Intramembrane Helix-Helix Interactions as the Basis of Inhibition of the Colicin E1 Ion Channel by Its Immunity Protein*

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It had previously been hypothesized that the ability of a small number of immunity protein molecules in the cytoplasmic membrane to confer protection against the lethal effects of a channel-forming colicin involves a complex stabilized by electrostatic or polar interactions between immunity protein, the colicin channel, and specific sites on the cytoplasmic membrane surface defined by the presence of the toi gene translocation proteins.

The hypothesis was tested (a) by constructing a hybrid colicin molecule, IaE1, containing the E1 channel domain, and the translocation and receptor domains of Ia, and (b) by altering charged residues in all peripheral regions of the immunity protein to neutral residues. It was concluded that the specificity of immunity protejue requires neither specific translocation proteins, nor a specific arrangement of charged residues of the immunity protein. (c) In addition, by making 65 site-directed mutations, “immunity by-pass” mutants were found at five different loci, Aa474, Ser477, His440, Phe443, and Gly444, on two proposed membrane-spanning helices of the open colicin channel, one hydrophobic (A471-A488) and one amphiphilic (V441-W460). The mutants in the hydrophobic helix showed a larger bypass effect. The “bypass” phenotype could be assayed by (i) cytotoxicity and (ii) K+ efflux in imm− cells caused by a bypass mutant but not wild-type colicin. It is concluded that the immunity protein exerts its specific effect through rapid lateral diffusion in the cytoplasmic membrane and helix-helix recognition and interaction with at least one hydrophobic and one amphiphilic trans-membrane helix of the colicin channel. Interaction with the amphiphilic helix implies that the immunity protein can react with the channel in the open state.

The channel-forming colicins are toxin-like molecules that exert their cytotoxic effect on susceptible Escherichia coli cells by forming a highly conductive ion channel in the cytoplasmic membrane that depolarizes the membrane (Gould and Cramer, 1977). The depolarizing channel results in inhibition of active transport, depletion of intracellular ATP and K+ levels (Phillips and Cramer, 1973; Kopecky et al., 1975), and subsequent cell death. The channel-forming colicin molecule, like other colicins and toxins, is divided into functional domains. The COOH-terminal third of the colicin E1 channel molecule possesses channel activity whose in vitro properties resemble those of the whole colicin molecule (Dankert et al., 1982; Bullock et al., 1983). The structure of the membrane-embedded voltage-gated ion channel includes (i) a hydrophobic segment identified as a helical hairpin in the structure of the soluble colicin A peptide (Parker et al., 1992) that may (Song et al., 1991) or may not (Parker et al., 1992) be an intrinsic part of the channel lumen; (ii) it also contains an amphiphilic helical hairpin whose voltage-dependent reversible insertion into the membrane may be responsible for channel gating (Merrill and Cramer, 1980). The COOH-terminal channel domain also contains the region of interaction with the protective immunity protein (Bishop et al., 1985), coded by the 4.2 megadalton ColE1 plasmid.

Immunity protein produced in association with the plasmid-encoded colicins protects the cell against the lethal action of the colicin with which it is produced and against exogenous homologous colicin produced and extruded into the medium by other cells (Bazaral et al., 1968). This protective mechanism is a unique aspect of the interaction of colicins on membranes, compared with other toxin and toxin-like molecules. Furthermore, because the immunity proteins of the channel-forming colicins E1 (Bishop et al., 1985), Ia, Ib (Mankovich et al., 1986), and A (Geli et al., 1988) are hydrophobic proteins and are known to be localized in the E. coli cytoplasmic membrane, the protective interaction between the proteins occurs within the two-dimensional space of this membrane. The orientation and topography of the immunity proteins of colicins A (Geli et al., 1989b) and E1 (Song and Cramer, 1991) have been determined. The colicin E1 immunity protein spans the membrane bilayer three times, with NH2 and COOH termini on its cytoplasmic and periplasmic sides, respectively (Song and Cramer, 1991).

Protection by the immunity proteins for colicins E2 (Schaller and Nomura, 1976) and E3 (Sidikaro and Nomura, 1974; Jakes, 1982) against the degradative effects of these colicins could be demonstrated in vitro in aqueous solution by the ability of the respective immunity proteins to react directly with the COOH-terminal domains of the respective colicins to prevent the inhibition of DNA and protein synthesis caused by colicins E2 and E3. In the case of the channel-forming colicins A and E1, it has thus far not been possible to demonstrate in vitro inhibition by immunity protein of colicin function (Geli et al., 1989b; Shirabe et al., 1993).

The problem of the mechanism of action of immunity protein on the channel-forming colicins is interesting because of: (i) the uniqueness of this mechanism; (ii) the ability of a small number of immunity protein molecules (≈10^5-10^6) located in the cytoplasmic membrane to prevent the formation by colicin of a functional and lethal channel in that membrane; (iii) the high specificity of the immunity protein produced in conjunction with each colicin for that colicin, in spite of the high degree of sequence identity between some of

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the channel domains (58% between colicin El and the closely related colicins Ia or Ib).

It was previously proposed that the ability of a small number of immunity protein molecules in the cytoplasmic membrane to exert its specific inhibitory reaction could result from a ternary protein reaction involving (i) the immunity protein, (ii) the channel domain of the colicin, and (iii) the tol or ton translocation proteins at unique sites in the membrane associated with the attachment of the latter (Song and Cramer, 1991). This hypothesis has been tested in the present study. It was found to be incorrect, but evidence has been obtained for direct interaction between the hydrophobic trans-membrane helices of the colicin El immunity protein and the trans-membrane helices of the channel domain of this colicin.

