Immunity proteins to pore-forming colicins: structure–function relationships

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Summary

Colicin A and B immunity proteins (Cai and Cbi, respectively) are homologous integral membrane proteins that interact within the core of the lipid bilayer with hydrophobic transmembrane helices of the corresponding colicin channel. By using various approaches (exchange of hydrophilic loops between Cai and Cbi, construction of Cbi/Cai hybrids, production of Cai as two fragments), we studied the structure–function relationships of Cai and Cbi. The results revealed unexpectedly high structural constraints for the function of these proteins. The periplasmic loops of Cai and Cbi did not carry the determinants for colicin recognition although most of these loops were required for Cai function; the cytoplasmic loop of Cai was found to be involved in topology and function of Cai. The immunity function did not seem to be confined to a particular region of the immunity proteins.

Introduction

Pore-forming colicins form voltage-dependent ion-channels in the cytoplasmic membrane of sensitive bacteria (Konisky, 1982). After these channels have formed, the cells are depleted of their intracellular K⁺ (Bourdinaud et al., 1990), active transport is inhibited (Cramer et al., 1983), and inorganic phosphate leaks out of the cells. The leakage causes intracellular ATP to be hydrolysed into ADP and AMP (Guirard et al., 1993).

Like many toxins, colicins are made up of functional domains, each of which is associated with particular functions in the overall activity (Baty et al., 1988). The $N$-terminal and the central domains of pore-forming colicins are involved in their transport through the *Escherichia coli* envelope; the $C$-terminal domain carries the ionophoric activity (Lazdunski et al., 1988; Pattus et al., 1990).

The crystal structure of the pore-forming domain of colicin A (ColA) in aqueous solution was obtained at 2.4 Å resolution (Parker et al., 1989; 1992). This domain consists of a hydrophobic hairpin (helices 8 and 9) surrounded by eight amphipathic $\alpha$-helices. In vitro experiments performed on model membranes at low pH indicate that if the membrane potential is missing, the pore-forming domain of ColA remains embedded at the surface of the membrane (Géli et al., 1992; Lakey et al., 1993; Duché et al., 1994). The hydrophobic hairpin of the homologous colicin E1 (ColE1) seems to assume a transmembrane orientation (Merrill et al., 1990; Rath et al., 1991; Zhang and Cramer, 1992; Shin et al., 1993). The structure of the membrane-spanning ion-channel has not been determined. It probably includes the hydrophobic hairpin and an amphiphilic hairpin that was identified in ColE1 as the voltage-sensitive region (Merrill and Cramer, 1990; Abrams et al., 1991; Song et al., 1991; Zhang and Cramer, 1992).

To prevent the colicin they release in the extracellular medium from killing them, colicin-producing strains also synthesize immunity proteins (Géli et al., 1986). According to their respective homologies, immunity proteins were classified into two groups (Schramm et al., 1988; Géli et al., 1989; Song and Cramer, 1991). The first group comprises immunity proteins to colicins A, B, and N, and the second comprises immunity proteins to colicins E1, 1a, and 1b. Colicin A and B immunity proteins show 38% identity and 39% conservative substitutions; their sequences can be aligned with the introduction of very few gaps.

Immunity proteins are integral inner membrane proteins (Goldman et al., 1985; Mankovich et al., 1986; Schramm et al., 1988; Géli et al., 1988; Pugsley, 1988). They interact with the pore-forming domain of the corresponding colicin (Mankovich et al., 1984; Bishop et al., 1985; Bénédicti et al., 1991; Géli and Lazdunski, 1992a). The topologies of colicin A and E1 immunity proteins (Cai and Cei, respectively) are known: Cai has four transmembrane regions and its $N$- and $C$-termini face the cytoplasm (Géli et al., 1989); Cei crosses the membrane bilayer three times (Song and Cramer, 1991).

The hydrophobic hairpin of the ColA pore-forming domain is the main determinant recognized by Cai; it was proposed that immunity protein function required helix–helix recognition within the lipid bilayer, involving the ColA hydrophobic hairpin (Géli and Lazdunski, 1992a, b). Another amphiphilic region of ColE1, defined

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as a transmembrane helix of the channel in the open state, appears to be involved in the interaction between ColEI and Cei (Zhang and Cramer, 1993). This is consistent with membrane helix-helix interactions being the basis for the immunity function.

