

Intracellular Immunization of Prokaryotic Cells against a Bacteriotoxin

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Received 4 August 1997/Accepted 3 December 1997

Intracellularly expressed antibodies have been designed to bind and inactivate target molecules inside eukaryotic cells. Here we report that an antibody fragment can be used to probe the periplasmic localization of the colicin A N-terminal domain. Colicins form voltage-gated ion channels in the inner membrane of *Escherichia coli*. To reach their target, they bind to a receptor located on the outer membrane and then are translocated through the envelope. The N-terminal domain of colicins is involved in the translocation step and therefore is thought to interact with proteins of the translocation system. To compete with this system, a single-chain variable fragment (scFv) directed against the N-terminal domain of the colicin A was synthesized and exported into the periplasmic space of *E. coli*. The periplasmic scFv inhibited the lethal activity of colicin A and had no effect on the lethal activity of other colicins. Moreover, the scFv was able to specifically inactivate hybrid colicins possessing the colicin A N-terminal domain without affecting their receptor binding. Hence, the periplasmic scFv prevents the translocation of colicin A and probably its interaction with import machinery. This indicates that the N-terminal domain of the toxin is accessible in the periplasm. Moreover, we show that production of antibody fragments to interfere with a biological function can be applied to prokaryotic systems.

Antibodies have long been used in biochemical science as in vitro tools for the identification, purification, and functional manipulation of target antigens; they have been exploited in vivo for diagnostic and therapeutic applications as well. In the past few years, several groups have demonstrated that recombinant antibody fragments can be produced by joining the C terminus of the heavy-chain variable fragment (V_H) to the N terminus of the light-chain variable fragment (V_L) by using a flexible polypeptide linker (19, 25). Such constructs, known as single-chain Fv (scFv) constructs, have binding activities similar to those of the native antibodies. The most common host for the engineering and production of scFv fragments is the microbial system, especially *Escherichia coli* (30, 33). With this system, the interactions between antibodies and their antigens or between catalytic antibodies and their substrates have been studied by site-directed mutagenesis (15, 31). Pharmacological tools were constructed by linking scFv with protein domains having various effector functions (21). Libraries of antibody fragments were constructed in lambda (29) or fd (2, 24) phages to select new antibodies directed against a wide variety of antigens, including highly conserved proteins.

Recently, scFvs were produced and targeted to various compartments of mammalian cells to interfere specifically with cellular functions and virus development (7, 28). This strategy, known as "intracellular immunization," has provided a powerful new family of molecules for gene therapy applications. These intracellular antibodies, termed "intrabodies," have been produced in other organisms, such as plants (1, 32).

Colicin A is a pore-forming bacteriocin that depolarizes the cytoplasmic membrane of sensitive *E. coli* cells. The mechanism of action of this toxin has three steps, each of which can be associated with a specific domain of the protein (4). The N-terminal domain is involved in the translocation of part of

the toxin from the outer to the inner membrane. The central domain is required for binding to the outer membrane receptor. The C-terminal domain has the pore-forming activity. The molecular mechanisms underlying colicin importation have been partly elucidated. The reception step requires outer membrane proteins. Translocation involved the bacterial proteins TolQ, TolR, TolA, and TolB (11). After binding to its receptor, colicin A unfolds to enter the cells (6, 12). It forms a pore in the inner membrane while still interacting with its receptor and its translocation machinery (13), and thus adopts an extended conformation across the cell envelope (6). Previous studies have suggested that colicin translocation may involve direct interaction with a component of the translocation machinery in the inner face of the outer membrane. However, most of these studies have been performed in vitro (5).