**MATERIALS AND METHODS**

*Strains and Plasmids—E. coli JM101 was used as the indicator strain for assay of cytotoxicity. JM101 was also transformed with pSKHY (+) imm to test immunity bypass function and with pSKHY (-) for comparison of cytotoxicity. The elements for immunity bypass in colicin El was cloned downstream from the 5'-end of the iia gene. pSKHY was also submitted to SacII digestion, generating the fragment encoding the colicin El channel domain and iia gene. pSKHY was also used for preparation of single strand DNA. The pBluescript I1 SK(+) (Stratagene, La Jolla, CA) was used for cloning, mutagenesis, and overexpression of the fragment. The plasmid for preparation of single strand DNA. The pBluescript I1 SK(+) (Stratagene, La Jolla, CA) was used for cloning, mutagenesis, and overexpression of the fragment.

**Table 1**

| Amino Acid Sequence | 5' | 3' |
|---------------------|----|----|
| E.coli | A | B |
| Ia | C | D |
| Ib | E | F |

*Arrow indicates trypsin cleavage site in colicin El that generates 19 residue COOH-terminal peptide (Dankert et al., 1982).*

*Residues of colicin El that are identical or quasi-identical to those of colicin Ia or Ib.

Sites of immunity bypass in colicin El channel domain. Numbers refer to sequence of colicin El.

**Overexpression and Purification of Colicin IaEl**—Because it is likely that the colicin Ia gene is also under control of the SOS system, E. coli strain IT3661 which is lexA−, was chosen for constitutive overexpression of hybrid colicin IaEl, and the region containing the regulatory elements upstream of the colicin Ia gene was left intact and included in the fragment for cloning. IT3661 was then transformed with pSKIaEl. This cell strain was incubated for 16 h in 2× YT medium before testing the cell lysate. A protein with M,~66,000, approximately the calculated molecular mass of the hybrid colicin, was shown by SDS-PAGE to be overexpressed.

**Site-directed Mutagenesis**—The method of Kunkel (1985) was followed and utilized strains MV1190 and CJ236 for construction of plasmids pSKIaEl. This cell strain was incubated for 2× YT medium before testing the cell lysate. A protein with M,~66,000, approximately the calculated molecular mass of the hybrid colicin, was shown by SDS-PAGE to be overexpressed.
**Colicin E1-Imm Protein Interaction**

**FIG. 1.** A, construction of the recombinant plasmid, pSKIaE1, encoding the translocation (T) and receptor binding (R) domains of colicin Ia and the channel-forming domain (C) of colicin E1 in a hybrid colicin IaE1. The plasmid also contains the gene for colicin E1 immunity (imm) protein. B, the DNA and amino acid sequences in the region of the SacII restriction site linking the plasmids pSKIa(−) (containing two copies of cia and cii genes) and pSKHY.

Val<sup>141</sup> → Cys, Asp<sup>146</sup> → Cys, Ser<sup>177</sup> → Ala, Ser<sup>177</sup> → Gln, Ser<sup>177</sup> → Lys, Val<sup>146</sup> → Arg, Thr<sup>146</sup> → Arg, and Lys<sup>310</sup> → Cys-Lys<sup>312</sup> → Asn in colicin E1 and Arg<sup>1</sup> → Gln, Asp<sup>97</sup> → Asn-Lys<sup>98</sup> → Gln, Arg<sup>29</sup> → Met-Lys<sup>34</sup> → Asn, Lys<sup>111</sup> → Met, and Asp<sup>177</sup> → Asn-Lys<sup>118</sup> → Gln-Lys<sup>118</sup> → Met in the immunity protein, were made as part of the present study.

**Oligonucleotide Synthesis—**All oligonucleotides used for the mutagenesis were constructed in the Laboratory for Macromolecular Structure at Purdue University. Mutants were made by using a separate oligonucleotide for each desired mutation. 19- and 30-mers were used for introduction of single base mismatches at one or two sites, and four sites, respectively. Oligonucleotides were directly used for mutagenesis.

**DNA Sequencing—**DNA sequencing for the screening of mutants was done in the Laboratory for Macromolecular Structure at Purdue University. Mutants were made by using a separate oligonucleotide for each desired mutation. 19- and 30-mers were used for introduction of single base mismatches at one or two sites, and four sites, respectively. Oligonucleotides were directly used for mutagenesis.

**Cytotoxicity Assay—**The cell strains, JM101 and JM101/pSK, used as the colicin E1 sensitive indicator, and JM101/pSKHY(−), used as imm<sup>−</sup> bypass indicator, were grown overnight to early log phase and 100 μl was spread on 2 X YT or 2 X YT (+75 μg/ml ampicillin) plates. From a 10 μg/ml stock solution of the colicin, 5-fold serial dilutions to a minimum colicin concentration of 3.2 or 16 ng/ml were assayed. The range between 10 μg/ml and 16 ng/ml was chosen because it was found that all mutants showed observable activities within this range. 20 μl from each dilution was spotted directly on a plate that had been overlaid with the indicator cells. Plates were incubated at 37 °C overnight (8–12 h), and the lowest dilution of mutant colicin that gave clearing of a spotted site was used to calculate specific activity.

