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An α-Helical Hydrophobic Hairpin as a Specific Determinant in Protein-Protein Interaction Occurring in *Escherichia coli* Colicin A and B Immunity Systems

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A collection of chimeric pore-forming domains between colicins A and B was constructed to investigate the specific determinants responsible for recognition by the corresponding immunity proteins. The fusion sites in the hybrid proteins were positioned according to the three-dimensional structure of the soluble form of the colicin A pore-forming domain. The hydrophobic hairpin of colicin pore-forming domains, buried in the core of the soluble structure, was the main determinant recognized by the integral immunity proteins. The immunity protein function may require helix-helix recognition within the lipid bilayer.

Pore-forming colicins kill bacteria by forming voltage-dependent channels in membranes of sensitive strains (21). On the basis of the structure of the pore-forming domain of colicin A (ColA) in aqueous solution and spectroscopic methods, a model of membrane insertion has been proposed (12, 19, 24, 27). This insertion is triggered by the spontaneous insertion in the bilayer of a hydrophobic helical hairpin buried in the water-soluble structure.

Colicin-producing strains protect themselves from autodestruction by synthesizing immunity proteins that inactivate the function of the pore-forming domain (3, 8, 9, 23, 25).

Pore-forming ColA and colicin B (ColB) follow different uptake pathways from their respective receptors in the outer membrane to their target (the inner membrane). ColB uses the TonB-dependent high-affinity transport system (16), whereas ColA enters via the Tol import system (4). Although the sequences of the pore-forming domains of ColA and ColB are similar (6) (57% of the residues are identical, and 17% are structurally related amino acids [Fig. 1]), the colicins have their own specific immunity proteins (ImmA and ImmB, respectively), which show 38% identity and 39% conservative substitutions. Both immunity proteins have four membrane-spanning segments, and their N and C termini are directed toward the cytoplasm (10).

We report that the hydrophobic hairpin of the colicin pore-forming domain is the major determinant required for the specificity of the recognition by the integral membrane immunity proteins.

MATERIALS AND METHODS

Construction of plasmids carrying *colA/colB* and *colB/colA* genes cloned in tandem. A 2.8-kb *Stu*I-*Hpa*I DNA fragment from pES7 (22) was cloned into *Sma*I of pUC12. The resulting plasmid, pUCBI9, was digested by *Eco*RV-*Hin*dIII to produce a 1.8-kb fragment including the DNA region encoding the pore-forming domains of ColB. This 1.8-kb DNA fragment was cloned into pColA9 (15) digested by *Eco*RV-*Hin*dIII. The resulting plasmid, pVAB, contained the complete *colA* gene with the truncated *colB* gene cloned in tandem. A 1.4-kb *Mam*I-*Bgl*II fragment from pColA9 was cloned into pUCBI9 digested by *Mam*I-*Bam*HI. The resulting vector, pVBA, contained the complete *colB* gene followed by a truncated *colA* gene, including the DNA region encoding the pore-forming domain of ColA. pVAB and pVBA, respectively, were used to produce the *colA/colB* and *colB/colA* chimeric genes.

Construction of the *colA/colB* and *colB/colA* chimeric genes. To construct chimeric genes, 5-μg samples of purified pVAB and pVBA, respectively, were linearized with *Mlu*I and *Mam*I (these restriction sites are located between the genes cloned in tandem) and treated with exonuclease III and mung bean nuclease according to the instructions of the supplier (Stratagene). Exonuclease III treatment was stopped every 30 s after 90 s of treatment. Treated plasmids were pooled and redigested with the restriction enzyme used for the initial linearization to reduce the wild-type background.

Selection of active hybrid colicins. Strain C600 was transformed with each of the treated plasmids. Transformants were picked up with toothpicks and transferred onto plates on which a sensitive indicator strain had been plated; the plates contained 300 ng of mitomycin C (inducer of the colicin expression) per ml. Active clones were selected, and their DNA was analyzed by restriction mapping. Homologous recombination resulted in deletions of 1,235 bp for pVAB and 1,446 bp for pVBA (18, 26). The chimeric genes of the selected clones were sequenced by the dideoxy-chain termination method.

