes, and (iii) reduced labeling of the colicin in liposomes by a phospholipid photoaffinity probe, showing that one or more of the residues following Asn-511 is necessary for both in vivo and in vitro activity and insertion into the bilayer. (iv) The truncated mutants also displayed an altered conformation at pH 6 that allowed greater binding and activity with liposomes at this pH relative to wild type. The cytotoxicity of single residue substitutions at Asp-509 showed a range of cytotoxicities, wild type > Ser-509 > Gln-509 > Leu-509, although none of these changes greatly affected the in vitro channel activity or pH dependence. Substitution of a neutral residue for either of the positively charged Lys residues in point mutants Lys-510 → Met or Lys-512 → Tyr also did not significantly affect in vivo or in vitro activity. However, the anion selectivity of the channel was significantly decreased by the latter mutations, indicating that Lys-510 and Lys-512 affect the ion selectivity, probably at the surface of the channel.

The colicin E1 molecule depolarizes and deenergizes the *Escherichia coli* cytoplasmic membrane by forming a monovalent ion-selective channel sufficiently conductive (Bullock et al., 1983) that H^+ pumping by oxidative metabolism cannot compete (Cramer et al., 1983). The channel-forming domain is contained in the carboxy-terminal region of the molecule (Dankert et al., 1982), although the minimum size of this carboxy domain that is competent for channel function is under debate (Liu et al., 1986). It is likely that the COOH-terminal domain is located in the interior of the water-soluble colicin E1 molecule, because of the greater hydrophobicity of the channel domain (Davidson et al., 1984a), its resistance to protease (Dankert et al., 1982; Ohno-Iwashita & Imahori, 1982), and the lack of reactivity in solution of its single Cys residue located 17 residues from the COOH terminus (Bishop et al., 1986). Thus, unfolding of the hydrophobic channel domain would be expected to occur at an early stage of channel formation in vitro, or after passage through the receptor and outer membrane in vivo. The 35-residue segment, Ala-475 through Ile-508, terminating 14 residues from the COOH terminus (enclosed by brackets in Figure 1), may serve as the initial hydrophobic anchor (Cleveland et al., 1983). It then seems reasonable to propose that 1 or more of the 3 charged residues immediately proximal on the COOH side to this 35-residue segment might have a function in membrane binding and control of channel ion selectivity and may also affect the folding of the soluble protein. These residues are the acidic Asp-509 and the positively charged Lys-510 and Lys-512. The role of these three charged amino acids near the COOH terminus of the colicin E1 channel, as well as the remaining segment before the COOH terminus, was tested by directed mutagenesis and functional assay, in vitro and in vivo, of the resulting proteins.

Materials and Methods

Strains and Media. Bacterial strains JM83 (Yanish-Perron et al., 1985), JM101 (Yanish-Perron et al., 1985), and RZ1032 (Kunkel, 1985), and *amber* suppressor cell strains Su1, Su2, and Su6, and *ochre* suppressor cell strain SuC (Bachmann, 1983) were grown and maintained as described previously (Shiver et al., 1987). The colicin derivative plasmid pDMS630 (Suit et al., 1985) harboring an ampicillin resistance gene was obtained from S. E. Luria and J. Suit (MIT, Cambridge, MA).

Mutagenesis. Site-directed mutagenesis was performed according to the method of Zoller and Smith (1983) as modified by Kunkel (1985) using M13 cloning vectors as described previously. The plasmid construct used for mutagenesis, designated as M13mPDJ30, was prepared by digestion of pCo1E1 (Chan et al., 1985) with *Eco*RI and *Xho*II restriction enzymes. The resulting mixture of two 0.6-kb fragments was ligated into M13tg130 (Kieny et al., 1983) in the polylinker cloning region using the *Eco*RI and *Bam*HI restriction sites. Recombinant plaques containing the distal portion of the *cea* gene were screened for the unique *Nru*I site lying anterior to *imm* (Figure 2). Asp-509 was changed to an *amber* mutant while Lys-512 was changed to an *ochre*

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                          340                       365
                          |                         |
E1 SNNRNAGIARVHEAEENLKKAQNNLLNSQIKDAVDATVSFYQTLTEKYGE
Ia KEKENIRNQLSGINQKIAEEKRKQDELKATKDAINFTTEFLKSVSEKYGA
Ib KEKENIRSQLADINQKIAEEKRKRDEINMVKDAIKLTSDFYRTIYDEFGK
A  QRQAEEAERQRQAMEVAEKAKDERELLEKTSELIAGMGDKIGEHLGDKYK
B  QRQEAENNAKDDFRVKKEQENDEKTVLTKTSEVIISVGDKVGEYLGDKYK

                          390                       415
                          |                         |
E1 KYSKMAQELADKSKGKKIGNVNEALAAFEKYKDVLNKKFSKADRDAIFNA
Ia KAEQLAREMAGQAKGKKIRNVEEALKTYEKYRADINKKINAKDRAAIAAA
Ib QASELAKELASVSQGKQIKSVDDALNAFDKFRNNLNKKYNIQDRMAISKA
A  AIAKDIADNIKNFQGKTIRSFDDAMASLNKITANPAMKINKADRDALVNA
B  ALSREIAENINNFQGKTIRSYDDAMSSINKLMANPSLKINATDKEAIVNA

                          440                       465
                          |                         |
E1 LASVKYDDWAKHLDQFAKYLKITGHVSFGYDVVSDILKIKDTGDWKPLFL
Ia LESVKLSDISSNLNRFSRGLGYAGKFTSLADWITEFGKAVRTENWRPLFV
Ib LEAINQVHMAENFKLFSKAFGFTGKVIERYDVAVELQKAVKTDNWRPFFV
A  WKHVDAQDMANKLGNLSKAFKVADVVMKVEKVREKSIEGYETGNWGPLML
B  WKAFNAEDMGNKFAALGKTFKAADYAIKANNIREKSIEGYQTGNWGPLML

                          490                       505
                          |                         |
E1 TLEKKAADAGVSYVVALLFSLLAGT          TLGIWGIAIVTGILC
Ia KTETIIAGNAATALVALVFSILTGS          ALGIIGYGLLMAVTG
Ib KLESLAAGRAASAVTAWAFSVMLGT          PVGIILGFAIIMAAVS
A  EVESWVLSGIASSVALGIFSATLGAYALSLGVPAIAVGIAGI LLAAVVG
B  EVESWVISGMASAVALSLFSLTLGSALIAFGLSATVVGFVGV VIAGAIG