Recently, we have found that a fusion protein comprising the pore-forming domain of ColA attached to a yeast mitochondrial sorting sequence was cytotoxic for the producing cell and that the lethality was inhibited within the membrane by Cai (Espesset et al., 1994, accompanying paper).

In this study, we addressed the role of the small hydrophobic loops (20 to 30 residues in length) of Cai in the protective function. These loops may interact directly with the hydrophilic loops of the membrane-spanning pore-forming domain of ColA or may influence the way in which the transmembrane helices of Cai functionally assemble. We also showed that Cai produced as two independent fragments did not assemble into a functional structure and that it was not possible to combine hydrophobic segments of two homologous immunity proteins to form a functional entity.

Results

Overproduction of Cai confers immunity to colicin B

We have previously observed that Cai conferred partial immunity to colicin B (ColB); in contrast, Cbi is highly specific for ColB (Geli and Lazdunski, 1992a). In order to determine the regions of Cai that are involved in ColA recognition, we performed NH₂OH mutagenesis on plasmid pIA1 (carrying the cai gene) and selected transformants insensitive to ColB (see the Experimental procedures). Three such clones were obtained whose insensitivities to ColB were correlated with the mutated pIA1 plasmid. The clones were also immune to high concentrations of ColA and sensitive to ColEI. Sequencing of the plasmid DNA of each immune clone showed that all mutations were located near to or in the Pribnow box of the cai promoter (Fig. 1A) (Lioubes et al., 1986); they were predicted to increase the promoter strength (Mulligan et al., 1984). Because no antibody directed against Cai was available, it was not possible to confirm that Cai had been overproduced from the mutated variants of pIA1. To determine whether cells overproducing Cai became immune to ColB, we used a Cai derivative (EpCai, encoded by plasmid pEpCai) whose production was inducible. In EpCai, the first 12 residues of Cai are replaced with a 30-amino-acid epitope recognized by the monoclonal antibody (mAb) 1C11 (Géli et al., 1993). We then analysed the kinetics with which C600 EpCai acquired immunity to ColA and ColB after the gene fusion had been induced (Fig. 1B): after 40 min, 100% of the cells were immune to ColA and 20% were immune to ColB; after 120 min, 90% of the cells were immune to ColB (Fig. 1B). By immunoblot analysis with the 1C11 mAb, we calculated that the amount of Ep-Cai required to protect the cells against ColB was 20-fold higher than that required to protect against ColA (not shown). The results indicated that cells overproducing Cai became immune to ColB and confirmed that Cai and Cbi were functionally related.

Fig. 1. A. Nucleotide sequence of the cai promoter region in NH₂OH-mutated pIA1 selected for insensitivity to ColB. The transitions are shown by arrows. The recognition and binding sequences for RNA polymerase are boxed with dotted lines (−35 sequence) and solid lines (Pribnow box), and +1 shows the start point of transcription (Lioubes et al., 1986).

B. Kinetics of acquisition of immunity to ColA and ColB after induction of EpCai production. C600 (pEpCai) was treated with mitomycin C (300 ng ml⁻¹) to induce the synthesis of EpCai and assayed for colicin sensitivity at the indicated times. The percentage of survival was expressed as the ratio of the absorbance (at 600 nm) of the culture incubated with ColA or ColB (1 mg ml⁻¹) to that of the control culture incubated with the dilution buffer. I, induced cells, NI, non-induced cells; ColA, percentage survival to ColA; ColB, percentage survival to ColB.
Immunity proteins to pore-forming colicins

Immunity is lost when the periplasmic L3 loop is exchanged between Cai and Cbi

An Arg-136/Asp substitution in the second periplasmic loop (L3) of Cai abolishes the immunity function (Géli et al., 1989) and the main ColA determinant for immunity recognition is located in the hydrophobic hairpin of ColA (Géli and Lazdunski, 1992a). To investigate whether L3 was involved in the specificity of colicin recognition, L3 of Cai was substituted for that of Cbi (Cai and Cbi sequences are aligned in Fig. 5 — see later). The resulting hybrid protein did not protect cells against low concentrations of either ColA or ColB (Fig. 2 and Table 1).