For the activity test, 3-ml samples of hybrid colicin-producing cultures were induced with 300 ng of mitomycin for 5 h. The cultures were centrifuged, and the pellets were resuspended in 100 μl of phosphate buffer (10 mM, pH 6.8) and sonicated for 15 min in a water-bath sonicator. Extracts were centrifuged for 15 min at 20,000 × *g*. Supernatants were used as starting material for the activity tests. Supernatants were diluted 1:10 serially from 1 to 10^{-5} and from 5 × 10^{-1} to 5 × 10^{-5} in phosphate buffer (10 mM, pH 6.8) containing bovine serum albumin (0.1 mg/ml). Samples (1 μl) of each dilution were spotted on a lawn of the indicator strain (optical density at 600 nm, 0.5). Procedures for the purification of ColB and ColA were adapted from those described

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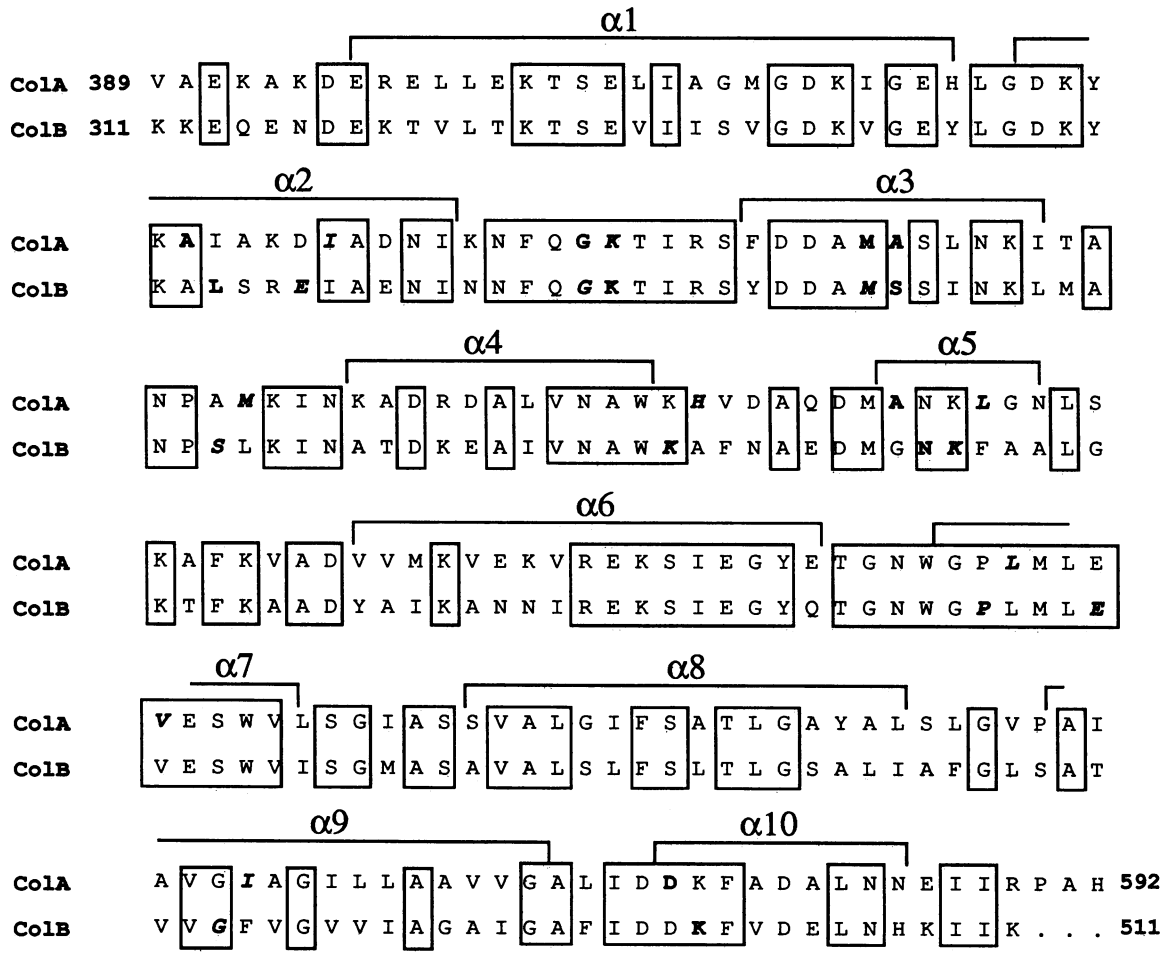


