JOURNAL OF BACTERIOLOGY, Sept. 1988, p. 4153–4160
0021-9193/88/094153-08$02.00/0

Vol. 170, No. 9

Effect of a Mutation Preventing Lipid Modification on Localization of the pCloDF13-Encoded Bacteriocin Release Protein and on Release of Cloacin DF13

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Received 19 February 1988/Accepted 6 June 1988

The pCloDF13-encoded bacteriocin release protein (BRP; M_r 2,871) is essential for the translocation of cloacin DF13 across the cell envelope of producing *Escherichia coli* cells. Overproduction of this BRP provokes lysis (quasilysis) of cells. Construction and analysis of a hybrid BRP–β-lactamase protein (BRP-Bla) demonstrated that the BRP contains a lipid modified cysteine residue at its amino terminus and is mainly located in the outer membrane. The significance of lipid modification for the localization and functioning of the BRP was investigated. Site-directed mutagenesis was used to substitute the cysteine residue for a glycine residue in the lipobox of the BRP and the BRP-Bla protein. The mutated BRP was unable to bring about the release of cloacin DF13 and could not provide the lysis (quasilysis) of host cells. However, the mutated BRP strongly inhibited the colony-forming ability of the cells, indicating that induction of the mutated protein still affected cell viability. In contrast to the wild-type BRP-Bla protein, the mutated BRP-Bla protein was mainly located in the cytoplasmic membrane, indicating that the mutation prevented the proper localization of the protein. The results indicated that lipid modification of the BRP is required for its localization and release of cloacin DF13, but not for its lethality to host cells.

Bacteriocin release proteins (BRPs) are low-molecular-weight proteins required for the translocation for newly synthesized bacteriocin molecules across both the cytoplasmic and outer membrane of producing *Escherichia coli* cells. The genes encoding a certain bacteriocin and its corresponding BRP are organized in a single operon and transcribed from the same promoter, which can be induced by activation of the host cell SOS response. Moderate induction of a bacteriocin operon results in strongly increased synthesis and release of the bacteriocin, whereas strong induction leads to lysis (marked decline in culture turbidity, also called quasilysis) of host cells due to the elevated level of BRP gene expression (9, 32).

Studies on the molecular basis of BRP-dependent bacteriocin export and cell lysis (quasilysis) have indicated that BRPs directly or indirectly activate the detergent-resistant phospholipase A, which leads to local permeabilization zones in the outer membrane and possibly also in the cytoplasmic membrane (3, 22, 34). These zones function in the release of bacteriocins and certain other cellular proteins, but might be deleterious to the cells when present in too large amounts. The precise role of BRPs in the translocation of bacteriocins across the cytoplasmic membrane as well as their effect on the integrity of this membrane are still poorly understood.

With the exception of a pColE9-J-encoded BRP, which seems to be involved in lysis but not in colicin export (15; R. James, personal communication), all known BRPs are highly similar in primary structure (4, 6, 9, 10, 29, 40). They contain a signal peptide with a Leu-X-Y-Cys sequence around the signal peptide cleavage site. This so-called lipobox, in which X and Y represent small neutral amino acids, is the consensus modification sequence for bacterial prolipoproteins (42). In these proteins, the cysteine residue is modified with diglyceride prior to their processing by signal peptidase II (SPase II) between the Y and cysteine residue. Subsequently, a third fatty acid is covalently linked to the N-terminal cysteine.

In a previous paper (23), we described the construction of a hybrid protein consisting of the almost complete pCloDF13-encoded BRP (amino-terminal part) fused to the mature portion of β-lactamase (carboxyl-terminal part). This BRP-Bla hybrid protein enabled us to study the acylation, processing, and subcellular localization of the BRP. The hybrid protein was shown to be modified with diglyceride and fatty acids, processed by SPase II, and located primarily in the outer membrane of *E. coli* cells (about 70%), whereas the remainder was found in the cytoplasmic membrane. A similar distribution was observed in *E. coli* minicells, which allow the detection of the mature BRP (31). The BRP-Bla protein can bring about the release of cloacin DF13, and overproduction of the hybrid protein leads to quasilysis of host cells (Luirink et al., submitted for publication).

In this study, we investigated the significance of lipid modification for the localization and functioning of the BRP. Site-directed mutagenesis was used to substitute the cysteine residue for a glycine residue in the lipobox of the BRP and BRP-Bla protein. The mutated BRP was unable to provoke the release of cloacin DF13 and lysis of host cells. However, strong induction of mutant BRP expression was still lethal for cells. The mutation prevented processing of the BRP-Bla hybrid protein. Unmodified BRP-Bla protein was mainly located in the cytoplasmic membrane, suggesting that the mutation prevented the proper localization of the BRP.

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TABLE 1. *E. coli* strains used in this study

Strain	Genotype	Source or reference
N3406	*thr leu thi lacY tonA supE*	38
FTP4170	Δ(*tonB trpAE*) *argE*(Am) *glyV55*	F. T. Pagel
BMH71-18	Δ(*lac proAB*) *supE thi mutL* F' *lacI*^{q} *ZΔM15 proAB*	19
JM103	Δ*lac pro thi strA supE endA sbcB15 hsdR4* F' *traD36 proAB lacI*^{q} *ZΔM15*	26
CE1303	F$^-$ *bio endA recA56 pldA*	8

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The various *E. coli* strains used in this study are listed in Table 1.

