An Unmodified Form of the ColE2 Lysis Protein, an Envelope Lipoprotein, Retains Reduced Ability to Promote Colicin E2 Release and Lysis of Producing Cells

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Site-directed mutagenesis was used to replace the codon for the N-terminal cysteine residue of pColE2-P9-encoded mature lysis protein (CelB) by an arginine codon. In contrast to the wild-type CelB protein, the product of the mutated gene, which has an altered signal peptidase cleavage site, was neither processed nor acylated. However, the mutant protein retained sufficient residual activity to cause partial, Mg²⁺-suppressible lysis and could activate envelope phospholipase A1-A2 and promote colicin release, albeit with reduced efficiency compared to the wild-type protein. We propose that the uncleaved signal peptide of the mutant protein acts as the functional equivalent of the fatty acyl groups normally linked to the N-terminal cysteine residue of the wild-type protein, thereby anchoring the protein in the cell envelope where it exerts its various effects.

INTRODUCTION

Colicins initially accumulate in the cytoplasm of producing *Escherichia coli* cells. Their release into the growth medium is totally dependent upon the expression of the lysis or bacterial release gene located downstream from the colicin structural gene in small, high copy number plasmids such as ColA, ColE1, ColE2, ColE3, ColD, ColN and CloDF13 (Pugsley, 1984; de Graaf & Oudega, 1986). Lysis gene expression causes activation of a chromosome-encoded phospholipase, with consequent changes in envelope lipid content, permeabilization of the outer membrane, release of colicin and periplasmic proteins (but not of most cytoplasmic or membrane proteins) and Mg²⁺-suppressible pseudolysis (Pugsley & Schwartz, 1984). Our characterization of the pColE2-P9 lysis gene (*celB*) indicated that it codes for a 27 amino acid membrane protein which is synthesized as a precursor with a 19 residue, N-terminal signal peptide (Cole *et al.*, 1985). The sequence of amino acids around the cleavage site in the precursor polypeptide (Ile-Leu-Ser-Ala↔Cys, where ↔ indicates the cleavage site) is similar to that found in precursors of bacterial lipoproteins in which the N-terminal cysteine is modified by ester- and amide-linked fatty acids (Wu, 1985). The sequence of mature CelB protein does not include a hydrophobic transmembrane domain which might anchor it in the cell envelope, although the fatty acyl groups attached to the N-terminal cysteine residue could perform this role, as they do in other lipoproteins.

In the present study, site-directed mutagenesis was used to convert the single cysteine codon, TGT, to CGT coding for arginine, and the effects of this change on CelB protein function were determined.

METHODS

Bacterial strains, plasmids and growth media. The strain used for most experiments was PAP105 [$\Delta(lac-pro)$ F' pAPIP502 (*lacI^{q1} ΔlacZM15 pro⁺ Tn10*); Pugsley & Schwartz, 1983b]. Strain BMH71-18 *mutL* (Kramer *et al.*, 1984) was the recipient strain for transfection with the products of site-directed mutagenesis

Abbreviation: ITPG, isopropyl β -D-thiogalactoside.

experiments. pAPIP226 is described in Pugsley & Schwartz (1983a). The media used were L broth and M63 minimal medium containing 0.8% (w/v) glucose (Miller, 1972), solidified where appropriate with 1.6% (w/v) Difco agar. Ampicillin (200 µg ml⁻¹), kanamycin (50 µg ml⁻¹) and tetracycline (15 µg ml⁻¹) were added as required. Colicin production was induced with 0.5 µg mitomycin C ml⁻¹; isopropyl β-D-thiogalactoside (IPTG; 1 mM) and cyclic adenosine monophosphate (1 mM) were used to induce *celB* or *celB1* expression in PAP105 derivatives carrying these genes under *lacZp* control in the pUC8 derivatives pCol25 and pCol26 respectively (see below).