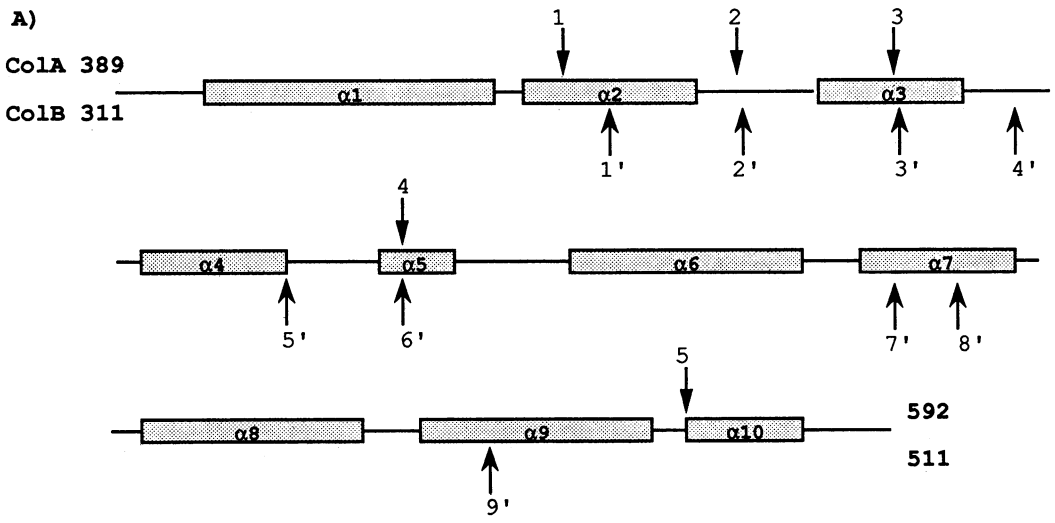
FIG. 1. Sequence alignment of the ColA and ColB pore-forming domains. Numbers refer to the residue positions on the entire colicin molecule. Homologous amino acids are boxed. The positions of the 10 α-helices of the ColA pore-forming domain refer to the X-ray crystallographic structure of soluble ColA (19). Residues in boldface type indicate the precise localizations of the junctions between pore-forming domains of ColA and ColB (Fig. 2).

previously (2, 20). Purified ColA and ColB (each at 1 mg/ml) were used as internal controls.

Construction of ColA- and ColB-immune strains. *immB* and *immA* pUC derivatives are from our laboratory and from V. Braun's laboratory, respectively (8, 23). The 1,359-bp *Pvu*II fragment of pColA9 was deleted to produce pIA1 (*immA* pBr derivative). An 800-bp *Eco*RI-*Hin*dIII DNA fragment from pES15 (23) carrying the *immB* gene was cloned into pIA1 cut with *Eco*RI-*Hin*dIII. The *immA* gene (Δ*Eco*RV-*Stu*I, 788 bp) of the resulting plasmid, pIAB, was deleted to produce pIB1, the *immB* pBr derivative. The titers of the killing activities of hybrid colicins on the immune indicator strains were determined as described above.

RESULTS

Construction of ColA/ColB and ColB/ColA chimeric proteins by homologous DNA recombination. To identify the specific determinants in ColA and ColB responsible for recognition by the membrane immunity proteins, a number of ColA/ColB and ColB/ColA chimeric pore-forming domains were generated and their killing activities against bacteria expressing either ImmA or ImmB proteins were tested. The hybrid colicins were constructed by homologous recombination of ColA and ColB genes (*caa* and *cba*, respectively). The DNA regions of *caa* and *cba* coding for their corresponding pore-forming domains can be aligned without introducing any gap; 415 identities out of 628 possible matches between bases were found. The pore-forming domains are 65% homologous at the protein level. Among 100 transformants that were tested for colicin activity, 48 expressed active colicin; of these, 8 were wild-type colicins. Among 40 chimeric genes coding for active proteins, 28 contained fixed deletions that resulted from homologous DNA recombination. Fusions occurred throughout the sequence except in helices 1, 6, and 8 (Fig. 2). Analysis of DNA homology between *caa* and *cba* revealed a correlation between the frequency of appearance of a hybrid protein and the level of homology upstream from the fusion site (data not shown). Hot spots of recombination were located between regions encoding helices 2 and 3 as well as within these regions. Sequenced inactive clones resulted either from large deletions or from codon-reading frame shifts. All ColA/ColB and ColB/ColA hybrid proteins encoded by the chimeric genes were dependent on Tol(A,B,Q,R) and TonB, respectively, for their uptake. This observation is consistent with