In this study, we have determined in vivo that the N-terminal domain of colicin A was accessible in the *E. coli* periplasm during toxin activity. Our approach involved exporting an antibody fragment directed against the N-terminal domain of the toxin into the periplasm of *E. coli*. We show that once exported, the antibody fragment was able to bind and specifically inactivate colicin A in vivo without affecting its receptor binding. The protection against colicin A conferred by interaction in vivo with the antibody fragment demonstrated that the N-terminal domain of colicin A passes through the outer membrane of *E. coli* and reaches the periplasm, leading to translocation of the C-terminal domain. Our results confirm that during pore formation, the N-terminal domain of colicin A is periplasmic, whereas the central domain is still bound to the cell surface. This work also demonstrates that despite the gel-like structure of the periplasm in which the lateral diffusion coefficients are 2 orders of magnitude smaller than those of the cytoplasm, an scFv fragment efficiently binds and inactivates its antigen.

MATERIALS AND METHODS

DNA techniques. cDNA was synthesized from mRNA isolated from the monoclonal antibody (MAb) 1C11-producing hybridoma cell line (27). V_H and V_L genes were amplified by PCR with the following oligonucleotides as primers: 5'-CATGCCATGACTCGCGCCAGCCGGCCATGGCCSARGTBMARC TKSWSARCWGG-3' and 5'-GDGTCAKMACRAYDTCACCTTTAGTACT ACCTTCACCTGAACCAGGTTTACCAGAACCTGAGGTAGAACCTG

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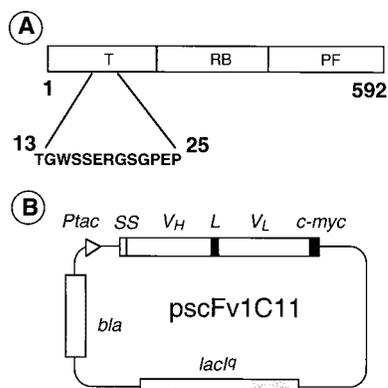


FIG. 1. Construction of the pscFv1C11 expression vector. (A) Position of the 1C11 epitope in the translocation domain of colicin A. T, RB, and PF, translocation, receptor-binding, and pore-forming domains of colicin A, respectively. The amino acid residues are abbreviated as follows: E, Glu; G, Gly; P, Pro; R, Arg; S, Ser; T, Thr; and W, Trp. (B) pscFv1C11 expression vector. The gene coding for the 1C11 scFv (V_H , L , V_L) was amplified by PCR and inserted into pPelB35PhoA'. The *bla*, *lacI^q*, *SS*, V_H , L , V_L , and *c-myc* genes encode the β -lactamase, *lac* repressor, signal sequence of pectate lyase of *Erwinia carotovora*, V_H domain, linker [(Gly₂Ser)₃], V_L domain, and c-myc tag, respectively. The scFv was under the control of the IPTG-inducible *tac* promoter (*Ptac*).

MRGAGACDGTGAS-3' and 5'-STCACHGTCTCYKCAGGTTCTACCTCA GGTCTGGTAAACCTGGTTCAGGTGAAGGTAGTACTAAAGGTGA DRTYGTIKMTGACHC-3' and 5'GAGTCATTCTGACTAGTCCCGTTTCA KYTCAVCTTKGTSCC-3', respectively. The *Nco*I and *Spe*I cloning sites are underlined. The resulting PCR fragments were assembled by the overlap PCR method as the 1C11 scFv gene (22). This gene was inserted between the *Nco*I and *Spe*I sites of pPelB35PhoA' (27) to give pscFv1C11. The nucleotide single-letter code is as follows: B, C or G or T; D, A or G or T; H, A or C or T; K, G or T; M, A or C; R, A or G; S, C or G; V, A or C or G; W, A or T; and Y, C or T.

