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Evidence that the Immunity Protein Inactivates Colicin 5 Immediately prior to the Formation of the Transmembrane Channel

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Determination and analysis of the nucleotide sequences of the activity, immunity, and lysis genes of colicin 5 assigned colicin 5 to the subclass of pore-forming colicins to which colicins 10, E1, Ia, Ib, and K belong. Mutational analysis of colicin 5 and exchange of DNA fragments between the most closely related colicins, colicins 5 and 10, and between their immunity proteins localized the regions that determine the reaction specificity between colicin 5 and its immunity protein to residues 405 to 424 of colicin 5, the region corresponding to the amphiphilic α-helix 6 of the similar colicins E1 and Ia. The specificity-conferring residues 55 to 58 and 68 to 75 of the immunity protein were localized in the cytoplasmic loop and the inner leaflet of the cytoplasmic membrane. The localization of the reactive regions of the immunity protein and the colicin close to the inner side of the cytoplasmic membrane suggests that the immunity protein inactivates colicin 5 shortly before the lethal colicin pores in the cytoplasmic membrane are opened.

Colicins are bactericidal proteins that are produced by about 50% of *Escherichia coli* isolates from humans. Colicins usually act only on *E. coli* strains because of the elaborate uptake system required for entry into sensitive cells. Their uptake is initiated by binding to colicin-specific receptor proteins located in the outer membrane (5, 6). After the colicins have bound to the receptor proteins, further translocation of the colicins is mediated by either the Ton or the Tol system. The Tol system consists of the proteins TolA, TolB, TolQ, and TolR (33); the Ton system consists of the proteins TonB, ExbB, and ExbD (4, 5, 21). For the Ton-dependent colicins, it has been shown that the electrochemical potential of the cytoplasmic membrane drives their uptake across the outer membrane (2, 10). Uptake-defective mutants with point mutations in *tonB* are suppressed by point mutations in receptors and in colicins, suggesting the interaction of TonB with these proteins (11). This conclusion is supported by the location of the suppressor sites in a consensus sequence (TonB box) close to the N terminus of the TonB-dependent receptors and colicins (1, 15, 20, 25).

Recently, a new colicin, designated colicin 5, has been described (3). Colicin 5 uptake is unusual because it requires the Ton system, although its cognate receptor Tsx is only known to function with the Tol system, as determined for the uptake of colicin K. An additional colicin, colicin 10, exhibited the same properties as colicin 5 (2a). A molecular genetic study of the colicin 10 determinant has revealed a strong homology between colicin 10 and colicin E1 in the domains with similar functions (18). Both colicins require the TolC protein for uptake (18), and colicin E1 forms pores in the cytoplasmic membrane (8), which can also be inferred for colicin 10. The amino acid sequences in the regions proposed to determine binding of colicin 10 to the Tsx receptor and uptake via the Ton system are different from those proposed to determine binding of colicin E1 to the BtuB receptor and uptake via the Tol system (18).

In this study, we investigated the colicin 5 determinant because it uses the same uptake system as colicin 10 and confers no cross-immunity to colicins 10 and E1, in contrast to the colicin 10 immunity protein, which cross-reacts with colicin E1. Since cross-immunity among colicins is unusual (in fact, closely related colicins can be differentiated by their specific immunities), it is of interest to unravel the similarities and differences among the immunity proteins and among the regions of the colicins that interact with the immunity proteins. By this means, one could expect to define the sites of the colicins and the immunity proteins responsible for their specific interactions. The understanding of the immunity to pore-forming colicins is still incomplete, since binding of these colicins to the immunity proteins has not been demonstrated. It is thought that pore-forming colicins are inactivated by the immunity proteins before they enter the cytoplasmic membrane or during pore formation. In this study, we identified the specific regions of interaction of the pore-forming colicin 5 and of the cognate immunity protein. According to the predicted transmembrane arrangement of the colicin and the immunity protein, the interacting sites are located at the inner side of the cytoplasmic membrane. This implies inactivation of the colicin at the very last moment before the cells are killed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are described in Table 1. All strains were grown in a medium composed of 1% Bacto tryptone–0.5% yeast extract–0.5% NaCl (TY), pH 7, or on TY agar plates. When necessary, media were supplemented with kanamycin at 50 μg/ml or chloramphenicol at 50 μg/ml. The ampicillin resistance of strains carrying *cfi-blaM* fusion genes was tested on TY agar plates supplemented with increasing concentrations of ampicillin (5, 25, 50, 100, 200, and 400 μg/ml).