**Purification of Wild Type and Mutant Colicin E1—**The DM1187 lexA<sup>+</sup> strain, which like the IT3661 strain produced colicin E1 constitutively, was used for overexpression of the cec gene (Song et al., 1991). DM1187/pSKEI(−) cells were grown overnight, harvested, resuspended in 50 mM sodium phosphate buffer, pH 7.0, with 2 mM EDTA, and broken in a French Press. The lysates was sedimanted at 10,000 × g for 15 min, the supernatants loaded on a Mono-S fast protein liquid chromatography column at 6 °C and eluted with an NaCl gradient. The purity of the resultant colicin E1 was routinely examined on an SDS-PAGE Phast gel system (Pharmacia LKB Biotechnology Inc.).

**Protein Assay—**Modified Lowry (Pierce Chemical Co. 23240X) and BCA assays (Pierce 232356) were used to determine protein concentration. Purified colicin was extensively dialyzed against distilled water and lyophilized to make a 2.0 mg/ml solution as an internal standard.

**Measurement of in Vivo K<sup>+</sup> Efflux—**Cells transformed with pSKHY (imm<sup>+</sup>) or with the empty vector pSK (imm<sup>−</sup>) as a control were inoculated into 2 X YT (+50 μg/ml ampicillin) medium. At early log phase, the cells were harvested, washed once with 0.4% glycerol, 0.5 mM KCl, 50 mM sodium phosphate, pH 7.0, resuspended in the same buffer at a cell density of 1.0 × 10<sup>9</sup>/ml, and incubated with...
stirring at 37 °C for about 20 min, to allow the cells to reaccumulate potassium. $K^+$ efflux caused by colicin E1 was measured with a potassium selective electrode (Orion model 93-14) immersed in 10 ml of the stirred cell suspension (Phillips and Cramer, 1973). Cytotoxicity of colicin added to the cell suspension was assayed from the same sample used for measurement of $K^+$ efflux. The colicin multiplicity ($m$) was determined from the survival ($S/S_0$) level, $m = \ln(S_0/S)$, of cells used in the $K^+$ efflux assay.

RESULTS

In order to explain the specificity of the colicin E1 immunity protein, and the ability of a small number ($10^2 - 10^3$) of immunity protein molecules to neutralize an inserted colicin channel molecule in all regions of the cell surface, it was proposed (a) that the immunity protein interacts in a ternary complex with the colicin channel domain and with the tolA and/or tolQ translocation proteins of the cell envelope that are localized in the cytoplasmic membrane (Song and Cramer, 1991). (b) It was also proposed that the extrinsic charged or polar segments of the immunity protein (Fig. 2A) would interact with extrinsic complementary regions of the channel domains and translocation proteins (Fig. 2B) in order to provide the specificity of the interaction. Given the membrane topography and orientation of the immunity protein (Song and Cramer, 1991) and the orientation of the tolA-tolQ proteins (Levengood and Webster, 1989; Song and Cramer, 1991), it was hypothesized that (i) the periplasmic L1 and COOH-terminal T2 regions of the immunity protein might interact with the large COOH-terminal domain of tolA and/or the periplasmic NH$_2$ terminus and exposed loops of tolQ, or (ii) the highly charged cytoplasmic segment L2, as well as the NH$_2$-terminal T1 of the immunity protein (Fig. 2A) might interact with the NH$_2$-terminal region of the tolA protein and/or the cytoplasmic loop and COOH terminus of the tolQ protein.

The requirements of the tol proteins and of extrinsic charges in the peripheral regions of the immunity protein for its function were tested as follows.

Construction and Characterization of Hybrid Colicin IaE1—

The requirement of a particular array of tol translocation proteins, or any individual tol protein, for expression of the function of colicin E1 immunity protein, was tested by making a hybrid colicin molecule, IaE1, whose translocation requires the tonB (Postle, 1990) instead of the tol gene products.

A protein with approximately the same electrophoretic mobility, $M_r 66,000$, of the hybrid colicin IaE1, containing the translocation and receptor domains of colicin Ia and the channel domain of colicin E1 (predicted molecular weight, 67,861), was overexpressed (cf., "Materials and Methods") in *E. coli* strain IT3661 lexA$^-$ and purified (Fig. 3, lane 3). It can be seen that colicin E1, with a molecular weight of 57,279 (Yamada et al., 1982), runs on the gel (Fig. 3, lane 5) with a substantially smaller $M_r$ value compared to the hybrid colicin in lane 3 (Fig. 3). The hybrid protein was purified by the same procedure used for wild type colicin E1, using a fast protein liquid chromatography Mono-S cation-exchange column, indicating that the protein has a similar chromatographic behavior. Western blotting showed that the protein could be recognized by the antibody against the colicin E1 COOH-

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**Fig. 2.** Topographical models. A, the colicin E1 immunity protein, with trans-membrane helices labeled H1-H3, and the peripheral regions NH$_2$-terminal T1, loop L1, loop L2, and COOH-terminal region T2; charged residues are circled, and residues mutated in the present work marked by an arrow. B, model of the channel domain of colicin E1 in the "open" state; region upstream of Lys$^{380}$ is accessible to several proteases, but probably also contains a membrane-bound segment (Zhang and Cramer, 1992).
toward the tolA-deficient strain tolA592, whereas colicin El showed no activity at a concentration of 10 pg/ml (Table IIA), showing the absence of the colicin El translocation domain in the hybrid colicin.