scFv expression and cell fractionation. A culture of *E. coli* JM101 harboring pscFv1C11 was grown in Luria-Bertani medium containing ampicillin (100 mg/liter) at 37°C to attain an A_{600} of 0.5. After 20 min of induction at 30°C with the indicated concentration of isopropyl- β -D-thiogalactopyranoside (IPTG) and with or without the indicated concentration of dithiothreitol (DTT), the cells were harvested by centrifugation at $4,000 \times g$ for 10 min (at 4°C). Cell fractionation was carried out by suspending the cell pellets in TES buffer (0.2 M Tris-HCl [pH 8.0], 0.5 mM EDTA, 0.5 M sucrose) (10 ml per liter of original culture). The cells were then subjected to a mild osmotic shock by addition of TES buffer, diluted 1:4 with H₂O. After incubation on ice for 30 min, the suspension was centrifuged ($2,000 \times g$, 10 min) to prepare the periplasmic fraction (supernatant) and spheroplast fraction (pellet).

ELISA. The enzyme-linked immunosorbent assay (ELISA) was performed as follows. Colicin A or bovine serum albumin was used to coat 96-well flat-bottom Nunc plates at a final concentration of 100 ng of protein/well. After blocking the plate with 5% milk in phosphate-buffered saline, the periplasmic preparations were added. Bound scFvs were detected with the anti-Myc-Tag 9E10 antibody. Horseradish peroxidase (HRP)-labelled rabbit anti-mouse immunoglobulin G (IgG) was used as the second antibody. Bound antibodies were detected by the addition of the HRP substrate, and the optical density was measured at 405 nm.

Colicin A activity. Cells were prepared and induced as described above and then washed, resuspended in 100 mM sodium phosphate buffer (pH 7.2) at a density of 5×10^{10} cells per ml, and kept on ice. Cells (2×10^9 /ml) were incubated for 5 min at 30°C in 100 mM sodium phosphate buffer (pH 7.2) containing glucose (0.2% [wt/vol]) and 0.5 mM KCl. Colicin A was added at a multiplicity of 100 (number of colicin molecules per cell), and the initial rate of K⁺ efflux was determined from the linear part of the K⁺ efflux curve as previously described (8). The rate of K⁺ efflux (V_{K^+} pscFv1C11) after colicin A treatment of cells harboring pscFv1C11 induced at the indicated concentration of IPTG was compared to that of *E. coli* JM101 cells (V_{K^+} JM101). The relative inhibition of K⁺ efflux was calculated as $[(V_{K^+} \text{ JM101}) - (V_{K^+} \text{ pscFv1C11})] / (V_{K^+} \text{ JM101})$.

RESULTS

Cloning of the 1C11 scFv. We investigated the ability of the 1C11 scFv to bind a 13-residue region (Thr¹³ to Pro²⁵) within the N-terminal domain of colicin A in vivo (Fig. 1A) (16). PCR-amplified genes encoding the V_H and V_L domains of the murine 1C11 antibody were joined by a DNA sequence coding

for a flexible polypeptide linker. The resulting construct encoding the 1C11 scFv was placed under the control of the *tac* promoter and was fused to the sequence encoding the pectate lyase B (PelB) signal peptide such that the recombinant protein would be translocated across the inner membrane into the periplasm (Fig. 1B). *E. coli* JM101 was transformed with the resulting plasmid, pscFv1C11, for scFv production.

The 1C11 scFv produced in the periplasm of *E. coli* is active in vitro. The production of an scFv fragment in *E. coli* often impairs growth and may cause lysis of the scFv-producing cells (26). This may be due to saturation of the export machinery when the recombinant protein is overproduced. To circumvent this major problem, we first investigated the effect of IPTG induction on cell growth. Growth of the transformed strain was affected after a 1-h induction period, even with a low concentration of inducer (20 μ M IPTG) and at low temperature (30°C). However, uninduced cells grew as rapidly as the untransformed parental cells (data not shown). Thus, to avoid nonspecific cell lysis, an induction time of 20 min was used in all experiments. The production and activity in vitro of the scFv in the periplasmic fractions were assessed by immunoblotting and ELISA. The relative concentration of scFv in the periplasmic extracts was estimated by immunoblot analysis with purified scFv as the standard. Without induction, a small amount of scFv (50 ng per 10^9 bacteria) was detected by immunoblotting (Fig. 2A). This amount was sufficient to give a significant signal in ELISA with purified colicin A (Fig. 2B). In the presence of 20 μ M IPTG, the amount of scFv produced in the periplasm was estimated to be 2.5 μ g per 10^9 bacteria, which corresponded to the maximum value obtained by ELISA (Fig. 2B). These results showed that some of the scFv molecules produced in the periplasm of *E. coli* were correctly folded, oxidized, and active in vitro.

