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Membrane Topography of ColE1 Gene Products: the Immunity Protein

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The topography of the colicin E1 immunity (Imm) protein was determined from the positions of Tn*phoA* and complementary *lacZ* fusions relative to the three long hydrophobic segments of the protein and site-directed substitution of charged for nonpolar residues in the proposed membrane-spanning segments. Inactivation of the Imm protein function required substitution and insertion of two such charges. It was concluded that the 113-residue colicin E1 Imm protein folds in the membrane as three *trans*-membrane α-helices, with the NH_2 and COOH termini on the cytoplasmic and periplasmic sides of the membrane, respectively. The approximate spans of the three helices are Asn-9 to Ser-28, Ile-43 to Phe-62, and Leu-84 to Leu-104. An extrinsic highly charged segment, Lys-66 to Lys-74, containing seven charges in nine residues, extends into the cytoplasmic domain. The specificity of the colicin E1 Imm protein for interaction with the translocation apparatus and the colicin E1 ion channel is proposed to reside in its peripheral segments exposed on the surface of the inner membrane. These regions include the highly charged segment Lys-66 to Lys-83 (loop 2) and the short (approximately eight-residue) NH_2 terminus on the cytoplasmic side, and Glu-29 to Val-44 (loop 1) and the COOH-terminal segment Gly-105 to Asn-113 on the periplasmic side.

Colicin E1 exerts its bactericidal effect by forming a highly conductive ion channel in the cytoplasmic membrane that depolarizes and deenergizes the cell (9). The channel domain, localized in the COOH-terminal third of the 522-residue colicin molecule, is susceptible to the protective effect of the 113-residue colicin E1 immunity (Imm) protein, a cytoplasmic membrane protein (3).

Six different channel-forming colicins and their Imm proteins have been identified (26, 28, 35–37). The action of an Imm protein is highly specific, meaning that the colicin E1 Imm protein can protect cells only from exogenous colicin E1 and not from any of the closely related channel-forming colicins. Knowledge of the arrangement and structure of the colicin E1 Imm protein in the membrane is ultimately essential for an understanding of the molecular basis of its protective function. The approaches available for studying membrane protein topography and orientation in the case of the *imm* gene product are presently restricted to those of molecular genetics, with Tn*phoA* and *lacZ* fusions (29–31), because of the small amount of protein made by the cell and the difficulty thus far in reconstituting the immunity function with purified Imm protein (9, 18).

Tn*phoA* random insertion mutagenesis takes advantage of the unique characteristics of bacterial transposable element Tn*5* (2) and a periplasmic protein, alkaline phosphatase (AP), encoded by the *phoA* gene. Tn*phoA* is a kanamycin-resistant derivative of Tn*5* that was constructed by inserting most of the *phoA* structural gene, without its signal peptide, close to the left end of Tn*5* (30). AP is made in the cytoplasm but is only active when exported into the periplasm (32, 33), perhaps because of obligatory dimer formation or greater stability (27, 29). The AP fusion technique has been used to describe the topography of many integral cytoplasmic membrane proteins (6, 19, 27, 31). The empirical rule that has been developed is that when insertions occur in an integral membrane protein with several membrane-spanning domains, the correct topography is preserved by the NH_2-terminal part of the protein. Fused AP then displays either high or low activity, depending on whether it is inserted on the periplasmic or cytoplasmic side of the membrane, thus providing information on the topography of the protein.

lacZ fusion takes advantage of β-galactosidase which, unlike AP, is active in the cytoplasm (29). In-frame *lacZ* fusions with a periplasmic domain of an integral membrane protein cause the *lacZ* gene product to be retained in the membrane, resulting in low β-galactosidase activity (14). The dependence of *lacZ* fusion activity on the *trans*-membrane orientation of the fusion site is thus complementary to that of *phoA* fusions (29), so that this method can often, but not always (19), provide an additional determinant of the topography of integral membrane proteins.

MATERIALS AND METHODS

Construction of plasmid pSL630. Plasmid pDMS630 (40), a ColE1 derivative with an ampicillin resistance gene (constructed by insertion of Tn*3* into ColE1) was digested with *Eco*RI and *Sma*I, each of which cuts at only one site. Two linear DNA fragments were generated, a 1.3-kb fragment that spans most of *cea* and a 10.5-kb fragment containing *imm*. These were blunt-ended by digestion with mung bean nuclease, self-ligated for 3 h at 16°C, and transformed into JM83. Colonies that grew on ampicillin were chosen randomly, plasmid DNA was isolated by a modified alkaline lysis method (34a), and a plasmid produced from self-ligation of the 10.5-kb fragment without the 1.3-kb fragment was isolated by digestion of the candidate plasmids with *Nru*I, which cuts at one site. This new construct, pSL630 (*cea imm*$^+$), was used to screen Tn*phoA* insertions into *imm*.

Tn*phoA* insertion mutagenesis. JM83/pSL630 was grown to log phase in 5 ml of 2× YT medium containing 16 g of tryptone (Difco) per liter, 10 g of yeast extract per liter, and 5 g of NaCl per liter and supplemented with 0.1 ml of 20% maltose. An aliquot (500 μl) of the cells was removed, centrifuged, and resuspended in 100 μl of 10 mM $MgCl_2$–5

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mM $CaCl_2$. The resuspended cells were incubated at room temperature for 10 min, λ:Tn*phoA* phage stock (kindly provided by B. Wanner) was added (25 μl, 10^{10}/ml), the mixture was incubated at room temperature for 30 min, several aliquots were removed, and 1 ml of fresh 2× YT medium was added to each of these aliquots. After incubation under agitation (2 h, 37°C), 40 μl of kanamycin stock (25 mg/ml) and 4 ml of fresh 2× YT medium were added to each aliquot, which was grown overnight. From the overnight cultures, plasmid DNA was isolated and transformed into a *phoA* strain, and the strain was spread on plates containing kanamycin (25 μg/ml) and 5-bromo-4-chloro-indolyl-phosphate (45 μg/ml).