Plasmids pACYC184 (5) and pJN67 (37) were used in the construction of pJL25 (see also Fig. 3). Plasmid pJL12, containing the BRP-Bla hybrid gene, has been described previously (23). Plasmid pINIIIA1 (25) was used for expression of the BRP and mutated BRP gene. Restriction fragments were cloned in the M13 phage vectors mp8 (26) and mp11 (27) for the construction of mutations and DNA sequencing. Plasmid pPI232, encoding detergent-resistant phospholipase A, has been described before (8).

Cells were routinely grown in Lab-Lemco broth (Oxoid Ltd., London, England) supplemented with 0.5% (wt/vol) sodium chloride and 0.5% (wt/vol) sodium lactate. When necessary, ampicillin (100 μg ml^{-1}), tetracycline (15 μg ml^{-1}), and/or chloramphenicol (30 μg ml^{-1}) was added to the culture medium to select for antibiotic resistance. When indicated, $MgCl_2$ was added to the culture medium to a final concentration of 20 mM to prevent BRP-dependent lysis of cells.

Chemicals and radiochemicals. Globomycin was a gift from M. Arai, Sankyo, Tokyo, Japan. Mitomycin C was purchased from Kyowa Co., Tokyo, Japan. Purified β-lactamase was a gift from J. Tommassen, Utrecht, The Netherlands. Fixed *Staphylococcus aureus* cells were purchased from Calbiochem-Behring, La Jolla, Calif. The radioisotopes were purchased from Dupont, NEN Research Products, Boston, Mass.

Oligonucleotide-directed in vitro mutagenesis. The mutagenic 17-mer 5' TTGCCTGACCTGCGACG 3' was used to mutate both the pCloDF13-derived BRP gene and the BRP-Bla hybrid gene at the same position. Mutagenesis was carried out according to the double primer method as described by Zoller and Smith (43), using *E. coli* BMH71-18 *mutL* as primary host. Putative mutants were identified by colony probing, using the 5'-^{32}P-labeled mutagenic primer at room temperature, followed by successive washes at increasing temperatures (43). To confirm that only the desired mutation had taken place, the DNA sequence of the mutated DNA fragment was determined by the dideoxy termination procedure of Sanger et al. (35), using *E. coli* JM103 as host for the M13 bacteriophage.

For mutagenesis of the BRP gene, a 0.4-kilobase *Bst*NI-*Cla*I fragment of pEV67, encompassing the complete BRP gene, was subcloned in M13mp8 (21). Following mutagenesis, the mutated BRP DNA fragment was isolated by digestion with *Eco*RI and *Hin*dIII; subsequently, the fragment was cloned into the polylinker of pINIIIA1.

For mutagenesis of the BRP-Bla hybrid gene, a 0.8-kilobase *Pst*I fragment of pJL12 carrying a 5'-terminal part of the BRP-Bla hybrid gene (23) was subcloned in M13mp11. Following mutagenesis, the fragment was recloned into pJL12 lacking the *Pst*I fragment.

Other recombinant DNA techniques. For isolation of plasmid DNA and M13 double-stranded DNA, the alkaline method of Birnboim and Doly (1) was used. Transformation of $CaCl_2$-treated cells was performed as described by Dagert and Ehrlich (7). All other basic DNA manipulations were carried out as described by Maniatis et al. (24). DNA-modifying enzymes were purchased from Bethesda Research Laboratories, Inc., Bethesda, Md., and Pharmacia, Uppsala, Sweden. All enzymes were used according to the instructions of the suppliers.

Immunochemical procedures. The hybrid BRP-Bla protein was detected by immunoblotting carried out essentially as described by Towbin et al. (36) and using a rabbit antiserum raised against purified β-lactamase. The amount of cloacin DF13 in culture supernatant fractions, broken cell suspensions, and subcellular fractions was determined with an enzyme-linked immunosorbent assay as described previously (38).

Labeling experiments. Labeling and pulse-chase labeling of whole cells and in vitro transcription/translation studies were carried out essentially as described previously (11, 23). Immunoprecipitation was carried out by the method of Hayashi and Wu (12), using an antiserum directed against β-lactamase. When indicated, globomycin dissolved in dimethyl sulfoxide (10 mg ml^{-1}) was added 5 min prior to labeling (final concentration, 100 μg ml^{-1}) to prevent processing by SPase II (13). Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 20) and autoradiography.

Subcellular localization. For isolation of subcellular fractions, cells were converted to spheroplasts essentially as described by Witholt et al. (41). Spheroplasts were collected by centrifugation, and the supernatant was taken as the periplasmic fraction. Subsequently, the spheroplasts were broken by short periods of sonication, and the membranes (cytoplasmic and outer membranes) were collected by centrifugation. This fraction was taken as the total membrane fraction. The supernatant fraction contained the cytoplasmic fraction. Cytoplasmic and outer membranes were separated and isolated by isopycnic sucrose density gradient essentially as described by Osborn et al. (30). After centrifugation, the sucrose density of each fraction was determined and samples of the various sucrose gradient fractions were analyzed by SDS-PAGE to examine the distribution of cytoplasmic and outer membrane proteins.