                    522
                    |
E1 SYIDKNKLNTINEVLGI
Ia ALIDESLVEKANKFWGI
Ib ALVNDKFIEQVNKLIGI
A  ALIDDKFADALNNEIIRPAH
B  AFIDDKFVDELNHKIIK
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FIGURE 1: Alignment from residues 316–522 of the sequences of COOH-terminal colicins E1, Ia, Ib, A, and B [adapted from Yamada et al. (1982), Varley and Boulnois (1984), Pattus et al. (1985), Mankovich et al. (1986), and Schramm et al. (1987)]. This domain includes all channel-forming COOH-terminal peptides of interest. The positions of the mutations at Glu(E)-468, Asp-(D)-509, Lys-(K)-510, and Lys(K)-512 are indicated. The hydrophobic segments of 35–49 residues common to these colicins are delineated by brackets. The sequence is enumerated for colicin E1.

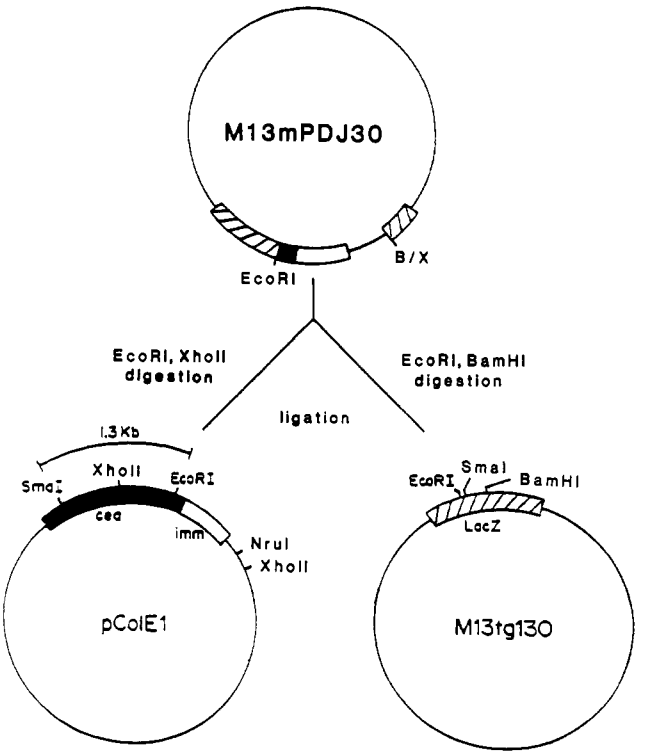

FIGURE 2: Construct of the M13 vector used for ColE1 mutagenesis.

mutant using synthetic oligonucleotides (5′-GTTTATTCTT*CT*AAATATAGGAGC-3′ and 5′-TTAAGTT*AATTCTTA-3′, respectively).

Lys-510 was changed to methionine by the method of Eckstein (Taylor et al., 198a,b) using a mutagenesis kit (Amersham) and a synthetic oligonucleotide (5′-TATTC*ATATCAATATAGG-3′).

Mutation candidates were initially identified by dot blot hybridization analysis (Zoller & Smith, 1983) and, because the mutatioms lie at the opposite end of the insert from the universal priming site in M13tg130, subsequently verified by using a modification of the dideoxy sequencing method (Sanger et al., 1977) developed for double-stranded templates (Chen & Seeburg, 1985), using the reverse sequencing primer for M13 vectors (754774, Boehringer Mannheim). The mutated *cea* fragments were reintroduced into pDMS630 by using the unique restriction enzyme sites *Nru*I and *Eco*RI and transformed into JM83 which yields truncated colicin molecules for *amb* and *och* mutations, or into suppressor cell strains Su1, Su2, Su6, or SuC, which substitute serine, glutamine, or leucine for *am* codons and tyrosine for *och* codons, respectively (Steege & Soll, 1979; Shiver et al., 1987). Following reconstitution of the colicin plasmid, each mutant was verified (a) by subcloning the *Eco*RI/*Nru*I fragment into M13tg131 for single-stranded dideoxy sequencing or (b) by sequencing the mutation within pDMS630 using the plasmid as a double-stranded template and an oligonucleotide primer complementary to a position upstream from the mutation (Chen & Seeburg, 1985).

Purification of Mutant Colicins. Colicin E1 was purified from JC411 or from JM83 cells harboring pDMS630 using a CM-Biogel ion-exchange column, as described by Shiver et al. (1987). Full-length mutant colicins were purified similarly. M_r 18 000 carboxy-terminal peptides containing the ion channel-forming domain were obtained by thermolysin digestion of purified wild-type and mutant colicins as desribed previously (Bishop et al., 1985). The truncated colicins produced by the *am* and *och* stop translation codons could not be purified in this manner and were purified by immunoaffinity chromatography as described below.

Hyperimmune anti-colicin E1 antibody was prepared from rabbits inoculated with highly purified colicin E1. The IgG fraction was purified from serum as described by Dankert et al. (1985): Whole blood was withdrawn from the rabbit, clotted overnight at 4 °C, and centrifuged at 2000*g* for 5 min to remove erythrocytes. The supernatant was adjusted to pH 5.0 with 3 M acetic acid, and 1 volume of octanoic acid (Sigma Chemical Co., St. Louis, MO) was added to 20 volumes of serum while rapidly stirring. The mixture was stirred at room temperature for 30 min and centrifuged at 30000*g* for 30 min. The resulting supernatant was slowly added to an equal volume of 100% saturated $(NH_4)_2SO_4$ while stirring and allowed to stand overnight at 4 °C. The precipitate was collected by centrifugation at 30000*g* for 30 min, washed twice with 50% saturated $(NH_4)_2SO_4$, resolubilized in one-tenth the original serum volume of coupling buffer (0.5 M NaCl and 0.1 M $NaHCO_3$, pH 9.0), and then dialyzed overnight vs coupling buffer. IgG concentrations were calculated by using an extinction coefficient of 1.34 mL/(mg·cm) at 280 nm (Hurn & Chantler, 1980).