Recombinant DNA techniques. Plasmid DNA was isolated with ion-exchange columns from Qiagen (Hilden, Germany). Standard methods were used for restriction endonuclease analyses, ligation, and transformation of plasmid DNA (22). Subclones of plasmid pHP20 were constructed with the vectors pBCSK+ and pBCKS+ (Stratagene, Heidelberg, Germany). Sequencing was performed by the dideoxy chain termination method (23) with an AutoRead sequencing kit (Pharmacia Biotech, Freiburg, Germany) and an ALF Automatic Sequenator (Pharmacia Biotech). Site-specific mutagenesis was performed by the PCR technique (12). The nucleotide sequence of the primer employed for the insertion of the *Bfr*I restriction site into the *cfa* gene is given as an example of the mismatch primers used for PCR (nucleotide replacement underlined): 5′-CTCGACTA GTGATCTTAAGCGACTTTG-3′. The sequences of the mutagenized frag-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Source or reference
ECOR5	Wild type (pCol5)	3
5K	*hsdR lacZ rpsL ser thi thr*	G. Schrempf
BL21	F^- *hsdS gal*	30
KS474	KS272 *degP41* (Δ*Pst*I-Kan^r)	29
MC4100	*araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 fibB5301 deoC1 ptsF25 rbsR*	M. Casabadan
AB2847	*aroB thi malT tsx*	B. Bachmann
HP45	5K *tonB*	18
C65	K10C4 *tolC*	34
pCol5	Natural plasmid carrying *cfa cfi cfl*	3
pCol10	Natural plasmid carrying *cta cti ctl*	18
pBCKS+	Phage T7 gene 10 promoter	Stratagene
pBCSK+	Phage T7 gene 10 promoter	Stratagene
pT7-3	Phage T7 gene 10 promoter	31
pJBS636	β-Lactamase fusion vector carrying the TEM β-lactamase gene *blaM*	9a
pHP10	pBCKS+ carrying *cta cti ctl*	18
pHP15	pBCKS+ carrying *cti ctl*	18
pHP20	pBCKS+ carrying *cfa cfi cfl*	This work
pHP21	pBCKS+ carrying *cfa-cta cti ctl*	This work
pHP22	pBCKS+ carrying *cta-cfa cfi cfl*	This work
pHP23	pBCKS+ carrying *cfa* (WAKHLE [residues 392–397]) *cfi cfl*	This work
pHP40	pBCKS+ carrying *cfi cfl*	This work
pHP41	pBCKS+ carrying *cfi-cti ctl*	This work
pHP42	pBCKS+ carrying *cti-cfi cfl*	This work
pHP44	pJBS636 carrying *cfi27-blaM*	This work
pHP45	pJBS636 carrying *cfi63-blaM*	This work
pHP46	pJBS636 carrying *cfi95-blaM*	This work
pHP65	pBCKS+ carrying *cfi63-blaM*	This work

ments were verified by DNA sequencing. For constructing *cfi-blaM* gene fusions, blunt-ended restriction sites were introduced into the *cfi* gene. After amplification by the PCR technique, the *cfi* gene fragments were cloned into the pJBS636 vector, forming *cfi-blaM* gene fusions. pJBS636, a β-lactamase fusion vector, was derived from the pJBS633 vector (7); pJBS636 also carries the T7 promoter and the multiple cloning site of plasmid pT7-7 (9a).

Radiolabeling of proteins. *cfi-blaM* gene fusions were transcribed under the control of the gene 10 promoter of phage T7 by T7 RNA polymerase, which was chromosomally encoded in *E. coli* BL21 and was under the control of the *lacI* repressor. Cells (2 ml) in the exponential growth phase (optical density at 578 nm, 0.4) were collected by centrifugation and then resuspended in 1 ml of a medium containing 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.1% NH_4Cl, 0.05% NaCl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 mM sodium citrate, 0.4% glucose, 20 mg of thiamine per liter, and 0.01% methionine assay medium (Difco Laboratories, Detroit, Mich.). T7 RNA polymerase synthesis was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside. After the culture was shaken for 60 min at 37°C, 20 μl of rifamycin (20 mg/ml, in methanol) was added to inhibit the *E. coli* RNA polymerase and the culture was shaken for an additional 30 min. Cells were then labeled by adding 185 kBq of [^{35}S]methionine and incubating the culture for 15 min at room temperature. Cells were sedimented by centrifugation, resuspended in 40 μl of sample buffer, and heated for 5 min at 100°C. Ten microliters was applied to a polyacrylamide gel (3% stacking gel, 15% running gel) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of 0.1% SDS. The dried gel was autoradiographed with Kodak X-Omat S100 film.

Colicin activity assays. Colicin activity was tested by spotting 10-fold dilutions of colicin crude extracts onto plates, containing 20 ml of TY agar, which were then overlaid with 3 ml of low-melting-point TY agar in which 0.1 ml of an overnight culture of the indicator bacterium had been suspended (15). Mutant strains were compared with their parent strains.

Purification of colicins 5 and 10. Colicins 5 and 10 were purified from *E. coli* W3110 cells harboring the natural plasmids pCol5 and pCol10, respectively; the recovery of active colicins from strains carrying natural plasmids was better than that from *E. coli* cells carrying cloned colicin genes. *E. coli* W3110 transformed with pCol5 or pCol10 was grown in TY medium to an optical density at 578 nm of 1 and subsequently treated with mitomycin C (400 ng/ml) for 3 h. Cells were collected by centrifugation, and the proteins remaining in the supernatant were precipitated with 50% $(NH_4)_2SO_4$. The precipitate was dissolved in 100 mM sodium phosphate (pH 7.2) and desalted with a PD10 gel filtration column (Pharmacia) equilibrated with 100 mM sodium phosphate buffer (pH 7.2). The eluate was centrifuged (10,000 × *g*, 10 min). The clear supernatant fluid was then applied to a Resource Q anion-exchange column (Pharmacia) and chromatographed by fast-performance liquid chromatography (Pharmacia) with a linear gradient of NaCl from 0 to 1 M in the sodium phosphate buffer. Each colicin appeared as a single peak that was eluted at about 0.25 M NaCl. The purity of colicins 5 and 10 was assessed by SDS-PAGE (13). To estimate the molecular masses of the colicins, their electrophoretic mobilities were compared with those of standard proteins by using a 10-kDa protein ladder (Gibco BRL, Eggenstein, Germany).

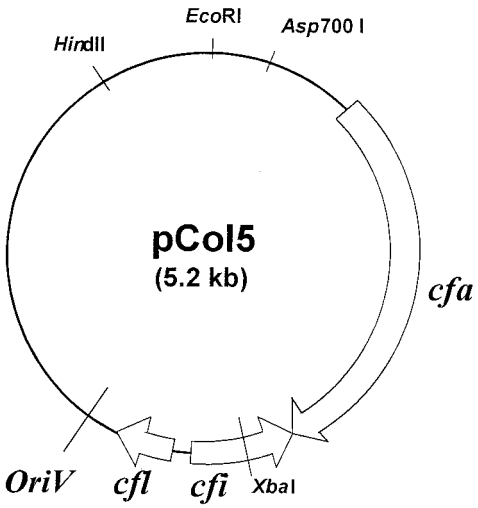

FIG. 1. Arrangement of the colicin 5 activity (*cfa*), immunity (*cfi*), and lysis (*cfl*) genes on the natural plasmid pCol5. The arrows indicate the transcription polarity. Restriction sites indicated are those used for cloning.