The presence of mutant and wild-type BRP-Bla protein in each fraction was determined by measuring the β-lactamase activity and by immunoblotting; β-lactamase activity was assayed spectrophotometrically as described by O'Callaghan et al. (28). Proteins present in periplasmic, cytoplasmic, and medium fractions were concentrated by trichloroacetic acid precipitation and washed with 90% ethanol prior to analysis by SDS-PAGE and immunoblotting.

The level of ampicillin resistance of cells was measured by a plate assay as described by Broome-Smith and Spratt (2) or a tube assay as described by Kadonaga et al. (16).

RESULTS

Construction of mutant BRP and mutant BRP-Bla. The amino-terminal cysteine residue of the mature portion of the pCloDF13-encoded BRP is modified with diglyceride and fatty acids. To investigate whether this acylation of the pCloDF13-encoded BRP is essential for its functioning, a

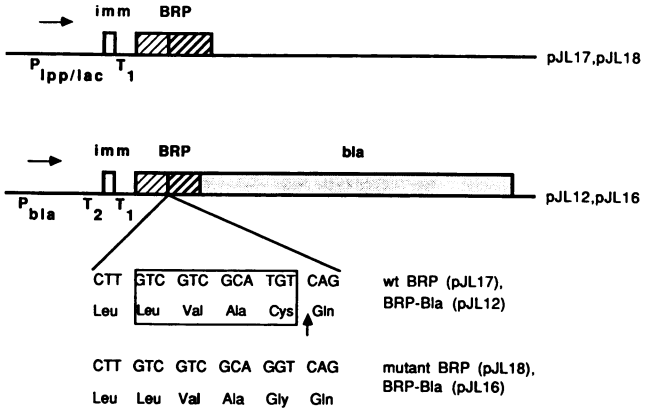

FIG. 1. Schematic presentation of pJL17, pJL18, pJL12, and pJL16. The mutation site is shown in detail. imm, 3' End of the gene encoding immunity protein; BRP, BRP gene; bla, part of the gene encoding the mature portion of β-lactamase; P_{bla}, β-lactamase promoter; $P_{lpp/lac}$, tandem lipoprotein/*lacZ* promoter-operator complex; T_1 and T_2, terminators of transcription derived from the cloacin operon. The horizontal arrows indicate the direction of transcription. The vertical arrow indicates the signal peptide cleavage site. The mutated nucleotide is underlined. The lightly hatched boxes represent the BRP signal sequence, whereas the heavily hatched boxes represent the mature part of the BRP.

mutation was introduced in the BRP gene by site-directed mutagenesis. The cysteine residue in the consensus sequence for prolipoprotein modification and processing was converted into a glycine residue (Fig. 1). Subsequently, the mutant BRP gene was subcloned under control of the *lpp-lac* promoter-operator system in the polylinker of the expression vector pINIIIA1. The resulting plasmid was designated pJL18. The wild-type BRP gene was cloned in an identical manner, which resulted in plasmid pJL17.

To examine the effects of the mutation of Cys^{+1} to Gly^{+1} on modification, processing, and subcellular localization, the BRP-Bla hybrid gene, contained in pJL12, was mutated in the same way as the BRP gene (Fig. 1). The resulting plasmid, containing the mutant BRP-Bla hybrid gene under control of the rather weak β-lactamase promoter, was designated pJL16.

Expression of the mutant BRP gene and lysis of host cells. Cultures of cells expressing the wild-type BRP show a marked decline in turbidity after strong induction. This decline in culture turbidity (quasilysis) is dependent on the high expression of the BRP and the presence of the so-called detergent-resistant phospholipase A and results from damaging of the cell membranes (9, 22). To investigate whether expression of the mutant BRP had an effect on the cell culture turbidity, cells of strain FTP4170 harboring pJL18 were cultured and induced with a high concentration of isopropyl-β-D-thiogalactoside (IPTG; Fig. 2A). A slight growth inhibition was apparent, but there was no marked decline in culture turbidity in the logarithmic phase of growth as observed with cells harboring pJL17, expressing the wild-type BRP. To study whether strong induction of the mutant BRP gene eventually results in cell death, cells harboring pJL18 from an induced culture were plated on solid broth lacking the inducer IPTG (Fig. 2B). Colony formation appeared to be inhibited, indicating that expression of the mutant BRP is potentially a lethal event. The wild-type BRP encoded by pJL17 showed a strong effect on colony-forming ability, probably due to lysis of induced cells (Fig. 2B). These results indicated that induction of the