Screening of Tn*phoA* insertions *imm* with correct orientations. Colony-containing plates were incubated for several days at 4°C; blue, faintly blue, and some randomly chosen white colonies were picked, inoculated separately into 3 ml of 2× YT medium for initial screening, and grown overnight; and 100 μl of each colony was spread on a 2× YT medium plate. To identify colonies without a functional *imm* gene resulting from a Tn*phoA* insertion in or near the *imm* gene of plasmid pSL630, we spotted purified colicin E1 protein directly on a lawn of candidate cells. Four or five spots (20 μl each) were applied by making 10-fold serial dilutions from 1 to 5 mg of colicin E1 per ml. Cells with a functional Imm protein are resistant to a colicin E1 concentration $>10^6$-fold above that which kills nonimmune cells. Colonies with plasmids that could not confer immunity to exogenous colicin E1 were identified from the clearing of zones to which colicin E1 had been applied, and the plasmid DNA was isolated. Restriction enzymes *Nru*I and *Eco*RI were chosen to map the selected plasmids. Plasmids having oppositely directed Tn*phoA* insertions in *imm* such that no in-frame fusions could be made with *imm* were identified by restriction analysis. Plasmids (e.g., pSL630:Tn*phoA*) having Tn*phoA* inserted with the correct orientation in *imm* were sequenced to identify the exact fusion junction.

DNA sequencing to identify the in-frame fusion junction. A synthetic oligonucleotide that had a matching sequence near the beginning of Tn*phoA* (19-mer corresponding to bases 15 through 33 of Tn*phoA*) was synthesized, purified with an oligonucleotide purification cartridge (400771; Applied Biosystems, Foster City, Calif.), and used as a primer for sequencing. Double-stranded DNA sequencing was performed directly on the candidate plasmids after denaturation of the plasmids with 2 M NaOH (8). The sequencing reaction was carried out with the Sequenase (modified T7 DNA polymerase) sequencing kit (70770; USB, Cleveland, Ohio) and [^{32}P]dATP (PB10204; Amersham, Arlington Heights, Ill.).

AP activity assay. An aliquot (1 ml) of an overnight culture was centrifuged and resuspended in an equal volume of 1 M Tris-Cl buffer (pH 8) (eight independent in-frame fusions at a time), 10 to 100 μl was removed, and the total volume of the latter was adjusted to 1 ml with 1 M Tris-Cl (pH 8). After 0.1% sodium dodecyl sulfate (SDS) (50 μl) and chloroform (100 μl) were added to each tube to cause lysis, the tubes were vortexed vigorously and incubated at 37°C (5 min) and 0.1 ml of 1% *p*-nitrophenol phosphate was added to each tube. The AP reaction was stopped (usually after 5 to 10 min) by the addition of 0.5 ml of 1 M K_2HPO_4 when a slightly yellow color began to develop in the most active mutant. The tubes were placed on ice, and the unbroken cells were removed by microcentrifugation (5 min) before measurement of the optical density of the supernatants at 420, 550, and 600 nm. The specific AP activities of the fusion proteins were calculated (7).

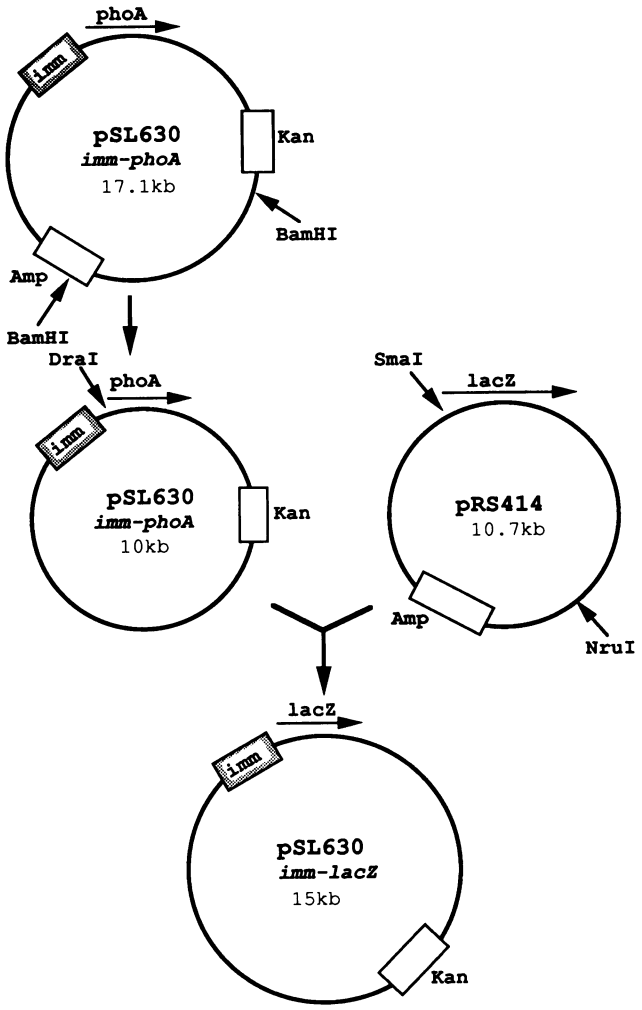

FIG. 1. Construction of the *imm-lacZ* fusion plasmids from the *imm-phoA* fusion plasmids. The original *imm-phoA* plasmids were cut with *Bam*HI and self-ligated to create smaller plasmids. The *lacZ* gene was cloned into the unique *Dra*I site of the smaller plasmids, resulting in the in-frame *imm-lacZ* fusions.

