JOURNAL OF BACTERIOLOGY, May 1989, p. 2673–2679
0021-9193/89/052673-07$02.00/0

Vol. 171, No. 5

pCloDF13-Encoded Bacteriocin Release Proteins with Shortened Carboxyl-Terminal Segments Are Lipid Modified and Processed and Function in Release of Cloacin DF13 and Apparent Host Cell Lysis

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Received 26 September 1988/Accepted 27 January 1989

By oligonucleotide-directed mutagenesis, stop codon mutations were introduced at various sites in the pCloDF13-derived bacteriocin release protein (BRP) structural gene. The expression, lipid modification (incorporation of [^{3}H]palmitate), and processing (in the presence and absence of globomycin) of the various carboxyl-terminal shortened BRPs were analyzed by a special electrophoresis system and immunoblotting with an antiserum raised against a synthetic BRP peptide, and their functioning with respect to release of cloacin DF13, lethality, and apparent host cell lysis were studied in Sup$^-$, *supF*, and *supP* strains of *Escherichia coli*. All mutant BRPs were stably expressed, lipid modified, and processed by signal peptidase II, albeit with different efficiencies. The BRP signal peptide appeared to be extremely stable and accumulated in induced cells. Full induction of the mutant BRPs, including the shortest containing only 4 amino acid residues of the mature polypeptide, resulted in phospholipase A-dependent and Mg^{2+}-suppressible apparent cell lysis. The extent of this lysis varied with the mutant BRP used. Induction of all mutant BRPs also prevented colony formation, which appeared to be phospholipase A independent. One shortened BRP, containing 20 amino acid residues of the mature polypeptide, was still able to bring about the release of cloacin DF13. The results indicated that the 8-amino-acid carboxyl-terminal segment of the BRP contains a strong antigenic determinant and that a small segment between amino acid residues 17 and 21, located in the carboxyl-terminal half of the BRP, is important for release of cloacin DF13. Either the stable signal peptide or the acylated amino-terminal BRP fragments (or both) are involved in host cell lysis and lethality.

Bacteriocins are plasmid-encoded toxic proteins which are among the few proteins released into the extracellular medium by *Escherichia coli*. The mechanism of bacteriocin release is unusual in two ways. First, the bacteriocin is synthesized without a cleavable signal peptide which could trigger its secretion across the cytoplasmic membrane. Second, a helper protein, the so-called bacteriocin release protein (BRP), is essential for translocation of the bacteriocin across both the cytoplasmic and outer membranes (6, 26).

The genes encoding the bacteriocin and its corresponding BRP are coordinately transcribed from a common promoter which is regulated by the SOS response (6, 15). Expression of the bacteriocin operon results in the synthesis and more or less specific release of the bacteriocin and also in inhibition of colony formation on broth agar plates (cell lethality), whereas full induction results in a marked decline in culture turbidity, called "lysis" (6). Initially, this "lysis" is not caused by a degradation of peptidoglycan but results from alterations in the bacterial outer membrane which are host strain dependent. The elevated level of BRP expression is responsible for the observed lethality and "lysis" of fully induced host cells. Divalent cations, such as Mg^{2+} and Ca^{2+}, in the culture medium suppress these effects of BRP expression but have no effect on the release of bacteriocin molecules (19, 29). The mechanism by which the BRP exerts these effects is still largely unknown. Phospholipase A activity in the outer membrane of *E. coli* is essential for both bacteriocin release and cell "lysis" (3, 19, 29).

All known BRPs are highly similar in primary structure (6, 26). They are relatively small proteins, molecular mass about 3,000 daltons (Da), which are synthesized as precursor molecules with a typical "lipobox" around the signal peptidase (SPase)-processing site (34, 37). Recently, evidence has been presented that the BRPs encoded by the pColA, pCloDF13, and pColE2-P9 plasmids are indeed lipoproteins which are processed by the prolipoprotein-specific SPase (SPaseII) (3, 20, 28). Nonlipidated forms of these BRPs, obtained by site-directed mutagenesis of the BRP genes, are more or less hampered in their processing and functioning (3, 17, 18, 28).

