A colicin A fragment containing the receptor binding domain can be directed to the periplasmic space in *E. coli* through gene fusion

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Received 13 March 1984

The central region of the colicin A polypeptide chain has been fused to the N-terminal part of β-lactamase through genetic recombination. This region comprising amino acid residues 70–335 confers on the hybrid protein the ability to protect sensitive cells from the lethal action of colicin A. Although colicin A belongs to the cytoplasmic compartment of *E. coli*, export of the hybrid protein to the periplasmic space was promoted by the signal peptide of β-lactamase.

Colicin *Export* *Receptor* *Hybrid protein*

1. INTRODUCTION

Colicin A is a channel-forming bactericidal protein of M_r 63 000 [1]: it exerts its lethal effect on the *E. coli* sensitive cell through depolarization of the inner membrane and consequent dissipation of the cellular energy level [2]. The formation of the dissipative channel across the inner membrane follows binding of the colicin molecule to a specific receptor located in the outer membrane. This receptor is composed of two proteins: Omp F and Btu B [3,4]. The functions responsible for receptor binding and lethality have been located in different domains of the colicin polypeptides [5–8]. The C-terminal domain of colicin A has been isolated and purified [9]. This polypeptide can produce channels in planar phospholipid bilayers [9] like colicin A [2] and thus is likely to be responsible for lethality. Furthermore, the complete nucleotide sequence of colicin A has been determined [1].

We demonstrate here that the domain of colicin A polypeptide chain responsible for receptor binding is located between amino acid residues 70 and 335 of the bacteriocin. This central domain of colicin A has been fused to the N-terminal part of β-lactamase through genetic recombination.

Evidence is presented that export of the hybrid protein can be promoted by the signal peptide of β-lactamase. To our knowledge, this is the first demonstration that a significant part of a cytoplasmic polypeptide chain can be expected to the periplasmic space.

2. MATERIALS AND METHODS

2.1. Materials

Restriction endonucleases were purchased from Boehringer and Bethesda Research Laboratory. [^{32}P]dATP and [^{35}S]methionine were obtained from Amersham. Mitomycin C was purchased from Sigma.

2.2. Bacterial strains, plasmids

E. coli K12 W3110, W3110 (ColA), RB113 and SK1592 (pBR322) have been described [10,11]. Cells were routinely grown in LB medium but

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00145793/84/$3.00

(A)

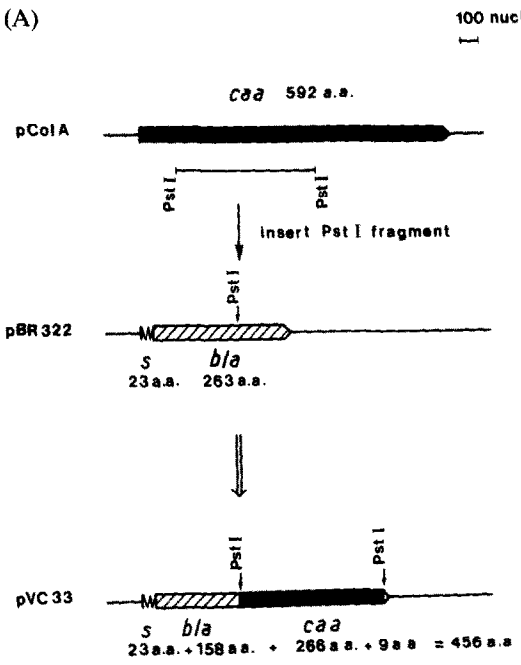

Fig.1. Construction of the *bla-caa* hybrid gene. (A) The plasmid pVC33, carrying *bla-caa* hybrid gene, was constructed by cloning the indicated *Pst*I restriction fragment of ColA plasmid into the *Pst*I site of the plasmid pBR322. (B) Reading frames and corresponding amino acid sequences around the *Pst*I sites. The numbering of amino acids starts from the N-terminus of colicin A [1] and pre-β-lactamase [18].

otherwise stated, as in [11]. The maxicell medium was as indicated by Sancar et al. [12].

2.3. *'Maxicell' technique, SDS gel electrophoresis, and DNA sequence analysis*

Proteins encoded by plasmids were detected as in [12]. ^{35}S-labeled proteins were analyzed on SDS–polyacrylamide slab gels and fluorography was carried out as in [10]. Nucleotide sequence analysis of recombined DNA was performed as in [1].