Five milligrams of purified IgG per milliliter of cyanogen bromide activated Sepharose 4B (Sigma Chemical Co.) was coupled overnight at 4 °C with slow agitation. The remaining active groups were blocked with a 0.2 M glycine, pH 8.0, wash for 2 h at room temperature. The gel was then dried through a sintered glass funnel, and the OD_{280} of the filtrate was measured to determine coupling efficiency. The gel was then washed alternately with 0.5 M NaCl, 0.1 M sodium acetate, pH 4, and coupling buffer and stored at 4 °C in 0.1 M phosphate and 0.01% NaN_3, pH 7.

Truncated colicins were purified as follows: L broth (1 L) was inoculated with a 5-mL overnight culture of JM83 containing pDMS630 harboring either *am*-509, *och*-512, or *am*-468 (Shiver et al., 1987) mutations, grown 2 h with vigorous agitation, induced with mitomycin C (to 0.5 μg/mL), and agitated slowly for 4 h at 37 °C. The cells were sedimented at 7500*g*, resuspended in 20 mL of 50 mM sodium borate at pH 9.5, and disrupted with a French press. About half of the supernatant from a 14500*g*, 10-min centrifugation was applied to a 6-mL anti-colicin E1 affinity column (Poly-Prep Chromatography Column, Bio-Rad, Richmond, CA) equilibrated in borate buffer and washed extensively. The column was then washed with 0.5 M NaCl and 50 mM borate, pH 9.5, solution to remove nonspecifically adsorbed material, and the colicin was eluted with 3 M NaSCN, pH 7.8. The eluted protein was immediately concentrated (Micro-Prodicon, Bio-Molecular Dynamics) and dialyzed vs 0.1 M phosphate, pH 7.0. Each of the buffers utilized above also contained 0.5 mM PMSF as a protease inhibitor which was found to be especially necessary for purifying *am*-468 protein. The protein was stored in small aliquots at −70 °C. The immunoaffinity column was washed with 4 M guanidine hydrochloride and 2 M NaCl, to remove residual protein, and equilibrated in 0.1 M phosphate and 0.01% NaN_3, pH 7.0. *am*-468 was the only truncated protein that did not purify to one or two definitive bands as visualized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).[1] This sample had about six components on the gel, with two major bands at positions corresponding to monomeric colicin E1.

Cytotoxicity. These experiments were performed as described previously (Shiver et al., 1987) using *Escherichia coli* B/1,5 as the colicin-sensitive indicator. Immune-purified wild-type colicin was used as the reference protein for all experiments with immune-purified mutants.

In Vitro Liposome and Bilayer Experiments. Channel-forming activities of wild-type and mutant colicins were determined by toxin-mediated Cl^- efflux from large, mostly unilamellar vesicles, formed from asolectin, as described previously (Shiver et al., 1987) with several modifications (Peterson & Cramer, 1987). Vesicles containing 0.1 M KCl, 10 mM DMG, and 1 mM $CaCl_2$, pH 5.0, were diluted to 0.1 mg/mL in 0.1 M choline nitrate, 0.1 M $NaNO_3$, 10 mM DMG, and 2 mM $CaCl_2$ (20-mL total volume) at the desired pH. Potassium diffusion potentials of −135 mV were established by addition of valinomycin to 15 nM from a methanolic stock solution. Colicin E1 was added to 3 ng/mL for measurements at pH 4. In order to quantify ion channel-forming activities for mutants which showed much less activity than wild type, protein was added until an activity similar to that of wild type was obtained. Triton X-100 (0.1%) was added at the end of Cl^- efflux measurements to release all residual entrapped Cl^- and thus to assure that all vesicle samples contained the same amount of Cl^-. Cl^- efflux was measured with a Cl^--sensitive electrode (Orion 94-17B) and a double-junction reference calomel electrode (Orion 90-02).

Photoaffinity labeling of colicin bound to liposomes was carried out as described in Xu et al. (1988).

Association of Colicin with Membrane Vesicles. Wild-type or mutant colicin protein (10 μg, an amount needed for visualization on Coomassie-stained gels) was mixed with 100 μL of membrane vesicles (20 mg of phospholipids/mL) and 1 mL of 100 mM KNO_3, 10 mM DMG, and 0.5 mM $Ca(NO_3)_2$ at the desired pH. After 30 min at room temperature, 1 M $CaCl_2$ was added (final concentration 20 mM Ca^{2+}) and incubated for 5 min, after which the aggregated liposomes were sedimented in a microfuge (10 min). Protein was recovered from both precipitate and supernatant fractions by using 2:1 chloroform–methanol [5:1 (v/v)] to extract the lipids, followed by precipitation of the wild-type or mutant proteins with TCA (15% final concentration). The precipitate was dissolved in Laemmli (1970) sample buffer for SDS–PAGE and stained with Coomassie Blue R-250 dye (Shiver, 1987).

Planar lipid bilayer experiments were performed as described previously (Shiver et al., 1987) using "solvent-free" membranes (Montal, 1974) formed from purified asolectin (type IV-S, Sigma) washed in acetone (Kagawa & Racker, 1971). The membranes were formed across a 200-μm-diameter hole in a sheet of Teflon pretreated with squalene that separated two 3-mL compartments machined from Lucite. Colicin or thermolytic peptide (M_r 18 000) was added to one side (cis) of the voltage-clamped membrane. The solutions were magnetically stirred. The voltages indicated were those of the trans compartment, the cis compartment set equal to 0 mV. Reversal potentials were determined in 1 and 0.1 M NaCl solutions (with 3 mM $CaCl_2$, 5 mM DMG, and 0.1 mM EDTA) buffered to pH 4.0, and calomel electrodes were used to contact the solutions through saturated KCl junctions. Colicin peptide was added to the cis compartment containing 1 M NaCl buffer, and channels were opened by applying negative voltages to the trans side. It was routinely checked that switching the voltage from one side of the reversal potential to the other led to a reversal in the direction of current. The pH dependence was obtained as described previously, using a trans-negative potential of −40 mV (Shiver et al., 1987).