Construction of β-galactosidase fusions. To test the reliability of the results obtained with eight in-frame *phoA* fusions, we introduced *lacZ* fusions at all *phoA* fusion sites by swapping the *phoA* gene with the *lacZ* gene (Fig. 1). pSL630:Tn*phoA* plasmids were digested with *Bam*HI, which cuts at two sites. The large fragment was self-ligated to create a 10-kb plasmid, which was ampicillin sensitive and kanamycin resistant because of the deletion of a 6-kb fragment that contained a large portion of Tn*3*. It also contained a single *Dra*I site located 254 bases away from the *imm-phoA* fusion site within the *phoA* structural gene. A new *lacZ*-based cloning vector, pRS414 (38), was cut with *Sma*I and *Nru*I, and a *lacZ*-containing 5-kb fragment of pRS414 was isolated and cloned into the unique *Dra*I site of pSL630:Tn*phoA*. Colonies harboring plasmids with a successful *lacZ* insertion were identified on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside after transformation of the ligated DNA into a *lacZ* strain.

β-Galactosidase assay. Cells carrying plasmids with an

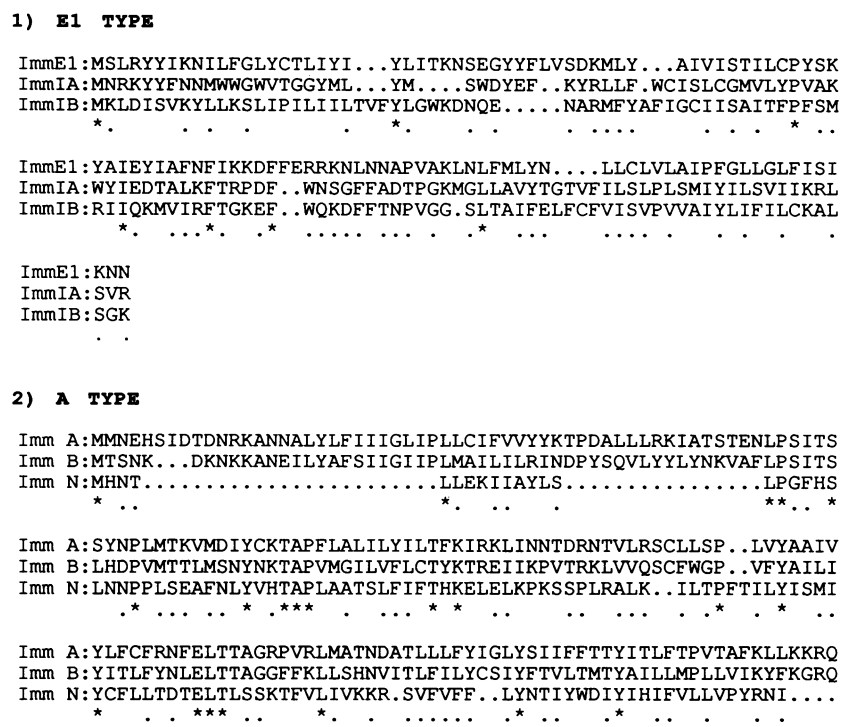

FIG. 2. Multiple sequence alignment of the E1- and A-type Imm proteins. Sequences were obtained from the published literature (26, 28, 35–37). The alignment program Clustal was run on the GCG DNA/Protein Sequence analysis package (University of Wisconsin, Madison). *, Match across all sequences; ·, Conservative substitution.

in-frame *lacZ* fusion in the *imm* gene were grown overnight. Each sample (1 ml) was centrifuged, resuspended in an equal volume of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol [pH 7.0]), and placed on ice; 50 μl was added to 950 μl of Z buffer; and 1 drop of 0.1% SDS and 2 drops of chloroform were added to each sample. The mixtures were vortexed vigorously and incubated at 30°C (15 min), *o*-nitrophenyl-β-D-galactopyranoside (0.2 ml of a 4-mg/ml solution) was added, and the suspensions were vortexed and incubated at 30°C. When a slightly yellow color developed, the reaction was stopped by the addition of 0.5 ml of 1 M Na_2CO_3. Unbroken cell debris was removed by microcentrifugation, the supernatant was removed gently, the optical densities at 420 and 550 nm were measured, and the specific activities were determined (34).

Site-directed mutagenesis. Mutants were generated with a new construct, pSKE1(−) (39), which has both *cea* and *imm* inserted into the multicloning site of the pBlueScript II SK(+) phagemid (Stratagene, La Jolla, Calif.). Single-stranded DNA for mutagenesis was isolated by infection of the cells harboring pSKE1(−) with helper phage M13K07 (42). Uracil-containing single-stranded DNA was obtained by growing pSKE1(−) in strain CJ236, an *ung dut* mutant. The mutagenesis reaction was carried out as described previously (24) and in the manual for the phagemid in vitro mutagenesis kit (170-3576; Bio-Rad) (2a).

Immunoprecipitation and pulse-chase experiments. Cells were grown overnight in M9 minimal medium supplemented with thiamine (1 μM) and ampicillin (50 μg/ml), diluted 50-fold into fresh minimal medium, and grown until an optical density at 420 nm of 0.7 was reached. At this point, 1.2 ml of cells was transferred into sterile microcentrifuge tubes and [^{35}S]methionine (6 μl, 800 Ci/mmol; Amersham) was added. For pulse experiments, 600 μl was removed from the cell suspension after 1 min, and for pulse-chase experiments, the remainder was incubated for 30 min with shaking after the addition of 100 μl of 0.3 M cold methionine; the cells from each sample were added to an equal volume of cold 10% trichloroacetic acid solution, incubated on ice for 5 min, and centrifuged. The sediment was washed twice with acetone, 30 μl of SDS solution (1% SDS, 50 mM Tris-Cl [pH 8.0]) was added, the mixture was boiled for 3 min, and a 50-fold dilution was made by the addition of Triton buffer (2% Triton X-100, 50 mM Tris-Cl [pH 8.0], 0.1 mM EDTA). Antibody to AP (kindly provided by B. Wanner) diluted 20-fold was added (3 μl) to each tube, which was incubated overnight at 4°C. To separate the antibody-labeled protein, we added protein A-positive *Staphylococcus aureus* cells (Boehringer Mannheim, Indianapolis, Ind.) resuspended in distilled H_2O (60 μl) to each tube and incubated the tubes at 0°C for 1 h with occasional swirling. Cells were sedimented by centrifugation and washed three or four times with Triton buffer and once with 10 mM Tris-Cl (pH 8), the sediments were dissolved in SDS sample buffer (0.08 M Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.0025% bromophenol blue) and separated by SDS-polyacrylamide gel electrophoresis (PAGE). Fusion proteins were visualized by autoradiography.