Oligonucleotide-directed mutagenesis of *celB*. Site-directed mutagenesis was done on an M13mp8 derivative (Messing & Vieira, 1982) carrying a 0.38 kb *Bgl*II–*Sau*3A1 restriction fragment including the *celB* gene and the transcription terminator of the colicin E2 operon (Pugsley & Schwartz, 1983b; Cole *et al.*, 1985). A 15 nucleotide mutagenic primer, GCCTGACGTGCAGAC (about 5 pmol), was annealed to the template (about 5 pmol) and extended with the Klenow fragment of DNA polymerase I (1 U) in the presence of T4 DNA ligase (80 U; New England Biolabs), deoxyribonucleoside triphosphates (0.5 mM), ATP (0.5 mM) and DTT (10 mM) at 16 °C for 16 h, essentially as described by Carter *et al.* (1985). Samples were used to transfect competent BMH71-18 *mutL* cells, and appropriate recombinants were identified by plaque hybridization (Benton & Davis, 1977). Hybridization was done for 1 h at 20 °C in a solution containing 6 × SSC, 10 × Denhardt's solution (Maniatis *et al.*, 1982), 0.2% (w/v) SDS and ³²P-labelled primer (2 × 10⁷ c.p.m.). Filters were rinsed with 6 × SSC (1 × SSC is 0.15 M-NaCl, 0.015 M-sodium citrate, pH 7.0) at 20 °C and exposed to X-ray film for 1 h to reveal the background pattern. Stringent washes were then done in 6 × SSC at 48 °C (i.e. 2 °C below the *T_d*) and filters reexposed. Plaques still exhibiting positive hybridization signals were purified and used for template preparation. The sequences of all putative mutant *celB* genes were confirmed by the modified dideoxy method (Biggin *et al.*, 1983) and in all cases the desired transition was found.

Other procedures. General molecular genetic techniques were as described by Maniatis *et al.* (1982). Basic procedures for labelling proteins with radioactive amino acids, for separating CelB protein by SDS-PAGE in highly cross-linked phosphate-buffered gels, and for lipid analysis were as described previously (Pugsley & Schwartz, 1983a, 1984; Cole *et al.*, 1985; Pugsley & Oudega, 1987). [³⁵S]Cysteine was used at 20 µCi ml⁻¹ (740 kBq ml⁻¹) in medium containing 0.05% methionine to reduce its metabolism into methionine. Lipoproteins were labelled with [³H]palmitate essentially as described by Pugsley *et al.* (1986). The label was added to cultures in M63 minimal medium containing 0.8% Casamino acids 10 min before the IPTG, and labelling was continued for a further 20 min. Total cell proteins were then precipitated with 10% (w/v) trichloroacetic acid, and the pellets were resuspended in methanol to remove lipids, then centrifuged, dried and resuspended in sample buffer for SDS-PAGE. For extraction with NaOH (saponification), the samples were resuspended in 1 M-NaOH in 90% (v/v) methanol and incubated for 1 h at 37 °C. The suspension was then pelleted by centrifugation and resuspended in sample buffer for electrophoresis. Control samples did not receive NaOH. For the fractionation of membranes, cells carrying pCol25 or pCol26 were labelled with [³H]palmitate or [³⁵S]methionine after IPTG induction and then lysed by sonication or repeated freeze-thawing in 50 mM-Tris/HCl (pH 7.4) containing 1 mM-MgSO₄ and 20 µg lysozyme ml⁻¹. Cell debris was then removed by centrifugation at 5000 *g* for 10 min, and membranes were pelleted at 130000 *g* for 4 h. The membranes were resuspended in 50 mM-Tris/HCl containing 1 mM-MgSO₄ and extracted with 1% (w/v) Triton X-100 for 20 min at 4 °C. Insoluble proteins were pelleted by high speed centrifugation as above. Triton-soluble proteins were precipitated with 10% (w/v) trichloroacetic acid. Duplicate membrane samples were layered onto sucrose gradients (35–55%, w/v) and centrifuged for 66 h at 130000 *g*. Fractions from the gradients were supplemented with 10 µg bovine serum albumin ml⁻¹ plus 0.1% (w/v) Triton X-100 and then precipitated with 10% (w/v) trichloroacetic acid. Precipitates were dissolved in sample buffer and examined by SDS-PAGE.