In the present study, site-directed mutagenesis was used to further study the structure-function relationship of the pCloDF13-encoded BRP and to investigate which part of the BRP is responsible for the release of cloacin DF13, lethality, and cell "lysis." Four suppressible nonsense mutations were introduced at different positions in the mature part of the BRP. The longest mutant BRP, containing the first 20 of the 28 amino acids of the mature protein, was found to be partly functional in the release of cloacin DF13 and cell "lysis." In contrast, the shortest mutant BRP, containing only four amino-terminal amino acids of the mature protein, could not bring about the release of cloacin DF13 but still caused Mg^{2+}-suppressible "lysis" of host cells, albeit with reduced efficiency. Furthermore, the effect of amino acid substitutions on the functioning of the BRP was studied by using various suppressor strains.

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MATERIALS AND METHODS

Bacterial strains, growth media, and plasmids. *E. coli* K-12 strains JM103 (21) and BMH71-18 *mutL* (13) were used for the construction of mutants and DNA sequencing. *E. coli* FTP4170 Δ(*tonB trpAE*) *argE*(Am) *glyV55* and the *supF* and *supP* suppressor strains FTP1330 and MDA2629, respectively, were a gift of F. T. Pagel, Houston, Tex. *E. coli* CE1303 (5) is defective in detergent-resistant phospholipase A activity and was used to test for BRP-dependent cell "lysis."

Several media were used for the culturing of the various strains. YT medium (23) was used for strains JM103 and BHM71-18. LB medium supplemented with 0.5% sodium lactate was used for routine culturing of bacteria and cloacin DF13 release experiments. M9 minimal medium (23), supplemented with 0.5% sodium lactate, was used for labeling experiments with a mixture of ^{3}H-amino acids. Protease peptone beef extract (34) was used for labeling studies with [^{3}H]palmitate. Ampicillin (100 μg/ml) or tetracycline (20 μg/ml) or both were added when required. When indicated, LB medium was supplemented with 20 mM $MgCl_2$ to reduce BRP-induced "lysis" of cells. Isopropyl-β-D-thiogalactoside (IPTG) was used to induce BRP gene expression.

The expression vector pINIIIA1 (22) was used for controllable and high-level expression of the BRP or mutant BRPs. The plasmid pJL25, a pACYC184 derivative coding for cloacin DF13, has been described previously (18).

Oligonucleotide-directed in vitro mutagenesis. Site-directed mutagenesis was performed with a derivative of M13mp8 carrying a 0.37-kilobase (kb) *Bst*NI-*Cla*I restriction fragment encompassing the complete pCloDF13-derived BRP gene (16). Mutagenesis was carried out by the double primer method as described by Zoller and Smith (38). The following mutagenic oligonucleotides were used for the introduction of a stop codon in various parts of the BRP gene encoding the mature portion of the BRP (the mutated nucleotides are underlined): 5′-TCCCGGATCTAGTTTGC-3′, position +5; 5′-TCCACCCTAAACATCCC-3′, position +10; 5′-AGGAG GACTATGGTGCC-3′, position +17; and 5′-CGTCAGTTA AGAGGAGG-3′ for a stop codon at position +21. The oligonucleotide 5′-GATCTTTTCTAGTTACCCGCG-3′ was used for the introduction of an *Hpa*I site at the 3′ terminus of the BRP gene. As a result of this last mutation, the Gln residue at position +28 of the mature BRP was changed to an Asn residue. Putative mutants were identified by colony probing with the 5′-end, ^{32}P-labeled mutagenic oligonucleotide and successive washings at increasing temperatures. To confirm that the desired mutation was constructed and to verify that no other mutations had occurred, the DNA sequence of the complete M13mp8 DNA insert was determined by the dideoxy termination method of Sanger et al. (30). Subsequently, the mutated inserts were isolated after digestion of the M13mp8 derivative with *Hin*dIII and *Eco*RI and ligated into the expression vector pINIIIA1 previously digested with the same restriction endonucleases. The resultant recombinant plasmids were designated pJL17 (containing the wild-type BRP gene), pJL17-5 (amber mutation affecting residue 5), pJL17-10 (amber mutation affecting residue 10), pJL17-17 (amber mutation affecting residue 17), pJL17-21 (ochre mutation affecting residue 21), and pJL22 (Gln-28 → Asn-28). All of these plasmids carry the (mutant) BRP gene under control of the tandem *lpp-lac* promoter-operator system (16).

Other recombinant DNA techniques. Plasmid DNA and M13 double-stranded DNA were isolated by the alkaline extraction method of Birnboim and Doly (2). Transformation of $CaCl_2$-treated cells was carried out by the method of Dagert and Ehrlich (4). All other basic DNA manipulations were carried out by the methods of Maniatis et al. (21). DNA-modifying enzymes were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md. and Pharmacia, Uppsala, Sweden, and used according to the instructions of the suppliers.