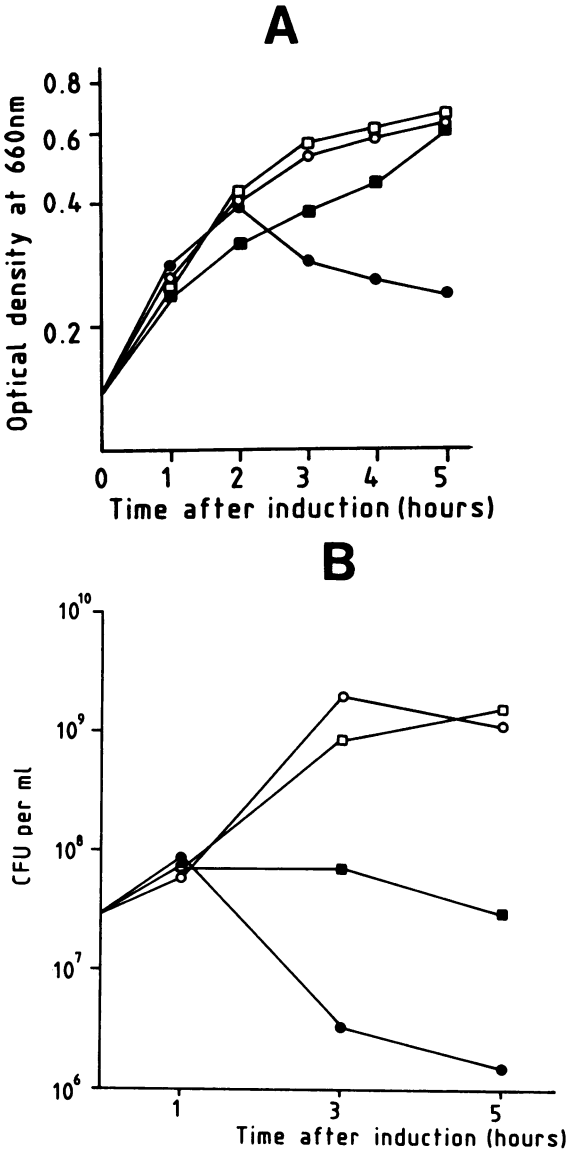

FIG. 2. Effect of induction with IPTG on growth (A) and colony-forming ability (B) of *E. coli* FTP4170 harboring pJL17 and pJL18. (A) Cells were grown in broth containing ampicillin, and IPTG was added at zero time. (B) Cells were cultured as in panel A, and at the indicated time points samples were taken, diluted, and plated on solid broth agar plates containing ampicillin but lacking IPTG. Symbols: ○, pJL17, no IPTG added; ●, pJL17, 1 mM IPTG; □, pJL18, no IPTG added; ■, pJL18, 1 mM IPTG.

mutated BRP gene still causes lethality of host cells, but without the concomitant cell lysis observed after induction of the wild-type BRP.

To investigate whether the lethality caused by the mutated BRP involves activation of the outer membrane detergent-resistant phospholipase A, plasmids pJL18 and pJL17 were transferred to strain CE1303. This strain is defective in detergent-resistant phospholipase A activity, and cultures of this strain expressing the wild-type BRP do not show the normally observed decline in culture turbidity (quasilysis; 22). Growth and induction experiments in liquid medium and on plates were carried out as described above. Strong induction of the BRP or the mutated BRP hardly affected the

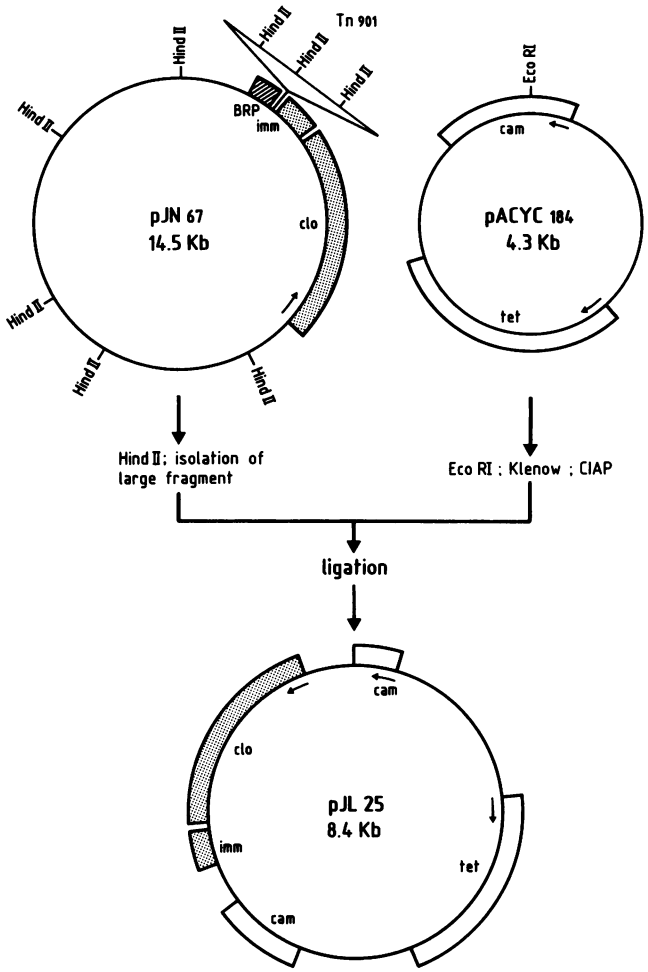

FIG. 3. Cloning strategy for construction of pJL25. Only the restriction endonuclease cleavage sites used are indicated. Arrows indicate the direction of transcription. clo, Cloacin gene; imm, immunity protein gene; BRP, BRP gene; tet, tetracycline resistance marker; cam, chloramphenicol resistance marker; CIAP, treatment with calf intestine alkaline phosphatase; Kb, kilobases.