RESULTS

*Site-directed mutagenesis of *celB**

To examine the role of acylation of the CelB protein in membrane perturbation, we decided to mutate the *celB* gene to remove the cysteine residue, the putative acylation site. Oligonucleotide-directed mutagenesis was used to convert the cysteine codon into an arginine codon. The mutant allele, *celB1*, was cloned under the control of the *lac* promoter in pUC8 (Vieira & Messing, 1982), and the resulting plasmid named pCol26. A control plasmid, pCol25, carrying the wild-type allele, was constructed in the same way.

Characterization of mutant lysis protein

Globomycin, a specific inhibitor of lipoprotein signal peptidase (Wu, 1985), was shown to prevent processing of pre-CelB protein (Cole *et al.*, 1985). This was considered as tentative

Fig. 1. Identification of wild-type CelB lysis protein encoded by pCol25 as a lipoprotein by the specific metabolic incorporation of [^3H]palmitate, and the absence of palmitate incorporation into a protein of the same size in cells carrying pCol26. *E. coli* cells were induced with IPTG to produce lysis protein which was labelled with [^3H]palmitate as described in Methods. Proteins were separated in highly cross-linked, phosphate-buffered polyacrylamide gels in the presence of SDS and detected by autoradiography.

evidence for CelB protein being a lipoprotein. Subsequently, a low molecular mass protein which could be labelled with [^3H]palmitate was specifically detected after expression of the *celB* gene carried by pCol25 (Pugsley *et al.*, 1987). Using cells carrying pCol25 grown in the presence of IPTG, we have shown that the band corresponding to CelB lysis protein in SDS-polyacrylamide gels is not detected after treatment of the samples with $0.1 \mu\text{g}$ proteinase K ml^{-1} , that about 60% of the [^3H]palmitate label is released by treatment with 1 M-NaOH in 95% methanol, and that the product co-migrates with authentic CelB protein labelled with ^{14}C -labelled amino acids (data not shown). These results provide strong evidence that the [^3H]palmitate-labelled product is acylated, mature CelB protein, and that about 40% of the palmitate is attached through an alkali-resistant amide bond. In other lipoproteins, palmitate is the only fatty acid linked via the amide bond to the N-terminal cysteine, while the two fatty acids attached through ester linkages via glycerol are of various types derived from the phospholipid pool (Jackowski & Rock, 1986). The fact that NaOH-resistant palmitate was found in CelB protein indicates, therefore, that it is processed in the same way as other bacterial lipoproteins.

The IPTG-inducible, low molecular mass protein was not detected in PAP105 (pCol26) cells (Fig. 1). To confirm that *celB1* was actually expressed, proteins produced by IPTG-induced cells

Fig. 2. Lysis proteins encoded by pCol25 (W) and pCol26 (M). Proteins produced by cells growing in the absence (-) or presence (+) of 1 mM-IPTG were labelled with ^{14}C -labelled amino acids (A), ^{35}S -methionine (B), ^{35}S -cysteine (C) or ^{35}S -palmitate (D). Labelling was continued for 10 min except for samples 3 and 6 (in the left-hand panel) which were pulse-labelled with ^{14}C -labelled amino acids for 2 min only. Proteins were separated in highly cross-linked polyacrylamide gels and detected by autoradiography. L, wild-type CelB lysis protein; L', mutant CelB lysis protein; X, ^{35}S -methionine-labelled putative stable CelB signal peptide detected only in the over-exposed gels (right-hand panel).