Tricine-SDS-PAGE and immunoblotting. Wild-type and mutant BRPs were separated by using highly crosslinked 16.5% polyacrylamide gels in tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (tricine-SDS-PAGE) as described by Schägger et al. (31). Immunoblotting was carried out essentially as described by Towbin et al. (35) on nitrocellulose paper with a small pore size (0.1 μm; Schleicher & Schüll, Dassel, Federal Republic of Germany). Blots were developed with a polyclonal mouse antiserum raised against a synthetic complete BRP peptide as the primary antibody and a horseradish peroxidase-conjugated goat anti-mouse immunoglobulin as the second antibody.

Radiolabeling of proteins. Exponentially growing cells (2×10^8 cells per ml) were induced with 1 mM IPTG. After 10 min, a mixture of ^{3}H-amino acids or [^{3}H]palmitate (30 Ci/mmol) was added to a final concentration of 30 and 50 μCi/ml, respectively, and incubation was continued for 30 min. When required, globomycin (a kind gift of M. Arai, Tokyo, Japan) dissolved in dimethyl sulfoxide (5 mg/ml) was added to a final concentration of 100 μg/ml 5 min prior to the addition of IPTG. To terminate labeling, cells incubated with ^{3}H-amino acids were collected by centrifugation and washed with phosphate-buffered saline. Labeling of cells with [^{3}H]palmitate was stopped by the addition of trichloroacetic acid to a final concentration of 10%. Subsequently, the cells were collected by centrifugation and washed twice with methanol to remove lipids. The labeled cells were dissolved in sample buffer and analyzed by SDS-PAGE. Finally, the gels were dried, and radiolabeled protein bands were visualized by fluorography.

Release of cloacin DF13. Five hours after induction with IPTG, the amount of cloacin DF13 released into the culture supernatant was determined by measuring the amount of cloacin DF13 in sonicated cell suspensions and in culture supernatant fractions. The amount of cloacin DF13 was determined with an enzyme-linked immunosorbent assay essentially as described previously (36).

RESULTS

Oligonucleotide-directed mutagenesis of the BRP gene. To study structure-function relationships of the pCloDF13-encoded BRP and to investigate which part of the protein is involved in cloacin DF13 release, lethality, and "lysis" of host cells, several mutations were created in the BRP gene by oligonucleotide-directed in vitro mutagenesis. Four suppressible nonsense mutations were introduced in the part of the BRP gene encoding the mature portion of the BRP (Fig. 1). As a result of these mutations, shortened BRPs of 4 (pJL17-5), 9 (pJL17-10), 16 (pJL17-17), and 20 (pJL17-21) amino-terminal amino acid residues were constructed. Furthermore, a mutant BRP gene was constructed in which the last codon, CAG, was changed to an AAC codon. This mutation resulted in an *Hpa*I restriction site at the 3′-terminal end of the BRP gene and in the substitution of the glutamine residue at position 28 into an asparagine residue in the BRP polypeptide (Fig. 1). The plasmid carrying this mutant BRP was called pJL22.