Computer analysis. Nucleotide and amino acid sequences were analyzed with the PC/GENE software package, with the programs CLUSTAL for performing multiple alignments, PALIGN for searching for homologies between proteins, and NALIGN for comparing nucleotide sequences.

Nucleotide sequence accession number. The nucleotide sequence reported in this study was deposited in the EMBL data bank under the accession no. X87835.

RESULTS

Cloning of the colicin 5 genes. The natural plasmid pCol5 (Fig. 1) was digested with *Eco*RI-*Hin*dII, and the restriction fragments were cloned into plasmid pBCKS+. Transformants of *E. coli* 5K were screened for colicin activity with colicin 5-sensitive untransformed *E. coli* 5K. A 4.7-kb fragment conferred colicin activity; a 2,166-bp *Bsp*HI-*Xho*II subfragment was completely sequenced (both strands). The nucleotide sequence contained three open reading frames, designated *cfa* (colicin 5 activity), *cfi* (colicin 5 immunity), and *cfl* (colicin 5 lysis) (Fig. 2). *cfa* and *cfl* had the same transcription polarity, and *cfi* had the opposite polarity (Fig. 1), an arrangement typical for the genes of all pore-forming colicins. *cfa*, *cfi*, and *cfl* encode proteins of 490 (M_r, 53,137), 96 (M_r, 11,498), and 43 (M_r, 4,556) amino acid residues, respectively. The electrophoretic mobility of purified Cfa in an SDS-polyacrylamide gel corresponded to an M_r of 57,000 (Fig. 3, lane 2), which is indistinguishable from the position of purified colicin 10 (Cta) (Fig. 3, lane 3).

Homology between colicins 5 and 10. Colicins 5 and 10 require the same proteins for import into sensitive cells (Table 2). Comparison of the Cfa protein (colicin 5) with the Cta protein (colicin 10) revealed a 93.7% overall identity (Fig. 4). Since the proteins have the same length, no sequence gaps had to be introduced to improve homology. The domains proposed to determine the TonB and TolC interaction (18) are com-