B)

		ColA/B					ColB/A								
		1	2	3	4	5	1'	2'	3'	4'	5'	6'	7'	8'	9'
activity against	*immA*	+	+	+	+	-	-	-	-	-	-	-	-	-	+
	immB	-	-	-	-	+	+	+	+	+	+	+	+	+	+/-

FIG. 2. (A) Location of the fusion sites in the hybrid pore-forming domains. Downward and upward arrows indicate the ColA/ColB and ColB/ColA fusion sites, respectively. (B) Activity (+) or lack of activity (−) against the *imm* indicator strain. Quantitative results are shown in Table 1.

previous evidence that the N-terminal domain of individual colicins determines the uptake pathway (1).

The hydrophobic α-helical hairpin of colicin pore-forming domains is the major determinant for immunity specificity. Extracts containing each hybrid colicin were prepared, and titers of the killing activity were determined by using the sensitive indicator strain C600. Plasmids with different copy numbers and therefore various levels of immunity protein expression were used as vectors for the *imm* genes (see Materials and Methods) (Table 1).

The results shown in Table 1 were obtained from three independent assays. When the activities of ColA and ColB were tested on *immA*$^+$ and *immB*$^+$ strains (Table 1), crossed immunity was observed with *immA*, which, in addition to conferring immunity to ColA, conferred partial immunity to ColB. In contrast, *immB* was highly specific for ColB.

The ColA/ColB fusions located in helices 2 to 5 (Fig. 2, Table 1) behaved identically with respect to *immA* and *immB* indicator strains: *immB* strains were fully protected, whereas *immA* strains were sensitive, although 10 times less sensitive than to ColA. These results suggest that the main determinant for ImmB recognition is located between helices 5 and 10 of the ColB pore-forming domain and that the main determinant for ImmA recognition is not located between helices 1 and 5.

There might be a minor determinant for ColA-ImmA recognition located upstream of helix 2 (Ala-424, ColA sequence), which could explain the two- to fivefold protection by ImmA between Ala-424/Leu-347 ColA/ColB fusion (Table 1) and a fusion containing the full-length pore-forming domain of ColB (Arg-426/Asn-303), which behaves like wild-type ColB.

When helix 10 was exchanged between ColA and ColB (Table 1), the hybrid protein behaved like ColA toward both the *immA* and the *immB* strains. This result implies that the main determinant for ImmA recognition does not contain helix 10.

All ColB/ColA fusions with fusion sites between helices 2 and 7 displayed a full ColA phenotype (protection by *immA*, activity on *immB*; Table 1). We conclude that the main specific determinants for ImmA recognition are located downstream of amino acid Val-525 (in the ColA sequence) in the middle of helix 7. All of these hybrids kill *immB* strains as well as ColA does. There is therefore no indication of minor *immB* recognition between helix 1 and the middle of helix 7. The fact that *immA* strains are even less sensitive to these hybrids than to ColA indicates that there is also no minor *immA* determinant upstream of helix 2, as suggested from the ColA/ColB hybrids.

The basis of the specificity should reside in the regions of low homology. Inspection of the aligned amino acid sequences shows 100% identity in helix 7 (Fig. 1). Therefore, the main determinant of immunity recognition could be shortened to extend from Leu-530 to Asp-577 of the ColA sequence. This region contains the hydrophobic hairpin (helices 8 and 9), which has been suggested to insert spontaneously into lipid bilayers in a potential-independent manner (14).