**TolA Translocation Protein Is Not Required for Action of Colicin E1 Immunity Protein**—The tolA-tolQ, and tonB proteins (Postle, 1990) are required for the translocation and in vivo activity of colicin E1 or Ia, respectively, and are anchored in the cytoplasmic membrane. The cell strain BW8983 (tonB<sup>-</sup>) in which the tolA and tonB translocation systems are functional and absent, respectively, is sensitive to colicin E1, but not to the hybrid IaE1 that contains the translocation and receptor binding domains for colicin Ia (Table IIB), confirming that the colicins IaE1 and E1 require the tonB and tolA systems, respectively, for translocation and activity. TolA592, in which the tonB and tolA systems are functional and non-functional, respectively, is sensitive to IaE1, but not to E1, again showing that colicins IaE1 and E1 require the tonB and tolA systems for activity. A slightly reduced cytotoxicity of the IaE1 hybrid toward the tolA<sup>-</sup> tonB<sup>-</sup> strain compared to the activity of colicin E1 toward the tolA<sup>+</sup> tonB<sup>-</sup> strain (Table IIA versus IIB) may be due to a slightly lower translocation efficiency of the colicin E1 channel domain by the Ia receptor and translocation domains.

The strain TolA592/pSKHY, in which colicin E1 immunity protein is present (imm<sup>+</sup>), is sensitive to neither colicin E1 nor colicin IaE1 (Table IIC), because colicin IaE1 is translocated by the tonB system, but its action was inhibited by E1 immunity protein in tolA592/pSKHY, it was concluded that structural interaction between colicin E1 and its immunity protein does not require the tolA translocation protein.

**Role of tolQ**—Some evidence was obtained for the lack of involvement of the tolQ protein in the action of immunity. Colicin E1 was completely inactive in a tolQ<sup>-</sup> strain (Table IID; Sun et al., 1986; Webster, 1991). The IaE1 hybrid possessed a weak activity, observed at concentrations ≥2.0 µg/ml (Table IID). However, this activity was not expressed in the presence of immunity protein (Table IIE), indicating that the tolQ protein was not involved in the expression of immunity.

**Modification of Peripheral Domains of Immunity Protein Does Not Affect Activity**—The colicin E1 immunity protein has been shown to span the membrane bilayer three times, with NH<sub>2</sub> and COOH termini on its cytoplasmic and periplasmic sides (Fig. 2A). It was hypothesized that the specificity of the immunity protein might reside in its peripheral segments containing charged or polar residues (Song and Cramer, 1991). This hypothesis was tested by site-directed mutagenesis of charges in all of the peripheral segments of the immunity protein. Charged residues in all peripheral segments including the terminal (T) and intermediate loop (L) regions, T1, L1, L2, and T2 (Fig. 2A) were changed to neutral residues as follows (Table III): Arg<sup>+</sup> → Glu<sup>-</sup>, in peripheral segment, T1, of the immunity protein (Fig. 2A), Asp<sup>7</sup>-Lys<sup>26</sup> → Asn<sup>-</sup>-Gln<sup>-</sup>, in segment L1, Arg<sup>25</sup>-Lys<sup>74</sup> → Met<sup>-</sup>-Asn<sup>-</sup>, in L2, Lys<sup>111</sup> → Met<sup>-</sup> in T2, and Asp<sup>7</sup>-Lys<sup>26</sup>, Lys<sup>111</sup> → Asn<sup>-</sup>-Gln<sup>-</sup>-Met<sup>-</sup> in segments L1, T2). The configuration of charged or polar residues in COOH-terminal segment T2 was of particular interest. This region had been implicated from mutagenesis studies to be important for the activity of the colicin A immunity protein (Geli et al., 1986, 1989), although it was previously noted that the structural requirements of the 113 residue E1 immunity protein having three membrane spanning α-helices could be very different from those of the 178 residue colicin A imm protein (Song and Cramer, 1991). Changing the only positively charged residue, Lys<sup>111</sup>, in the colicin E1 immunity protein COOH terminus (segment T2) to a neutral Met had no effect on the function of the imm protein, both in a single point mutant and in a triple mutant that also included changing 2 basic residues, Asp<sup>27</sup> and Lys<sup>26</sup>, in the L1 region to neutral residues Asn and Gln (Table III). The 2 positively charged residues Arg<sup>25</sup>-Lys<sup>74</sup> in the highly charged cytoplasmic loop L2 were mutated to neutral Met<sup>-</sup> and Asn<sup>-</sup>, but this double mutant also showed full activity. Removal of the single charged residue, Arg<sup>25</sup>, at the cytoplasmically located NH<sub>2</sub> terminus was also without effect. Thus, it was concluded that a particular arrangement of charged or polar residues in the peripheral segments of the colicin E1 immunity protein was not critical for activity.

