Identification of residues in the putative TolA box which are essential for the toxicity of the endonuclease toxin colicin E9

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E colicins are plasmid-coded, protein antibiotics which bind to the BtuB outer membrane receptor of *Escherichia coli* cells and are then translocated either to the outer surface of the cytoplasmic membrane in the case of the pore-forming colicin E1, or to the cytoplasm in the case of the enzymic colicins E2–E9. Translocation has been proposed to be dependent on a putative TolA box; a pentapeptide (DGSGW) located in the N-terminal 39 residues of several Tol-dependent colicins. In this study, site-directed mutagenesis was used to change each of the residues of the putative TolA box of colicin E9 to alanines. In the case of the two glycine residues, the resulting mutant proteins were indistinguishable from the native colicin E9 protein in a biological assay; whereas the other three residues when mutated to alanines resulted in a complete loss of biological activity. Mutagenesis of two serine residues flanking the putative TolA box, Ser34 and Ser40, to alanines did not abolish the biological activity of the mutant colicin E9, although the zones of growth inhibition were hazy and slow to form. The size of the zone of inhibition was significantly smaller than the control in the case of the Ser40Ala mutant. The ColE9/Im9 complex was isolated from the three biologically inactive TolA box alanine mutants. In competition assays all three mutant protein complexes were capable of protecting sensitive *E. coli* cells against killing by the native ColE9/Im9 complex. On removal of the Im9 protein from the three mutant ColE9/Im9 complexes, all three mutant colicins exhibited DNase activity. These results confirm the importance of the putative TolA box in the biological activity of colicin E9, and suggest that the TolA box has a role in the translocation of colicin E9.

Keywords: E colicins, translocation, protein–protein interaction, TolA protein, BtuB receptor

INTRODUCTION

Colicins are plasmid-encoded antibacterial proteins that are classified into groups on the basis of the cell surface receptor to which they bind. The E colicins, for example, all bind to the product of the chromosomal *btuB* gene, which is an essential component of the high-affinity transport system for vitamin B₁₂ in *Escherichia coli* (Di Masi *et al.*, 1973). Each E colicin plasmid codes for the production of a specific immunity protein which protects the producing cell against the cytotoxic activity of its colicin, located at the C-terminus of the protein, by binding to this domain (Jakes *et al.*, 1974; Sidikaro &

Nomura, 1974; Wallis *et al.*, 1992a, b). Based on immunity tests (Watson *et al.*, 1981; Cooper & James, 1984) the E group colicins have been subdivided into nine types (ColE1–ColE9), and these fall into one of three cytotoxic classes: membrane-depolarizing (or pore-forming) agents such as ColE1 (Cramer *et al.*, 1990), DNases such as colicins E2, E7, E8 and E9 (Lau *et al.*, 1992) and RNases such as colicins E3, E4, E5 and E6 (Masaki *et al.*, 1992). The DNase- and RNase-type E colicins constitute a group called the enzymic E colicins (James *et al.*, 1996).

After binding to specific receptors in the outer mem-

brane, colicins are translocated across the outer membrane in a process which is mediated by the Ton (Braun, 1995) or the Tol system (Lazdunski, 1995). The TonB–ExbB–ExbD complex facilitates the flow of energy from the cytoplasm to the outer membrane for the energy-dependent transport of ferric siderophore complexes, vitamin B₁₂ and group B colicins (Braun, 1995). It has been proposed that an energized conformation of the TonB protein opens channels in the outer membrane, formed by receptor proteins like FepA, which acts as a receptor for colicins B and D and for ferric siderophores, or BtuB, which is a receptor for vitamin B₁₂.