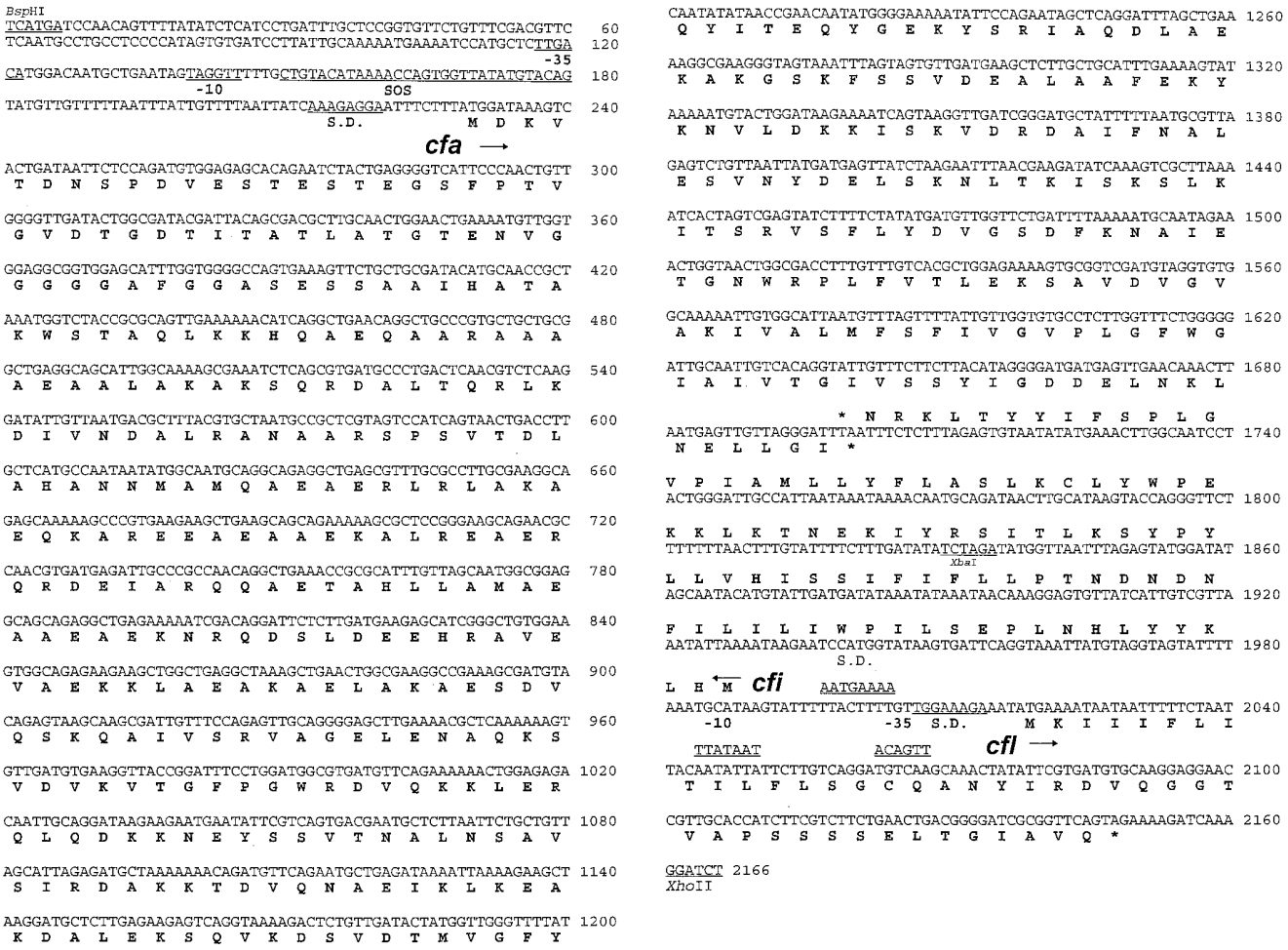

FIG. 2. Nucleotide sequences of *cfa*, *cfi*, and *cfI*. The −10 and −35 promoter regions, the SOS boxes, and the Shine-Dalgarno sequence (S.D.) are indicated.

pletely identical in the amino acid and nucleotide sequences. The domains presumably involved in binding to Tsx (18) are 99.1% identical in the two colicins. In contrast, the C-terminal pore-forming domains display only 83.7% identity. In particular, a region comprising 38 residues (residues 387 to 424) contains different amino acids at 22 sites (partly underlined in Fig. 4).

Since the C-terminal region of colicins usually interacts with the respective immunity proteins (6), the different sequences might determine the various immunity specificities (Table 2). This region of 38 amino acid residues is displayed in Fig. 5, in which the corresponding region of colicin E1 is included for comparison because of the cross-reaction of Cti with colicin E1. (However, the colicin E1 immunity protein does not cross-react with Cta [18]).

Determination of the region in colicin 5 that determines the immunity specificity. The sequence WAKHLE (D) in colicins 10 and E1 (Fig. 5) reads LSKNLT in colicin 5. To see whether these sequences determine the cross-reaction of Cti with colicins 10 and E1 and the lack of cross-reaction with colicin 5, LSKNLT of colicin 5 was converted to WAKHLE by PCR. Modified colicin 5 was fully active but retained specificity for Cfi (Table 2). Therefore, the region downstream of WAKHLE or LSKNLT was exchanged between colicins 5 and 10, taking advantage of a *Bfr*I restriction site in *cta*; a *Bfr*I site was introduced into *cfa* by PCR (after lysine number 404 [marked by an

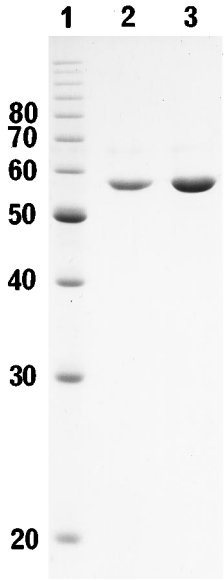

FIG. 3. SDS-PAGE of purified colicin 5 (lane 2) and colicin 10 (lane 3). The molecular masses (in kilodaltons) of standard proteins are given in lane 1.

TABLE 2. Sensitivities of *E. coli* strains to colicins 5 and 10 and their chimeras

Strain (genotype)	10-fold dilution resulting in clear (turbid) zone[a]				
	Col5	Col10	Col5 (WAKHLE)	Col5-Col10	Col10-Col5
5K (wild type)	2 (3)	2 (3)	2 (3)	1 (3)	2 (3)
AB2847 (*tsx*)	(0)	(0)	ND	ND	ND
HP45 (*tonB*)	r	r	ND	ND	ND
C65 (*tolC*)	r	r	ND	ND	ND
5K, pHP40 (*cfi*$^+$)	(1)	2 (3)	(1)	1 (2)	(0)
5K, pHP15 (*cti*$^+$)	2 (3)	(0)	2 (3)	r	2 (3)

[a] The last 10-fold dilutions which resulted in clear zones of growth inhibition and those resulting in turbid zones (in parentheses) are listed. For example, 2 indicates that the colicin solution could be diluted 10^2-fold to yield clear zones. Abbreviations: Col5 and Col10, colicins 5 and 10, respectively; Col5 (WAKHLE), colicin 5 in which the sequence LSKNLT was replaced with WAKHLE; Col5-Col10, hybrid protein between N-terminal colicin 5 and C-terminal colicin 10; Col10-Col5, hybrid protein between N-terminal colicin 10 and C-terminal colicin 5; ND, not determined; r, resistant.

arrow in Fig. 5]). The cell extract containing the colicin 5-colicin 10 hybrid protein was somewhat less active than the extract containing colicin 10-colicin 5, which was as active as colicin 5 (Table 2). Cti conferred immunity to colicin 5-colicin 10, and Cfi conferred immunity to colicin 10-colicin 5 (Table 2), which localized the immunity region of both colicins to the segment downstream of residue 404. This confines the immunity-specifying determinants of both colicins to the region in which they differ from residue 405 to residue 424.