Results

Mutagenesis. *am*-509, Met-510, and *och*-512 mutations were obtained at frequencies of greater than 90% and approximately 60% and 5%, respectively, as determined by dot blot hybridization analysis. The *Eco*RI/*Xho*II pCol E1 fragment was not stable in M13tg130 with rearrangements frequently observed to occur shortly after *cea*. Therefore, growth of phage was limited to 5 h after infection of *E. coli*. Each mutation was confirmed by using both single- and double-stranded templates, except for Met-510 which was checked only by the latter technique, both before and after reconstitution of the *cea* gene as described previously.

Purification of Mutant Proteins. Full-length mutant colicin molecules were purified to near-homogeneity by CM-Biogel chromatography (Figure 3A). In some samples, a major contaminating band of protein was observed in variable quantities migrating before the intact colicin on SDS–PAGE gels (e.g., lane 5, Q-509). The M_r 18 000 thermolytic peptides were obtained with a purity comparable to wild type (e.g., Figure 3A, lane 6, peptide from the Ser-509 mutant). Normally, ~10 mg of purified wild-type protein was obtained for each liter of culture while approximately 4, 3, 6, and 1 mg of mutant colicin was recovered from the same culture volumes for Ser-, Gln-, and Leu-509 and Tyr-512, respectively. This indicates that the suppressor strains Su1, Su2, Su6, and SuC exhibited approximately 40, 30, 60, and 10% suppression of the mutant *am* or *och* stop codons, since (a) the relative levels of production were in accordance with the relative suppression levels reported previously for these strains (Gallucci & Garen, 1966; Miller & Albertini, 1983) and (b) the amount of truncated *am*-509 and *och*-512 proteins made in the nonsuppressor JM83 strain was much larger relative to wild type.

[1] Abbreviations: DMG, dimethylglutaric acid; EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

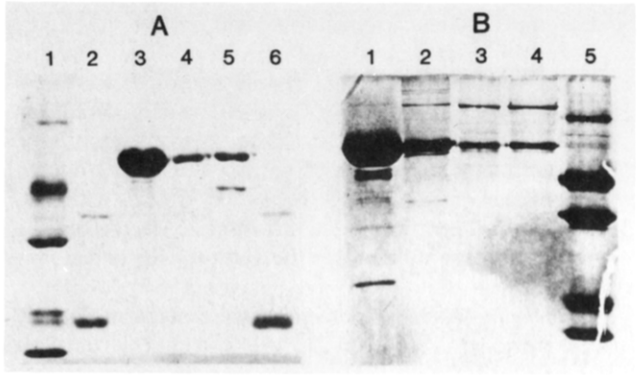

FIGURE 3: SDS–PAGE documentation of the purity of mutant proteins and M_r 18 000 thermolytic peptide derived from the mutants. (A) lane 1, molecular weight standards; lane 2, M_r 18 000 colicin E1 (10 μg); lane 3, colicin E1 (60 μg); lane 4, S-509 mutant colicin (18 μg); lane 5, Q-509 mutant colicin (16 μg); lane 6, the M_r 18 000 peptide of S-509 (9 μg). The minor band seen above the purified S-509 peptide is the M_r 40 000 thermolytic peptide fragment that also contains the channel-forming domain. The M_r 18 000 peptide of mutant Q-509 was also run on this gel and showed a single band, although it stained weakly (not shown). (B) Lane 1, colicin E1 (60 μg); lane 2, immune-purified colicin E1 (18 μg); lane 3, immune-purified *am*-509 and lane 4, *och*-512 (12 μg of each); lane 5, molecular weight standards (BSA, 68K; ovalbumin, 43K; carbonic anhydrase, 30K; β-lactoglobulin, 18K; lysozyme, 14K).

The truncated colicins could not be isolated in this manner, and an immunoaffinity column was utilized to purify these proteins. Figure 3B shows that two protein bands were obtained by this procedure for both wild-type and truncated colicins, one migrating at the same position as colicin and one corresponding to a size that a colicin dimer would exhibit (lanes 2–4). A Western blot showed that these "dimer" bands consisted of colicin E1, which is not surprising since the colicin antibody was used to purify the protein. The dimer was totally converted to monomer by treatment with 5% mercaptoethanol (data not shown). The dimer must then involve intermolecular disulfide cross-linking. The dimer band had a variable intensity relative to the monomer band (up to 50%). Faint dimer bands were also observed with overloaded gels of native colicin purified by CM-Biogel chromatography (Figure 3B, lane 1) but never to the level seen with the immune-purified colicin.

Cytotoxicity. The cytotoxic activities of each of the colicin point-mutant colicins were compared to the wild type (Table IA,B). Wild-type colicin purified by immunoaffinity chromatography had an activity comparable (50%) to that of colicin E1 purified by CM-Biogel chromatography (Table IA). Substitution of a neutral residue, Met and Tyr, respectively, for the Lys at position 510 or 512 either did not affect the activity or affected it only slightly (Table IB). The activity of the colicin mutated at position 509 depended on the particular substitution. The more polar substitutions displayed a higher activity: Ser-509 showed the highest activity (45% relative to the wild type); the Gln-509 and Leu-509 activities were 20 and 4%, respectively. These activities were representative of at least two independent protein preparations and were well-preserved by storage of the proteins at −70 °C, except for Gln-509 which lost activity with time of storage for both cytotoxicity and in vitro function. The reduced cytotoxicity shown by this mutant was not due to storage, because only newly prepared protein was assayed.

The truncated proteins obtained from the *am*-509 and *och*-512 mutations showed dramatic losses in cytotoxicity (Table IC). No activity was observed at 20 ng/mL, a concentration at which wild-type colicin killed more than 99% of the indicator strain. At 200 ng/mL, some killing by each truncated mutant was observed, from which it was determined that the upper limit of activity was 3% with respect to wild-type colicin. with the truncated mutant *am*-468, which is missing the last 55 amino acids (Shiver et al., 1987), no cytotoxic activity was observed at any concentration of pure or crude protein used, in assays of colony-forming ability or in spot tests which are a more sensitive monitor of cytotoxicity. This result also indicated that no detectable read-through translation of the *am* codon occurred.