Table 1. Exchange of hydrophilic loops between Cai and Cbi and correlation with the immunity activity.

| Immunity proteins and mutant forms | ColA Concentration (mg ml⁻¹) | ColB Concentration (mg ml⁻¹) |
|-----------------------------------|------------------------------|------------------------------|
|                                   | 10⁻¹ | 10⁻² | 10⁻³ | 10⁻⁴ | 10⁻⁵ | 10⁻¹ | 10⁻² | 10⁻³ | 10⁻⁴ | 10⁻⁵ |
| No immunity                       | +    | +    | -    | -    | -    | +    | +    | +    | -    | -    |
| Cai                               | +    | +    | +    | ±    | -    | +    | +    | +    | -    | -    |
| Cbi                               | +    | +    | +    | +    | +    | +    | +    | +    | -    | -    |
| Cai + Cbi                         | +    | +    | +    | +    | ±    | -    | -    | -    | -    | -    |
| Cai (L1)                          | +    | +    | -    | -    | -    | +    | +    | +    | +    | ±    |
| Cai (L2)                          | +    | +    | +    | ±    | -    | +    | +    | +    | +    | ±    |
| Cai (L3)                          | +    | +    | +    | ±    | -    | +    | +    | +    | +    | ±    |
| Cai (L1' + L3)                    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    |
| Cai (L2 + L3)                     | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    |

a. One microlitre of each colicin dilution was spotted onto a lawn of the indicator strain; + indicates a plaque of growth inhibition and thus a lack of activity of immunity protein, - indicates no clearing corresponding to active immunity protein, and ± indicates a turbid plaque of growth inhibition.

b. Brackets indicate the replaced regions of Cai (see Fig. 2). The immunity gene and its variants were carried by a pBR derivative (pA2) in C600.
The major part of the first periplasmic loop (L1*, see Fig. 2) of Cai was also exchanged by the corresponding region of Cbi, but the hybrid immunity protein protected against CoiA and CoiB exactly like the authentic Cai (Table 1). However, when four residues (Ile–Glu–Gly–Arg) were inserted after Arg-46 of Cai (in L1), the immunity function was completely lost (not shown). Moreover, a Cai/Cbi hybrid comprising L1* and L3 from Cbi did not confer immunity against either CoiA or CoiB (Fig. 2 and Table 1). Therefore, although L1* was found to be sensitive to residue substitution only, L3 appeared to be totally required in order for the immunity protein to be functional.

The cytoplasmic L2 loop is required for immunity function

L2 loops of Cai and Cbi are rich in positive charges and it has been shown that the way in which membrane proteins are topologically organized requires such positive charges (von Heijne, 1986). An Arg-92/Asp mutation in L2 of Cai was reported to result in an intermediate immunity level (Géli et al., 1989). We therefore investigated whether L2 was only involved in the topology of Cai or also had a role in the immunity function.

L2 of Cai was replaced with that of Cbi (Fig. 2). The hybrid protein protected against 100-fold less CoiA than did the native Cai (Table 1). To study the topology of this hybrid protein, a tagged inducible fusion protein (EpVBG7) was constructed: it consisted of EpCai fused to alkaline phosphatase (PhoA) in L3 (see the Experimental procedures). L2 of EpVBG7 was then replaced with that of Cbi. The PhoA activities of cells producing each protein, EpVBG7 and the modified EpVBG7, were compared: the activities were found to be identical for both proteins (not shown) and reflected the fact that L2 was able to promote the export of the PhoA moiety of EpVBG7. This identity indicated that the gross organization of the protein in the membrane was probably not affected when L2 was replaced with that of Cbi. Nevertheless, the hybrid immunity protein was unable to take up a functional structure, despite its overall topology being similar to that of the native protein.

A Cai/Cbi hybrid containing L2 and L3 of Cbi was also constructed: it had no protective activity, which was not surprising in view of the effect of the L3 substitution described above.