**Immunity “Bypass” Mutants in the Colicin E1 Channel**

![Fig. 3. SDS-PAGE of purified colicins IaE1 and E1. Lanes: 1 and 4, molecular weight markers, 2, whole cell lysate of IT3661/pSKIAE1; 3, purified colicin IA; 5, purified colicin E1. Molecular mass (in parentheses) markers are: phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa).](image-url)
Immunity (imm)/protein and site-specific mutants: 1.0 0.2 0.04 80 16 3.2

Immunity (imm)/protein and site-specific mutants

| Immunity (imm)/protein and site-specific mutants | Colicin E1 conc.* | Immm*/Immm* (efficacy of bypass)* |
|-----------------------------------------------|------------------|----------------------------------|
| imm*                                          | mg/ml            | ng/ml                            |
| imm*                                          | -                | +                                |
| Arg7 → Gln (T1)*                              | -                | -                                |
| Asp7 → Asn-Lys*                               | -                | -                                |
| Arg7 → Met-Lys*                               | -                | -                                |
| Lys11 → Met (T2)                              | -                | -                                |
| Asp7 → Asn-Lys*                               | -                | -                                |
| → Met (L1,T2)                                 | -                | -                                |

TABLE IV

Cytotoxicity of site-directed mutants of colicin E1 localized in helices H2, H3 (Fig. 2B) in the presence of the immunity protein

| Colicins* Indicator strain | Colicin conc. | Immm*/Immm* (efficacy of bypass)* |
|----------------------------|---------------|----------------------------------|
| Wild-type imm*             | 100 100 10 0 2 0 0 0.01 0.001 0.003 | ImmM*/ImmM* (efficacy of bypass)* |
| Wild-type imm*             | 100 100 10 0 2 0 0 0.01 0.001 0.003 | ImmM*/ImmM* (efficacy of bypass)* |
| His460 → Arg imm*          | 100 100 10 0 2 0 0 0.01 0.001 0.003 | ImmM*/ImmM* (efficacy of bypass)* |
| Phe443 → Lys imm*          | 100 100 10 0 2 0 0 0.01 0.001 0.003 | ImmM*/ImmM* (efficacy of bypass)* |
| Gly444 → Lys imm*          | 100 100 10 0 2 0 0 0.01 0.001 0.003 | ImmM*/ImmM* (efficacy of bypass)* |
| Ala474 → Glu imm*          | 100 100 10 0 2 0 0 0.01 0.001 0.003 | ImmM*/ImmM* (efficacy of bypass)* |
| Ser477 → Arg imm*          | 100 100 10 0 2 0 0 0.01 0.001 0.003 | ImmM*/ImmM* (efficacy of bypass)* |
| Lys477 → imm*              | 100 100 10 0 2 0 0 0.01 0.001 0.003 | ImmM*/ImmM* (efficacy of bypass)* |

- Table III

**Domains**—The approximate topography of the hydrophobic helical hairpin of the colicin E1 channel domain (helices H3-H4, Fig. 2B) was previously mapped by the relative cytotoxicity pattern within a large set of site-directed substitutions of charged for non-polar residues at 26 different positions in the hydrophobic region of the channel domain (Song et al., 1991). These mutants showed diminished cytotoxic activity toward sensitive imm* cells compared to wild-type colicin E1.

**Discussion**

In a previous study of the topography and orientation of the colicin E1 immunity protein in the cytoplasmic membrane, the following mechanism for specific interaction of immunity protein with colicin channel domain was proposed (Song and Cramer, 1991): (a) immunity protein, present in a low copy number (<10) per cell, diffuses laterally in the membrane until the specific sites of interaction including the colicin channel domain are found; (b) the specific sites are the positions of entry into the membrane of the imported colicin channel, which would be defined by sites of apposition with the tolA and/or tolQ translocation proteins that would

TABLE III

Effect on function of immunity protein of mutagenesis of the charged residues to neutral residues in the peripheral segments of the immunity protein (Fig. 2A).

| Immunity (imm)/protein and site-specific mutants | Colicin E1 conc.* |
|------------------------------------------------|------------------|
| imm*                                          | mg/ml            |
| imm*                                          | ng/ml            |
| Arg7 → Glu (T1)*                              | -                |
| Asp7 → Asn-Lys*                               | -                |
| Arg7 → Met-Lys*                               | -                |
| Lys11 → Met (T2)                              | -                |
| Asp7 → Asn-Lys*                               | -                |
| → Met (L1,T2)                                 | -                |

- All mutant and wild type immunity protein carrying plasmids, and pSK (amp') cloning vector (as imm* control to monitor colicin activity) were introduced into strain JM101. Colicin E1 activity was assayed on ammp' 2×YT plates. Assays were performed twice. + indicates clear zone and lack of activity of immunity protein; - signifies no indication of activity associated with wild-type protein; nt, not tested.

- Brackets denote peripheral regions of imm protein shown in Fig. 2A; this mutant and all those in this column have the imm* genotype.

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Cytotoxicity of site-directed mutants of colicin E1 localized in helices H2, H3 (Fig. 2B) in the presence of the immunity protein

- Table IV

**Tables**

| Colicins* Indicator strain | Colicin conc. | Immm*/Immm* (efficacy of bypass)* |
|---------------------------|---------------|----------------------------------|
| Wild-type imm*            | 100 100 10 0 2 0 0 0.01 0.001 0.003 | ImmM*/ImmM* (efficacy of bypass)* |
| Wild-type imm*            | 100 100 10 0 2 0 0 0.01 0.001 0.003 | ImmM*/ImmM* (efficacy of bypass)* |
| His460 → Arg imm*         | 100 100 10 0 2 0 0 0.01 0.001 0.003 | ImmM*/ImmM* (efficacy of bypass)* |
| Phe443 → Lys imm*         | 100 100 10 0 2 0 0 0.01 0.001 0.003 | ImmM*/ImmM* (efficacy of bypass)* |
| Gly444 → Lys imm*         | 100 100 10 0 2 0 0 0.01 0.001 0.003 | ImmM*/ImmM* (efficacy of bypass)* |
| Ala474 → Glu imm*         | 100 100 10 0 2 0 0 0.01 0.001 0.003 | ImmM*/ImmM* (efficacy of bypass)* |
| Ser477 → Arg imm*         | 100 100 10 0 2 0 0 0.01 0.001 0.003 | ImmM*/ImmM* (efficacy of bypass)* |
| Lys477 → imm*             | 100 100 10 0 2 0 0 0.01 0.001 0.003 | ImmM*/ImmM* (efficacy of bypass)* |