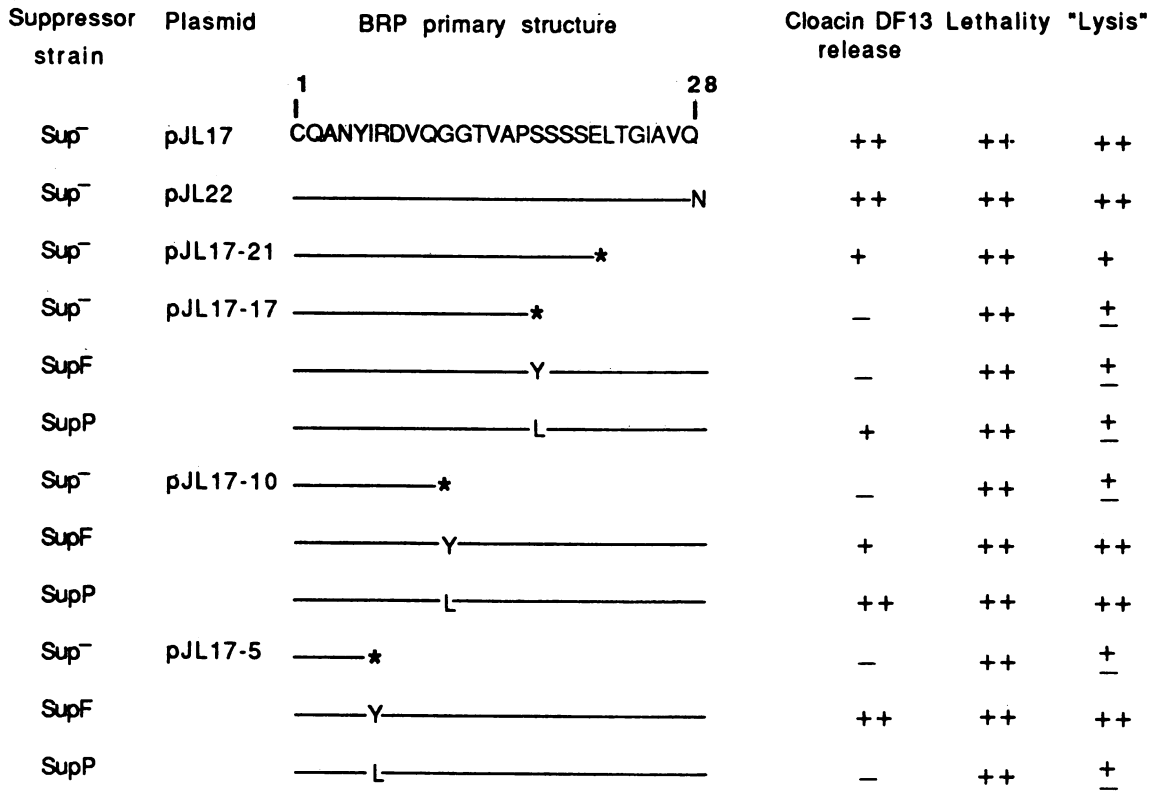

FIG. 1. Primary structure and functioning of mutant BRPs. The primary structure of the mature BRP encoded by pJL17 and the mutant BRPs encoded by the indicated plasmids is given in the one-letter code in the center. The position of a stop codon mutation is indicated by an asterisk. The suppressor strains in which the various plasmids were expressed are indicated at the left; Sup⁻, strain FTP4170; SupF, strain FTP1330; SupP, strain MDA2629. Data on the activities of the various mutant BRPs are presented on the right. The release of cloacin DF13 was determined with cells harboring both pJL25 and one of the indicated mutant BRP plasmids. Cells were cultured in broth supplemented with 20 mM $MgCl_2$, ampicillin, and kanamycin. Mitomycin (induction of cloacin DF13; 500 ng/ml) and IPTG were added in the early logarithmic growth phase, and incubation was continued for 5 h. Various concentrations of IPTG were tested for the induction of the (mutant) BRP, and a suboptimal concentration that did not cause any "lysis" was selected. Symbols: ++, about 50% of the cloacin DF13 produced was released during a 5-h induction period; +, about 20% release; −, less than 5% release. The amount of bacteriocin produced was comparable for each culture. Lethality was studied by measuring the colony-forming ability of host cells on broth agar plates containing ampicillin and various concentrations of IPTG. Portions (5 μl) of a 10^5-diluted stationary-phase culture harboring the indicated plasmid were spotted, and the plates were incubated for 20 h at 37°C. Symbols: ++, complete inhibition of colony formation (complete loss of viability) on plates containing >60 μM IPTG. "Lysis" (marked decline in culture turbidity) of cells harboring one of the indicated plasmids was studied in broth supplemented with ampicillin and after induction with various concentrations of IPTG added in the early logarithmic growth phase. Symbols: ++, "lysis" during moderate induction conditions (40 μM IPTG); +, "lysis" only after strong induction (100 to 1,000 μM IPTG); ±, intermediate effect on culture turbidity under strong induction conditions (100 to 1,000 μM IPTG).

Expression and identification of mutant BRPs. The identification of the pCloDF13-encoded BRP by SDS-PAGE has proven to be rather difficult because of its small size (24, 25). Recently, superior resolution of relatively small proteins (between 1 and 30 kDa) was achieved by using a three-layer discontinuous SDS-PAGE system with tricine instead of glycine as the trailing ion (31). This gel system was used for the detection of the pCloDF13-derived BRP and constructed mutant BRPs in induced cells of strain FTP4170 (Fig. 2A). Based on their relative molecular mass, the mature forms of the wild-type BRP and the shortened BRP encoded by pJL17-21 could be detected on a stained gel. Furthermore, precursors polypeptides of the pJL17-17- and pJL17-10-encoded mutant BRPs were detected, but a pJL17-5-encoded BRP derivative polypeptide could not be identified on the stained gel, possibly due to the presence of other, cell-derived proteinaceous material of low molecular mass. The indicated protein bands could not be detected in noninduced cells. Calculations based on the intensity of the stained bands indicated that the BRP was expressed in induced cells to about 10^5 molecules per cell.