Separate transmembrane fragments of Cai do not lead to immunity activity

Several polytopic proteins produced as fragments are functional because the transmembrane elements reassociate within the membrane (Popot, 1993). As L2 seems to be essential in order for Cai to assemble functionally or for Cai to recognize CoiA, Cai produced as two independent fragments should not be active.

A DNA sequence (DE1) containing a stop codon, a Shine–Dalgarno sequence, and a start codon was inserted in plasmid pEpCai between codons of cai corresponding to Asn-96 and Asn-97 (in L2); as a result, Cai should be expressed in two fragments: Ep-F(13-96) and F(97-178) (Fig. 3A). Cells transformed by the resulting plasmid (pEpCaiDE1) were completely sensitive to CoiA after production of both fragments had been induced (Fig. 3B); this confirmed that L2 was required for the immunity protein to be functional. To check that the fragments were expressed and membrane associated, we transferred the DE1 sequence into pEpVBG7: the modified plasmid (pEpVBG7DE1) encoded Ep-F(13-96) and a hybrid protein in which the PhoA protein was fused to the F(97-173) fragment (F(97-173)-PhoA) (Fig. 4A). The two fragments were produced; Ep-F(13-96) was found to be membrane associated but we were not able to probe its orientation by protease treatment because L1 and L3 of Cai are fully resistant to externally added protease in EDTA-treated cells (V. Géli, unpublished). PhoA activity was detected when F(97-173)-PhoA was produced (Fig. 4B); thus, at least the first transmembrane fragment of F(97-178) was correctly assembled into the membrane.
**Cai function requires the integrity of its transmembrane helices**

We then tested whether a functional hybrid immunity protein could be constructed from the hydrophobic stretches of different immunity proteins. Cbi/Cai hybrids were generated by homologous recombination. Hybrid proteins resulting from homologous recombination are perfect fusions (see the Experimental procedures). After cells had been transformed with plasmids encoding recombinant immunity proteins, clones insensitive to CoIA were selected: the fusion sites of all 70 selected clones were located upstream of Leu-30 of Cai (Fig. 5). All these hybrid proteins conferred levels of immunity that were higher even than those of the native Cai (Table 2), presumably because the fusion genes were under the control of the cbi promoter, which is stronger than the cai promoter.

The DNA regions encoding hybrid immunity proteins of 20 clones sensitive to CoIA were also sequenced: most of them resulted from frameshifting. However, three

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**Fig. 4. Cai fragments are membrane associated.**

A. Predicted membrane topologies of Ep-F(13-96) and F(97-133)-PhoA.

B. Immunolocalization of Ep-F(13-97). Cells bearing plasmid pEpCai and pEpVBG7DE1 were treated for two hours with 300 ng ml⁻¹ mitomycin C to induce the production of EpCai and Ep-F(13-97), respectively. The membrane fraction of each induced culture was prepared and the proteins were subjected to SDS-PAGE and immunoblot analysis with the 1C11 mAb. Lane 1, EpCai; lane 2, Ep-F(13-97).

C. PhoA activity of F(97-133)-PhoA. Cells bearing plasmid pEpVG7 (encoding Ep-F(13-133)-PhoA) or pEpVBG7DE1 were treated as described above and their PhoA enzymatic activities were measured.

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**Fig. 5. Location of the fusion sites in the Cbi/Cai hybrids.** Residues in bold indicate the junctions between Cbi and Cai. Identical amino acids are boxed. Transmembrane helices are labelled H1, H2, H3, and H4 according to the Cai topology.
clones carried plasmids encoding perfect hybrid immunity proteins; the fusion sites of these inactive Cbi/Cai hybrids were located in L1, H2 and L3, respectively (Fig. 5, Table 2).

Only two transformants insensitive to ColB were obtained; the fusion sites were located in the extreme C-terminal part of Cbi (Fig. 5). These hybrid proteins displayed the same immunity activities as Cbi (Table 2).

These results indicated that only the first two-thirds of H1 of Cai were able to be replaced by the corresponding region of Cbi to give an active immunity protein. Interestingly, immunity was conserved when the regions Cai(1-30) or L1* were exchanged, although immunity was lost when the region Cai(1-60) was exchanged (see above and Fig. 5, Table 1). This suggested that the region Cai(30-Leu-Cys-Ile-Phe-Val-Val-Tyr-37) contained determinants for ColA recognition or that this region was specifically required for interaction with another transmembrane hydrophobic helix of Cai.