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**Discussion**

In a previous study of the topography and orientation of the colicin E1 immunity protein in the cytoplasmic membrane, the following mechanism for specific interaction of immunity protein with colicin channel domain was proposed (Song and Cramer, 1991): (a) immunity protein, present in a low copy number (<10) per cell, diffuses laterally in the membrane until the specific sites of interaction including the colicin channel domain are found; (b) the specific sites are the positions of entry into the membrane of the imported colicin channel, which would be defined by sites of apposition with the tolA and/or tolQ translocation proteins that would

**Discussion**

Tests of Hypotheses for the Protective Immunity Protein Interaction

In a previous study of the topography and orientation of the colicin E1 immunity protein in the cytoplasmic membrane, the following mechanism for specific interaction of immunity protein with colicin channel domain was proposed (Song and Cramer, 1991): (a) immunity protein, present in a low copy number (<10) per cell, diffuses laterally in the membrane until the specific sites of interaction including the colicin channel domain are found; (b) the specific sites are the positions of entry into the membrane of the imported colicin channel, which would be defined by sites of apposition with the tolA and/or tolQ translocation proteins that would
Colicin E1-Imm Protein Interaction

TABLE V

Location of mutations in colicin E1 channel domain with no effect on immunity bypass

| Helix H1* | Interhelix H1-H2 loop | H2 | H2-H3 loop | H3 | H3-H4 loop | H4 | COOH-terminal segment |
|-----------|----------------------|----|------------|----|------------|----|----------------------|
| Trp424 → Lys | Gly230 → Arg | Asp466 → Cys | Leu460 → Lys | Ala461 → Lys | Gly699 → Glu | Ile608 → Lys | (Lys619 → Cys, Lys612 → Asn)* |
| Leu428 → Lys | His340 → Cys | Val468 → Lys | Ala462 → Lys | Ala467 → Lys | Thr460 → Asp | Thr460 → Asp | |
| Gin430 → Lys | His240 → Glu | Ser469 → Lys | Ser577 → Gly | Thr470 → Lys | Gly698 → Glu | Gly698 → Glu | |
| Phe431 → Lys | His240 → His | Ile400 → Lys | Ser577 → Ala | Leu467 → Lys | Gly698 → Ala | Gly698 → Arg | |
| Tyr434 → Lys | His240 → Cys | Leu400 → Lys | Tyr470 → Lys | Gly699 → Ala | Val179 → Asp | Ile497 → Lys | |
| Thr438 → Lys | Val464 → Cys | (Lys476 → Met) | Val400 → Asp | Val400 → Asp | Ala480 → Lys | Ala480 → Lys | |
| (Lys403 → Met) | Ser424 → Lys | Lys405 → Met | Ala471 → Lys | Ala471 → Lys | Ile497 → Met | Ile497 → Lys | |
| Lys406 → Met |^b | ^b | Leu476 → Lys | Leu476 → Arg | Leu476 → Arg | Leu476 → Arg | |
| ^b | | | Leu477 → Arg | Leu477 → Arg | Leu477 → Arg | |

*See Fig. 23.
^b Double mutant.
^c Double mutant with second mutation, C505 → A.
^d Triple mutant with C505 → A.

form a ternary reaction complex with immunity protein and channel domain; (c) the formation of the ternary complex would be mediated by the extramembrane loops of these proteins and electrostatic or polar interactions, as suggested by the critical role of the H3-H4 interhelix region in the colicin A immunity protein (Geli et al., 1986) and the unique multicharge region in interhelix loop L2 of the colicin E1 imm protein (Fig. 24).

The studies reported in the present study on retention of protective immunity function (i) in laE1 hybrid colicin acting on tonB'tolA' or tonB'tol Q' cells (Table II), (ii) with site-directed mutants of immunity protein in which charged residues were removed in each of the extramembrane loops T1, L1, L2, and T2 (Table III), and (iii) through the existence of bypass mutations in the trans-membrane helical domains of the colicin channel, indicate that hypotheses (b) and (c) above are incorrect.