(B)

bla-caa junction:

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                                          Pst I
bla-caa : GAG  CGT  GAC  ACC  ACG  ATG  CCT  GCA | GGC  CAG  CCG  ACC  ATG  AAC  GGA
          CTC  GCA  CTG  TGG  TGC  TAC  GGA  CGT  CCG  GTC  GGC  TGG  TAC  TTG  CCT

fusion
protein : Glu  Arg  Asp  Thr  Thr  Met  Pro  Ala  Gly  Gln  Pro  Thr  Met  Asn  Gly

β-lacta-
mase    : Glu  Arg  Asp  Thr  Thr  Met  Pro  Ala  Ala  Met  Ala  Thr  Thr  Leu  Arg
                                     182

coli-
cin A   : Gly  Lys  Val  Ile  Ile  Asn  Ala  Ala  Gly  Gln  Pro  Thr  Met  Asn  Gly
                                     70
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caa-bla junction:

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                                          Pst I
caa-bla : TCT  CAG  GCT  AAT  AAT  ATC  CTG  CAG  CAA  TGG  CAA  CAA  CGT  TGC  GCA  AAC  TAT  TAA
          AGA  GTC  CGA  TTA  TTA  TAG  GAC  GTC  GTT  ACC  GTT  GTT  GCA  ACG  CGT  TTG  ATA  ATT

fusion
protein : Ser  Gln  Ala  Asn  Asn  Ile  Leu  Gln  Gln  Trp  Gln  Gln  Arg  Cys  Ala  Asn  Tyr  End

coli-
cin A   : Ser  Gln  Ala  Asn  Asn  Ile  Leu  Gln  Asn  Ala  Arg  Asn  Glu  Lys  Ser  Ala  Ala
                                     335
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2.4. Immunoprecipitation

Antisera were prepared and imunoprecipitation was carried out as in [10,13]. The antiserum directed against TEM-I β-lactamase was a generous gift from G. Cesarini. All antisersa were preincubated with whole cells to remove eventual traces of anti-lipopolysaccharide antibodies.

2.5. Preparation of subcellular fractions

Cells were harvested by centrifugation and converted to spheroplasts as in [14]. The spheroplasts were centrifuged; the supernatant contained the periplasmic fraction. Spheroplasts were then resuspended and lysed and cellular membranes were pelleted by centrifugation; the supernatant contained the cytoplasmic fraction. Osmotic shock fluids were prepared as in [15].

2.6. Assay of cell survival in cell protection experiments

Binding of the hybrid protein to sensitive cells was assayed by measuring the degree of inhibition of bacteriocidal activity of native colicin A. A fixed amount of cells (5×10^8/ml) was incubated with various amounts of the bacterial extract obtained after centrifugation of lyzed spheroplasts. The incubation was carried out for 5 min at 37°C and 1 ng/ml colicin A was then added. After a further incubation of 10 min at 37°C, SDS (0.5 mg/ml) was added, and the incubation was continued for 10 min. The absorbance of the cell suspension at 600 nm was then measured and the percentage of survival was calculated as in [16].

3. RESULTS AND DISCUSSION

3.1. Construction of hybrid gene (bla-caa) *encoding the central region of colicin A polypeptide chain*

The complete nucleotide sequence of the structural gene for colicin A has been determined [1]. Comparison of the deduced amino acid sequence with that of colicin E1 indicated that a homologous region extending over 10 amino acid residues existed in the central part of the two colicins [1]. Since both proteins are known to interact with the Btu B protein and since the receptor binding domain has been located in this polypeptide region [6,8] for colicin E1, the prospect of finding the receptor binding domain of colicin A in the central region of the polypeptide chain was good.

A hybrid gene encoding the signal sequence of β-lactamase as well as the central part of colicin A polypeptide was constructed. A 0.9 kb *Pst*I restriction fragment from ColA plasmid, containing half of the colicin A gene (*caa*) [1,17], was cloned into the unique *Pst*I site of pBR322, in the β-lactamase gene (fig.1A). This cloning does not alter the reading frame of the *Pst*I restriction fragment of *caa* (fig.1B). It resulted in the formation of a hybrid *bla-caa* gene. The nucleotide sequence of the recombinant plasmid, named pVC 33, was checked at the junction region and found to correspond to that expected (fig.1B,2).