**Discussion**

It has been previously shown that it was possible to replace the N- and C-terminal cytoplasmic ends of Cai by unrelated amino acid sequences without affecting its function (Géli et al., 1988). Various single point mutations in the polar loops of Cai, L2, and L3 decrease the immunity function (Géli et al., 1989), whereas those of the ColE1 immunity protein (Cei) tolerate a high degree of substitution (Song and Cramer, 1991; Zhang and Cramer, 1993). However, the main determinant necessary for colicins A, B, and E1 to be recognized by immunity proteins is located in the transmembrane regions of their pore-forming domains (Géli and Lazdunski, 1992a; Bénédetti et al., 1992; Zhang and Cramer, 1993). Lateral diffusion of the immunity proteins in the membrane would ensure rapid recognition of colicin molecules (Zhang and Cramer, 1993).

Moreover, we have recently shown that, when produced in *E. coli*, a chimeric protein comprising the ColA pore-forming domain fused to a mitochondrial intermembrane space sorting sequence formed a channel in the inner membrane of the cells; the channel formed independently of the Tol proteins and it was inhibited by Cai (Espesset et al., 1994, accompanying paper). Thus, it appears that immunity proteins interact within the core of the lipid bilayer with the channel in the open state (Géli and Lazdunski, 1992a; Zhang and Cramer, 1993). However, this does not exclude a role for the polar regions of Cai.

The results reported in this study indicated that: (i) Cai overproducing cells became immune to ColB; (ii) L1* and L3 of Cai and Cbi did not carry, by themselves, the determinants for colicin recognition, although most of these loops were required for Cai function; (iii) when L2 of Cai was replaced with that of Cbi the immunity function was lost but this was not due to modified topology; (iv) Cai produced as two membrane fragments was not functional; and (v) fusion sites of functional Cbi/Cai hybrids were located upstream of Leu-30 of Cai. These results showed that although Cai and Cbi are homologous (38% identity and 39% conservative substitutions), only limited regions were able to be exchanged without affecting function.

Two roles can be proposed for the hydrophilic loops of Cai and Cbi: (i) they may interact on both sides of the cytoplasmic membrane with different parts of the colicin molecule to prevent the pore-forming helices from assembling; (ii) they may allow the transmembrane helices of Cai to assemble functionally. These two roles may or may not be mutually exclusive.

The first proposition implies that assembly of Cai into the membrane would not be perturbed if its hydrophilic loops

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**Table 2. Immunity activity of Cbi/Cai hybrid proteins.**

| Immunity protein | Fusion site | ColA (mg ml⁻¹) | ColB (mg ml⁻¹) |
|------------------|-------------|---------------|---------------|
|                   |             | 1 10⁻¹ | 10⁻² | 10⁻³ | 10⁻⁴ | 1 10⁻¹ | 10⁻² | 10⁻³ | 10⁻⁴ |
| No immunity      | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |
| Cai              | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |
| Cbi              | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |
| Cbi/Cai          | (19/23) | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |
| Cbi/Cai          | (22/26) | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |
| Cbi/Cai          | (24/28) | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |
| Cbi/Cai          | (25/30) | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |
| Cbi/Cai          | (57/61) | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |
| Cbi/Cai          | (75/79) | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |
| Cbi/Cai          | (129/133)| ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |
| Cbi/Cai          | (189/172)| ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |

a. The colicin activity was analysed as described for Table 1.
b. Brackets indicate the positions of the fusion sites in the hybrid immunity proteins (see Fig. 4).
were exchanged. When L2 of Cai was replaced with that of Cbi, the PhoA activity of the modified EpVBG7 protein was not affected; thus it seemed that the topology of Cai was not affected when its polar regions were replaced with those of Cbi. Furthermore, if the polar regions of Cai interacted with the ColA pore-forming domain in the open state, this domain would be expected to expose regions on the cytoplasmic side of the inner membrane to interact with L2 of Cai. However, studies on the gating of ColE1 channels (Slatin et al., 1986; Raymond et al., 1986; Jakes et al., 1990; Abrams et al., 1991) and ColE1 topographic studies (Song et al., 1991; Zhang and Cramer, 1992) indicate that the loops of ColE1 connecting the four putative transmembrane helices on the cytoplasmic side of the inner membrane are very short and uncharged. As ColA is homologous to ColE1, it is unlikely that its cytoplasmic loops interact with L2 of Cai. Thus we favour the second proposition: the hydrophilic loops may allow the transmembrane helices of Cai to assemble functionally.