The Tol-dependent translocation pathway is used both for the uptake of group A colicins (colicins A, E1 to E9, K, L, N and S4) and for the DNA of filamentous phages (Lazdunski, 1995). Four gene products are involved, *tolQ*, *tolR*, *tolA* and *tolB*, which are clustered at 17 min on the *E. coli* chromosomal map (Webster, 1991). The TolA, TolB, TolQ and TolR proteins are required to translocate colicins A, E2–E9, K and N; whilst only the TolC, TolA and TolQ proteins are required to translocate colicin E1. The nucleotide sequence of the *tol* genes has been determined and the Tol proteins have been localized in the *E. coli* cell envelope. The TolQ, TolR and TolA proteins are associated with the cytoplasmic membrane. TolQ contains three membrane-spanning segments (Kampfenkel & Braun, 1993). TolA and TolR are anchored to the cytoplasmic membrane by their N-terminal domains, whilst TolB is principally located in the periplasm, but is associated with the outer membrane (Isnard *et al.*, 1994), through an interaction with the peptidoglycan-associated protein (Pal) (Bouveret *et al.*, 1995). TolC is an outer-membrane protein which is able to form pores *in vitro* (Benz *et al.*, 1993) and is also involved in the secretion of α -haemolysin (Wandersman & Delepelaire, 1990) and colicin V (Gilson *et al.*, 1990).

The TolA protein is believed to perform a central role in the Tol-dependent translocation pathway (Levengood *et al.*, 1991). It is composed of three domains: the N-terminal domain of some 40 amino acids which anchors the protein in the cytoplasmic membrane, a central domain of some 260 amino acids which spans the periplasmic space, and a C-terminal domain of some 120 amino acids. Interactions between the N-terminal domain of both colicin A and E1 with the C-terminal domain of TolA have been demonstrated *in vitro* by Western blotting of total cell proteins of *E. coli* cultures that overproduce the TolA protein (Bénédetti *et al.*, 1991). After renaturation of the proteins on the filter and incubation with colicins, binding to the TolA protein was detected by using specific antibodies. The results showed that the Tol-dependent colicins A and E1 were able to interact with TolA, whilst the TonB-dependent colicin B was not. The C-terminal region of TolA was required for the colicin binding to occur, although the isolated N-terminal domain of colicin A, corresponding to the translocation domain, was sufficient for binding. It is tempting to speculate that the *in vitro* interaction

between TolA and colicins A and E1 may be of importance in the translocation process *in vivo*.

The N-terminal translocation domains of group B colicins contain a pentapeptide motif, called the 'TonB box', which is also found in the FepA and BtuB receptor proteins (Schramm *et al.*, 1987). Mutations in the TonB box of group B colicins (or in their receptors) reduce colicin uptake (Mende & Braun, 1990), and can be suppressed by mutations in TonB (Braun, 1995). Such allele-specific extragenic suppression indicates physical and functional interaction between group B colicins, their receptors and TonB. In contrast to the situation with group B colicins, very little is known about the role of specific residues of the translocation domain itself in the uptake of group A colicins. A number of hybrid colicins have been constructed by recombining colicin domains of Tol- and Ton-dependent colicins. The properties of these hybrid colicins clearly demonstrate that the translocation pathway is defined by the N-terminal domains (Bénédetti *et al.*, 1992). N-terminal fragment swaps between colicin E1 and colicin 10, pore-forming colicins which use the Tol and the Ton uptake systems respectively, confirmed that the Tol dependence of colicin E1 uptake was located in residues 1–34 (Pils & Braun, 1995a). Comparison of the Tol-dependent colicins E1, E2 and E3 revealed a consensus pentapeptide sequence DG(T/S)G(S/W), located in the various colicins between residues 30 and 39, at a similar position to the TonB box of Col10. This pentapeptide has been referred to as the 'TolA box' (Pils & Braun, 1995a), although there is no direct experimental evidence that these specific amino acids are essential in the translocation process. One intriguing question is the difference between the translocation of pore-forming colicins and the enzymic E colicins (James *et al.*, 1996); the former are translocated to the outer surface of the cytoplasmic membrane, whilst the latter have to be present in the cytoplasm to exhibit their cytotoxic activity. In this work we have investigated the involvement of a number of amino acids, including the putative TolA box residues, in the biological activity of the enzymic colicin E9.