1C11 scFv-producing cells are protected against colicin A. The ability of the recombinant antibody to protect the scFv-producing cells against colicin A was assessed in vivo. The addition of colicin A to sensitive cells induces an efflux of cytoplasmic K⁺, which correlates with pore-forming activity (8). The maximum K⁺ efflux is reached with about 400 colicin molecules per cell (13). Bacteria were washed extensively prior to the addition of colicin to remove any scFv released by cell lysis or leakiness. The K⁺ efflux induced by the addition of colicin A to transformed cells (at a multiplicity of 100) was 90% lower than that of untransformed cells. This effect was observed irrespective of the concentration of IPTG used for induction (Fig. 2C). As little as 50 ng of periplasmic scFv per 10^9 bacteria, produced as a result of promoter leakiness, was sufficient to prevent pore formation almost completely. Cells producing the 1C11 scFv were thus protected against colicin A. We carried out the following experiments to demonstrate that the inhibition of colicin A activity was not due to scFv release into the medium. Proteins in the supernatant of the washed cells (induced with 0 or 100 μ M IPTG) were precipitated with trichloroacetic acid, and the total amount of scFv present was estimated by immunoblotting. No signal was detectable, showing that there was less than 10 ng of scFv per 10^9 bacteria in the supernatant (data not shown). Moreover, transformed cells whether uninduced or induced with 100 μ M IPTG, were protected against colicin A even when 10^5 colicin A molecules were added per cell (Fig. 2C). Under these conditions, any scFv fragments released into the medium would not be able to block all colicin molecules.

DTT-reduced 1C11 scFv does not inhibit colicin A activity. To verify that the inhibition of colicin A activity was specifically dependent on 1C11 scFv production, we used a reducing agent to inactivate the recombinant antibody fragment. Periplasmic

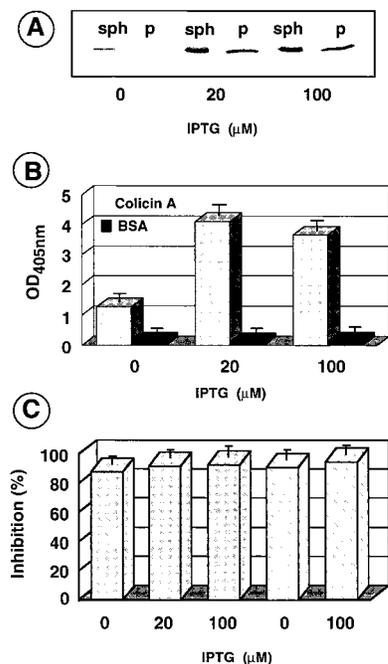


FIG. 2. Effect of periplasmic 1C11 scFv on colicin A activity. (A) Subcellular location of the 1C11 scFv produced in *E. coli*. Cells were grown at 37°C to an A_{600} of 0.5 and then induced with the indicated concentration of IPTG for 20 min. The amount of scFv in the periplasmic fraction (p) and spheroplast fraction (sph) was estimated by immunoblotting with MAb 9E10 (directed against the c-myc tag). (B) 1C11 scFv binding in the periplasmic fraction in vitro. An ELISA was used to measure the binding activity of the 1C11 scFv in the periplasmic fraction. Colicin A or bovine serum albumin was used to coat the plates at a final concentration of 100 ng of protein/well, and then the plates were treated with periplasmic fractions. HRP-labelled rabbit anti-mouse IgG was used as the second antibody. Bound antibodies were detected by the optical density (OD) at 405 nm. (C) In vivo inhibition of colicin A activity by 1C11 scFv produced in sensitive cells. 1C11 scFv-producing cells and control cells were prepared and induced as described above and then were suspended in 100 mM sodium phosphate buffer (pH 7.2). Colicin A was added at a multiplicity (number of colicin molecules per cell) of 100 (shaded bars) or 10^5 (hatched bars), and the initial rate of K^+ efflux was determined from the linear part of the K^+ efflux curve. The relative inhibition of K^+ efflux was calculated as described in Materials and Methods. Mean values and standard errors are shown; three independent experiments were performed for each set of conditions.