Expression of the BRP and mutant BRPs was also studied by immunoblotting with a mouse polyclonal antiserum raised against a synthetic peptide encompassing the complete mature BRP (Fig. 2B). The antiserum reacted strongly with three pJL17-encoded polypeptides of approximately 6, 5, and 3 kDa. These polypeptides were identified as the acylated precursor, the nonacylated precursor, and the acylated mature BRP, respectively (see also below). The intensity of the three polypeptide bands was about the same, which suggested that newly synthesized BRP precursor polypeptides are slowly acylated and processed.

Mutant BRP polypeptides could not be detected by immunoblotting. Apparently, the shortened polypeptides have lost a strong antigenic determinant which is probably located at the carboxyl-terminal end of the protein. Computer-aided analysis of the BRP protein structure confirmed the presence of such an antigenic determinant. The antiserum still reacted

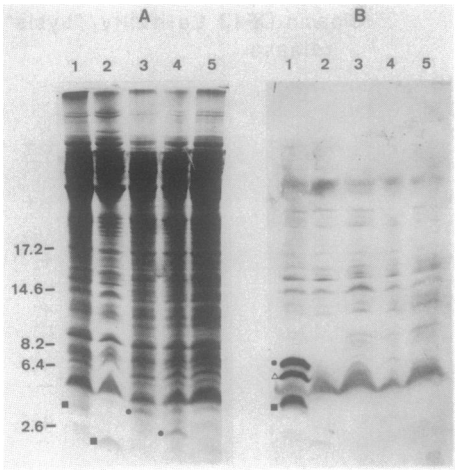

FIG. 2. Expression and identification of the wild-type and shortened BRPs. (A) Tricine-SDS-PAGE, Coomassie brilliant blue staining; (B) immunoblotting. Cells of FTP4170 harboring pJL17 (lane 1), pJL17-21 (lane 2), pJL17-17 (lane 3), pJL17-10 (lane 4), and pJL17-5 (lane 5) were cultured in broth containing 20 mM $MgCl_2$ and ampicillin and induced for 3 h with 1 mM IPTG. The cells were collected, washed, and suspended in sample buffer. The equivalent of 0.4 OD_{660} units (about 5×10^8 cells) was applied to each lane. The relative molecular masses of marker proteins are indicated at the left (in kilodaltons). Symbols: ●, lipid-modified BRP precursor; △, unmodified BRP precursor; ■, mature form of the BRP.

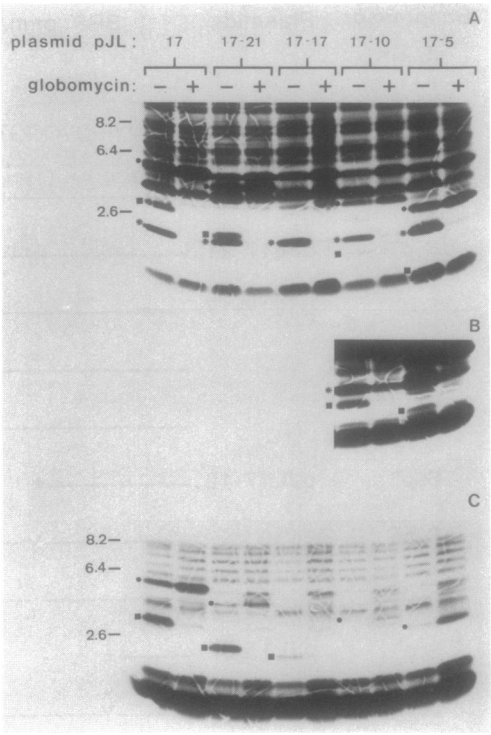

FIG. 3. Lipid modification and processing of mutant BRPs. Cells of FTP4170 harboring one of the indicated plasmids were labeled for 30 min in the absence or presence of globomycin with a ^{3}H-amino acid mixture (A and B) or with [^{3}H]palmitate (C). Part B represents an overexposure of the bottom right-hand corner of part A. The lower part of the fluorograms is shown. The symbols for the various BRP-related polypeptides are the same as indicated in the legend to Fig. 2; *, stable BRP signal peptide. The molecular masses of marker proteins are indicated (in kilodaltons) at the left of parts A and C.

strongly with a BRP-Bla hybrid protein (results not shown), which lacked only the last three carboxyl-terminal amino acid residues of the mature BRP (20). Apparently, amino acid residues 21 to 25 are required for recognition of the BRP by the antiserum.