The hybrid protein produced has therefore the signal sequence and the N-terminal 158 amino acid residues of β-lactamase [18], 266 amino acid residues from colicin A [1] and 9 additional amino acid residues from the construction (before the first stop codon) (fig.1B). This protein should have an M_r of 50791. It is under the control of the *bla* promoter and is thus rather weakly expressed. It could not be detected upon SDS–polyacrylamide gel electrophoresis of total cell proteins. Therefore, proteins encoded by pVC 33 were analyzed in the maxicell system [12]. A protein of about 50 kDa was found to be synthesized (fig.3A, lane 3). This protein was immunoprecipitated by antisera directed against either colicin A or β-lactamase which evidenced that the correct hybrid protein was produced (fig.3B, lanes 5 and 7).

3.2. Evidence for partial export of the hybrid protein

Since the hybrid protein comprised the signal sequence of β-lactamase, its compartmentalization was checked. Cell fractionation showed that about 50% of the hybrid protein was exported to the periplasmic space. Partial export was previously reported for ovalbumin [19] and insulin [20] produced in *E. coli*. The hybrid protein that remained in the cytoplasm was stable (fig.3B, lanes 5 and 7) whereas that transferred to the periplasmic space was cleaved by periplasmic proteases. A 26-kDa polypeptide, immunoprecipitated by anti-colicin A serum (fig.3B, lane 4) and a 28-kDa polypeptide immunoprecipitated by the anti-β-lactamase serum (fig.3B, lane 6) were found in the periplasmic fluid. Immunoprecipitation of the fusion fragments from the periplasmic space indicated that

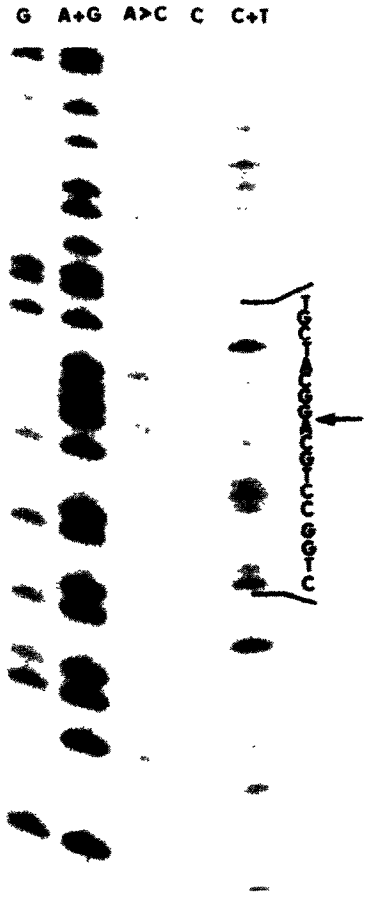

Fig.2. Nucleotide sequence at the junction between *bla* and *caa* DNA fragments. The plasmid DNA was digested with *Cla*I (there are 2 Cla I sites, one in pBR322 and one in *caa*) and labeled with [^{32}P]dATP as in [1]. The labeled DNA was cleaved with *Hinc*II and the 343 bp *Cla*I/*Hinc*II fragment was purified and used for sequencing. Only the region around the *Pst*I site (indicated by an arrow) is shown.

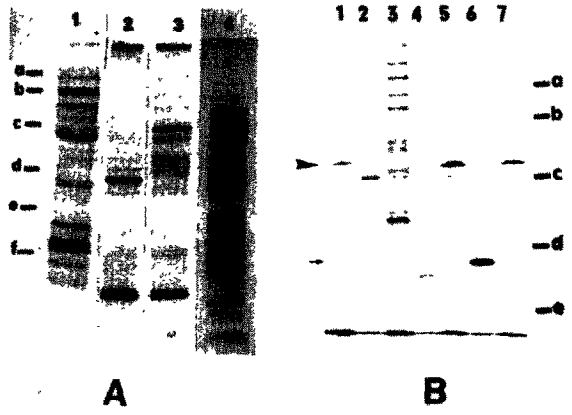

Fig.3. Expression and localization of the hybrid protein encoded by pVC33. (A) Expression of pVC33 in the maxicell system. Cells treated as in [12] were labeled for 2 h in the presence of [^{35}S]methionine and proteins were analyzed by SDS–polyacrylamide gel electrophoresis and fluorography. Lanes: (1) background control with RB113 cells, (2) RB113 (pBR322), (3) RB113 (pVC33), (4) RB113 (pColA). (B) Cell fractionation on W3110 (pVC33) (lanes 2–7). Lanes: (1) the position of the hybrid protein RB113 (pVC33), (2) total proteins from osmotic shock fluid; (3) total proteins from cytoplasmic compartment, immunoprecipitated proteins by the anti-colicin A serum, (4) from the periplasmic fluid and (5) from the cytoplasm, immunoprecipitated proteins by the anti-β-lactamase serum, (6) from the periplasmic fluid and (7) from the cytoplasm. Arrows indicate the migrations of the native hybrid protein (►) and of the periplasmic degradation product immunoprecipitated by the anti-β-lactamase serum (➞). Calibrated proteins (a–f) have been described [1].