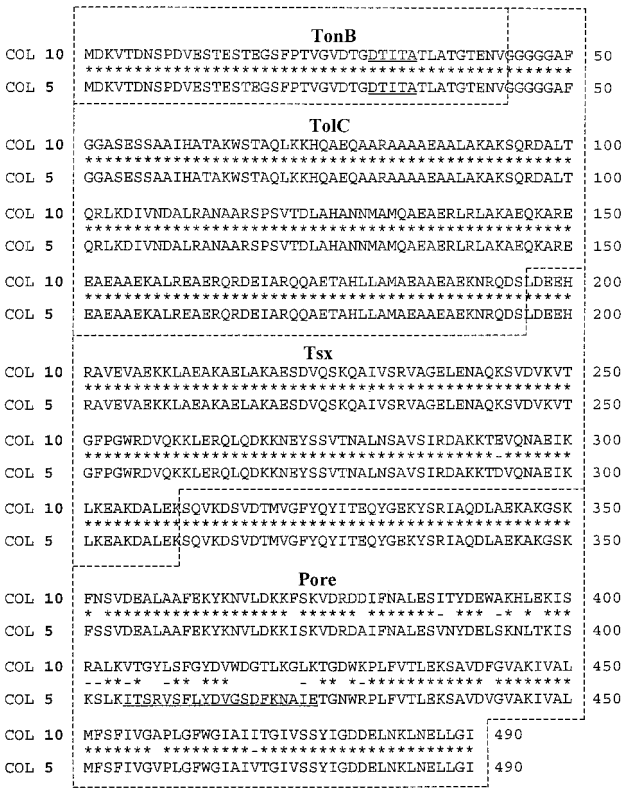

FIG. 4. Comparison of the colicin 5 (Cfa) and colicin 10 (Cta) amino acid sequences. Identical residues are marked by asterisks; dashes indicate similar residues. The regions proposed to be involved in the TonB-, TolC-, and Tsx-dependent uptake across the outer membrane and the pore-forming region in the cytoplasmic membrane are boxed. The sequences conferring immunity specificity to colicin 5 and colicin 10 are underlined. The postulated TonB boxes DTITA are underlined.

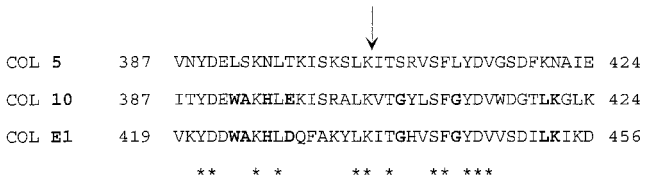

FIG. 5. Demonstration of the sequence similarity of colicins 10 (residues 387 to 424) and E1 (residues 419 to 456) (residues marked in bold letters) and of the sequence dissimilarity of colicin 5, which does not cross-react with Cti. The arrow indicates the site where the colicin 5 and colicin 10 fragments were exchanged to identify the region that specifically interacts with the related immunity protein. Asterisks indicate residues identical among all three colicins; points show residues with similar properties.

Determination of the regions in the immunity proteins that recognize colicins 5 and 10. Cfi and Cti exhibit the greatest identity (65.6%) among the immunity proteins of pore-forming colicins (Fig. 6). Cfi and Cti contain three nonhomologous regions that are potential candidates for the specific interaction with Cfa and Cta, respectively. To determine which of the nonhomologous regions specifies recognition of the cognate colicin, both immunity genes were cleaved by *Nsp*I, which cuts at the same position in *cfi* and *cti*. Exchange of the fragments, which allowed the exchange of two of the three nonhomologous regions, resulted in the hybrid proteins Cfi-Cti and Cti-Cfi, with fusion sites after histidine 40 (marked by an arrow in Fig. 6). *E. coli* 5K transformed with pHP41 *cfi-cti* was immune to added colicin 10, and transformants carrying pHP42 *cti-cfi* were immune to colicin 5 (Table 3), showing that one or both of the nonhomologous regions determine the respective immunity specificities against colicins 5 and 10.

The immunity protein inactivates the colicin at the inner side of the cytoplasmic membrane. The hydropathy profile of the Cfi immunity protein, like that of the Cti immunity protein (18), shows three hydrophobic segments that are predicted to form three α-helices across the cytoplasmic membrane (Fig. 7). To support this model, hybrid proteins between the colicin 5 immunity protein and β-lactamase (Bla) were constructed. Only fusions at sites located in the periplasmic space should allow cells to grow as single colonies on nutrient agar plates supplemented with 5 μg of ampicillin per ml (7). The Cfi-Bla hybrid in the N-terminal periplasmic loop (residue 27 of Cfi in L1 of Fig. 7) conferred resistance to 100 μg of ampicillin per ml, whereas cells expressing the Cfi-Bla hybrid in the cytoplasmic loop (residue 63 in L2 of Fig. 7) formed no colonies on plates containing 5 μg of ampicillin per ml. Cells expressing Cfi to which Bla was fused at the second-to-last amino acid (residue 95), predicted to be located in the periplasm, unexpectedly did not form colonies on plates containing 5 μg of ampi-

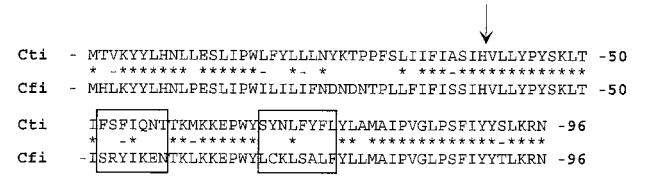

FIG. 6. Comparison of the amino acid sequences of the colicin 5 (Cfi) and colicin 10 (Cti) immunity proteins. The sequences identified as specifying the reactions with the colicins are boxed. The arrow indicates the site of fragment exchange. Asterisks indicate identities between the two colicins; dashes indicate similar residues.

TABLE 3. Immunities of *E. coli* strains to colicins 5, 10, and E1

Strain (genotype)	10-fold dilution resulting in clear (turbid) zone[a]		
	Col5	Col10	ColE1
5K	2 (3)	2 (3)	3 (4)
5K, pHP40 (*cfi*$^+$)	(1)	2 (3)	3 (4)
5K, pHP15 (*cti*$^+$)	2 (3)	(0)	0 (1)
5K, pHP41 (*cfi-cti*$^+$)	2 (3)	(0)	0 (1)
5K, pHP42 (*cti-cfi*$^+$)	(1)	2 (3)	3 (4)
5K, pHP44 (*cfi27-blaM*)	2 (3)	2 (3)	ND
5K, pHP45 (*cfi63-blaM*)	2 (3)	2 (3)	ND
5K, pHP46 (*cfi95-blaM*)	(1)	2 (3)	ND
KS474	2 (3)	2 (3)	ND
KS474, pHP40 (*cfi*$^+$)	(1)	2 (3)	ND
KS474, pHP44 (*cfi27-blaM*)	2 (3)	2 (3)	ND
KS474, pHP45 (*cfi63-blaM*)	2 (3)	2 (3)	ND
KS474, pHP46 (*cfi95-blaM*)	(3)	2 (3)	ND

[a] See footnote to Table 2 for details.