METHODS

Plasmids, bacterial strains and media. *E. coli* JM83 was used as the host strain for cloning and mutagenesis. *E. coli* BL21(DE3) was used as the host strain for the expression vector pET21a (Novagen), which has a strong, IPTG-inducible T7 polymerase promoter and a polyhistidine tag (His-tag) to facilitate the purification of overexpressed proteins. All cultures were routinely grown in Luria–Bertani (LB) broth, or on plates of LB agar, supplemented where required with ampicillin (100 µg ml⁻¹). Plasmid pRJ358, which encodes the colicin E9 structural gene (*ceaI*) and the Im9 immunity gene (*ceiI*), has previously been described (Garinot-Schneider *et al.*, 1996).

DNA manipulation. Restriction enzymes and T4 DNA ligase were purchased from Boehringer Mannheim or New England Biolabs. Digestion of DNA with restriction endonucleases, electrophoresis of restriction fragments, ligation of DNA

fragments and transformation into *E. coli* were carried out as described by Sambrook *et al.* (1989).

PCR. PCR reactions were carried out in 50 µl volumes using 1 unit of *Taq* polymerase with 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, using an Amplitron II thermal cycler.

Colicin production tests. Test cultures were stabbed into plates of LB agar containing ampicillin and were incubated overnight at 37 °C before exposure of the plates to chloroform vapour for 15 min. After allowing residual chloroform to evaporate for 1 h, the plates were overlaid with 4 ml molten 0.7% (w/v) non-nutrient agar containing 50 µl of a standing overnight culture of the colicin-sensitive indicator strain *E. coli* JM83(pUC18). After incubation of the plates at 37 °C for at least 6 h the plates were inspected for a clear zone of growth inhibition around the test culture. Each culture was tested independently at least three times.

Total cell protein extracts of colicin-producing cultures. Standing overnight cultures were diluted 100-fold in 5 ml LB broth containing ampicillin and shaken at 37 °C for 2 h ($OD_{600} \sim 0.2\text{--}0.4$). At this point 2.5 ml of culture was transferred to a second flask containing mitomycin C ($0.5 \mu\text{g ml}^{-1}$). About 2 h after induction with mitomycin C, when the OD_{600} was more than 1.0, 2 ml of the culture from each flask was centrifuged at 13 000 *g* for 30 s. The cell pellets were then resuspended in 100 µl 1× SDS-loading buffer [25 mM Tris/HCl pH 6.8, 1% (w/v) SDS, 5% (w/v) glycerol, 0.1% bromophenol blue], boiled for 5 min and re-centrifuged. The supernatants (total cell proteins) were stored at -20 °C until required.

SDS-PAGE. SDS-PAGE gels (10% or 15%, w/v, acrylamide) were prepared and loaded with samples of total cell proteins, or of purified proteins, using the mini-Atto gel system following the protocol recommended by the manufacturer. The SDS-PAGE gels were stained for proteins with Coomassie blue for 1 h and then de-stained.

PCR site-directed mutagenesis. A mutagenic primer was used in a PCR reaction together with forward PCR primer S30, which is complementary to a 20 bp sequence located 105 bp upstream of the start codon of the *ceaI* gene in the template plasmid pNP11. After isolation of the first PCR product with a Qiaex II kit (Qiagen), a second PCR reaction was performed using the first PCR product as a 'megaprimer' together with the reverse PCR primer S19, which is complementary to a sequence at 612 bp of the *ceaI* gene in pNP11. The second PCR product containing the desired mutation was then purified, restricted with *NdeI* and *EcoRV*, and cloned in pNP11 restricted with the same enzymes. The nucleotide sequence of the complete *NdeI-EcoRV* restriction fragment was determined, using a Pharmacia ALF automated DNA sequencer, to confirm that the expected mutation had been made.