Lateral Diffusion of Immunity Protein: K+ Efflux in the Presence of Immunity Protein

In the absence of a mechanism that would convey direction to the immunity protein produced in low copy number, the above hypothesis (a) of lateral diffusion of imm protein in the cytoplasmic membrane to the reaction sites is still regarded as correct. The measured half-time for K+ loss from the cell population, after a delay attributed to binding and translocation, is ~60 s, similar to a half-time for efflux of 40 s predicted from an intracellular K+ concentration of 0.25 M in 0.15 M osmolar medium (Richey et al., 1987), an intracellular volume of 5 x 10^-16 liter (Neidhardt, 1987), respectively, and a single channel conductance of ~10^5 K+/s (Fig. 4). The mean-square distance, \( r^2 = 4Dt \), over which a membrane-bound protein with a diffusion constant of ~10^-9 m^2 s^-1 (Cramer and Knaff, 1991) can diffuse during the lag time of 20-40 s that precedes channel formation (Fig. 4) is approximately 10^-7-10^-8 cm^2. This area is greater than or equal to that of the cytoplasmic membrane surface, consistent with the concept that a single immunity protein molecule has a reasonable probability of finding the colicin channel molecule in the cytoplasmic membrane before the channel opens or before the open channel has allowed the efflux of a large amount of intracellular K+. The existence of an immunity bypass site on amphiphilic channel helix H2, which has been shown to insert into the membrane upon imposition of a membrane potential (Merrill and Cramer, 1990), implies that immunity protein can react with the channel in the open state.

Mechanism of Specific Interaction of Immunity Protein with Colicin E1 Channel Domain

Direct Interaction—The ability of the colicin E1 immunity protein reaction to accommodate the tonB as well as the tol translocation proteins implies that these macromolecule translocation systems do not have any specific role in the recognition mechanism of immunity protein with the channel-forming colicin. As for colicins E2 and E3, immunity protein...
must directly interact with colicin E1, although in the case of the latter, the interaction involves the channel domain in the membrane.

Based on (i) the importance of the extramembrane region between helices H3-H4 in colicin A immunity protein analogues to peripheral segment T2 in the colE1 immunity protein (Fig. 2A) for immunity protein function (Geli and Lazdunski, 1989), (ii) the high proportion (8 out of 18 residues) of charged amino acids in loop L2 of the ColE1 imm+ protein (Fig. 2A), and (iii) precedent for stabilization of an oligomeric integral membrane protein complexes by favorable electrostatic interactions (Szczepaniak et al., 1991), it was proposed that the specificity of immunity protein recognition could occur through polar or electrostatic interactions between the extramembrane loops of the immunity protein and the target channel domain (Song and Cramer, 1991). This hypothesis appears wrong.

Neutralization by mutagenesis of (i) the only charge in extramembrane segments T1 and T2, (ii) of the only charges in loop L1, or (iii) of two of the four consecutive charges at positions 71-74 in loop L2 (Fig. 2A), had no effect on immunity protein function (Table III). Substitution of a charged residue at position 39 in loop L2 or at positions 43 or 45 near the H2-L2 boundary was also without effect (Song and Cramer, 1991).

Although these data do not rule out the involvement of the polar extramembrane segments in immunity protein-colicin E1 recognition, they suggest that the segments of these proteins that are intrinsic to the membrane, the trans-membrane α-helices, may carry information necessary for the recognition. A somewhat similar conclusion was also obtained in a fusion protein study of the immunity interaction with hybrid colicin A-colicin B (Geli and Lazdunski, 1992), in which the region of the channel domain that reacts with immunity protein was inferred to start a short distance on the NH2-terminal side of the hydrophobic α-helices (i.e. corresponding to a start position some residues on the NH2-terminal side of Lys470 in the model shown in Fig. 2B) (Geli and Lazdunski, 1992).

**Immunity Bypass Mutants**—In order to localize the sites of interaction of the channel domain of colicin E1 with immunity protein, a search was made for the existence of immunity bypass mutants in the collection of 29 site-directed non-polar → charged residue mutants that was used to map the membrane topography of the Ala471-Ile488 hydrophobic domain, and to infer that it is a hydrophobic helical hairpin (Song et al., 1991). The existence of mutations in this region that can bypass immunity function strongly implies interaction within the bilayer. Most of these mutants possess low cytotoxicity (Song et al., 1991). Mutants were studied that had a lowered but readily measurable activity in an imm− strain and were examined for increased activity in an imm− strain. The 2 residues at which mutants with the largest bypass effect could be found were Ala471 and Ser477 in hydrophobic helix H3 of the colicin E1 channel domain (Fig. 2B). The Ala471 → Glu, Ser477 → Arg, and Ser477 → Lys mutants had cytotoxic activities approximately 5-, 25-, and 3000-fold lower than wild-type when assayed on the imm+ indicator (Table IV; cf., Song and Cramer, 1991). These mutants were able to bypass the imm− protective function at concentrations 225, 5.0, and 1.0 times the minimum concentration needed to demonstrate cytotoxic activity on imm− cells, in contrast to the wild-type colicin that does not show cytotoxicity when added to imm− cells at 200 μg/ml, a concentration 7 × 104 greater than needed for activity with imm− cells (right-hand column, Table IV).

Charge-substitution mutants at 27 other sites in the Ala471-Ile488 hydrophobic H3-H4 helices were found to be negative for the immunity bypass phenotype (Table V). Because of the 3-residue spacing between the bypass mutants at positions 474 and 477, and the possibility of the bypass phenotype being associated with α-helical periodicity (see below), particular attention was paid to mutants offset by 3-4 residues from positions 474 and 477. This subset included mutants Ala471 → Lys, Val485 → Asp, Ala481 → Asp and Ser486, Leu487 → Arg in hydrophobic helix H3 (Table V). Fourteen of the 15-17 residues in helix H4, including 5 at the same approximate depth in the membrane as Ala471 and Ser477 in the model of Fig. 2B, have been tested and found to be bypass-negative (Table V). Thus, the potential for bypass of immunity function in the hydrophobic helical hairpin appears to be localized in residues 474 and 477 of helix H3. Substitution of a large and/or basic residue at positions 477 and 440 appears to be required for immunity bypass, as Ser477 → Ala or Gly and His440 → Cys or Glu mutants are unable to bypass immunity (Table V).