fractions of cells preincubated with various concentrations of DTT during expression were tested by immunoblotting and ELISA. Surprisingly, DTT treatment resulted in loss of the 1C11 scFv from the periplasmic fractions (Fig. 3A). Presumably, the reduced 1C11 scFv fragments were aggregated or degraded by periplasmic proteases. The same periplasmic fractions had a lower ELISA signal than the control without DTT, demonstrating the loss of the 1C11 scFv (Fig. 3B). The same conditions were used for in vivo experiments. As expected, preincubation of the scFv-producing cells with various concentrations of DTT prior to the addition of colicin A resulted in lower levels of toxin inhibition by the scFv fragment (Fig. 3C). Thus inhibition of colicin A activity required the presence of functional oxidized scFv.

The periplasmic 1C11 scFv specifically inactivates colicin A. We then tested whether the inhibition of colicin A activity by the 1C11 scFv was due to an indirect effect on the pore-forming colicins. We tested the activity of colicins E1 and B on the 1C11 scFv-producing strain. Colicins E1 and B are ionophoric colicins with different import machineries. Colicin E1, like colicin A, uses the Tol machinery, whereas colicin B uses the Ton machinery (for review, see reference 18). Neither the

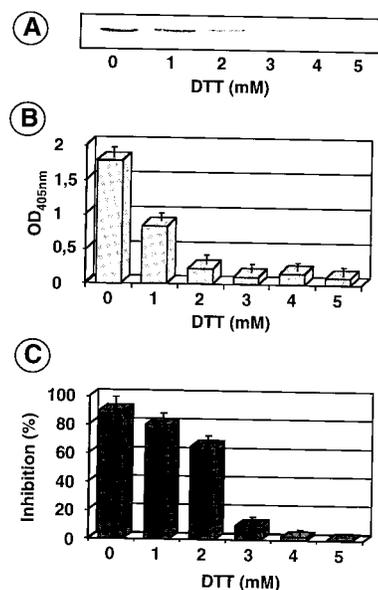


FIG. 3. Effect of DTT on periplasmic 1C11 scFv. (A) Amount of 1C11 scFv in periplasmic extracts. Periplasmic extracts were prepared as described in the legend to Fig. 2A and Materials and Methods, except that cells were incubated with the indicated concentration of DTT during induction (20 μ M IPTG). (B) In vitro binding of periplasmic 1C11 scFv. Periplasmic extracts containing the 1C11 scFv were tested by ELISA. Bars indicate the optical density (OD) at 405 nm. (C) In vivo binding of the 1C11 scFv. The inhibition of colicin A activity was determined as described in the legend to Fig. 2C and Materials and Methods, except that uninduced cells were incubated with the indicated concentration of DTT for 20 min before harvesting. Bars indicate the percentage of inhibition. Mean values and standard errors are shown; three independent experiments were performed for each condition.

activity of colicin E1 nor that of colicin B was inhibited by the scFv fragment (Fig. 4). Thus, the inhibition of colicin A activity involved specific interaction between the scFv fragment and the toxin. Moreover, a different scFv, scFv 5A4, directed against cortisol (27), had no effect on colicin A activity (Fig. 4).