Protein purification and protein determinations. The protocol for His-tag protein purification of the ColE9/Im9-His-tag complexes was adapted from that provided in the manufacturer's (Novagen) user manual. Fifty millilitres of LB broth (containing $50 \mu\text{g ampicillin ml}^{-1}$) was inoculated with 0.5 ml of an overnight culture of *E. coli* BL21(DE3) containing the mutant plasmid and incubated at 37 °C with shaking. At an OD_{600} of 0.6 overexpression was induced by adding IPTG to a final concentration of 1 mM. Growth was continued for a further 2–3 h. The cultures were centrifuged for 15 min at 10 000 r.p.m. The cells were washed in 15 ml ice-cold binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris/HCl, pH 7.5), and the cell pellet was stored at -80 °C for 24 h. The cell pellet was resuspended in 4 ml ice-cold binding buffer,

treated with 200 µl lysozyme (20 mg ml^{-1}) for 30 min at 37 °C, and then subjected to freezing and thawing (three times) to break the cell walls. Nucleic acids were degraded with DNase I and RNase A, and the bacterial suspension was centrifuged at 14 300 r.p.m. for 20 min. The post-centrifugation supernatant was filtered through a 0.45 µm membrane. A 1.0 ml Ni^{2+} column was charged with 5 ml charge buffer (50 mM NiSO_4), and then equilibrated with 3 ml binding buffer. The prepared cell extract was then loaded on to the column. The column was washed with 8 ml binding buffer, 4 ml wash buffer (60 mM imidazole, 500 mM NaCl, 20 mM Tris/HCl, pH 7.5) and the colicinE9/Im9 complex was eluted with elution buffer (1 M imidazole, 500 mM NaCl, 20 mM Tris/HCl, pH 7.5). The elution was monitored by Bradford assay of aliquots at 595 nm and by running samples on SDS-PAGE. Although considerable amounts of the ColE9/Im9 complex were eluted with the wash buffer, the purity of the ColE9/Im9 complex recovered in the elution buffer was found to be significantly higher (see Fig. 3). Protein-containing fractions were dialysed exhaustively against water and the protein lyophilized prior to storage at -20 °C. The yield of protein from a typical purification was 20–30 mg (l bacterial culture)⁻¹.

Preparation of free colicin. Colicin E9 was purified in the absence of Im9-His-tag, by eluting from the Ni^{2+} column under denaturing conditions, with binding buffer containing 6 M guanidine hydrochloride. Guanidine hydrochloride denatures the ColE9/Im9-His-tag complex, thus removing the colicin whilst leaving Im9-His-tag bound to the column. Colicin E9 was then renatured by dialysis against water. Previous studies have shown that guanidine-hydrochloride-denatured colicin E9 can be refolded by dialysis and that full biological activity is restored to the colicin (Wallis *et al.*, 1992a). The presence of Im9-His-tag-free colicin E9 was determined by SDS-PAGE. Purified Im9 was a gift from C. Giffard, University of East Anglia.

Receptor binding assay of ColE9/Im9 complexes. An overnight culture of sensitive *E. coli* JM83(pUC18) cells was diluted 100-fold into 100 ml LB broth containing ampicillin, and grown at 37 °C to an OD_{600} of 0.1. Aliquots (10 ml) were then transferred to clean, sterile flasks and the cells were incubated with 1.2 µg ColE9/Im9-His-tag and either 1.2, 12, or 120 µg mutant ColE9/Im9-His-tag at 37 °C for at least 5 h. The concentration of ColE9/Im9-His-tag used was the lowest concentration which significantly affected the growth of *E. coli* JM83(pUC18), as judged by measuring the increase in OD_{600} . Cells containing 1.2 µg native or mutant colicin only, or no colicin at all, were included as controls. The OD_{600} of each sample treatment was taken at 30 min intervals to monitor cell viability. Each competition assay was performed at least twice with each mutant ColE9/Im9-His-tag complex being tested.

DNase activity assay. DNase activity assays were done as previously described (Wallis *et al.*, 1992a). Each digest contained 200 ng pUC18 DNA and colicin E9 (610 ng; 10 pmol), in the presence and absence of Im9 (98 ng; 10 pmol), and was repeated at least twice.