Conversely, the periplasmic loops of Cai may interact with ColA on the periplasmic side of the inner membrane. Indeed, Cai was inactivated when both L1 and L3 were replaced with those of Cbi. However, the activity of Cei is not affected even if its peripheral domains are extensively modified. This suggests that these regions are not critical for activity (Zhang and Cramer, 1993). In contrast, hydrophobic intramembrane helix–helix interactions are important for both ColA and ColE1 (Géli and Lazdunski, 1992a; Zhang and Cramer, 1993).

The differences in permissivity with regard to alterations of peripheral loops in Cai and Cei may reflect structure–function relationships. The structures of these proteins require that: (i) the transmembrane helices assemble either into a three-α-helix bundle (Cei) or four-α-helix bundle (Cai), and (ii) these bundles should be able to interact with hydrophobic helices of colicins. The structural constraints imposed on helix orientation should be much less marked with a three-α-helix bundle than with a four-α-helix bundle. Thus the bundle of Cai helices may be destabilized when its peripheral loops are modified, whereas the Cei 3-helix bundle may be more robust.

Cai produced as two membrane fragments was unable to assemble into a functional protein. Transmembrane fragments of several proteins behave as autonomous folding domains (Köster and Braun, 1990; Popot and Engelman, 1990; Bibi and Kaback, 1990; Lemmon et al., 1992; Kahn and Engelman, 1992; Maggio et al., 1993). These transmembrane regions may undergo tight intermolecular associations that may not be modified when the domains function. In contrast, because intramembrane hydrophobic interactions are involved in the function of immunity proteins (Géli and Lazdunski, 1992a; Zhang and Cramer, 1993), inter-helix affinities within immunity proteins may not be very high so that the helices can interact with the hydrophobic helical hairpins of the pore-forming colicins. This is consistent with independent membrane fragments of Cai not being able to assemble into a functional complex and with L2 being required. Alternatively, proteins with only four transmembrane segments may have a reduced probability of assembling correctly when produced as two separate fragments.

The protective function of the Cbi/Cai hybrids indicates that the hydrophobic regions of Cai and Cbi cannot be combined although they are homologous. The results also suggest that the last seven amino acids of H1 of Cai (residues 30 to 36) are specifically required for function. These data suggest that either the functional packing of Cai requires H1 to interact with another Cai transmembrane region or that H1 specifically recognizes ColA.

Immunity protein mutants able to protect against colicin mutants which bypass the wild-type immunity protein may help better define how Cai and pore-forming helices interact.

**Experimental procedures**

**Strain and plasmids**

*E. coli* C600 was the recipient strain for all plasmids and was also used as a sensitive indicator strain for colicin cytotoxicity. Plasmids pIA1, pIB1, and pIAB carry cai, cbi, and both cbi and cai in tandem, respectively (Géli and Lazdunski, 1992a). Plasmid pVL1 (Géli et al., 1988) was cut by NcoI–Smal, treated with *E. coli* polymerase Klenow fragment, and re-ligated to give pEPsEAI. Plasmid pEPsEAI carries the cai gene under the control of the ColA gene-inducible promoter (Lioubès et al., 1986); the protein encoded by pEPsEAI (EpCAI) also carries an amino-terminal 'tag' recognized by mAb 1C11 (Géli et al., 1988, 1993). EpCAI is fully functional (Géli et al., 1993). Plasmid pEPVBG7 was constructed by replacing the BglII–HindIII DNA fragment of pEPsEAI with the BglII–HindIII DNA fragment of pVBG7 (Géli et al., 1989); the protein encoded by pEPVBG7 has the PhoA enzyme fused to L3 of EpCAI. ColA and ColB are encoded by pE4 and pUCBIB, respectively (Duché et al., 1993; Géli and Lazdunski, 1992a).