Fig. 3. Effect of production of wild-type (pCol25; □, ■) and mutant (pCol26; ○, ●) lysis proteins on growth of *E. coli* K12 in L broth containing 1 mM-IPTG (to induce lysis gene expression) with (■, ●) or without (□, ○) 20 mM-Mg²⁺.

carrying pCol26 were labelled with [³⁵S]methionine, [³⁵S]cysteine or ¹⁴C-labelled amino acids, and these were compared with proteins produced by cells carrying pCol25. Wild-type CelB protein was clearly identified in [³⁵S]cysteine labelled cells and less clearly identified when ¹⁴C-labelled amino acids were used as the protein label. CelB protein was not detected in [³⁵S]methionine-labelled cells (Fig. 2). This last result was expected because the only methionine present in the *celB* gene product is the initiator methionine of the signal peptide, and this is proteolytically removed during processing and maturation of the CelB protein. Wild-type CelB protein was not detected in cells pulse-labelled for 2 min with [³⁵S]methionine or ¹⁴C-labelled amino acids (lanes 3 and 6 in Fig. 2A), confirming our previous failure to detect unprocessed, unmodified CelB protein (Cole *et al.*, 1985). The product of the *celB1* allele could not be detected in [³⁵S]cysteine-labelled cells (as expected from the predicted absence of cysteine from this protein), but was found in extracts from cells labelled with [³⁵S]methionine or ¹⁴C-labelled amino acids (Fig. 2). The presence of methionine in the *celB1* gene product, which was apparently larger than wild-type CelB (Fig. 2) protein suggests that the *celB1* gene product was not processed, and therefore retained its signal peptide.

[³⁵S]Methionine-labelled CelB1 protein was stable for at least 60 min, indicating that signal peptide cleavage occurred very slowly, if at all. When membranes were separated by isopycnic sucrose gradient centrifugation, [³⁵S]methionine-labelled CelB1 protein was found in both the inner membrane protein- and the outer membrane protein-containing fractions. It was, however, totally insoluble in Triton X-100, which normally solubilizes *E. coli* inner membrane proteins (data not shown). [³H]Palmitate-labelled wild-type CelB protein was also found in sucrose gradient fractions corresponding to both inner and outer membranes, and was only partially solubilized by Triton X-100 (data not shown), as observed previously with ¹⁴C-labelled CelB protein (Cole *et al.*, 1985).

Activity of the *celB* gene product

Cells carrying pCol25 (*celB*⁺) underwent partial lysis about 35 min after the addition of IPTG to exponentially growing cultures (Fig. 3), as reported with cells carrying similar plasmids (Pugsley & Schwartz, 1983b). Cells carrying pCol26 (*celB1*) also consistently 'lysed' when

Fig. 4. Complementation of the *celB*::Tn5 mutation in plasmid pAPIP226 by mutant or wild-type *celB* genes present on plasmid pCol26 and pCol25 respectively. *E. coli* strain PAP105 carrying the appropriate plasmids was grown to early exponential phase in L broth. Mitomycin C ($0.5 \mu\text{g ml}^{-1}$) was added to induce colicin production, and then 1 h later, IPTG (1 mM) was added to induce lysis gene expression. Samples, removed from the cultures after a further 60 or 150 min incubation, were centrifuged to pellet the cells and proteins in the cell-free medium were precipitated with 10% (w/v) TCA. The cell pellets and TCA-precipitated proteins were dissolved in Tris/HCl buffer (50 mM, pH 7.2) containing 2% (w/v) SDS and 12% (v/v) glycerol, heated to 100°C for 5 min and examined by PAGE in Tris glycine-buffered 9% (w/v) acrylamide gels containing SDS. Lanes 1 and 2, cells carrying pAPIP226 and pCol26 after 60 and 150 min of induction with IPTG respectively; lanes 3 and 4, cells carrying pAPIP226 and pCol25 after 60 and 150 min of induction respectively; lanes 5 and 6, medium from cells carrying pAPIP226 and pCol25 after 60 and 150 min induction respectively; lanes 7 and 8, medium from cells carrying pAPIP226 and pCol25 after 60 and 150 min respectively; lanes 9 and 10, medium from cells carrying pCol25 (9) or pCol26 (10) only after 150 min of induction with IPTG. The samples loaded on the gels were equivalent to 200 μl of culture. Note that the presence of a doublet colicin E2 band is due to proteolysis (Pugsley, 1983) and does not indicate a precursor-mature protein transition.

treated with IPTG, but only after about 100 min (Fig. 3). In both cases, lysis was almost completely abolished when the medium was supplemented with 20 mM- MgSO_4 (Fig. 3). The same effect of MgSO_4 was reported for cells expressing the wild-type *celB* allele under either colicin operon or *lac* promoter control (Pugsley & Schwartz, 1984; Pugsley *et al.*, 1987). Since MgSO_4 does not prevent lysis caused by other agents (lytic bacteriophage infection, penicillin treatment), this result implies that mutant CelB1 protein induces the same type of partial lysis as is induced by wild-type CelB protein.