RESULTS

Alignment of the N-terminal domains of Tol-dependent colicins

Comparison of the Tol-dependent colicins E1, E2 and E3 revealed a consensus pentapeptide sequence DG(T/S)G(S/W), located in the various colicins be-

forming colicin K (GGTGA) (Pilsl & Braun, 1995b) shows much less sequence homology to the putative TolA box of the enzymic E colicins. The only similar pentapeptide sequence in the N-terminal fragment of the pore-forming colicin N, another member of the Tol-dependent group A colicins, appears to be SGAGS. We therefore decided to determine the significance of the five residues of the putative TolA box in the biological activity of colicin E9.

To facilitate the isolation and characterization of site-directed mutants of the putative TolA box of colicin E9, we constructed plasmid pRJ364, which is derived from pRJ358 (Garinot-Schneider *et al.*, 1996) by the introduction of an *EcoRV* site at 490 bp of the *ceal* gene, using PCR site-directed mutagenesis. An *NdeI* site was also introduced at the initiation codon of the *ceal* gene, together with a *SacI* site at 993 bp. The resulting plasmid pNP11 (Fig. 2) was then used as the template for mutagenesis of the putative TolA box residues, and for the biological characterization of the resulting mutants.

We have recently shown the value of alanine scanning mutagenesis of mutations localized in the DNase domain of colicin E9 which abolish the biological activity of colicin E9. Two out of six random mutations which abolished the biological activity of colicin E9 gave fully active colicins when the corresponding residues were mutated to alanine (Garinot-Schneider *et al.*, 1996), possibly because the original mutations in these two

Fig. 3. His-tag purification of two of the mutant ColE9/Im9-His-tag complexes. The purification of the 61555 Da colicin E9 and the 10649 Da Im9-His-tag proteins as a complex was confirmed by SDS-PAGE on a 15% gel. Lane 1, marker proteins (66.0, 45.0, 36.0, 29.0, 24.0, 20.1, 14.2 kDa); lanes 2 and 7, total cell proteins from a culture producing the Asp35Ala and the Trp39Ala mutant colicin respectively; lanes 3 and 8, eluate from the binding buffer step; lanes 4 and 9, eluate from the wash buffer step; lanes 5 and 10, eluate from the elution buffer step; lane 12, sample of the native ColE9/Im9-His-tag complex. The relative mobilities of the colicin E9 and Im9-His-tag proteins are identified on the right.

Table 1. Colicin phenotype of alanine mutants of colicin E9

The residue shown in colicin E9 was mutated to alanine, using site-directed mutagenesis. The colicin phenotype of the resulting colicin was determined in a stab test. The size of the zone of inhibition encoded by the mutant plasmids is quantified with respect to that encoded by plasmid pNP11 (* indicates that although the zone of inhibition was similar in size to that of the control, it was hazy and slow to form; —, no zone of inhibition).

Residue	Phenotype
None	+++++
Ser34	+++++*
Asp35	—
Gly36	+++++
Ser37	—
Gly38	+++++
Trp39	—
Ser40	+++*
Ser52	+++++
Trp56	+++++

mutants disrupted the structure of the protein. Since the Ser37Phe mutation which abolished the biological activity of colicin E3 (Escuyer & Mock, 1987) might have a significant effect on protein structure, we made a Ser37Ala mutant of colicin E9 in pNP11, using PCR mutagenesis. The Col⁻ phenotype of the Ser37Ala mutant colicin confirmed the importance of this residue to the biological activity of colicin E9. SDS-PAGE of total cell proteins from a mitomycin-C-induced culture confirmed that the Ser37Ala mutant colicin is induced at similar levels to the native colicin E9 by mitomycin C, and is not inherently unstable (data not shown).

Mutagenesis of the other four residues of the putative TolA box revealed that converting either of the two Gly residues (Gly36 and Gly38) to Ala had no effect upon the biological activity of the mutant colicin E9, whilst mutation of Asp35 or Trp39 to Ala completely abolished the biological activity of the mutant colicin E9 (Table 1).