RESULTS

Alignment of Imm proteins of channel-forming colicins. The Imm proteins can be divided into two groups on the basis of the sizes of the hydrophobic anchor regions of the channel domains of the respective colicins. The E1 type includes the

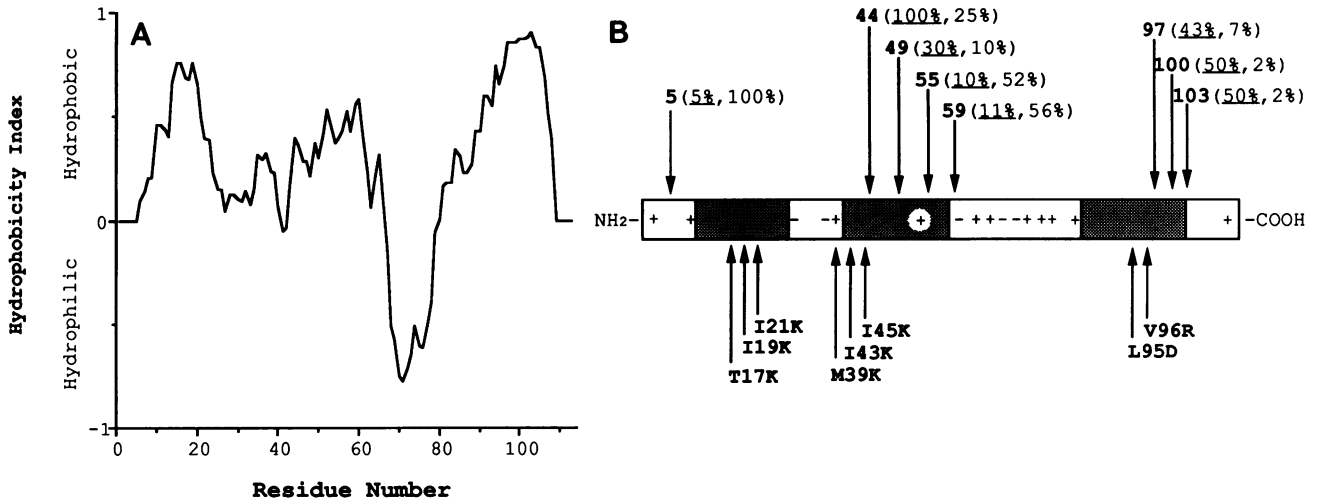

FIG. 3. (A) Hydrophobicity analysis of the colicin E1 Imm protein. The hydropathy plot was calculated with the consensus index for amino acid hydrophobicity of Eisenberg (13), with an averaging interval of 11 residues. (B) Distribution of hydrophobic putative membrane-spanning domains and sites for *phoA* fusion and site-directed mutagenesis. The hydrophobic segments long enough to span the membrane are indicated by hatched boxes, spanning residues 9 to 28, 39 to 60, and 84 to 105. Positions with a charged residue are indicated in their approximate locations with + and −. The numbers above the box indicate the *phoA* and *lacZ* fusion sites, with the numbers in parentheses showing the relative AP (first number) and β-galactosidase (second number) activities at the fusion sites. The exact values of the specific activities of the AP and β-galactosidase fusions, with standard deviations, were as follows (three and two trials, respectively): 5, 11 ± 5, 734 ± 70; 44, 207 ± 67, 180 ± 45; 49, 62 ± 20, 72 ± 0.5; 55, 22 ± 3, 380 ± 144; 59, 23 ± 2, 412 ± 131; 97, 88 ± 13, 42 ± 11; 100, 103 ± 13, 16 ± 1; and 103, 103 ± 10, 10 ± 8. The arrows below the box indicate the approximate sites for site-directed mutagenesis, with the mutational change indicated by the one-letter code (D, aspartate; I, isoleucine; L, leucine; K, lysine; M, methionine; R, arginine; T, threonine; V, valine).

E1, Ia, and Ib Imm proteins, with an average size of 113 residues. The A type includes the A, B, and N Imm proteins, of which the A and B Imm proteins are significantly longer (178 and 175 residues, respectively). Sequence alignment of these two groups of Imm proteins shows that the Imm proteins are most divergent near the NH_2 terminus, a segment between residues 14 and 35 of the colicin E1 Imm protein (Fig. 2). The central region, Lys-66 to Lys-74, with a high charge density and the long COOH-terminal hydrophobic segment, Leu-85 to Asn113, are relatively well conserved.

Prediction of membrane-spanning domains of colicin E1 Imm proteins. The amino acid sequence of the colicin *imm* gene product (3) and the distribution of hydrophobic residues (hydropathy) in the protein are shown in Fig. 2 and 3A, respectively. There are three prominent regions of hydrophobicity (hatched regions in Fig. 3B) approximately spanned by residues 9 to 28, 43 to 62, and 84 to 105. The last two hydrophobic spans are separated by a polar loop (L1; Fig. 4) including a highly charged (seven charges in nine residues) sequence, Lys-66 to Lys-74, that appears as a pronounced minimum in the hydropathy plot (Fig. 3A). This analysis suggests the existence of three membrane-spanning helices (H1 to H3) in the colicin E1 Imm protein (Fig. 4). Such a hypothesis must be supported by the results of biochemical or genetic tests. Such tests could also provide information on the orientation of the colicin E1 Imm protein in the membrane, which cannot be predicted by hydropathy analysis.