Modification and processing of mutant BRPs. To investigate further whether the shortened BRP polypeptides are expressed, lipid modified, processed, and stable, induced cells of *E. coli* FTP4170 harboring pJL17 or a derivative plasmid were labeled with a mixture of ^{3}H-amino acid residues or with [^{3}H]palmitate in the presence or absence of globomycin (Fig. 3). Addition of globomycin blocks the action of SPaseII, which specifically cleaves acylated lipoprotein precursors (8, 10) and results in the accumulation of acylated prolipoproteins in the *E. coli* cytoplasmic membrane (11). The processing of the wild-type BRP precursor and all mutant BRP precursors appeared to be sensitive to globomycin (Fig. 3A and B). This indicated that all of these precursor forms are subjected to thio-ether modification, followed by cleavage of the signal sequence by SPaseII.

A polypeptide of about 2 kDa appeared to be present in cells not treated with globomycin and disappeared upon globomycin treatment. This polypeptide could not be detected in noninduced cells and was not labeled when cells were incubated with [^{3}H]palmitate (Fig. 3C). Apparently, this polypeptide represents the cleaved signal peptide of the wild-type and mutant BRPs. Since the signal peptide and the various precursor forms were still clearly detectable after 30 min of labeling, this experiment shows that the BRP signal peptide is surprisingly stable and that the processing of the various precursors is a rather slow process.

The processed and mature forms of most of the mutant BRPs were expressed rather well and stably maintained in the cells (Fig. 3A and B). The level of expression and stability of the processed and mature form of the pJL17-17-encoded shortened BRP could not be deduced from the labeling experiments involving ^{3}H-amino acid residues, since it comigrated with the stable signal peptide (Fig. 3A). However, labeling of cells harboring pJL17-17 with [^{3}H]palmitate (Fig. 3C) or with [^{35}S]cysteine (not shown) indicated that this shortened BRP was stably expressed, albeit at a lower level than the other mutant BRPs.

The extent of acylation of the precursor and mature forms of the wild-type and mutant BRPs was deduced from the relative fluorogram intensities after labeling with [^{3}H]palmitate (Fig. 3C). Both the mature and precursor BRP polypeptides were labeled efficiently with [^{3}H]palmitate, as has been shown previously for the BRP–β-lactamase (Bla) hybrid protein (20). The mature form of the pJL17-21-encoded mutant BRP was labeled much more effectively than its precursor form, which suggests that in this shortened BRP, [^{3}H]palmitate is incorporated more efficiently via amide linkage than via thio-ether linkage (10). The mature form of the shortened BRP encoded by pJL17-17 was weakly labeled, whereas its precursor could not be detected. The opposite appeared to be true for the pJL17-10-encoded mutant BRP. The pJL17-4-encoded precursor was strongly labeled, but its mature form could not be detected due to the presence of labeled phospholipids at the bottom of the gel. However, the position of this mature polypeptide on the fluorogram (Fig. 2B) indicated that it was acylated. The pJL22-encoded mutant BRP could not be distinguished from wild-type BRP in any of the experiments described above (not shown).

In summary, the experiments presented so far show that all mutant BRPs are expressed, lipid modified, processed by SPaseII, and stably maintained in the cells, albeit with different efficiencies.

Functioning of mutant BRPs. The physiological role of the pCloDF13-encoded BRP is to bring about the translocation of cloacin DF13 across the envelope of producing cells (6). However, the BRP also has a negative effect on the integrity of host cells when it is produced in too large amounts. This is demonstrated by the inability of moderately induced cells to form colonies on solid medium (lethality) and by a rapid decline in turbidity ("lysis") of fully induced cultures.