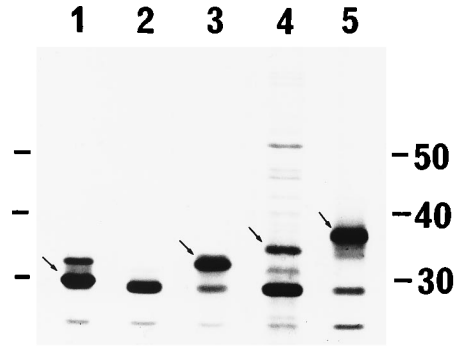

FIG. 8. SDS-PAGE of [^{35}S]methionine-labeled Cfi27-Bla (lane 3), Cfi63-Bla (lane 4), and Cfi95-Bla (lane 5) expressed in *E. coli* BL21 (indicated by arrows) transformed with plasmids pHP44, pHP45, and pHP46, respectively. Lane 1 shows the precursor and the processed form of β-lactamase (indicated by an arrow) expressed by plasmid pT7-3, and lane 2 shows the neomycin phosphotransferase expressed by the vector used, plasmid pJBS636.

cillin per ml. The cells lysed, and the released β-lactamase protected nearby cells so that growth zones with a high density of cells appeared on plates. However, these growth zones did not appear on plates containing the 200 μg of ampicillin routinely used to identify in-frame β-lactamase hybrid proteins with cytoplasmic fusion sites (7); the zones appeared with cells that expressed the Cfi63-Bla hybrid in the cytoplasmic loop.

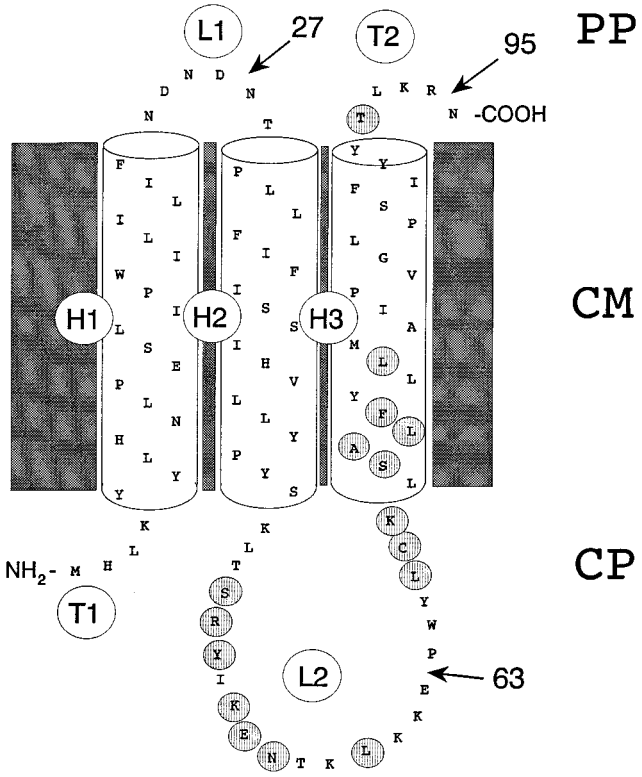

FIG. 7. Predicted arrangement of the colicin 5 immunity protein in the cytoplasmic membrane of *E. coli*. The shaded residues specify interaction of the immunity protein with colicin 5. H1, H2, and H3 denote the transmembrane α-helices, L1 and L2 denote loops, and T1 and T2 correspond to the N- and C-terminal ends, respectively. PP, periplasmic space; CM, cytoplasmic membrane; CP, cytoplasm. Arrows indicate the locations of the fusion sites of the constructed Cfi-Bla hybrid proteins.

Cells expressing Cfi95-Bla grew in zones at a high density on plates containing 50 μg of ampicillin per ml and grew less well on plates containing 100 μg of ampicillin per ml. Apparently, Cfi95-Bla conferred a lower level of ampicillin resistance than did Cfi63-Bla, although sequencing of the *cfi95-blaM* fusion site confirmed the expected in-frame fusion.

To examine proteolytic degradation, Cfi95-Bla was expressed in *E. coli* KS474 *degP*, which lacks the periplasmic DegP protease (29), which has been shown to cleave abnormal periplasmic proteins (28). KS474 transformed with plasmid pHP46 (*cfi95-blaM*) formed colonies on plates containing 50 μg of ampicillin per ml; no colonies of KS474 cells expressing Cfi63-Bla with a cytoplasmic fusion site formed on such plates. These data confirmed the hypotheses that active Cfi95-Bla was formed and that single colonies could grow in the presence of ampicillin, which indicates a periplasmic location of the fusion site.

To examine the degradation of Cfi95-Bla in *degP*$^+$ cells, *cfi95-blaM*, *cfi63-blaM*, and *cfi27-blaM* were cloned downstream of the phage T7 promoter and transcribed by the T7 RNA polymerase in *E. coli* BL21. [^{35}S]methionine-labeled Cfi27-Bla, Cfi63-Bla, and Cfi95-Bla (Fig. 8, lanes 3 to 5, respectively) were present on the autoradiograph after SDS-PAGE. No evidence for inhibition of Cfi95-Bla synthesis or degradation was found. In fact, the Cfi95-Bla band gave a stronger signal than did the bands of the other hybrid proteins, and pulse-chase labeling did not reveal Cfi95-Bla degradation (data not shown). The increase in ampicillin resistance was related to the *degP* mutant phenotype since the parent, *E. coli* MC4100 *degP*$^+$ pHP46 *cfi95-blaM*, and the other *degP*$^+$ strain tested, *E. coli* BL21 pHP46, were as sensitive to ampicillin as *E. coli* 5K pHP46 was.