Tn*phoA* insertion mutagenesis. Random Tn*phoA* insertion mutagenesis with λ:Tn*phoA* generated eight in-frame and at least five out-of-frame fusions within *imm*, detected by screening of more than 200 colonies. The in-frame insertion sites are at amino acid residues Tyr-5, Val-44, Leu-49, Tyr-55, Tyr-59, Leu-97, Pro-100, and Leu-103, covering most of *imm*. All in-frame fusions, except that at Tyr-5, were obtained from colonies that turned blue in the presence of 5-bromo-4-chloro-indolyl-phosphate. The colony derived from a fusion at Tyr-5 remained unchanged in color. The fusion at Val-44 rapidly turned the colony dark blue, and a blue "halo" indicative of active AP slowly developed around the colony in a few days.

The relative AP activities from the eight in-frame fusions were measured simultaneously with log-phase cells. These activities and the relative β-galactosidase activities resulting from *lacZ* fusions are shown in Fig. 3B as the first and second numbers, respectively, in the parenthesis. The fusion at Tyr-5 yielded the lowest AP activity. The fusion at Val-44 yielded the highest AP activity, which was 20-fold greater than that yielded by the fusion at Tyr-5. The AP activities decreased gradually for the fusions at residues 49, 55, and 59. The remaining three fusions, near the COOH terminus at residues 97, 100, and 103, yielded AP activities approximately 10-fold higher than that yielded by the fusion at residue 5. This pattern of AP activities at the above-described fusion sites would be consistent with a model of membrane topography in which the colicin E1 Imm protein has three *trans*-membrane α-helices, with the NH_2 terminus on the cytoplasmic side of the membrane (Fig. 4).

The three-helix model implies the following. (i) The NH_2 and COOH termini are positioned on opposite sides of the cytoplasmic membrane. (ii) Two segments of the protein, residues 29 to 42 in L1 connecting the first and second membrane-spanning domains and residues 105 to 113 at the COOH terminus (T2), extend into the periplasm. An extrinsic highly charged segment, Lys-66 to Lys-74, containing seven charged residues in L2 extends into the cytoplasmic domain (Fig. 4). This model would be consistent with the

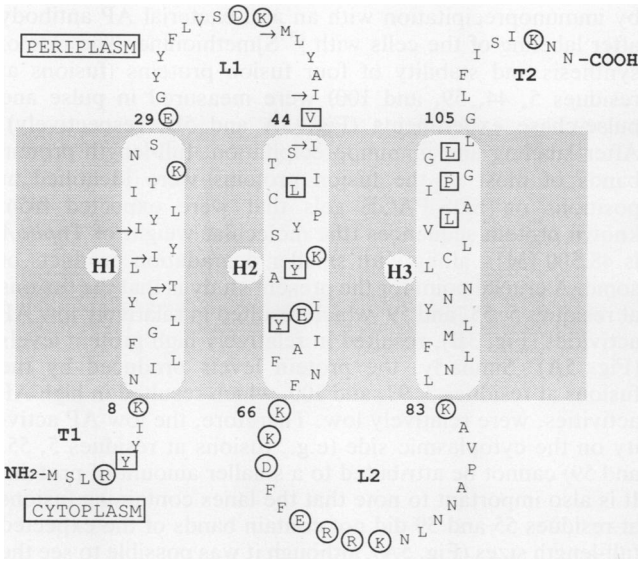

FIG. 4. Topographical model of the colicin E1 immunity protein. The model shows three *trans*-membrane helices, in accordance with the prediction from the hydropathy analysis, with the NH_2 and COOH termini on the cytoplasmic and periplasmic sides, respectively. All charged residues are circled, and the fusion sites are boxed. Following the notation of Geli et al. (17), the three putative *trans*-membrane α-helices are labeled H1 to H3; the loops connecting H1-H2 and H2-H3 on the periplasmic and cytoplasmic sides of the membrane are labeled L1 and L2, respectively; and the NH_2 and COOH termini are labeled T1 and T2, respectively. The model has three membrane-spanning helices, with H1 and H2 having one and two charges in the membrane-spanning regions, respectively.

hydropathy analysis of the colicin E1 Imm protein (Fig. 3A) and a four-helix model proposed for the colicin A Imm protein (16, 17). The colicin A Imm protein (178 versus 113 residues) includes a large loop (L3) on the periplasmic side of the membrane and a 4th helix (H4), with the COOH terminus (T2) on the cytoplasmic side of the membrane (17).

The low AP activity from the fusion at Tyr-5 might be thought to arise from the very short NH_2-terminal portion of the colicin E1 Imm protein that forms a hybrid protein with AP. Thus, it could be argued that the NH_2 terminus actually faces the periplasm but that the NH_2-terminal five residues of the colicin E1 Imm protein attached to the AP moiety form a segment that is not able to transport the bulky AP portion (48 kDa) of the hybrid protein across the membrane, resulting in low AP activity. Attempts to find in-frame fusions between residues 5 and 44 that might clarify this problem were in vain. After screening and sequencing of a large number (>100) of randomly picked colonies, Tn*phoA* transpositions repeatedly occurred at the same sites. This result indicates there is a limit to the number of in-frame fusions that can be isolated with Tn*phoA*, the insertion of which seems to show a preference for certain sequences. To further test the validity of the model based on the *imm-phoA* fusions, we constructed *lacZ* fusions at all *phoA* fusion sites and tested the existence of the *trans*-membrane helix (H1), Asn-9 to Ser-28, by using site-directed mutagenesis to change nonpolar to charged residues in this segment.