To investigate whether the mutant BRPs still function in the release of cloacin DF13, cells of strain FTP4170 harboring pJL17 or a derivative, mutant plasmid were transformed with a second plasmid, pJL25. This plasmid is compatible with pJL17 and derivative plasmids and directs the synthesis of cloacin DF13 under transcriptional control of its own mitomycin-inducible promoter, but lacks the BRP gene. This so-called binary vector system allows the separate induction and control of the synthesis of BRP and production and release of cloacin DF13 (16–18). The double transformants were moderately induced, and the production and release of cloacin DF13 were measured in the absence of "lysis." The results of these experiments are summarized in Fig. 1. Cells producing the wild-type BRP, the mutant BRP encoded by pJL22, or the shortened BRP encoded by pJL17-21 excreted significant amounts of the bacteriocin. However, virtually no cloacin DF13 was released from cells expressing the other shortened BRPs, not even when the expression of the mutant BRPs was fully induced.

The effects of the various mutant BRPs on cell lethality and "lysis" were also investigated (Fig. 1). Induction of all mutant BRPs strongly affected the colony-forming ability of moderately and fully induced host cells, similar to the results obtained with the wild-type BRP (16–18). Comparable results were obtained when *E. coli* CE1303, defective in detergent-resistant phospholipase A activity, was used as a host. This indicated that BRP-induced lethality of host cells is a phospholipase-independent event.

Moderate induction of the wild-type and mutant BRP encoded by pJL22 caused cell "lysis." The pJL17-21-encoded BRP, which contains 20 amino acid residues of the mature protein, also caused "lysis" of cells, but only after full induction. In contrast, the shortened BRPs containing only 16, 9, and 4 amino acid residues of the mature sequence only slightly affected the culture turbidity under high-level induction conditions (intermediate "lysis"). Addition of $MgCl_2$ to the culture medium prevented the observed "lysis" caused by the various mutant BRPs, whereas no "lysis" could be detected when *E. coli* CE1303 was used as the host (not shown). Apparently, the molecular mechanism responsible for host cell "lysis" is the same for the wild-type and the mutant BRPs.

Functioning of mutant BRPs in suppressor strains. The effects of point mutations affecting residues 5, 10, and 17 of the mature BRP on release of cloacin DF13 and cell "lysis" was studied by using two different (*supF* and *supP*) amber suppressor strains (Fig. 1). Although suppression of amber mutations is not always complete, the experiments indicated that substitution of the serine residue at position 17 of the pJL17-17-encoded BRP by a tyrosine residue had no effect on the phenotype of this mutant BRP, whereas substitution of the serine residue by a leucine residue resulted in significantly increased bacteriocin-releasing capacity. In contrast, the substitution of the glutamine residue at position 10 (pJL17-10) with a tyrosine or a leucine residue nearly restored the biological activities of the mutant BRP. Finally, suppression of the amber mutation at position 5 in the *supF* strain resulted in wild-type BRP with the expected activities. However, transition of the tyrosine residue at position 5 to a leucine residue did not affect the phenotype of the mutant BRP. Unfortunately, other suppressor strains could not be tested because of poor growth, especially when the binary vector system was used.

The results presented above suggest that the first 20 amino-terminal amino acid residues of the mature BRP are sufficient for bringing about a significant release of cloacin DF13. The shortest mutant BRP containing only 4 amino acid residues of the mature BRP still caused the same level of lethality as the wild-type BRP. The tyrosine residue at position 5 and the serine at position 17 appear to be important for the proper functioning of the BRP in cloacin DF13 release. The glutamine residue at position 10 is less important, since it can be exchanged for a tyrosine or leucine without significantly affecting the functioning of the BRP.

DISCUSSION

In this paper we describe the construction and analysis of carboxyl-terminal-shortened BRPs. Various observations have indicated that the carboxyl-terminal segment of this type of protein is not essential for functioning in bacteriocin release or host cell "lysis." For instance, the replacement of nine carboxyl-terminal amino acid residues of the pColE2- and pColE3-encoded BRPs by one glycine residue did not affect their "lysis" phenotype (33). A hybrid protein consisting of the pCloDF13-encoded BRP lacking three carboxyl-terminal amino acid residues fused to the mature part of β-lactamase was still functional both in extracellular export of cloacin DF13 and in cell "lysis" (17, 18). A comparable hybrid protein in which the pColN-encoded BRP was fused to β-galactosidase also retained biological activity (27). Another observation is that the amino-terminal region of most known BRPs is highly similar in primary structure and that they also share local sequence similarities with essential peptides of certain icosahedral coliphage lysis proteins (6, 14). In contrast to the amino-terminal segments, the carboxyl-terminal regions show significant differences in primary structure (6, 14). Finally, the pColE9-J-encoded BRP contains only 26 amino acid residues, including its putative signal peptide, but is functional in causing cell "lysis" but not colicin release (12; R. James, personal communication).