TABLE 1. Hybrid colicins[a]

Colicin	Residue positions	Fusion site (amino acid)	Activity[b]	Protection factor[c]			
				immA$^+$	*immA*$^{++}$	*immB*$^+$	*immB*$^{++}$
ColB		389	10^5	10	50	Full	Full
ColA/ColB	426/303	380	10^5	10	50	50,000	Full
ColA/ColB	424/347	424	10^5	50	100	100,000	Full
ColA/ColB	438/361	438	10^5	50	100	100,000	Full
ColA/ColB	448/371	448	10^5	50	100	100,000	Full
ColA/ColB	483/406	483	10^4	50	100	100,000	Full
ColA/ColB	577/500	577	10^5	500	1,000	10	50
ColA		592	10^5	500	1,000	1	5
ColA		311	10^5	500	1,000	1	5
ColB/ColA	350/429	350	10^4	5,000	10,000	1	5
ColB/ColA	360/439	360	10^4	5,000	10,000	1	5
ColB/ColA	370/449	370	10^4	5,000	10,000	1	5
ColB/ColA	381/460	381	10^4	5,000	10,000	1	5
ColB/ColA	397/476	397	10^4	5,000	10,000	1	5
ColB/ColA	406/485	406	10^4	5,000	10,000	1	5
ColB/ColA	442/521	442	10^4	5,000	10,000	1	5
ColB/ColA	446/525	446	10^4	5,000	10,000	1	5
ColB/ColA	483/562	483	10^5	1	5	500	5,000
ColB		511	10^5	10	50	Full	Full

[a] The positions of hybrid colicins with respect to the ColA pore-forming domain structure are shown in Fig. 2, except for ColA/ColB 426/303, which does not result from homologous recombination.

[b] Maximal dilution of colicin extract for which a plaque of growth inhibition of the indicator strain is detected.

[c] The protection factor is equal to the activity against the *imm*$^-$ strain divided by the activity against the *imm*$^+$ strain. Immunity genes on immune indicator strains are carried either by pBr derivatives (*imm*$^+$) or pUC derivatives (*imm*$^{++}$). Full, the *imm* indicator strain is not killed by undiluted colicin extract.

The above conclusions were strengthened by analysis of the killing activity of the Gly-483/Ile-562 ColB/ColA hybrid protein, which has a fusion site in the first 33% of helix 9 (Fig. 2). The *immA* indicator strains were completely sensitive to this protein, and the *immB* indicator strains were partially sensitive (Table 1). Gly-483/Ile-562 killed *immA* strains even better than ColB did and was at least 200-fold more active than ColB on the *immB* (pBr) strain. However, the *immB* strains retained some protection against this hybrid protein (Gly-483/Ile-562 killed the *immB* [pBr] strain 500-fold less efficiently than did ColA). These activity results provide direct evidence for the role of helices 8 and 9 in the recognition process.

DISCUSSION

The strong specificity of the recognition between a colicin and its immunity protein suggests a direct interaction between the two proteins. All of the data presented so far indicate that ImmA and ImmB proteins recognize the same region on the polypeptide chain of the ColA and ColB pore-forming domains that comprises helix 8 to the end of helix 9. The strong homology between the pore-forming domains of ColA and ColB on the one hand and their immunity proteins on the other hand strongly suggests that the ColA and ColB pore-forming domains fold into very similar three-dimensional structures (5). Therefore, it is expected that protein-protein recognition occurs in the same areas of the molecules, even though different motifs are recognized to ensure specificity. The partial crossed immunity with ImmA supports this hypothesis. It has been hypothesized that the hydrophobic helical hairpin of the ColA pore-forming domain spontaneously inserts into the membrane bilayer and that the peripheral amphipatic helices have their axes parallel to the plane of the bilayer (the umbrella model [19]). However, recent fluorescence measurements (13) and nuclear magnetic resonance studies on the effect of the ColA pore-forming domain on *E. coli* lipids (11) indicate that in the absence of membrane potential, the hydrophobic hairpin does not insert as a perpendicular hairpin but remains packed with the surface helices. The question arises as to whether α-helices 8 and 9 insert into the membrane upon membrane potential application. If so, the immunity protein could prevent the potential-dependent insertion of the helical hairpin that would follow the insertion of the voltage-responsive segments (17). The localization of the interaction between the ColA immunity protein and ColA remains to be determined. The understanding of the structure of the colicin pore-forming domain in the membrane in the presence of a membrane potential will help us understand with what region of the immunity protein helices 8 and 9 are interacting. The search for mutants of the immunity protein able to protect against the chimeric colicin (BBA 9) will also contribute to the understanding of the molecular interaction between pore-forming colicins and their respective immunity proteins.