turbidity of cultures, as expected. However, induction of both BRPs caused inhibition of colony formation on broth agar plates. A comparable effect on cell viability (colony formation) was observed when strain CE1303 harboring pJL18 was complemented with plasmid pPI232, which codes for the detergent-resistant phospholipase A (8). These results indicated that the effect on cell viability caused by the mutant BRP does not involve induction of the detergent-resistant phospholipase A.

Release of cloacin DF13. To study whether the mutant BRP was still able to bring about the release of cloacin DF13, a plasmid was constructed encoding cloacin and its immunity protein, but lacking the BRP gene (Fig. 3). A 4.1-kilobase *Hin*dIII fragment, containing the genes encoding cloacin and its immunity protein as well as the promoter of the bacteriocin operon was isolated from pJN67. This plasmid is a pCloDF13 derivative with an insertion of transposon Tn*901* between the genes encoding immunity protein and BRP. Subsequently, this 4.1-kilobase fragment was cloned in the polymerase-treated *Eco*RI site of pACYC184. The resulting plasmid, pJL25, is compatible with pJL17 and pJL18, which are derivatives of the pColE1-derived pINIIIA1 expression

TABLE 2. Effect of the BRP mutation on release of cloacin DF13

Plasmid[a]	Concn of inducer (μM IPTG)[b]	Culture turbidity after 5 h (660 nm)	Cloacin DF13 production (μg ml^{-1})[c]	Cloacin DF release (%)[d]
pJL17	0	0.57	44.1	3.4
	40	0.49	59.0	51.3
	1,000	0.36	28.3	61.5
pJL18	0	0.52	43.4	1.1
	40	0.53	50.3	1.4
	1,000	0.57	54.2	1.0

[a] *E. coli* FTP4170 (pJL25) was used as host for the indicated plasmids. Cells were cultured in broth supplemented with ampicillin, tetracycline, and 20 mM $MgCl_2$.

[b] Mitomycin C (500 ng ml^{-1}) and IPTG (indicated concentration) were added at the early exponential growth phase to induce the synthesis of cloacin DF13 and BRP, respectively.

[c] The total amount of cloacin DF13 produced was determined 5 h after induction started by measuring the amounts present in the cells and in the culture supernatant fraction. The total production of cloacin DF13 in cells harboring pJL17 after induction with 1,000 μM IPTG was lower than after moderate induction, due to the effect of BRP expression on cell viability.

[d] Cloacin DF13 released into the medium is given as a percentage of the total amount (amounts in cells and in medium) of cloacin DF13 produced in the culture.

vector. Plasmid pJL25 was transferred to *E. coli* FTP4170. With this recombinant strain, a high yield of cloacin DF13 production was obtained upon induction of the bacteriocin promoter with mitomycin C without concomitant lysis of host cells, due to the absence of the BRP gene. A double transformant of strain FTP4170, harboring both pJL18 and pJL25, was used to study the effects of simultaneous induction of BRP and cloacin DF13 synthesis on cell growth and bacteriocin release. BRP synthesis was induced with IPTG, whereas the synthesis of cloacin DF13 was strongly induced with 500 ng of mitomycin C ml^{-1} (Table 2). The results showed that high induction of the mutant BRP gene did not result in the release of cloacin DF13, nor did it affect cell growth. In contrast, moderate induction of the wild-type BRP gene was sufficient for significant release of cloacin DF13 without apparent cell lysis.

Synthesis and processing of the mutant BRP-Bla. The synthesis and processing of the pCloDF13-encoded BRP is difficult to study in whole cells of *E. coli* due to its low molecular mass, which makes it almost impossible to visualize the polypeptide. Therefore, the effect of the $Cys^{+1} \rightarrow Gly^{+1}$ mutation on synthesis and processing was studied with the mutant BRP-Bla hybrid protein (see also reference 23). Cells of *E. coli* N3406 harboring pJL12 or pJL16 were labeled in the presence and absence of globomycin, and the immunoprecipitated hybrid proteins were analyzed by SDS-PAGE (Fig. 4A). When cells with pJL12 were labeled with [^{35}S]methionine, the mature form of the hybrid protein was easily identified (lane 1). After labeling in the presence of globomycin, the precursor of the hybrid protein was detected, as expected (lane 2; see also reference 23). This precursor migrated as a double band (23; see below). In contrast, following labeling in the presence and absence of globomycin, the mutant hybrid protein was detected as a double band (lanes 3 and 4) which migrated slightly faster than the precursor of the wild-type BRP-Bla protein. This suggested that the mutation prevented processing of the hybrid protein. The difference in mobility of the precursors of the wild-type BRP-Bla and mutated BRP-Bla protein is probably caused by lack of lipid modification of the