In order to study the effects of the mutated *celB* gene on colicin release, plasmids pCol25 or pCol26 were introduced into strain PAP105 carrying pAPIP226 (pColE2-P9 *celB*::Tn5). This strain produces colicin E2 but does not release it into the medium due to the absence of CelB protein (Pugsley & Schwartz, 1983a). Plasmids carrying *celB*⁺ under *lacZp* control can complement the *celB*::Tn5 mutation and promote colicin release (Pugsley & Schwartz, 1983b;

Fig. 5. Changes in lipid composition as a consequence of the induction of mutant or wild-type *celB* lysis genes in *E. coli* strain PAP105 carrying pCol26 or pCol25 respectively. Cells were grown to early exponential phase in L broth containing [14 C]acetate to label lipids, centrifuged, washed and resuspended in fresh medium. IPTG (1 mM) was then added to induce lysis gene expression. Samples were removed from the culture at various times and lipids were extracted from the cells and analysed by thin layer chromatography. FFA, free fatty acids; CL, cardiolipin; PE, phosphatidyl ethanolamine; PG, phosphatidyl glycerol; LPE, lysophosphatidyl ethanolamine. The uneven migration of samples on the left of the plate is an artefact, and does not indicate changes in the structure of the lipids concerned.

this paper Fig. 4). Similarly, the *celB1* allele in pCol26 also complemented the *celB*::Tn5 mutation in pAPIP226, although colicin release, like lysis, occurred more slowly than with the wild-type *celB* allele and substantial amounts of colicin E2 remained cell-associated (Fig. 4). No colicin release occurred until after 4 h of induction when pUC8 was used in place of pCol25 or pCol26. We conclude that the *celB1* gene product is active in promoting colicin release, albeit less-so than the product of the wild-type gene.

Wild-type CelB protein activates envelope phospholipase A1–A2, an essential step in outer membrane permeabilization leading to colicin release (Pugsley & Schwartz, 1984). CelB1 protein was also observed to activate phospholipase, although changes in envelope phospholipid composition occurred more slowly and were less extensive than with the wild-type CelB protein (Fig. 5). No changes in phospholipid composition were induced by expression of either mutant or wild-type CelB protein in a strain carrying a mutation in *pldA*, the structural gene for detergent-resistant phospholipase A1–A2 (data not shown; see Pugsley and Schwartz, 1984).

DISCUSSION

Several features of the wild-type CelB lysis protein could contribute to the activation of phospholipase and to the specific export of colicin across the inner membrane. These include the

removal of the signal peptide, the apparent high degree of stability of the signal peptide after its removal from wild-type pre-CelB (Pugsley & Schwartz, 1983*a, b*; this paper Fig. 2), the conversion of the N-terminal cysteine to glyceryl-cysteine, the addition of fatty acids by ester linkages to the glycerol moiety and by amide-linkages to the free amino group of the cysteine, and the membrane location of the mature protein. Most of these features of the CelB protein depend on the presence of the cysteine residue at position +1; its replacement represented an obvious approach to probing structure-function relationships.