β-Galactosidase fusions. The *lacZ* fusions were constructed by a swap in which the linearized intact *lacZ* gene fragment was cloned at the unique *Dra*I site of the *imm-phoA* plasmid, located 254 bases from the fusion site toward the *phoA* structural gene (Fig. 1). In an integral membrane protein spanning the bilayer several times, fused β-galactosidase activities vary depending on the fusion site and are expected to be complementary to fused AP activities because β-galactosidase is active in the cytoplasm and cannot be translocated to the periplasm (14, 29).

All of the *imm-lacZ* fusion plasmids turned colonies blue on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside although there were considerable differences in intensities. The β-galactosidase activities were qualitatively complementary to the AP activities, except perhaps for the relationship between the *imm-lacZ* fusions at residues 44 and 49 (Fig. 3B). The average value for these two fusions seems slightly out of order, but the difference is not statistically meaningful. The highest β-galactosidase activities were obtained from the *imm-lacZ* fusions at residues 5 and 59, consistent with an orientation of these residues on the cytoplasmic side of the membrane, as predicted from the Tn*phoA* data. The COOH-terminal *imm-lacZ* fusions at residues 97, 100, and 103 yielded low activities, implying a periplasmic localization of the COOH terminus, consistent with the Tn*phoA* data and the proposed model (Fig. 4).

Site-directed mutagenesis. The rationale of the mutagenesis experiments was to substitute charged for hydrophobic residues in the segment attributed to hydrophobic H1 and thus to provide a second test of the existence of this putative *trans*-membrane helix. Changes of hydrophobic to charged residues should have a more destabilizing effect if the residues are buried in the membrane bilayer than if they are surface exposed (39), thus resulting in a greater impairment of the immunity function unless the altered surface-exposed residues are functionally or structurally important. As discussed in the accompanying paper (39), the number of inserted charges required to destabilize the helix is expected to depend on the length of the helix. The lengths of *trans*-membrane segments H1, H2, and H3 are proposed to be approximately 20, 20, and 21 residues, respectively (Fig. 4). The absence of charged residues on either side of the predicted membrane-spanning domain of H2 and on the COOH end of H3 (Fig. 4) indicates that these *trans*-membrane segments could be longer. H2 also contains two charges, Lys-54 and Glu-58, separated by four residues, suggesting that Lys-54 and Glu-58 can readily form an ion pair whose net charge would be much smaller than that of either Lys-54 or Glu-58 alone (22).

Six single lysine substitutions were made at hydrophobic residues Thr-17, Ile-19, Ile-21, Met-39, Ile-43, and Ile-45. The first three mutations were predicted to occur in H1, and the next three were predicted to occur in L1 connecting H1 and H2 or on the periplasmic side of H2 (Fig. 4). The mutants with the substitution and addition of a single charge in H1 (Ile-19 → Lys and Ile-21 → Lys) and within or on the edge of H2 (Ile-43 → Lys and Ile-45 → Lys) did not show a decrease in Imm protein function when tested with colicin E1 (Table 1). The most concentrated colicin samples (1 mg/ml; no dilution) were not cytotoxic. H2 and putative H1 are evidently sufficiently stable so that substitution of a single charged residue does not disrupt their structures.

The effect of the substitution of two charges in H3 and H1 was tested. Double mutants (Ile-19 → Lys and Ile-21 → Lys [H1] and Leu-95 → Asp and Val-96 → Arg [H3]) that showed a large decrease in Imm protein activity were constructed. Indicator cells in each case were not protected against 1-μg/ml and higher concentrations of the colicin (Table 1). Even in these mutants, the protective function of the Imm

TABLE 1. Effect on cytotoxicity of single and double lysine mutations in the *imm* gene coding for helices H1 through H3 and L1 (Fig. 4)

ColE1 plasmid (no. of trials)	Cytotoxicity[a] at the following colicin concn (mg/ml):					
	1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Wild type (*imm*$^+$)	−	−	−	−	−	−
Wild type (*imm*)	+	+	+	+	+	+
17 T→K	−	−	−	−	−	−
19 I→K	−	−	−	−	−	−
21 I→K	−	−	−	−	−	−
39 M→K	−	−	−	−	−	−
43 I→K	−	−	−	−	−	−
45 I→K	−	−	−	−	−	−
19 I→K + 21 I→K (5)	+	+	+	+	−	−
21 I→K + 39 M→K (2)	+	+	+	−	−	−
39 M→K + 45 I→K (3)	+	+	+	−	−	−
95 L→D + 96 V→R (5)	+	+	+	+	−	−

[a] Assayed as described in Materials and Methods. +, Complete clearing of the spot (no protection by the Imm protein); −, no clearing (protection by the Imm protein).

protein could be expressed when the colicin was diluted sufficiently, to 100 and 10 ng/ml, as shown in Table 1.

Two other double mutants were also constructed to compare the effect of a charge substitution on a residue in the proposed membrane-spanning domain with the effect of one on a residue predicted to be in a peripheral loop. Met-39 was predicted to be in peripheral L1. Double mutants were constructed with a substitution of Lys for Met-39 and a charge substitution in H1 (Ile-21 → Lys) or H2 (Ile-45 → Lys). These double mutants also showed a decrease in cytotoxicity with a 10^{-2} dilution of colicin, but this decrease was 10-fold lower than that of the double mutants with a charge substitution in H3 or H1. The lesser effect of the charge substitution on residue 39 was consistent with its peripheral location. The measurable loss of function over that obtained with the Ile-21 → Lys mutation alone may be attributable to the difficulty in translocating the extra lysine residue in L1 across the membrane (43). The greater loss of function with the insertion of two charges at residues 19 and 21 and at residues 95 and 96 is consistent with the location of residues 19 and 21 in H1 (Fig. 4).