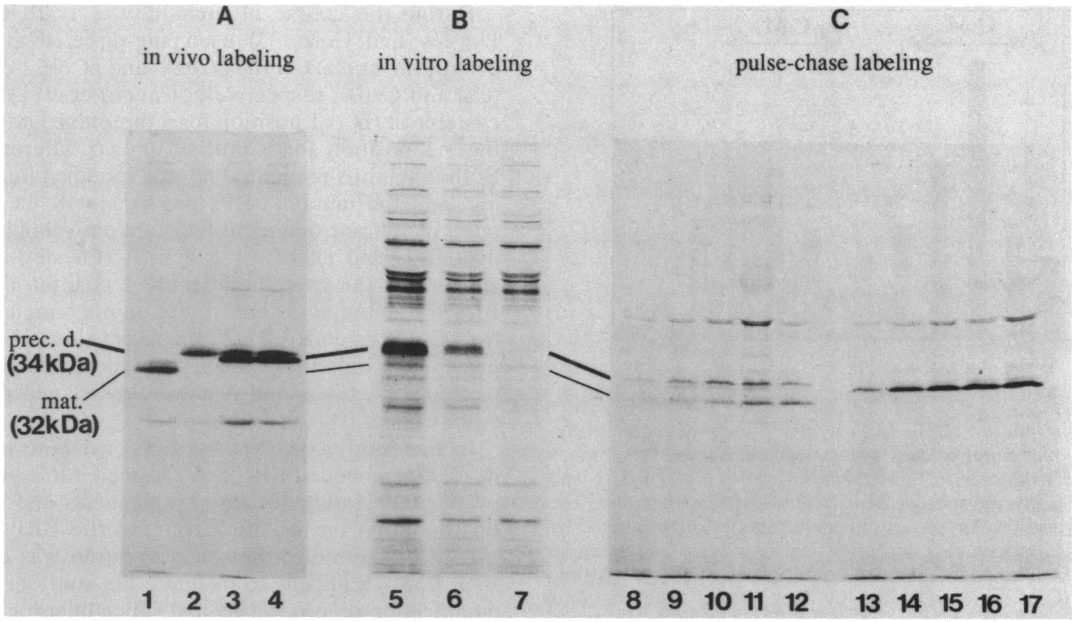

FIG. 4. Autoradiogram of [^{35}S]methionine-labeled and immunoprecipitated BRP-Bla hybrid protein and mutant hybrid protein. (A) Cells of N3406 harboring pJL12 or PJL16 were labeled for 5 min in the presence or absence of globomycin. Lane 1, pJL12, no globomycin; lane 2, pJL12 plus globomycin; lane 3, pJL16, no globomycin; lane 4, pJL16 plus globomycin. (B) In vitro transcription/translation products directed by pJL12 (lane 5) and pJL16 (lane 6). Lane 7 shows the result of a control experiment without DNA added. The in vitro system was tested for the absence of processing of the murein lipoprotein. (C) Pulse-chase labeling of cells of N3406 harboring pJL12 (lanes 8 to 12) or pJL16 (lanes 13 to 17). Cells were pulse-labeled for 15 s (lanes 8 and 13) and chased for 15 s (lanes 9 and 14), 30 s (lanes 10 and 15), 45 s (lanes 11 and 16), and 60 s (lanes 12 and 17). The precursor and mature species of the (mutant) BRP-Bla protein are connected with bars to indicate their positions on the various gel panels. prec. d., Precursor doublet of the BRP-Bla protein and mutant hybrid protein; mat., mature form of the BRP-Bla protein; kDa, kilodaltons.

latter protein. The intensity of the upper and lower bands of the precursor doublets varied in different experiments (see discussion).

To further study whether or not the mutated BRP-Bla hybrid protein is processed and to investigate the relationship between the upper and lower bands of the mutant BRP-Bla precursor doublet, two experiments were carried out.

First, in vitro transcription/translation studies were carried out with pJL12 and pJL16 (Fig. 4B). Both the BRP-Bla and the mutant hybrid proteins migrated at the position of the precursor of the wild-type hybrid protein. Both proteins still migrated as a double band. Since processing of precursors does not take place in this type of in vitro experiment, this indicated that there is no precursor-product relationship between the two counterparts of the doublet bands.

Second, a pulse-chase experiment was carried out at 30°C (Fig. 4C). The precursor of the BRP-Bla protein was detected after pulse-labeling for 15 s and was chased into the mature form (lanes 8 through 12). In contrast, the precursor of the mutant hybrid protein could not be chased into a mature form (lanes 13 through 17). Also, a comparable pulse-chase experiment was carried out at 37°C, in which the chase period was extended up to 10 min. Virtually the same results were obtained (not shown).

Taken together, the results indicated that the mutation prevented processing of the precursor of the hybrid protein. Apparently, SPase II does not recognize the mutated signal peptide cleavage site anymore, and alternative processing by, for instance, SPase I does not occur.