Three additional, although less pronounced, sites for bypass mutants were found in an additional set of site-directed substitutions, His440 → Arg, Phe443 → Lys, and Gly444 → Lys, that were constructed in the polar and somewhat amphiphilic helix H2. These mutants have a cytotoxic activity relative to wild-type that is, respectively, unchanged, 5-fold smaller, and 5-fold lower when assayed on the imm+ indicator. They are able to bypass immunity function in imm− cells at concentrations approximately 1.7 × 104, 3.1 × 104, and 6.3 × 104, respectively, of the minimum concentration needed for activity with imm− cells (reciprocals of latter numbers shown in right-hand column, Table IV). The existence of bypass mutants on helix H2 implies that the protective interaction with the immunity protein can occur after the amphiphilic helical hairpin has inserted into the membrane (Merrill and Cramer, 1990) and initiated the opening of the channel. The two sets of sites on helices H2 and H3 of the colicin channel domain are on opposite sides of the membrane according to the model of Fig. 2B. As can be seen from the aligned sequences of colicins E1, Ia, and Ib (Table I), the residues His440, Phe443, Gly444, and Ala474 are at non-conserved sites. This suggests that the specificity of the colicin E1-immunity protein interaction may be partly determined by these non-homologous or non-conserved residues.

**Protein-Protein Recognition via Trans-membrane Helices**—From the above data and discussion, it was concluded that the mechanism of the specific inhibitory complex of the immunity protein with the colicin E1 channel domain involves (i) lateral diffusion in two-dimensional space of the highly dIute immunity protein in the membrane, and (ii) recognition and formation of an interprotein helix complex by interaction at least one of the three helices of the immunity protein with two of the helices, H2 and H3, of the colicin E1 channel domain (Fig. 5). The distribution of the bypass mutants at positions 440, 443, and 444 on helix H2, and at 474 and 477 on helix H3, suggests that one face of each helix is involved in helix-helix interaction with the immunity protein. Precedents for the existence of important helix-helix interactions in the assembly of polytopic membrane proteins such as bacteriorhodopsin and lactose permease, in the formation of a tightly associated dimer of glycophosphin A, and in proposed mechanisms of receptor-mediated trans-membrane signaling, have been reviewed (Bormann and Engelman, 1992). The helix-helix interactions could be mediated by salt bridges, hydrogen bonds, or specific packing and interactions of non-polar residues, as in the case of glycophosphin A (Lemmon et al., 1992). Because the bypass mutants occur on both a hydro-
the helices of the channel while the latter are bound at the membrane surface. Thus, it is proposed in the present work that immunity-channel colicin interaction involves helix-helix interaction of the immunity protein when the colicin channel helices are inserted into the membrane bilayer (Zhang and Cramer, 1992).

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Fig. 5. Scheme for mechanism of action of immunity protein with the colicin E1 channel showing lateral diffusion of imm protein in the cytoplasmic membrane and ultimate specific helix-helix interaction of hydrophobic helices of immunity protein (Fig. 2A) with membrane-spanning helices (H2, H3, Fig. 2B) of the colicin channel domain.

Hydrophobic and polar helix, all of these possibilities for the interface interaction exist at the present time. However, because the mutants with the more pronounced bypass effects occur on the mostly hydrophobic helix H3, hydrophobic interaction between channel and immunity protein seems most likely. The degree of hydrophobicity of the three helices of the immunity protein is H3 > H2 = H1. A relatively high polarity of H1 was indicated by full retention of imm protein function when a lysine was substituted at positions 17 or 19 of these general considerations of helix polarity, it is presently hypothesized that helix H3 of the immunity protein is the most likely participant in the inhibitory complex.

A fusion protein study of the principle determinants for immunity protein recognition in colicin A-colicin B hybrids indicated that the analogous hydrophobic hairpin, H3-H4, in colicins A and B are for immunity (Geli and Lazdunski, 1992). The present study of immunity recognition in the colicin E1 channel (i) is in agreement that the interaction between the channel colicin and immunity protein is direct, and (ii) was able to localize particular interactions involved in immunity recognition. (iii) Two strongly interacting residues were found in helix H3, but (iv) no such residues were selected in hydrophobic helix H4. Instead, (v) amphiphilic helix H2 was found to contain 3 residues involved as determinants of immunity recognition, although more weakly. (vi) It was proposed by Geli and Lazdunski (1992) that the potential trans-membrane helices of the channel that interact with the hydrophobic immunity protein do so while bound at the membrane surface. However, a long-lived surface-bound state of the channel helices contradicts the FTIR dichroism measurements made with both colicins E1 (Rath et al., 1991) and colicin A (Goormaghtig et al., 1991) channel domains. It is also difficult to understand in the model presented by Geli and Lazdunski (1996) how the trans-membrane hydrophobic helices of the immunity protein can interact with the helices of the channel while the latter are bound at the membrane surface. Thus, it is proposed in the present work that immunity-channel colicin interaction involves helix-helix interaction of the immunity protein when the colicin channel helices are inserted into the membrane bilayer (Zhang and Cramer, 1992).