In changing the cysteine residue to arginine, we hoped to replace the cleavage site recognized by lipoprotein signal peptidase by one recognized by signal peptidase I (also called leader peptidase; see Pugsley & Schwartz, 1985). This would have allowed us to determine the biological effects of a CelB protein which would probably not have been anchored in the cell envelope by its fatty acyl groups (see Introduction). However, pre-CelB1 protein was not processed by signal peptidase I (Fig. 2). This may have been due to the fact that the sequence around the potential cleavage site was not recognized by signal peptidase. Arginine is the N-terminal residue of *E. coli* K12 alkaline phosphatase and the structurally equivalent lysine is present at position +1 in several exported proteins (Watson, 1984). A detailed survey of charge distributions in precursors of a large number of exported or secreted proteins has shown, however, that prokaryotic exported proteins tend to have acidic residues just after the cleavage site. von Heijne (1986) suggests that a dipolar structure with a positive net charge difference between the N terminus of the signal peptide and the region just after the cleavage site may be important for efficient export and processing. Wild-type pre-CelB protein has a net charge difference of +2 between these regions (see Cole *et al.*, 1985). The introduction of Arg in place of Cys+1 reduces this net charge difference to +1.

An alternative explanation for the failure of signal peptidase I to process pre-CelB1 protein may be that its small size (46 residues) makes it a poor substrate for this enzyme, possibly because the precursor cannot fold into the correct conformation to allow signal peptidase I access to the cleavage site.

CelB1 protein retains only two of the characteristic features of the wild-type protein, namely its membrane location and the stability of the signal peptide, which, in the mutant, remains attached to the mature part of the polypeptide. The fact that the mutant protein retains a low level of activity for all the functions known for the mature wild-type CelB protein (promotion of lysis, translocation of colicin E2 across the cytoplasmic membrane and activation of phospholipase A1-A2), indicates that the modified cysteine residue of mature wild-type CelB protein is not completely indispensable for any of these phenomena, although the wild-type, fatty acylated protein is clearly more effective than the mutant, unmodified derivative lacking this cysteine residue.

One possible explanation for at least some effects of *celB* expression is that the accumulation of the apparently stable signal peptide (Pugsley & Schwartz, 1983*a, b*) in the cell envelope perturbs membrane structure sufficiently to activate phospholipase. The signal peptide of the major 'Braun' lipoprotein is unstable, and is rapidly degraded after cleavage from pre-lipoprotein (Ichihara *et al.*, 1986; Novak *et al.*, 1986). Mutant CelB lysis protein with its unprocessed signal peptide might be expected to have the same effect as the processed but stable signal peptide of wild-type CelB protein.

According to current models for signal-peptide-dependent export of envelope proteins in *E. coli* (Pugsley & Schwartz, 1985; Wickner & Lodish, 1985), the signal peptide inserts into and remains within the cytoplasmic membrane. Mature lysis protein is found in both inner and outer membranes (Oudega *et al.*, 1984; Cole *et al.*, 1985; this paper). Therefore, if current models also apply to lysis protein export, the signal peptide would be expected to remain in the cytoplasmic membrane whatever the final location of the mature, acylated lysis protein, and mutant, unacylated lysis protein should remain anchored in the inner membrane by its unprocessed signal peptide. Our cell fractionation data indicated, however, that mutant lysis protein was also found in both inner and outer membranes. This implies that the signal peptide can reach the outer membrane, a possibility not previously considered likely even though junctions between the inner and outer membranes have often been proposed as sites of protein export in Gram-

negative bacteria (see Pugsley & Schwartz, 1985). This interpretation relies on the accurate prediction of lysis protein location by cell fractionation techniques. Tommassen (1986) has already cited several situations in which such techniques are not reliable, and we have found that lipoprotein localization cannot always be accurately determined by physical fractionation (unpublished data). Thus, we believe that other methods should be sought to determine the location of wild-type and mutant lysis proteins in the cell. On the other hand, we note that phospholipase A1-A2 is an outer membrane protein (Nishijima *et al.*, 1977), and would be expected, therefore, to be activated by agents (e.g. mutant lysis protein) affecting the outer membrane.

One other result deserves some comment; namely, the observation that wild-type CelB protein seems to be much more abundant than was previously assumed (see Pugsley & Schwartz, 1983*a, b*). Note, in particular, that CelB protein is by far the most intensely [³H]palmitate-labelled lipoprotein (Fig. 1). If this reflects the abundance of the protein, it may be possible to purify sufficient amounts to conduct *in vitro* studies on its action.

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