The loss of biological activity of the Asp35Ala and the Trp39Ala mutant colicins was not due to differences in induction or stability (Fig. 3). To confirm the importance of the putative TolA box residues, we also mutated the flanking residues Ser34 and Ser40 to alanine residues. Neither of these mutations abolished the biological activity of colicin E9 (Table 1), but there was a noticeable difference, compared with the native colicin/Im9 complex, in that the zones of inhibition formed in the stab test were hazy and slow to appear. In the case of the Ser40Ala mutant, the zone of inhibition was significantly smaller than that of the control. An alternative pentapeptide sequence, DGRGH, located at residues 5–9, which showed some homology to the putative TolA box was found, but mutagenesis showed that residues 5–9 have no special role in the translocation process of colicin E9. The Arg7Ala mutation had a slight effect on the colicin phenotype, resulting in a slightly smaller zone of inhibition in the stab test. The only other significant homology in Fig. 1 was between residues 52 and 64 (SG(V/I)HWGGGSGHGN) in the enzymic E colicins with residues 34–46 of colicin K (SGVHWGGGSGHGN), which has been previously reported (Pils & Braun, 1995b). We therefore decided to mutate Ser52 and Trp56 of colicin E9 to Ala residues. The resulting mutant colicins were as active as the control colicin E9 in stab tests (Table 1).

Purification of the mutant ColE9/Im9 complexes

We have shown previously that a polyhistidine tail on Im9 facilitates the purification of the 15 kDa DNase domain of colicin E9, when both proteins are co-expressed in T7 expression vectors (Wallis *et al.*, 1994; Garinot-Schneider *et al.*, 1996). To facilitate the purification of the ColE9/Im9 complex, we therefore introduced an *Xho*I site in place of the stop codon of the *cei* gene of plasmid pNP11 during a PCR reaction. The 2012 bp *Nde*I-*Xho*I PCR fragment, containing the *cei* and *cei* genes, was then cloned into plasmid pET21a restricted with the same enzymes. The resulting plasmid, pCS4, was then transformed into *E. coli* BL21(DE3). IPTG-induced cultures of *E. coli* BL21(DE3) containing pCS4 were shown to overexpress the 61555 Da colicin

Fig. 4. Competition assay of the three mutant ColE9/Im9-His-tag complexes. Results of the competition assay are shown for two of the TolA box mutants, Asp35Ala (b) and Trp39Ala (c), and are compared with that of the His575Ala mutant (a) of colicin E9, which is biologically inactive due to a mutation in the DNase domain (Garinot-Schneider *et al.*, 1996). The results with the Ser37Ala mutant have previously been shown (Garinot-Schneider, 1996), and are very similar to the other two TolA box mutants.

E9 protein, together with a 10649 Da Im9 protein which contained two extra residues and a polyhistidine tail at the C-terminus. Soluble protein extracts from these cultures were used to purify the ColE9/Im9-His-tag complex. The 1345 bp *NdeI-NcoI* fragment of each of the three alanine mutants of the putative TolA box which abolish the biological activity of colicin E9 was sub-cloned into pCS4 restricted with *NdeI* and *NcoI*. *E. coli* BL21(DE3) cultures containing the resulting plasmids were then induced with IPTG and the mutant ColE9/Im9-His-tag complexes were purified (Fig. 3).

The mutant ColE9/Im9-His-tag complexes as eluted from the nickel affinity column were judged to be >90% pure from the Coomassie-stained gels, thus showing the value of this rapid purification strategy.