To address the potential influence of the 1C11 scFv on colicin A binding, this toxin was incubated at a multiplicity of 400 with uninduced cells harboring pscFv1C11 for 1 min. This is longer than required for its full translocation (12). Colicin E1, which uses the same receptor, was then added at a multiplicity of 400. The activity of colicin E1 was inhibited by 90%

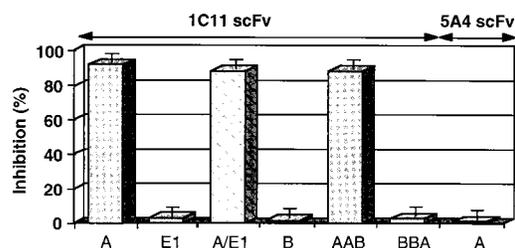


FIG. 4. 1C11 scFv specificity. 1C11 and 5A4 scFv-producing cells were prepared as described in the legend to Fig. 3C, except that cells were not incubated with DTT. Inhibition of colicin A, E1, B, AAB, and BBA activity in 1C11 scFv-producing cells and inhibition of colicin A activity in 5A4 scFv-producing cells were determined as described in the legend to Fig. 2C (shaded bars). Cells producing the 1C11 scFv were also incubated with colicin A at a multiplicity of 400. After 1 min, colicin E1 was added at the same multiplicity, and the K^+ efflux was determined as described above (hatched bar). Bars indicate the percentage of inhibition. Mean values and standard errors are shown; three independent experiments were performed for each condition.

(Fig. 4). This result shows that colicin A was still in contact with its receptor when colicin E1 was added to the cells. Thus, the binding of colicin A to its receptor was not affected by the periplasmic scFv.

The periplasmic 1C11 scFv interacts with the colicin A N-terminal domain. Finally, we tested whether the scFv fragment interacted *in vivo* with the N-terminal domain of colicin A. The pore-forming activities of two hybrid colicins were analyzed. Colicin AAB possesses the N-terminal and central domains of colicin A fused to the pore-forming domain of colicin B, whereas colicin BBA possesses the N-terminal and central domains of colicin B and the pore-forming domain of colicin A (17). As expected, colicin AAB, but not colicin BBA, activity was inhibited by the scFv fragment. From these results, we concluded that colicin A activity was specifically inhibited by 1C11 scFv in the cell periplasm.

DISCUSSION

Intrabodies are a new generation of antibodies able to fix and inactivate target molecules inside eukaryotic cell compartments. In this study we used an intrabody to determine the intracellular location of the colicin A N-terminal domain in the *E. coli* envelope.

We have cloned DNA sequences encoding the V_H and V_L domains of the murine MAb 1C11. This MAb recognizes a sequence from the colicin A N-terminal domain (16). The heavy- and light-chain regions of MAb 1C11 were connected by a flexible linker which started at the carboxyl end of the heavy-chain Fv domain and ended at the amino terminus of the light-chain Fv domain. The resulting gene encoded the 1C11 scFv domain in the form of a single-chain antigen-binding protein. The folding and assembly of an antigen-binding site require the formation of intrachain disulfide bonds. The periplasm of *E. coli* is particularly useful for the heterologous production of recombinant proteins, because disulfide bond formation is catalyzed by the Dsb system (3). Thus, we fused this scFv gene to a sequence encoding the pectate lyase B (PelB) signal peptide. This recombinant antibody was targeted to the periplasmic space. However, as previously described for other scFv fragments, most of the 1C11 scFv molecules aggregated in the cytoplasm or in the periplasm (26). However, the ELISA signal obtained with periplasmic fraction of cells producing the 1C11 scFv demonstrated that soluble periplasmic 1C11 scFv molecules were correctly folded and active. Interestingly, incubation of cells with DTT during 1C11 scFv production resulted in the loss of soluble scFv fragments from the periplasmic space. The unfolded scFv fragments were either degraded or aggregated.