So far, we know that the second periplasmic loop (L3 in Fig. 3) is required for the function of the immunity protein (10). In addition, we recently found that only the first transmembrane segment could be functionally exchanged between ImmA and ImmB and that specific exchange of the second periplasmic loop cannot be done without a loss of function (7a). It is tempting to speculate that the function of the immunity protein requires specific determinants located in the hydrophobic segments in addition to the second periplasmic loop.

As indicated in Table 1, *immA* strains confer greater immunity to ColB/ColA fusion proteins than to ColA. Although the activities of the purified ColB/ColA (360/439; the numbers refer to amino acid residue positions) fusion protein and ColA were similar in a plate assay with a sensitive indicator strain, the in vivo activities tested with a K^+ probe

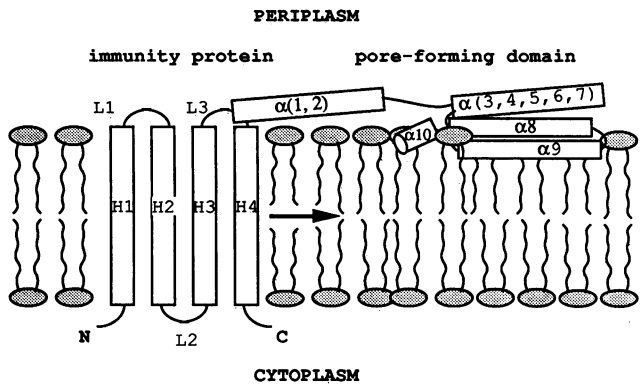


FIG. 3. The major determinant for immunity specificity is located between α-helical hydrophobic helices 8 and 9. For clarity, only the C-terminal pore-forming domain of ColA is represented. The α-helices are not shown embedded in the membrane but are displaced vertically upward from their predicted positions. According to the new model of ColA membrane insertion by Lakey et al. (13), the hydrophobic hairpin remains surface localized in the absence of potential application. Membrane potential causes insertion of further helices (17). At one of these two stages, the hydrophobic helices of ImmA might interact with the α-helices 8 and 9 to prevent the formation of the channel. The ImmA topology is represented as reported previously (10). Immunity transmembrane helices are labeled H1 to H4; the two periplasmic loops and the cytoplasmic loop are labeled L1, L3, and L2, respectively. It is not known whether the immunity protein freely diffuses within the membrane or interacts with a component common to the ColA and ColB uptake pathways.

for their ability to induce an efflux of cytoplasmic K^+ were different. For purified ColA, ColB, and ColA/ColB fusions, the initial rates of efflux saturated between 100 and 300 added molecules, depending on the colicin which was tested, whereas between 2,000 and 3,000 molecules of purified ColB/ColA (360/439) were necessary to reach saturation. However, the values of the maximum initial rates were similar for all tested proteins (10a). It is thus possible that for some unknown reason ColB/ColA (360/439) is less efficient than ColA for entry into the bacterium, but this effect is not observed when ColB/ColA (360/439) is tested on a sensitive indicator strain with an overnight plate assay. It is noteworthy that all ColB/ColA fusions have the same behavior. This could explain why $ImmA^+$ strains protect better against ColB/ColA fusions than against ColA (the number of efficient ColB/ColA molecules is lower). On sensitive indicator strains like C600 or on $ImmB^+$ strains, this lower efficiency of ColB/ColA molecules for their entry is insignificant.

The results reported in this paper also show that immunity specificity depends mainly on the pore-forming domain of the hybrid colicin and not on its uptake pathway. Thus, either immunity proteins do not interact with the colicin uptake systems or immunity proteins are able to recognize both Tol and Ton systems through a component common to the two uptake pathways (7).

With the availability of the ColA pore-forming domain structure, ColA-ImmA interactions may provide new insights into the functioning of antagonists of ion channels and interactions between proteins in lipid bilayers.

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