Channel Activity of Mutant Colicins Assayed with Liposomes. Each of the mutants with single residue changes, Ser-509, Leu-509, Gln-509, Met-510, and Tyr-512, showed activities comparable to wild type for mediation of Cl^- efflux from vesicles at pH 4.0 (Table IIB), the approximate acidic pH value at which maximum activity is observed (Davidson et al., 1984a,b, 1985; Shiver et al., 1987). Gln-509 had the

Table I: Cytotoxicity of Colicin E1 Mutants Compared to the Wild Type

protein	quantity (ng/mL)	rel multiplicities[a]
(A) wild type		
colicin E1	20	1.0
colicin E1[b]	20	0.50 (n = 1)[c]
(B) point mutants		
Ser-509	20	0.45 ± 0.01 (n = 2)
Gln-509	20	0.20 ± 0.07 (n = 3)
Leu-509	20	0.04 ± 0.05 (n = 2)
Met-510	20	1.0 (n = 2)
Tyr-512	20	0.57 (n = 1)
(C) truncated mutants		
am-468[d]	20 or 200	0 (n = 2)
am-509	20	0 (n = 3)
am-509	200	<0.03[e] (n = 1)
och-512	20	0 (n = 3)
och-512	200	<0.03[e] (n = 1)

[a] Multiplicity, m, is defined as the hit number assuming exponential, $\exp(-m)$, inactivation of the cell population. Cytotoxicities are expressed as the ratio of the multiplicity to that of the wild-type colicin. The uncertainty is expressed as the standard deviation from the mean. The typical survival level of the cell population exposed to 20 ng/mL wild-type colicin is $\exp(-7)$. [b] Colicin E1 purified by immunoaffinity chromatography. The activities indicated above for the immune-purified truncated colicins, *am*-468, *am*-509, and *och*-512, are relative to activity for this protein. [c] "n" is the number of trials. [d] Properties of this mutant described in Shiver et al. (1987). [e] The multiplicity of wild type was 13 at 200 ng/mL cells, which is not in the linear part of a killing curve, so that 0.03 is a maximum estimate for the activity of the mutant.

Table II: Channel Activity of Colicin Mutants Assayed with Membrane Vesicles

protein	initial rate of Cl^- efflux (% wt)	ratio of initial rates of Cl^- efflux, ±valinomycin[a]
(A) wild type		
colicin E1[b]	100	4.2
colicin E1[c]	100	4.3
(B) point mutants		
Ser-509	185 ● 10 (n = 2)	3.3
Leu-509	81 ± 5 (n = 2)	6.1
Gln-509	55 ± 3 (n = 2)	3.6
Met-510	78 ± 5 (n = 2)	4.3
Tyr-512	93 ± 5 (n = 2)	5.3
(C) truncated mutants		
am-468	2 ± 1 (n = 4)	3.0
am-509	14 ± 1 (n = 3)	3.6
och-512	14 ± 1 (n = 3)	3.4

[a] Applied potential was −135 mV with valinomycin addition, pH 4.0. [b] An efflux rate of 7.6×10^4 Cl^- s^{-1} (E1 molecule)$^{-1}$ was measured for wild type using 3 ng/mL protein (0.1 mg/mL lipid) with the diffusion potential of −135 mV. [c] Wild-type colicin purified by immunoaffinity chromatography.

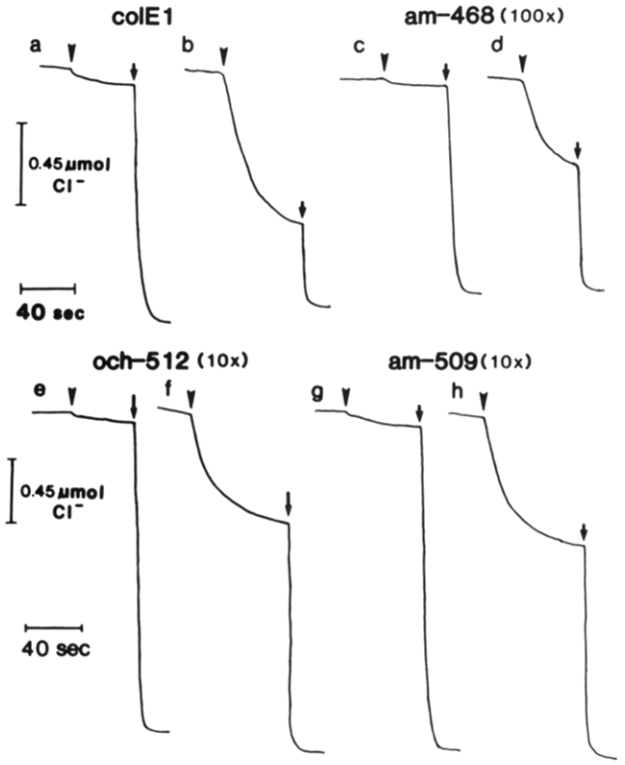

FIGURE 4: Relative channel activity of truncated mutant colicin. *am*-468, *am*-509, and *och*-512 protein-mediated Cl^- efflux from asolectin vesicles at pH 4.0 and an applied K^+ diffusion membrane potential of −135 mV. Colicin protein was added at the location of the first arrow on each trace, and Triton X-100 was added at the second arrow in order to release all encapsulated Cl^-. Colicin concentrations (nanograms per milliliter): wild type, a (0.3), b (3.0); *am*-468, c (30), d (300); *och*-512, e (3), f (30); *am*-509, g (3), h (30).

lowest activity, which decreased with time of storage at −70 °C, but was still 55% of wild-type activity when measured soon after purification. Immune-purified colicin E1 showed no loss in activity relative to colicin purified by CM-Biogel (Table IIA). However, immune-purified *am*-509 and *och*-512 were approximately 10-fold less active relative to wild-type colicin, while *am*-468 was less active by 2 orders of magnitude (Figure 4; Table IIC). Control experiments indicated that the small residual activity of the *am*-468 mutant may be affected by the nonspecific background.

All of the proteins showed between 3- and 6-fold enhancement of ionophoretic activity after addition of valinomycin to establish a potassium diffusion potential. The three truncated proteins were the least voltage dependent of all samples tested, both mutant and wild type, implying that some of the chloride efflux may be due to lysis of vesicles by these mutants.