One of the major aims of this paper was to demonstrate that cells expressing the 1C11 scFv were protected against colicin A, suggesting that an scFv fragment produced in the periplasm of *E. coli* can bind and inactivate its target molecule *in vivo*. The promoter was leaky enough to produce enough scFv fragment to inactivate toxin activity almost completely. The specificity of this interaction was demonstrated in several ways: by hybrid colicins, other scFv fragments, and scFv inactivation by DTT. Periplasmic secretion has been used to produce and purify active proteins. However, in all cases, the fragments have been purified or recovered in a standard buffer by osmotic shock. Conditions in the periplasm of bacteria are very different from those in the blood or in a buffer. It has been suggested that this compartment has a gel-like structure, and is filled with a peptidoglycan matrix with large pores (23). The lateral diffusion coefficients are 2 orders of magnitude smaller than those of the cytoplasm, and periplasmic proteins are much less

mobile (10). We thus tested whether the protection was really due to periplasmic activity of the antibody fragment. Our results, including cell protection in the presence of a large excess of colicins, exclude involvement of any released scFv fragments in the protection process. This shows that an scFv fragment can efficiently bind its antigen in the periplasmic space and demonstrates the potential use of intracellular antibodies.

However, the aim of this study was to investigate the colicin A translocation process. The colicin A import machinery is composed of periplasmic and membrane proteins. These proteins are preferentially located in the contact sites between the inner and outer membranes and are stabilized by colicin A (20). Direct interactions between the N-terminal domain of colicin A and the C-terminal domain of TolA have been described *in vitro* (5), and recently the N-terminal domain of colicin E3, which uses the same import machinery as colicin A, has been cross-linked *in vivo* to the periplasmic TolB protein (9). We demonstrate here that the presence of an scFv fragment directed against the N-terminal domain of colicin A inactivates the toxin. But which step of the process is affected by this interaction? We first determined that colicin A binding was not affected by the 1C11 scFv. Indeed, colicin A competed with colicin E1 for binding. In a previous paper, we demonstrated that 400 mutant colicin A molecules were required to block 90% of colicin E1 binding, whereas 1,000 mutant colicin A molecules were required to block the translocation step of colicin E1 (13). Here, we show that 400 colicin A molecules gave 90% inhibition of colicin E1 activity on the scFv-producing cells. Colicin A molecules were thus interacting with their receptor and impeding colicin E1 binding. This result demonstrates that colicin A binding was not affected by the presence of the scFv fragment. We then investigated whether the scFv fragment could affect the activity of the C-terminal domain. Previous work has demonstrated that the C-terminal domain of colicin A, without the other domains in *E. coli* periplasm, is able to exert its lethal effects (14). Moreover mutant colicin A molecules with deletions in their N-terminal domains are inactive *in vivo* but form channels in phospholipid planar bilayers (4). This indicates that the C-terminal domain does not require the N-terminal domain after translocation to function. It is thus very improbable that an interaction between the scFv and the N-terminal domain of colicin A directly affects the formation and action of the transmembrane channel because of the C-terminal domain. In the presence of the scFv, colicin A was unable to form pores. Although our data do not directly demonstrate it, it is very probable that the scFv prevented interaction between colicin A and its import machinery, the Tol system, an interaction already demonstrated *in vitro* for the TolA protein.

The production of antibody fragments has been proposed as a method for analyzing the biological activity of antigens in eukaryotic cells (7). As demonstrated here, this approach can be extended to prokaryotic cells for the study of the biological function of antigens. This novel approach in bacterial studies made it possible for us to demonstrate that colicin A exposes its N-terminal domain in the *E. coli* periplasm during the complex process that leads to cell death.

ACKNOWLEDGMENTS

This work was supported by The Centre National de la Recherche Scientifique.

We are grateful to V. Géli, S. Slatin, D. Missiakas, and H. Bénédetti for critical review of the manuscript.

P.C. and J.F. contributed equally to this work.

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