Subcellular localization. In a previous paper the subcellular localization of the BRP-Bla hybrid protein was studied (23). About 70% of the protein was detected in the outer membranes and the remainder was mainly located in the cytoplasmic membrane, whereas minor amounts were found in the periplasm and cytoplasm. This localization was in agreement with the localization of the BRP studied in *E. coli* minicells (31). Cells expressing the hybrid protein were resistant to ampicillin, indicating that the β-lactamase portion of the fusion protein protruded into the periplasm (23).

The localization of the mutant BRP-Bla protein was studied by determining the β-lactamase activity of subcellular fractions of strain N3406(pJL16) and by immunoblotting. About 70% of the total β-lactamase activity was located in the total membrane fraction, whereas, in contrast to the wild-type BRP-Bla protein, a significant β-lactamase activity (about 30% of the total activity) was detected in the periplasmic fraction. When the total membrane fraction was separated into cytoplasmic and outer membrane fractions, most of the activity (about 90%) was found to be associated with the cytoplasmic membranes, whereas the wild-type BRP-Bla protein was mostly found in the outer membranes (about 75%; Fig. 5). This distribution was confirmed by immunoblotting of pooled membrane fractions (Fig. 6). These experiments showed that the mutation prevented the proper localization of the hybrid protein.

The mutant hybrid protein could not be detected in the periplasm by immunoblotting (not shown). Possibly, the β-lactamase activity found in the periplasmic fraction is derived from unstable β-lactamase fragments generated by proteolytic degradation of mutant hybrid proteins.

The 50% lethal dose of ampicillin for cells harboring pJL16 did not differ from that for cells harboring pJL12, expressing the wild-type BRP-Bla protein. Apparently, as described for the wild-type BRP-Bla protein, the β-lactamase portion of

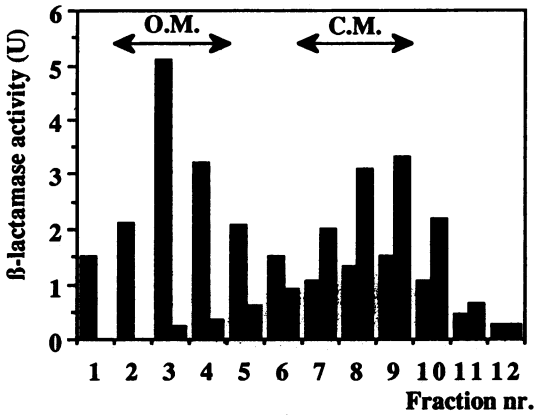

FIG. 5. Distribution of BRP-Bla hybrid protein and mutant hybrid protein over outer and cytoplasmic membranes. Membranes were isolated from cells of N3406 harboring pJL12 or pJL16 and separated by isopycnic sucrose density gradient centrifugation. The amount of hybrid protein present in each fraction was estimated by measuring the β-lactamase activity. The activity is expressed in arbitrary units. The positions of the outer (O.M.) and cytoplasmic membranes (C.M.) are indicated by the arrows. The fractions containing the outer membranes had a sucrose density of 1.23 to 1.25 g ml^{-1} and contained >90% of the major outer membrane protein markers. Fractions containing cytoplasmic membranes had a density of 1.15 to 1.18 g ml^{-1}. Symbols: Black bars, BRP-Bla protein; hatched bars, mutant hybrid protein.

the mutant hybrid protein is fully active and thus protrudes into the periplasm.

DISCUSSION

NH_2-terminal cysteine has a central role in the modification and processing of (pro)lipoproteins. During biosynthesis of lipoproteins, a glyceryl moiety is attached to the sulfhydryl group in the cysteine residue, followed by O-acylation of glyceryl cysteine. Subsequently, the modified prolipoprotein is processed by SPase II and the free NH_2 group of cysteine is N-acylated by a fatty acid, which results in a fully modified and processed lipoprotein (42).

In a previous paper, we presented evidence that the pCloDF13-encoded BRP is a lipoprotein which is processed by SPase II. A BRP-Bla hybrid protein was used to facilitate the study of BRP modification and processing since we were not able to visualize the BRP due to its low molecular mass.

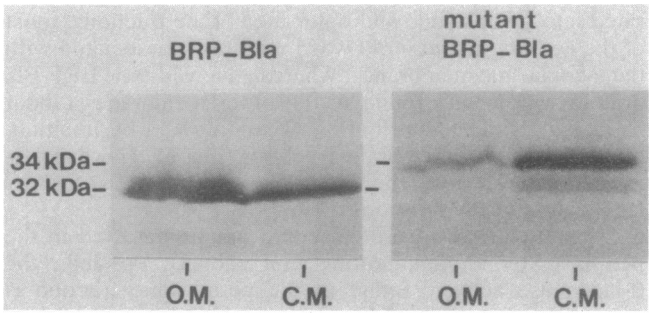

FIG. 6. Immunoblotting of BRP-Bla hybrid protein and mutant hybrid protein present in outer and cytoplasmic membranes. Membranes of cells expressing wild-type BRP-Bla (pJL12) and mutant BRP-Bla (pJL16) proteins were separated as described in the legend to Fig. 5, and outer and cytoplasmic membranes were pooled. O.M., Outer membranes; C.M., cytoplasmic membranes.