Decreased Insertion of Truncated Colicin into Liposomes. The low activity in vivo and in vitro of the colicin molecules truncated at positions 509 and 512 was not due to an inability to associate with liposomes. As measured by cosedimentation, extraction of the protein from the liposomes, precipitation of that in the supernatant, and quantitation of the resulting protein concentration by SDS–PAGE, the truncated colicins bound to liposomes to the same extent as wild type to liposomes at pH 4.0 (Figure 5A). The use of trypsin as a probe for protein not inserted into the liposomes also did not discriminate between wild-type and the truncated proteins (data not shown). However, it was found that the specific labeling of the truncated proteins by an azido-labeled phospholipid photoaffinity probe added to the vesicles was smaller than that of the immune-purified wild type (Table III). Thus, the truncated

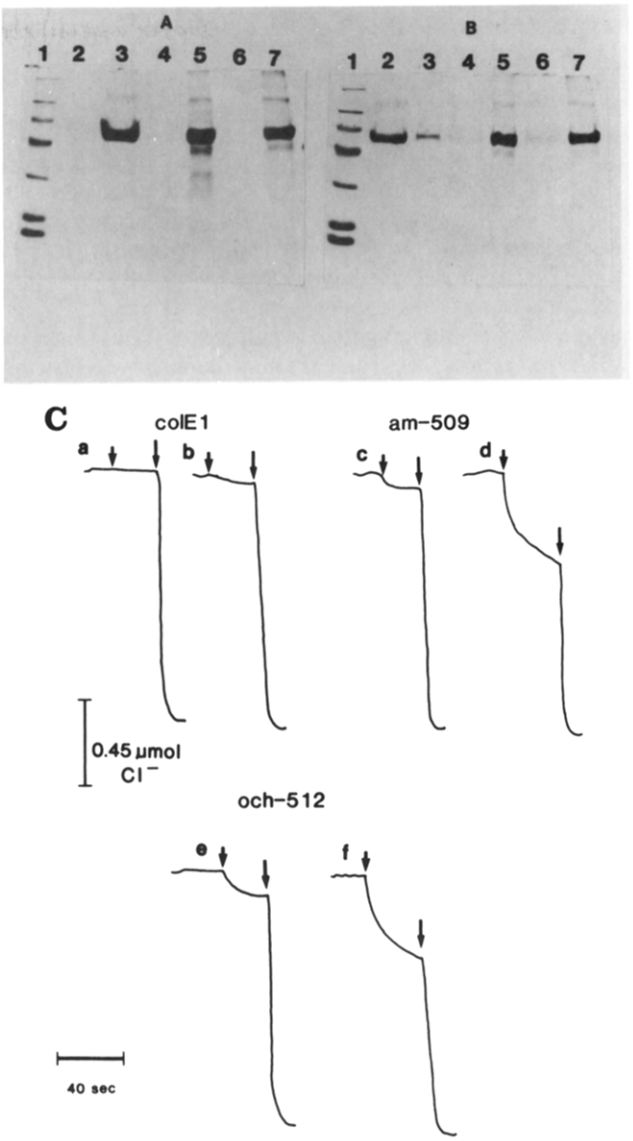

FIGURE 5: Binding of *och*-512 and *am*-509 protein to asolectin vesicles at (a) pH 4 and (B) pH 6 relative to wild-type colicin. Binding was measured by cosedimentation with liposomes. Protein bound to liposomes and remaining in supernatant was recovered as described under Materials and Methods. Lanes 1–7: standards (1), supernatant and sediment for wild type (2, 3), for *am*-509 (4, 5), and for *och*-512 (6, 7). (C) Relative activity for Cl^- efflux from liposomes of mutant truncated colicins compared to wild type at pH 6.0. Colicin protein and Triton X-100 (0.1%) were added at the location of the first and second arrows, respectively, on each trace. Colicin concentrations (nanograms per milliliter): wild type, a (6.0), b (1400); *am*-509, c (24), d (300); *och*-512, e (24), f (300).

Table III: Labeling of Colicin E1 and COOH-Terminal Lysine Mutants in Artificial Membranes by [^{14}C]Azidophospholipid[a]

protein	labeling (cpm)	rel binding (%) (pH 4.0)
E1 wild type	523 ± 78 (n = 2)	100
am-509[b]	169 ± 73 (n = 2)	32
och-512[b]	191 ± 78 (n = 2)	36

[a] 1-Myristoyl-2-[[12-(4-azido-2-nitrophenyl)amino]dodecanoyl]-*sn*-glycero-3-[^{14}C]phosphocholine. [b] The cpm values for *am*-509 and *och*-512 were corrected for slight differences in the amount of protein recovered as determined by scanning the polyacrylamide gels. These differences were less than 20%.

proteins did not insert into the membrane bilayer to the same extent as did the wild type.

Binding and Activity of Truncated Mutants Are Greater than Wild Type at pH 6.0. At pH 6.0, the truncated mutant

Table IV: Parameters for the Dependence, log (dI/dt) = a(pH) + b, of Channel Activity on pH[d]

peptide, 0.1 M NaCl	a	b	r^{2}[b]
wild type (D-509)	−(1.51 ± 0.20)[c]	8.7 ± 1.1	0.57
Leu-509	−(1.25 ± 0.20)	7.2 ± 1.0	0.60
Ser-509	−(1.32 ± 0.13)	6.8 ± 0.6	0.71
Gln-509	−(1.49 ± 0.10)	8.4 ± 0.5	0.80

[a] Data was fit and parameters determined by linear regression. dI/dt is given in units of 10^{-10} A/min. [b] The square of the regression coefficient. [c] Uncertainties are expressed as standard errors.

polypeptides bound to liposomes to a greater extent than the wild type (Figure 5B), which is known to bind weakly at pH values >5.0 (Davidson et al., 1984b, 1985). Whereas the channel activity of the truncated mutants was 10-fold less than the wild type at pH 4.0, its activity was greater than that of wild type at pH 6.0 (Figure 5C). An explanation for the increased association of the truncated proteins compared to the wild type at pH 6.0 would be that at this pH the mutants have a larger solvent-exposed hydrophobic surface than does the wild type. Support for such a structural change in the mutants was obtained by the finding that the truncated proteins partitioned into the detergent, Triton X-114, to a greater extent at pH 6.0 than did the wild type (Merrill et al., unpublished results).

Planar Bilayer Experiments. The qualitative features of all the mutants with single residue changes were the same as the wild type. Thus, the channels turned on for negative voltages and off for positive voltages, activity increased as the pH of the cis compartment was lowered (Bullock et al., 1983), and steady state was not reached when the pH was ≥4.0.