**Hydroxylamine mutagenesis of pIA1 and selection of ColB-immune clones**

Hydroxylamine mutagenesis was performed as described (Silhavy et al., 1984). Briefly, 5 µg of purified plasmid pIA1 DNA was incubated for 3 h at 65°C in a total volume of 500 µl containing 100 mM phosphate buffer, pH 6, 1 mM EDTA, and 0.5 M NH₂OH. The reaction was stopped by passing the mix through a spin column of Sephadex G50 (Pharmacia). The DNA was precipitated and resuspended in H₂O at a concentration of 200 ng ml⁻¹. C600 competent cells (0.1 ml) were transformed by electroporation with 200 ng of mutagenized plasmid. The cells were incubated for 45 min in 500 µl of LB medium and then 2 µg of purified ColB was added. Cells were incubated for 10 more min and were plated on ampicillin Luria–Bertani plates. Plasmids were isolated from selected transformants and used to transform C600 cells. The killing
activity of ColB was tested on the transformants to check that the immune phenotype was plasmid-linked. Plasmids of immune clones were sequenced.

Substitution of Cai hydrophilic loops L1, L2, and L3 for the homologous loops of Cbi

The CbiI restriction site of plA1 was destroyed to obtain plA2. AccI, Ccl, and Eco47lll restriction sites were created by Accl, ClaI, AflW, or AflW-BglII. AflW-t-BglII, or Plasmid plA2 was cut either by >iccl + C/al, or Plasmid plA2 was cut either by >iccl + C/al, or Hpal + Eco471ll. 0g/tl and Hpal are natural sites of plA2.

Production of Cai as two independent fragments

A pair of oligonucleotides (5'-TAC ATT AAT GAG CGG TAT TCA CAA GTA CTG TAT TAC CTG AAC AAA GTC GCA TTC CTG CCA T-3'; 5'-CGA TGG CAG GAA TGC GAC TTT GTT GTA CAG GTA TAC TAC TAC TTG TGA ATA CAG CTC ATT AAT AGT GT-3'); (5'-TATA AGA ACT CGC GAT ATC ATT AAA CCG GTA ACG GTT AAA TTA GTC GTC A-3'; 5'-GA TCT GAC GAC TAA TTT ACG CTT AAC GTA CTG CCA TTA TTT AAT GAT ATC GGC AGT TC-3'), and (5'-ACT ACA GCT GGC GGA TTT GAC TAA TTT ACG CGT TAC CGG TTT AAT GAT ATC GT-3); (5'-TAC ATT AAT GAG CCG TAT TCA CAA GTA CTG CCA TTA TTT ACG CTT AAC GTA ATG CAT AAT GTC CCA AGT GC-3') was introduced into plA2 cut with Hpal + Eco47lll. Ogl/tl and Hpal are natural sites of plA2.

Construction of Cbi/Cai hybrid proteins

Plasmid plAB (5 ng) carrying cbi and cai in tandem was linearized with EcoRV (this restriction site is located between the two genes), treated with exonuclease III and mung-bean nuclease as previously described (Géli and Lazdunski, 1992a), and used to transform strain C600. Transformants were tooth-picked onto plates on which 100 μl of purified ColA or ColB solution (20 μg ml⁻¹) had been spread. ColA- or ColB-sensitive and -insensitive clones were identified. Their DNAs were analyzed by restriction mapping. Homologous recombination between cbi and cai resulted in a fixed deletion (Tomassen et al., 1985). The hybrid genes resulting from homologous recombination were selected and sequenced.

Miscellaneous

Published methods were used for ColA and ColB purification (Cavard and Lazdunski, 1979; Pressler et al., 1986), liquid and plate assays for colicin cytotoxicity (Cavard and Lazdunski, 1979; Géli and Lazdunski, 1988; 1992a), site-directed mutagenesis (Baty et al., 1987), DNA sequencing (Sequenase sequencing kit protocol), and the alkaline phosphatase assay (Manoil and Beckwith, 1985). EpCai and EpVBG7 were expressed as described by Géli et al. (1988).

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