export had occurred. Cleavage of the Bla-Caa protein occurred near the fusion joint. Indeed, a 17.4-kDa fragment of β-lactamase was contained in the hybrid protein and a 28-kDa fragment was immunoprecipitated by the anti-β-lactamase serum from the periplasmic fluid (fig.3B, lane 6). However, part of the chimeric polypeptide in its colicin A region appeared to be digested since a 26-kDa fragment instead of an expected 30-kDa one was immunoprecipitated by anti-colicin A serum (fig.3B, lane 4).

Colicin A is normally produced on free polysomes [21] and exclusively detected in the cytoplasm of producing cells in electronmicroscopy [22]. A conclusion of this study is that part of the colicin A polypeptide can be directed to the periplasm by the signal sequence and the N-terminal region of β-lactamase. To our knowledge, a cytoplasmic protein has never been directed to the periplasmic space in *E. coli* by genetic recombination. In particular, none of the protein fusions between alkaline phosphatase or the maltose-binding protein and β-galactosidase has succeeded in transferring this protein into this cell compartment [23].

The second conclusion of this work is that the β-lactamase-colicin A hybrid protein is unstable in the periplasmic space. The hybrid protein was probably cleaved around the fusion region but part of the colicin A polypeptide was also digested.

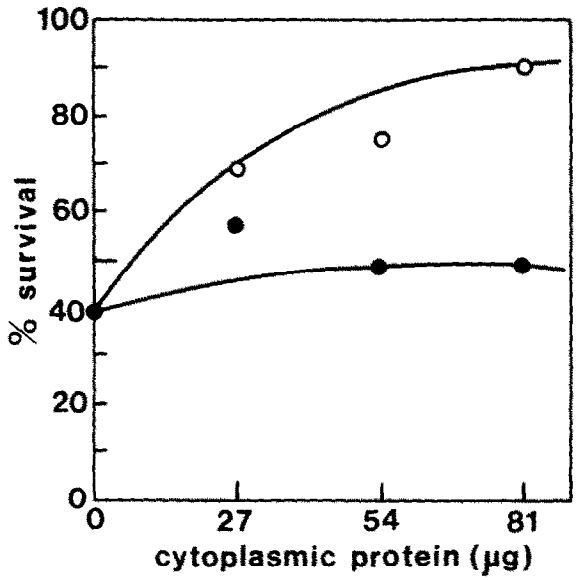

Fig.4. The hybrid protein contains the receptor-binding domain of colicin A. Cells sensitive to colicin A were pre-incubated with various amounts of cytoplasmic proteins from W3110 (pVC33) (○---○) and W3110 (pBR322) (●---●) and then with colicin A as indicated in section 2. The percentage of survival was plotted as a function of the amount of cytoplasmic protein added. The concentration of colicin A was chosen such that about half of the cells was killed in the absence of protection.

3.3. The receptor-binding domain is located in the central region of colicin A polypeptide

The hybrid protein constructed did not exert any lethal effects on sensitive cells but could protect these cells against the lethal action of colicin A. The protection of the cell population caused by the adsorbed hybrid protein was monitored through survival (fig.4). A given amount of colicin A that killed about 60% of the cells was used. The cells were either preincubated with growth medium or with a cytoplasmic extract containing the hybrid protein. Almost full protection of sensitive cells could be obtained when sufficient extract was provided.

This result demonstrated that the region from amino acid residue 71 to 335 in the colicin A polypeptide contained the receptor-binding domain. This region was correctly folded in the hybrid protein since it could still bind the receptor located in the outer membrane. As previously reported regions 234–243 of colicin E1 and 223–232 of colicin A are homologous [1]. Since both colicins are supposed to interact with the Btu B protein and since the receptor-binding peptide in colicin E1 has been located between amino acid residues 230 and 370 [8] it is tempting to speculate that the homologous peptides mentioned above are involved in Btu B binding. Site-directed mutagenesis experiments are planned to test this hypothesis.

ACKNOWLEDGEMENTS

This research has been supported by the CNRS (ATP microbiologie), INSERM (CRL 821022) and the Fondation pour la Recherche Médicale. We would like to thank Martine Chartier for skilled technical assistance and Monique Payan for help in the preparation of this manuscript.

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