(A) pH Dependence. It has been hypothesized that the pH dependence of in vitro activity in the acidic pH region arises from titration of acidic residues in the channel-forming domain (Davidson et al., 1984a; Shiver et al., 1987). The relative pH dependence of activity was measured on planar membranes for three mutants at position 509, since this residue borders the hydrophobic domain, and an acidic amino acid residue is present in the corresponding or neighboring position in all other channel-forming colicins sequenced to date (Figure 1). log (dI/dt) (I = current) was plotted as a function of pH, and the data were fit by linear regression (Table IV). For all three mutants, the pH dependence ("a" in Table IV) was not significantly ($p < 0.05$) different from wild type as determined from a Student's t test on the means of the slope of the regression line. Kinetically, Gln-509 showed activity comparable (cf. value of "b" in Table IV) to wild type, whereas Leu-509 and Ser-509 were less active.

(B) Reversal Potentials. As reversal potentials of colicins vary with the lipid composition of the membrane (Raymond et al., 1985; Bullock & Cohen, 1986) and asolectin is a heterogeneous lipid mixture, reversal potentials were measured with the same batch of lipid mixture, as described previously (Shiver et al., 1987) and illustrated in Figure 6. The reversal potentials of the wild-type and the 509 mutants were the same at pH 4.0, with the effective permeability to Cl^- calculated according to the Goldman–Hodgkin–Katz equation (Hodgkin & Katz, 1949) as about 5.0 times that of Na^+. However, the mutations at positions 510 and 512, that substituted in each case a neutral residue for a positively charged Lys residue, caused a decrease in the Cl^-/Na^+ selectivity to 3.4 and 2.8 for the Lys-510 → Met and Lys-512 → Tyr mutants, respectively (Table V).

Truncated Peptides. *am*-509 and *och*-512 were tested on planar membranes at pH 4.0. They were similar to the wild-type protein in that they turned on for negative potentials and turned off for positive potentials. However, for *am*-509, the planar membranes invariably broke for currents in excess of 10^{-10} A. *och*-512 was similar, except currents could reach about 10^{-9} A before the membrane broke. While it was not possible to quantify the activity of these peptides because they were lytic, qualitatively these truncated mutants exhibited reduced kinetic activity.

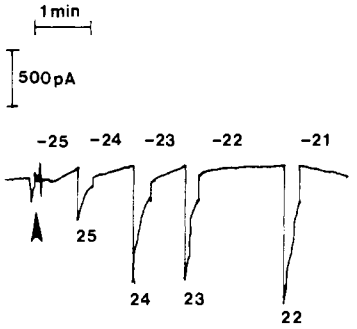

FIGURE 6: Determination of reversal potential. At the arrow, 0.66 μg/mL Tyr-512 mutant protein was added to the high-salt side of a voltage-clamped asolectin planar membrane separating 1 M NaCl, 10 mM DMG, 3 mM $CaCl_2$, and 0.1 mM EDTA, pH 4.0, from 0.1 M NaCl, 10 mM DMG, 3 mM $CaCl_2$, and 0.1 mM EDTA, pH 4.0. Voltages were applied as indicated (in millivolts). After each negative voltage, a positive potential pulse was applied to turn channels off. For this membrane, the direction of current flow reversed between −21 and −23 mV, and the reversal potential was determined as −22 mV.

Table V: Reversal Potentials and Ionic Selectivity for Wild-Type and Mutant Colicin Peptides

peptide	reversal potential (mV)	ion selectivity (P_{Cl^-}/P_{Na^+})[a]
(I) wild type	−31.0 ± 1.0 (n = 5)	4.9
(II) mutants at Asp-509		
Leu-509	−30.0 ± 0.7 (n = 5)	4.6
Ser-509	−31.7 ± 0.6 (n = 7)	5.1
Gln-509	−30.6 ± 0.6 (n = 4)	4.8
(III) mutants at Lys-510, Lys-512		
Met-510	−24.7 ± 4.0 (n = 13)	3.4
Tyr-512	−20.9 ± 4.4 (n = 8)	2.8

[a] Calculated according to the Goldman–Hodgkin–Katz voltage equation (Hodgkin & Katz, 1949). Reversal potentials were measured for asolectin bilayers separating 1 M NaCl, 10 mM DMG, 3 mM $CaCl_2$, and 0.1 mM EDTA, pH 4.0, from 0.1 M NaCl, 10 mM DMG, 3 mM $CaCl_2$, and 0.1 mM EDTA, pH 4.0.

DISCUSSION

Requirements for Activity at the COOH Terminus. (i) The loss of activity with *och*-512 shows that 1 or more of the last 11 residues is essential for activity. The report that thermolysin can cleave the last 10 residues and not impair function (Ohno-Iwashita et al., 1982) implies that Lys-512 is critical for function in the absence of the 10 COOH-terminal residues. The reduced activity observed for the truncated peptides is consistent with the observation that high concentrations of carboxypeptidase Y added to the cis side of a planar membrane abolished activity of open channels (Cleveland et al., 1983). (ii) Because the effect of the Lys-510 → Met or Lys-512 → Tyr mutations on cytotoxicity and in vitro channel function was small, the positive charge at position Lys-510 or -512 is not essential if the rest of the COOH-terminal chain is intact. This result resembles the finding that a decrease of one or two positive charges at the NH_2 terminus of the *E. coli* lipoprotein signal peptide has little effect on its insertion and cleavage (Lehnhardt et al., 1986).

Defect in the am-509 and och-512 Truncated Proteins. The initial hypothesis regarding the two Lys residues near the COOH terminus was that they were required for proper binding and insertion. A simple assay for binding at the pH 4.0 optimum, cosedimentation with liposomes, showed no difference between wild type and the two truncated mutants. This assay is not quantitative: It is difficult to measure a dissociation constant because the colicin concentration can be varied over only a small range, since at low concentrations unlabeled protein cannot be detected and at high concentrations the vesicles tend to precipitate. Therefore, insertion of the colicin molecule into liposomes was measured by reacting with a phospholipid photoaffinity probe with the azido group positioned at the end of dimyristoyl (14-carbon) phospholipid added to the liposomes. The truncated mutants were labeled to the extent of 30–35% of wild type. The simplest interpretation of these data is that the one-third efficiency of labeling represents one-third as much mass of the truncated molecules, relative to the wild type, inserted into the bilayer center. Thus, the loss of 11 COOH-terminal residues, including Lys-512, or 14 COOH-terminal residues, including both Lys-510 and Lys-512, results in the bound colicin inserting less completely into the membrane.