Cfi27-Bla and Cfi63-Bla conferred no immunity to Cfa on *E. coli* 5K transformants (Table 3). In contrast, *E. coli* 5K expressing Cfi95-Bla was as immune to added Cfa as was *E. coli* 5K expressing Cfi (Table 3).

DISCUSSION

The amino acid sequences of Cfa and Cfi are most homologous to the respective amino acid sequences of the activity, immunity, and lysis proteins of colicins 10, E1, Ia, Ib, and K (14, 18, 19, 32, 35). Therefore, data on pore formation of colicins E1 (8), Ia (16), and K (24) and their interaction with the respective immunity proteins are most likely valid for co-

licin 5 and its immunity protein. The predicted transmembrane arrangement of the colicin 5 immunity protein (Cfi) is similar to the model proposed for the colicin E1 immunity protein. The topography of the colicin E1 immunity protein is supported by Tn*phoA* and *lacZ* fusions and by site-directed substitutions of charged residues for nonpolar residues in the proposed membrane-spanning regions (27). The colicin E1 immunity model predicts three transmembrane helices, the N-terminal end localized in the cytoplasm, and the C-terminal end localized in the periplasm. In agreement with the predicted membrane topology of Cfi, the β-lactamase activities of the Cfi27-Bla and Cfi63-Bla hybrid proteins localized L1 to the periplasm and L2 to the cytoplasm (Fig. 7). Although the level of β-lactamase activity of Cfi95-Bla was lower than that of Cfi27-Bla, growth of single colonies in the *degP* strain demonstrated a periplasmic fusion site, implying that residue 95 is in the periplasm as the model proposes. However, only the *degP* strain containing *cfi95-blaM* formed single colonies on plates containing ampicillin, despite the lack of Cfi95-Bla degradation in *degP*$^+$ cells. Since sequencing of the *cfi95-blaM* fusion site and SDS-PAGE of radioactively labeled Cfi95-Bla demonstrated the correctness of the fusion, we assume that the immunity protein prevented the folding of β-lactamase into its native conformation. A small proportion was active and was partially degraded by DegP so that the level of resistance was not high enough to allow growth of single colonies. In the *degP* mutant, this small proportion remained active and conferred resistance to ampicillin.

The model assigns the amino acids responsible for the specific reaction with colicin 5 (shaded residues in Fig. 7) to the cytoplasm and to the inner side of the cytoplasmic membrane. This implies that the immunity protein does not inactivate the colicin in the periplasmic space and thus does not prevent it from entering the cytoplasmic membrane. This conclusion is supported by the location of the interacting region in colicin 5. The specificity-determining region was localized to residues 405 to 424. The homologous region in colicin Ia is located immediately C terminal to the "swinging gate," the segment, comprising at least residues 511 to 541, that is translocated across a planar lipid bilayer upon application of a transmembrane voltage (26). Translocation of this segment is associated with opening of the channel. In colicin A, the region equivalent to the Cfi specificity region is contained in helix 6 of the X-ray structure obtained with a crystallized C-terminal fragment (17). This helix is proposed to be inserted into the cytoplasmic membrane upon opening of the channel (17). Since these topographic data probably also apply for colicin 5, the immunity protein interacts with regions on the colicin that are located in the inner leaflet of the cytoplasmic membrane. This proposal also implies that helix 6 is translocated farther into the cytoplasmic membrane than has been previously proposed (26). However, the membrane topology of pore-forming colicins of the colicin 5 type may differ in detail from those of the colicin A type. It was shown that the immunity proteins of colicins A (9) and E1 (36) interact with the transmembrane helices of the related colicins, from which it was inferred that they bind to the colicins in the open state. We favor the idea that the immunity proteins inactivate the pore-forming colicins when the helices are inserted into the cytoplasmic membrane shortly before the channels are opened.

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REFERENCES

1. **Bell, P. E., C. D. Nau, J. T. Brown, J. Konisky, and R. J. Kadner.** 1990. Genetic suppression demonstrates interaction of TonB protein with outer membrane transport proteins in *Escherichia coli*. J. Bacteriol. **172:**3826–3829.
2. **Bradbeer, C.** 1993. The proton motive force drives the outer membrane transport of cobalamin in *Escherichia coli*. J. Bacteriol. **175:**3146–3150.

2a. **Bradley, D. E.** Personal communication.

3. **Bradley, D. E., and S. P. Howard.** 1992. A new colicin that adsorbs to outer membrane protein Tsx but is dependent on the *tonB* instead of the *tolQ* membrane transport system. J. Gen. Microbiol. **138:**2721–2724.
4. **Braun, V.** 1989. The structurally related *exbB* and *tolQ* genes are interchangeable in conferring *tonB*-dependent colicin, bacteriophage, and albomycin sensitivity. J. Bacteriol. **171:**6387–6390.
5. **Braun, V.** 1995. Energy-coupled transport and signal transduction through the gram-negative outer membrane via TonB-ExbB-ExbD-dependent receptor proteins. FEMS Microbiol. Rev. **16:**295–307.
6. **Braun, V., H. Pilsl, and P. Groß.** 1994. Colicins: structures, modes of action, transfer through membranes, and evolution. Arch. Microbiol. **161:**199–206.
7. **Broome-Smith, J. K., and B. G. Spratt.** 1986. A vector for the construction of translational fusions to TEM β-lactamase and the analysis of protein export signals and membrane protein topology. Gene **49:**341–349.
8. **Cramer, W. A., F. S. Cohen, A. R. Merrill, and H. Y. Song.** 1990. Structure and dynamics of the colicin E1 channel. Mol. Microbiol. **4:**519–526.
9. **Espesset, D., P. Piet, C. Lazdunski, and V. Geli.** 1994. Immunity proteins to pore-forming colicins: structure-function relationships. Mol. Microbiol. **13:** 1111–1120.