During the course of our study, Cavard et al. (3) and Pugsley and Cole (33) used site-directed mutagenesis to change the lipobox of the pColA- and pColE2-encoded BRPs (Cal and CelB), respectively. Cavard et al. (3) replaced the cysteine at the +1 position for a threonine and the alanine at the −1 position for a proline in two different constructs. Both mutations prevented normal modification and processing, and the mutant BRPs proved not to be functional in outer membrane detergent-resistant phospholipase A-dependent lysis and export of colicin A. Pugsley and Cole (33) substituted the cysteine at the +1 position of the pColE2 BRP for an arginine. This mutation prevented processing of the pColE2-encoded BRP precursor. However, this mutant protein was able to stimulate the export of colicin E2, albeit to a lesser extent, and it caused a delayed phospholipase A-dependent lysis of cells.

In this study, the NH_2-terminal cysteine residue of the pCloDF13-encoded BRP was changed into a glycine residue by oligonucleotide-directed mutagenesis and the effects of this mutation on the functioning of the BRP were investigated. Furthermore, the same mutation was created in the BRP-Bla hybrid gene to facilitate the study of the effects of modification on processing and subcellular localization. The experiments showed that the mutant BRP was not functional with respect to release of cloacin DF13 and lysis of host cells. These results agree mostly with those obtained by Cavard and co-workers. However, induction of the mutant BRP gene caused lethality of host cells as observed after growing of induced cells on broth agar plates, but this lethality was found to be independent of detergent-resistant phospholipase A.

In contrast to the wild-type BRP-Bla protein, the mutant BRP-Bla protein was not processed. We assume that the constructed mutation prevents processing of the mutant BRP by SPase II and also by SPase I, despite the creation of the putative SPase I cleavage site Ala-Gly (39). Alternative processing by SPase I has been shown to take place with one of the mutant pColA BRPs ($Cys^{+1} \rightarrow Thr^{+1}$). The mutant pColE2 BRP was not processed either, despite the suitable SPase I cleavage site created in this protein (39).

Expression of the mutant BRP-Bla protein resulted in a doublet protein band migrating slightly faster than the wild-type BRP-Bla precursor. Both bands of the doublet represented the mutant precursor as confirmed by pulse-chase experiments and in vitro transcription/translation studies. The formation of doublet bands is not an unusual feature for (mutant) pre-β-lactamase and is believed to be the result of differences in conformation (18).

Most of the mutant BRP-Bla precursor protein was found to be associated with the cytoplasmic membranes. This is in contrast to the wild-type mature BRP-Bla protein which is mainly located in the outer membranes. Unfortunately, expression of the hybrid BRP-Bla gene is too low to confirm these results by immunoelectron microscopy studies (23). However, we feel that the data obtained by "conventional" fractionation methods are reliable, taking the very low abundance of the hybrid proteins into account. The most plausible explanation for the aberrant localization of the mutant hybrid protein is that the uncleaved signal sequence anchors the protein in the cytoplasmic membrane, as has been observed earlier for uncleaved pre-β-lactamase (17, 18). Like the β-lactamase portion of the wild-type BRP-Bla protein (23), the β-lactamase portion of the mutant hybrid protein probably protrudes into the periplasm since this protein is fully active in the protection of cells against

ampicillin. Cytoplasmic (pre) β-lactamase is enzymatically inactive (16).

We assume that the results obtained with the mutant BRP-Bla hybrid protein can be extrapolated to the mutant BRP. Therefore, the lack of processing and the aberrant subcellular localization might explain why this protein is unable to bring about the release of cloacin DF13 and lysis of cells.

The pCloDF13-encoded BRP possesses three functions that can be distinguished. It can bring about the release of cloacin DF13, it can cause a marked decline in culture turbidity (lysis) in liquid medium, and it inhibits colony formation of induced host cells on solid medium. Based on our results and those of others (3, 33), we assume that the lipid moiety is essential for targeting of the BRP to the outer membrane and that the mature polypeptide structure is required for activation of the detergent-resistant phospholipase A. Furthermore, the lethality (inhibition of colony formation) caused by the BRP is a phospholipase-independent effect which might involve the BRP signal sequence. Lipid modification does not seem to be essential for proper localization and functioning of the pColE2-encoded BRP (33). Possibly, the peptide part of this BRP also has affinity for the outer membrane as suggested for unmodified Braun's prolipoprotein (14). The BRP signal sequence, which seems to be extremely stable for the pColA- and pColE2-encoded BRPs, could play a role in killing of host cells. The accumulation of this sequence or the interaction between the signal sequence and the cytoplasmic membrane or both could be deleterious to the cells. Recently, we observed that a mutant BRP, containing only four amino-terminal amino acid residues, still causes lethality of host cells on plates, but is unable to cause lysis of cells in liquid medium and to bring about release of cloacin DF13 (submitted for publication). We are currently investigating the role of the pCloDF13 BRP signal sequence in more detail by fusing it directly to the mature part of β-lactamase.

ACKNOWLEDGMENT

Part of this investigation was financially supported by Public Health Service grant GM-28811 from the National Institutes of Health to H.C.W.

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