AP levels and stabilities. Each fusion protein was identified by immunoprecipitation with an anti-bacterial AP antibody after labeling of the cells with [^{35}S]methionine. The rate of synthesis and stability of four fusion proteins (fusions at residues 5, 44, 59, and 100) were measured in pulse and pulse-chase experiments (Fig. 5A and 5B, respectively). After labeling and immunoprecipitation, full-length protein bands of most of the fusion proteins were identified at positions on SDS-PAGE gels that were expected from known protein sequences (the molecular weight of Tn*phoA* is 48,500 [31]), along with smaller degradation products of some. A crucial point for the present study is that the fusions at residues 5, 55, and 59, which resulted in relatively low AP activities (Fig. 3B), resulted in relatively high protein levels (Fig. 5A). Similarly, the protein levels produced by the fusions at residues 5, 97, and 100, which resulted in high AP activities, were relatively low. Therefore, the low AP activity on the cytoplasmic side (e.g., fusions at residues 5, 55, and 59) cannot be attributed to a smaller amount of protein. It is also important to note that the lanes containing fusions at residues 55 and 59 did not contain bands of the expected full-length sizes (Fig. 5A), although it was possible to see the full-length residue 55 and 59 fusion protein bands weakly when the cells were labeled for 1 min and rapidly precipitated (data not shown). Although the amounts of fusion proteins at residues 97 and 100 were small, bands corresponding to the full-length proteins could be seen (Fig. 5A).

It was also observed that the AP moieties that could not be translocated into the periplasm (fusions at residues 5, 55 [not shown], and 59) were degraded faster than were the ones that could be exported (fusions at residues 44, 97, 100, and 103) (Fig. 5B). After a 30-min chase, protein bands on the gels resulting from fusions at residues 5 and 59 had almost completely disappeared, indicating the unstable nature of these fusion proteins. On the other hand, the residue 44 fusion protein was more stable and showed a weak full-sized band after a 30-min chase. Although the residue 100 fusion protein was degraded into a smaller product, it was stable enough to be seen after a 30-min chase (Fig. 5B).

The problems of varying protein levels among different Tn*phoA* fusions and of high and different rates of protein turnover have been noted previously (19, 27, 29). It has been proposed that a higher level of protein turnover in the cytoplasm might contribute to lower levels of AP activity (27). This correlation was also noted in the present study (Fig. 5B). On the other hand, the average level of protein

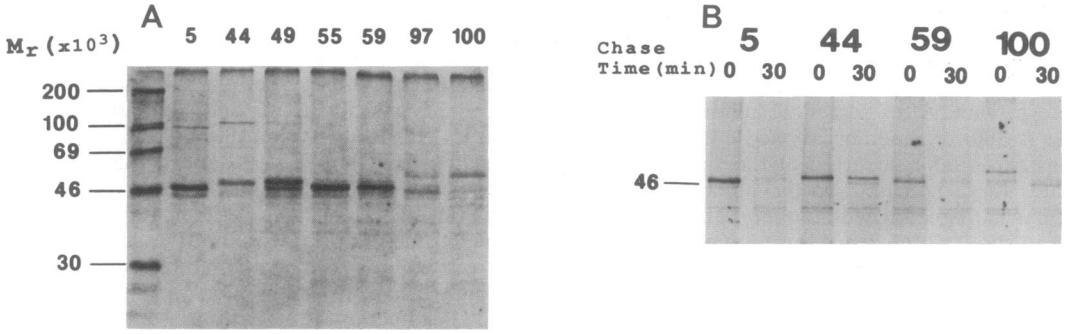

FIG. 5. (A) Identification of *imm-phoA* fusion proteins. Cells were labeled with [^{35}S]methionine for 2 min and immunoprecipitated with an anti-bacterial AP antibody. The immunoprecipitates were run on SDS-PAGE gels and autoradiographed. The leftmost lane contains molecular weight standards: myosin (200,000), phosphorylase *b* (97,000), bovine serum albumin (69,000), ovalbumin (46,000), and carbonic anhydrase (30,000). The molecular weight of Tn*phoA* is 48,500 (33). Residues are indicated above the lanes. (B) Stability of the residue 5, 44, 59, and 100 *imm-phoA* fusion proteins. Cells were labeled for 2 min and chased for 30 min, and the immunoprecipitates were separated on SDS-PAGE gels and autoradiographed.

resulting from fusions at residues 5, 55, and 59 was not reduced relative to the other fusions (Fig. 5A). The somewhat smaller size of the fusions at residues 5, 55 and 59 (Fig. 5A) may not be critical, since the proteolytic cleavage is near the fusion junction, the AP appears to be intact, and the size of the AP, if exported, should be sufficient for activity (19).

DISCUSSION

The Imm protein produced in association with the channel-forming colicins can protect the host cell against the action of exogenous colicin produced by its neighbors. This protective function is a unique aspect of the action of these colicins on membranes as compared with that of other toxinlike molecules. The protective mechanism of the Imm proteins of the channel-forming colicins is different from that of a direct interaction of the respective Imm proteins with colicin E2 or E3 (5). The protective function of the Imm proteins of the channel-forming colicins has been studied for colicins A, E1, and Ia. The intriguing nature of the problem arises from the following facts. (i) Approximately 10^2 immunity molecules per cell, located in the inner membrane (3, 16, 44), can prevent the formation of a lethal ion channel in the inner membrane via a colicin molecule randomly adsorbed to the outer membrane receptor and translocated across the cell envelope to the inner membrane. (ii) The immunity effect is exerted against the COOH-terminal domain of the colicins, which contains the channel function (3, 11). Despite the appreciable sequence identity of the 150 to 200 residues in the channel domains of the six different channel-forming colicins (A, B, E1, Ia, Ib, and N) and the very high (60 to 80%) identity between the Ia and Ib Imm proteins, the action of an Imm protein is specific for the Col plasmid from which it is derived. (iii) In the case of colicins E1 and A, it appears that the immunity function cannot be observed in a reconstituted system with liposomes (9, 18).