Altered Conformation at pH 6.0. It is known that both binding and activity of wild-type colicin and channel-forming peptide have a pronounced acidic pH dependence, so that binding and activity are very much greater at pH 4.0 than at pH 6.0. Although the truncated mutant has a binding insertion defect at pH 4.0, unlike wild type its association with liposomes is similar at pH 6 and pH 4. It is proposed that the explanation for this inversion is that the wild type requires an acidic pH to break a salt bridge, allowing the COOH-terminal hydrophobic domain with a high membrane affinity to be unmasked, as documented for diphtheria toxin (Sandvig & Olsnes, 1981) and the hemagglutinin glycoprotein of influenza virus (Wiley & Skehel, 1987). However, the hydrophobic domain is already exposed at pH 6 in the truncated mutants. In support of this hypothesis, at pH 6 the truncated colicins partition into nonionic detergent (Triton X-114) to a significant extent, whereas the wild type does not (Merrill et al., unpublished results). In addition, the dimer form observed particularly with the truncated mutants is probably an indicator of this exposed hydrophobic domain, since the dimers arise from intermolecular disulfide formation. The colicin has only one cysteine at position 505 which is near the end of the hydrophobic domain. This cysteine is normally shielded from solvent in the wild-type molecule (Bishop et al., 1986), so that intermolecular disulfide-linked dimers are only formed in the wild type when it is denatured.

Effect of Lys-510 and Lys-512 on Ion Selectivity of the Channel. These two Lys residues are expected to reside in the polar phase (Davidson et al., 1984a) on the cis side of the inserted channel in the polar phase since the channel activity (Cleveland et al., 1983) and channel protein size are affected by *cis*-carboxypeptidase Y (Xu et al., 1988). Mutation of either of these residues to a neutral residue, Lys-510 → Met or Lys-512 → Tyr, has the qualitative effect that would be anticipated from simple electrostatic considerations; i.e., the selectivity or affinity of the channel for anions is decreased relative to that for cations. Thus, these positively charged residues are believed to be situated at the end of the hydrophobic channel anchor, at the "mouth" of the channel lumen, where they concentrate anions (Figure 7). Two residues within the hydrophobic anchor domain, Thr-501 and Gly-502, have also been found to affect the channel selectivity (Shirabe et al., 1988). The insensitivity of the reversal potential to mutational changes at Asp-509 demonstrates that this residue, although it is a neighbor of Lys-510, is not important for determining the selectivity of open channels. Asp-509 is the first residue on the COOH side of the 35-residue hydrophobic domain, and it is probably situated at the aqueous interface of the membrane bilayer rather than in the polar medium forming the "mouth" of the channel.

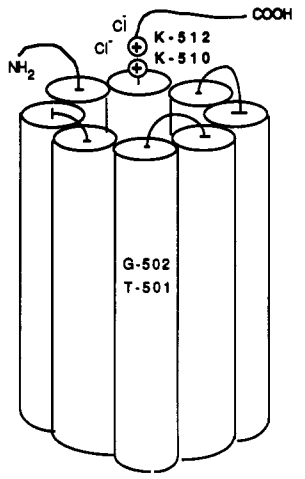

FIGURE 7: Representation of the colicin E1 channel showing Lys-510 and Lys-512 at the mouth of the channel where it is inferred that they influence the ion selectivity of the channel, the Lys residues increasing the affinity for anions (e.g., Cl^-, as shown). The other residues in the channel domain known to affect the ion selectivity, Thr-501 and Gly-502 (Shirabe et al., 1988), are also shown. The model is not intended to make any statement about the number of trans-membrane peptides but does imply a monomeric channel as discussed in Peterson et al. (1987).

Role of Asp-509 or of Several Carboxylic Residues in the Acidic pH Dependence. Asp-509 is one of five conserved acidic amino acids, along with residues 373, 388, 408, and 468, in the channel-forming domain starting at number 350 in the aligned sequences (Figure 1). Although an acidic residue is present at position 509 in colicins E1, Ia, Ib, A, and B (Table I), titration of Asp-509 alone is not a major factor in the pH dependence of colicin E1 activity with artificial membrane vesicles. Differences in the means of the slope of the regression line fitting the titration data were not statistically different. However, the mean slope of the regression line for wild type on the batch of asolectin in these studies (performed in 0.1 M NaCl) was 1.51. This slope was 1.48 (in 0.1 M NaCl) and 1.52 (in 1 M NaCl) when performed on a different batch of asolectin (Shiver et al., 1987). Thus, it would appear that in spite of the documented quantitative variabilities in the activity and voltage dependence of colicin E1 (Bullock et al., 1983; Bishop et al., 1986) the pH dependence of the protein can be quantitatively determined. If this is the case, then the smaller means of the regression lines for Leu-509 and Ser-509 indicate that protonation of the Asp at position 509 promotes channel formation. However, the small effect at Asp-509, as well as Glu-468 (Shiver et al., 1987), argues that protonation of several residues is responsible for the large dependence of binding and activity on pH values in the acidic region. In addition, since the order of the means for the mutants (Leu < Ser < Gln) was the same as for substitutions for glutamate at position 468 (Shiver et al., 1987), the possibility cannot be ruled out that any differences are due to properties other than titration of the substituted residues, such as side chain hydrophobicity.

Question about the Concept of an Independent Channel Domain. Isolation of peptides from the COOH-terminal third

of the molecule with appropriate channel activity (Dankert et al., 1982), a receptor binding peptide from the center of the molecule (Brunden et al., 1984), similar kinds of peptide isolation in the other E-type colicins and other channel colicins, and diphtheria toxin [reviewed in Cramer et al. (1983) and Davidson et al. (1984)] have led to the concept of an independent domain for receptor binding and channel formation in colicin E1 and the other channel-forming colicins. The low cytotoxic activity of the Leu-509 mutant (Table I) suggests the possibility that the COOH terminus is also needed in some way for translocation across the cell envelope since the in vitro activity of this mutant is not greatly altered (Table II). A similar situation has been observed for a mutation at Gly-439, also in the channel-forming domain (Shirabe et al., 1988).

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