In order to study the structural requirements of these BRPs more systematically, we introduced stop codons at four different positions in the pCloDF13-derived BRP gene by oligonucleotide-directed mutagenesis. The longest mutant BRP peptide, missing eight carboxyl-terminal amino acid residues, still caused cell "lysis" and release of cloacin DF13, albeit with reduced efficiency. The three shorter BRP peptides, missing 11 to 24 amino acid residues, were unable to bring about the release of cloacin DF13 but still caused an intermediate decline in culture turbidity after strong expression. The mutant and wild-type BRPs did not differ in their ability to block colony formation on broth agar plates containing an inducing agent. Apparently, the release of cloacin DF13 requires the presence of a complete or almost complete mature BRP, whereas smaller segments of the BRP are enough to cause lethality and "lysis." The discrepancy in the requirements for cell "lysis" and cloacin DF13 release once more indicate that these two events are not necessarily

coupled (6, 16). This idea is supported by recent experiments of Suit and Luria (32), who isolated *E. coli* mutants which are resistant to "lysis" caused by expression of the colicin E1 BRP, whereas the release of colicin E1 and periplasmic enzymes was not affected. Phospholipase A was present but was apparently not activated by BRP expression in any of these mutants.

Labeling experiments revealed that all mutant BRPs are expressed, lipid modified, and processed by SPaseII, albeit with different efficiencies. The cleaved signal peptide was shown to be extremely stable, as has been observed for the pColA- and pColE2-encoded BRP signal peptides (3, 28).

Considering the structure and functioning of the shortest mutant BRP, we infer that the accumulation of either the acylated tetrapeptide or the stable signal peptide (or both) is responsible for the observed lethality. It is conceivable that cleaved signal peptides accumulate in the cytoplasmic membrane and either disrupt the lipid bilayer structure, interfere with the normal export pathway of secreted proteins, or form large, ion-permeable aggregates which affect the membrane energy metabolism. Unprocessed mutant BRP precursors, in which the cysteine residue at position 1 is substituted with a glycine residue, still cause "lethality," indicating that cleavage is not essential for this effect (17, 18). Another possible explanation is that the acylated tetrapeptide itself is responsible for the observed lethality and intermediate cell "lysis" which is Mg^{2+} suppressible and detergent-resistant phospholipase A dependent. In that case, we expect that the acylated tetrapeptide is localized in or near the outer membrane. The putative role of the BRP signal peptide is currently being studied in more detail by gene fusion and in vitro experiments.

The mutant BRP containing 20 amino acid residues of the mature BRP is still functional in cloacin DF13 release, whereas the mutant BRP of 16 residues is not. Possibly, the rather hydrophilic region between residues 16 and 20 is involved in recognition of cloacin DF13 molecules. However, at present, it is not known whether a direct interaction between a given bacteriocin and its BRP is required for the more or less specific export of the bacteriocin. The fact that no specific region of colicin A is required for its extracellular release argues against such a direct molecular interaction (1).

Studies by Ghrayeb and Inouye (9) with a hybrid protein consisting of a part of the murein lipoprotein and β-lactamase indicated that only 9 amino acid residues of the mature lipoprotein are sufficient for normal acylation, processing, and subcellular localization. Comparable results were obtained by d'Enfert and Pugsley with a hybrid protein consisting of the signal peptide plus 11 amino acid residues of the mature pullulanase and alkaline phosphatase (7). The smallest mutant BRP described in this paper is still acylated and processed by SPaseII, which indicates that the signal peptide plus four amino acid residues of the mature BRP are sufficient for recognition by the prolipoprotein modification and processing enzymes. This also suggests that the presence of a β-turn structure immediately following the cleavage site might not be a prerequisite for acylation and processing of prolipoproteins, as was suggested by Giam et al. (10). We suppose that the signal peptide plus a cysteine residue at position 1 are sufficient for acylation and processing of prolipoproteins. This idea was confirmed by directly fusing the BRP signal peptide plus the Cys-1 residue to the mature portion of β-lactamase. Preliminary experiments showed that this hybrid protein is lipid modified and processed (unpublished results).

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