The above-described data imply that the specificity of the interaction of immunity at least partly arises from a specific interaction with the colicin translocation or adhesion apparatus that extends to the cytoplasmic membrane (1). The gene products known to be required for the translocation of colicin E1 across the cell envelope are the BtuB, TolA, TolC, and TolQ proteins (12, 20, 21, 25, 41) and possibly the TolR protein. The TolA and TolQ proteins are believed to be inserted into the cytoplasmic membrane and to span it with one and three α-helices, respectively (4, 25).

The topography and orientation of the colicin E1 Imm protein in the cytoplasmic membrane, as deduced from the Tn*phoA* and *lacZ* fusions, are consistent with the principle of positive charge distribution of translocated protein loops with ≤70 residues (43), according to which there should be fewer positive charges on the translocated than on the nontranslocated side (43). The folding pattern of the colicin E1 Imm protein shown in Fig. 4 has two and eight positive charges on the periplasmic and cytoplasmic sides of the membrane, respectively. The orientation of the NH_2 terminus is the same as that inferred for the colicin A Imm protein (17).

Some of the differences in Imm protein specificity may arise from the large differences in structure shown in the present work between the colicin (and, by inference, the colicin Ia and Ib) Imm proteins with three *trans*-membrane α-helices and the colicin A and B Imm proteins with four such helices. Thus, extrinsic L3 between H3 and H4 of the colicin A Imm protein contains three positive charges (a total of five charged residues) and has been inferred from mutagenesis studies to be important for immunity function, along with H4 and the COOH terminus (15, 17). L3 appears to be quite different in the colicin E1 Imm protein, in which it contains about nine residues and only one charge. H4 and the last 65 residues of the colicin Imm protein are absent in the colicin E1 Imm protein.

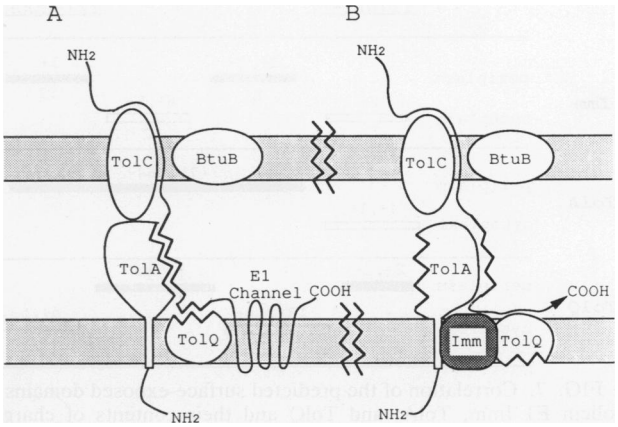

FIG. 6. (A) Model of colicin E1 translocation across the membrane in the absence of the colicin E1 Imm protein. (B) Model of the blockage of the colicin E1 COOH-terminal channel domain by the Imm protein through interaction with the TolA and/or TolQ protein.

A major difference between the Imm proteins of colicins N and A that may partly account for their specificities is the truncated NH_2 terminus of the former, which results in the absence of T1 and most of H1 (17).

Part of the specificity of the colicin E1 Imm protein probably arises from an interaction with the translocation apparatus. Interaction with the Tol proteins is a more precise and testable hypothesis than is interaction with adhesion zones, because the proteins in the latter have not been identified and the existence of these zones as structural entities has been questioned (23). Even though there are relatively few colicin E1 Imm proteins in the cytoplasmic membrane, the diffusion constant in the membrane ($\sim 10^{-9}$ to 10^{-10} cm^2/s [10]) would allow a root mean square migration on the membrane surface of at least 500 nm in 10 s, so that the probability of an encounter and association with the colicin translocation apparatus is high.

We suggest (Fig. 6) that the colicin E1 Imm protein interacts both with extrinsic segments of the TolA and/or TolQ proteins that are localized in the cytoplasmic membrane (4, 25) and with the colicin E1 channel domain. The charged residues in the extrinsic segments of the colicin E1 Imm protein are hypothesized to be responsible for the specific interactions with the *tol* gene apparatus and the colicin E1 channel domain. A working hypothesis is that periplasmic L1 and the COOH terminus (T2) of the colicin E1 Imm protein may interact with the large, highly charged COOH-terminal domain of the TolA protein and/or with the periplasm-exposed NH_2 terminus (T1) and L2 of the TolQ protein (Fig. 7), as well as with some residues among the 10 positive and 8 negative charges of the channel domain that are believed to be exposed on the periplasmic side of the membrane. Highly charged L2 and/or the NH_2 terminus (T1) of the colicin E1 Imm protein facing the cytoplasm may interact with the NH_2 terminus (T1) of the TolA protein and/or L1 and the COOH terminus (T2) of the TolQ protein of the translocation apparatus.

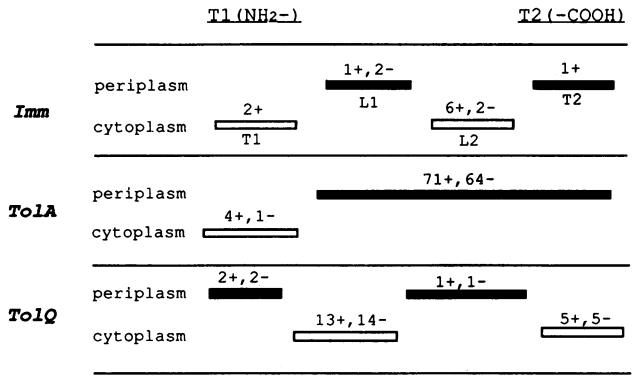

FIG. 7. Correlation of the predicted surface-exposed domains of colicin E1 Imm, TolA, and TolQ and their contents of charged amino acids. The black and white bars indicate the regions exposed to the periplasmic and cytoplasmic sides, respectively. The numbers over each bar indicate the total numbers of positive and negative charges in the segments. The orientation of the TolQ protein was inferred from sequence and hydropathy analyses, as well as from the principle that the translocated side of the protein contains a smaller number of positive charges (43).

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