9a. **Focareta, T.** Unpublished results.

10. **Hancock, R. E. W., and V. Braun.** 1976. Nature of the energy requirement for the irreversible adsorption of bacteriophages T1 and ϕ80 to *Escherichia coli*. J. Bacteriol. **125:**409–415.
11. **Heller, K. J., R. J. Kadner, and K. Günter.** 1988. Suppression of the *btuB*451 mutation by mutations in the *tonB* gene suggests a direct interaction between TonB and TonB-dependent receptor proteins in the outer membrane of *Escherichia coli*. Gene **64:**147–153.
12. **Killmann, H., R. Benz, and V. Braun.** 1993. Conversion of the FhuA transport protein into a diffusion channel through the outer membrane of *Escherichia coli*. EMBO J. **12:**3007–3016.
13. **Lugtenberg, B., J. Meijers, R. Peters, P. van der Hock, and L. van Alphen.** 1975. Electrophoretic resolution of the major outer membrane proteins of *Escherichia coli* K-12 into four bands. FEBS Lett. **58:**254–258.
14. **Mankovich, J. A., C.-H. Hsu, and J. Konisky.** 1986. DNA and amino acid sequence analysis of structural and immunity genes of colicins Ia and Ib. J. Bacteriol. **168:**228–236.
15. **Mende, J., and V. Braun.** 1990. Import defective colicin B derivatives mutated in the TonB box. Mol. Microbiol. **4:**1523–1533.
16. **Nogueira, R. A., and W. A. Varanda.** 1988. Gating properties of channels formed by colicin Ia in planar lipid bilayer membranes. J. Membr. Biol. **105:**143–153.
17. **Parker, M. W., J. P. Postma, F. Pattus, A. D. Tucker, and D. Tsernoglou.** 1992. Refined structure of the pore-forming domain of colicin A at 2.4 A resolution. J. Mol. Biol. **224:**639–657.
18. **Pilsl, H., and V. Braun.** 1995. Novel colicin 10: assignment of four domains to the TonB- and TolC-dependent uptake via the Tsx receptor and to pore formation. Mol. Microbiol. **16:**57–67.
19. **Pilsl, H., and V. Braun.** 1995. Strong function-related homology between the pore-forming colicins K and 5. J. Bacteriol. **177:**6973–6977.
20. **Pilsl, H., C. Glaser, P. Groß, H. Killmann, T. Ölschläger, and V. Braun.** 1993. Domains of colicin M involved in uptake and activity. Mol. Gen. Genet. **240:**103–112.
21. **Postle, K.** 1993. TonB protein and energy transduction between membranes. J. Bioenerg. Biomembr. **25:**591–601.
22. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
23. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
24. **Schein, S. J., B. L. Kagan, and A. Finkelstein.** 1978. Colicin K acts by forming voltage-dependent channels in phospholipid bilayer membranes. Nature (London) **276:**159–163.
25. **Schöffler, H., and V. Braun.** 1989. Transport across the outer membrane of *Escherichia coli* K12 via the FhuA receptor is regulated by the TonB protein of the cytoplasmic membrane. Mol. Gen. Genet. **217:**378–383.
26. **Slatin, S. L., X. Q. Qiu, K. Jakes, and A. Finkelstein.** 1994. Identification of a translocated protein segment in a voltage-dependent channel. Nature (London) **371:**158–161.
27. **Song, H. Y., and W. A. Cramer.** 1991. Membrane topography of ColE1 gene products: the immunity protein. J. Bacteriol. **173:**2935–2943.

28. **Strauch, K. L., and J. Beckwith.** 1988. An *Escherichia coli* mutation preventing degradation of abnormal periplasmic proteins. Proc. Natl. Acad. Sci. USA **85:**1576–1580.
29. **Strauch, K. L., K. Johnson, and J. Beckwith.** 1989. Characterization of *degP*, a gene required for proteolysis in the cell envelope and essential for growth of *Escherichia coli* at high temperature. J. Bacteriol. **171:**2689–2696.
30. **Studier, F. W., and B. A. Moffat.** 1986. Use of bacteriophage T7 RNA-polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189:**113–130.
31. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82:**1074–1078.
32. **Varley, J. M., and G. J. Boulnois.** 1984. Analysis of a cloned colicin Ib gene. Complete nucleotide sequence and implications for regulation of expression. Nucleic Acids Res. **12:**6727–6739.
33. **Webster, R. E.** 1991. The *tol* gene products and the import of macromolecules into *Escherichia coli*. Mol. Microbiol. **5:**1005–1011.
34. **Whitney, E. N.** 1971. The *tolC* locus in *Escherichia coli* K12. Genetics **67:**39–53.
35. **Yamada, M., Y. Ebina, T. Miyata, T. Nakazawa, and A. Nakazawa.** 1982. Nucleotide sequence of the structural gene for colicin E1 and predicted structure of the protein. Proc. Natl. Acad. Sci. USA **79:**2827–2831.
36. **Zhang, Y. L., and W. A. Cramer.** 1993. Intramembrane helix-helix interactions as the basis of inhibition of the colicin E1 ion channel by its immunity protein. J. Biol. Chem. **268:**10176–10184.