Characterization of the mutant ColE9/Im9 complexes

Since the three alanine mutants we had constructed had mutations in the putative TolA box sequence, we would ideally wish to test the three mutant ColE9/Im9 complexes in an *in vivo*, or *in vitro* assay of translocation. Unfortunately, the only assay of this type is the *in vitro* demonstration of an interaction between the N-terminal domain of either colicin A or E1 with the C-terminal domain of TolA (Bénédetti *et al.*, 1991). Under the same experimental conditions there is no interaction between the N-terminal domain of the enzymic colicin E3 with TolA (H. Bénédetti, personal communication). This may reflect a difference in the Tol-dependent translocation of pore-forming and enzymic E colicins. We therefore carried out (1) an *in vivo* assay to confirm that the mutant ColE9/Im9-His-tag complexes were still capable of binding to the BtuB receptor, and (2) an *in vitro* assay to confirm that the mutant ColE9/Im9-His-tag complexes were still capable of DNase activity. The former is performed by a competition assay in which a mutant ColE9/Im9 complex is used to protect *E. coli* cells against cell killing by the native ColE9/Im9-His-tag complex (Fig. 4). Each of the three purified mutant ColE9/Im9-His-tag complexes was able to protect *E. coli* cells. As a control in this experiment we used a His575Ala mutant ColE9/Im9-His-tag complex, which is biologically inactive due to a mutation in the DNase domain (Garinot-Schneider *et al.*, 1996). The three TolA box mutants were capable of protecting *E. coli* cells at lower relative concentrations than the His575Ala mutant (Fig. 4). This may be due to blocking of the BtuB receptor as a result of the TolA box mutant proteins not being translocated. The three TolA box mutant proteins thus appear to have no defect in binding to the BtuB receptor and are therefore assumed to have no gross defect in their three-dimensional structure as a result of the alanine mutations. The latter assay is performed using a plasmid nicking assay (Wallis *et al.*, 1992a, b, 1994) and shows that each of the three purified mutant colicin E9 proteins, when separated from Im9-His-tag, were capable of DNase activity. In all cases the DNase activity was inhibited by the presence of Im9 protein in the assay (data not shown).

DISCUSSION

The results of this study show clearly that three of the five residues of the putative TolA box (Pilsel & Braun, 1995a) are essential for the biological activity of colicin E9. The phenotype of the Ser37Ala mutant confirms the conclusion of earlier work with a Ser37Phe mutant of colicin E3 (Escuyer & Mock, 1987). Alanine scanning mutagenesis of a total of 14 of the first 56 residues of colicin E9 revealed that only the three residues identified in the TolA box completely abolish the biological

activity of colicin E9. Mutation of Ser34 and Ser40 to alanine results in colicin zones of inhibition which are slow to form and are hazy in comparison to the control, thus indicating some effect on biological activity. The zone of inhibition of the Ser40Ala mutant was also significantly smaller than that of the control culture. Although these results provide support for the hypothesis that the TolA box pentapeptide and the flanking Ser residues are important for biological activity, there is only indirect evidence that they are involved in Tol-dependent translocation. However, since we have demonstrated that the three mutant proteins are capable of binding to the BtuB receptor, and also exhibit DNase activity, it is certainly suggestive that mutations located in this domain of the protein are defective in translocation. We cannot at this stage exclude the possibility that the three TolA box mutants are biologically inactive because they become chemically and/or functionally labile within the bacterial cell. However, the large number of other mutations made in this region which do not affect biological activity, perhaps makes this an unlikely explanation. A more direct assay of the interaction of colicin E9 with proteins of the *E. coli* Tol-dependent translocation system will be required to answer this question unequivocally.

It is interesting that the suggested TolA box sequence of colicin K (GGTGA) contains an alanine residue at the fifth position (Pilsel & Braun, 1995b). From our data with colicin E9, this would be expected to have a dramatic effect on the biological activity of the colicin. The only similar pentapeptide sequence in colicin N (SGAGS) also contains an alanine residue at the third position. Alanine scanning mutagenesis of the putative TolA boxes of colicin K and colicin N would be very informative. Progress in our understanding of the translocation of colicins will require knowledge of the three-dimensional structures of the translocation domain of Tol-dependent colicins, as has recently been obtained for the TonB-dependent colicin Ia (Wiener *et al.*, 1997), together with the development of an *in vitro* or *in vivo* assay of translocation. The alanine mutants described in this work will be of value in dissecting the role of any interaction with proteins of the translocation machinery in the biological activity of colicin E9.

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