Modification, Processing, and Subcellular Localization in *Escherichia coli* of the pCloDF13-Encoded Bacteriocin Release Protein Fused to the Mature Portion of β-Lactamase

JOEN LUIRINK, TAKESHI WATANABE, HENRY C. WU, FREEK STEGEHUIS, FRITS K. DE GRAAF, AND BAUKE OUDEGA

Department of Molecular Microbiology, Biological Laboratory, Vrije Universiteit, 1081 HV Amsterdam, The Netherlands, and Department of Microbiology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814-4799

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A fusion between the pCloDF13-derived bacteriocin release protein and β-lactamase was constructed to investigate the subcellular localization and posttranslational modification of the bacteriocin release protein in *Escherichia coli*. The signal sequence and 25 of the 28 amino acid residues of the mature bacteriocin release protein were fused to the mature portion of β-lactamase. The hybrid protein (Mₚ, 31,588) was expressed in minicells and whole cells and possessed full β-lactamase activity. Immunoblotting of subcellular fractions revealed that the hybrid protein is present in both the cytoplasmic and outer membranes of *E. coli*. Radioactive labeling experiments in the presence or absence of globomycin showed that the hybrid protein is modified with a diglyceride and fatty acid and is processed by signal peptidase II, as is the murein lipoprotein. The results indicated that the pCloDF13-encoded bacteriocin release protein is a lipoprotein which is associated with both membranes of *E. coli* cells.

Bacteriocins are plasmid-encoded antibiotic proteins which are produced and released by bacteriocinogenic bacterial strains (17). The release of bacteriocins in *Escherichia coli* is dependent on the expression of the gene encoding the so-called bacteriocin release protein (BRP). This gene is located in the various bacteriocin operons downstream from the inducible bacteriocin promoter and the bacteriocin structural gene. Induction of bacteriocin operons by activation of the host cell SOS response not only results in increased synthesis and release of bacteriocin, but also, in some instances, in lysis of host cells (7, 30). The elevated level of BRP gene expression is primarily responsible for the observed lysis (19a, 32).

The BRPs encoded by pColE1 (kil product), pColE2-P9 (cellB product), pColE3-CA38 (hic product), pColA-CA31 (cal product), and pCloDF13 (gene H product) have been sequenced and were found to share considerable sequence homology (3, 6, 7, 12, 26, 40). Furthermore, the BRPs seem to be functionally interchangeable (31). In contrast to their respective bacteriocins, the BRPs are synthesized as precursor molecules with an amino-terminal signal sequence and, like the murein lipoprotein, contain the consensus tetrapeptide which constitutes the recognition site for modification and processing enzymes (42). All mature BRPs, except for the pColA-CA31-encoded BRP, have been localized in the cell envelopes of induced cells (6, 12, 15). The colicin A release protein might be excreted into the medium together with colicin A (3). Subcellular localization studies in *E. coli* minicells showed that the pCloDF13-encoded BRP is present in both the cytoplasmic and outer membranes, but the majority (about 75%) was found in the outer membrane (28). The mode of action of the BRPs is still poorly understood.

Recent experiments have indicated that induction of BRP synthesis results in activation of detergent-resistant phospholipase A, which in turn might cause local permeabilization of the outer membrane by the accumulation of lysophosphatidylethanolamine (20, 33). It has been proposed that bacteriocin molecules use these permeabilization zones for their release in a semispecific manner and that strong induction of phospholipase A results in lysis of cells (7). Translocation of bacteriocin across the cytoplasmic membrane is also dependent on BRP gene expression, but the mechanism is unknown.

We are investigating the processing, posttranslational modification, subcellular localization, and functioning of the pCloDF13-encoded BRP. Research on this polypeptide is difficult because of its low molecular mass, about 2,900 daltons for the mature protein, and the inability to detect the polypeptide in whole cells. To circumvent these problems, we constructed a hybrid gene containing the almost complete pCloDF13 BRP gene at its 5' end and a portion of the *bla* gene encoding mature β-lactamase at its 3' end. Experiments are presented here that indicate that the hybrid gene product is modified with a diglyceride and fatty acid, processed by the signal peptidase also involved in processing of the murein lipoprotein (SPase II), and located in both the cytoplasmic and outer membranes of *E. coli*. The implications of these results for the functioning of the BRP are discussed.

MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** The bacterial strains used in this study were *E. coli* N3406 (38) and *E. coli* DS410 (9). Plasmid pTG2 (16) was used as the cloning vector. The pCloDF13 derivative plasmid pEV67 has been described previously (12). M9 minimal medium (22) supplemented with 0.4% succinate, thiamine (100 µg/ml), L-leucine (50 µg/ml), and L-threonine (50 µg/ml) was used for labeling studies involving [3H]glycerol and [3H]methionine. Difco Proteose Peptone beef extract medium (35) was used for labeling studies involving [3H]palmitate. When necessary,
ampicillin (100 μg/ml) or tetracycline (20 μg/ml) was added to the culture medium to select for antibiotic resistance.

Chemicals and radiochemicals. The radioisotopes were purchased from New England Nuclear Corp., Boston, Mass., and Amersham Corp., Amersham, United Kingdom. Fixed *Staphylococcus aureus* cells were purchased from Calbiochem-Behring, La Jolla, Calif. Globomycin was a gift from M. Arai, Sanky Co., Tokyo, Japan. Purified β-lactamase was a gift from J. Tommassen, Utrecht, The Netherlands.

Immuno blotting. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out as described by Laemmli (18). Immunoblotting was carried out essentially as described by Towbin et al. (37) with antibodies directed against purified β-lactamase. Anti-β-lactamase serum was raised in rabbits.

Determination of 50% lethal dose. Ampicillin sensitivity was tested by the 50% lethal dose test described by Kadonaga et al. (16).

Recombinant DNA techniques. Plasmid isolation, transformation, gel electrophoresis, and electroelution of DNA fragments were carried out by standard procedures (21). T4 DNA ligase and the restriction endonuclease *FnuDII* were purchased from New England BioLabs, Inc., Beverly, Mass. All other restriction endonucleases and DNA-modifying enzymes were purchased from Boehringer Mannheim Corp., Mannheim, Federal Republic of Germany. All enzymes were used according to the instructions of the suppliers.

Isolation of subcellular fractions. For the isolation of the various subcellular fractions, cells were made into spheroplasts 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 cytoplasmic and outer membranes (total membrane fraction) were collected by centrifugation (19); the supernatant fraction was taken as the cytoplasmic fraction. The total membrane fractions were treated with the detergents Triton X-100 and benzyl alcohol (10), and cytoplasmic and outer membranes were separated by isopycnic sucrose density gradient centrifugation as described by Osborn et al. (27). Proteins present in periplasmic and cytoplasmic fractions were concentrated by trichloroacetic acid precipitation and washed with acetone before SDS-polyacrylamide gel electrophoresis and immunoblotting. β-Lactamase activity in the various fractions was assayed spectrophotometrically as described previously (25). As controls, the activities of β-galactosidase (22) and alkaline phosphatase (2) were measured as cytoplasmic and periplasmic markers, respectively.

Immunocytochemical labeling with cryosections. Immunocytochemical labeling with protein A-gold complexes on ultrathin cryosections of *E. coli* cells was carried out as described previously (36).

Labeling experiments. Isolation and labeling of minicells with [35S]methionine, as well as the analysis of labeled polypeptides by SDS-polyacrylamide gel electrophoresis and autoradiography, were carried out as described by Moot et al. (23). Proteolytic processing in minicells was inhibited by the addition of 8.5% (vol/vol) ethanol (29).

Exponentially growing cells (2 × 10^8 cells per ml) were labeled with [3H]glycerol (9.0 Ci/mmol) or [3H]palmitate (30 Ci/nmol) at 50 μCi/ml for 30 min or with [35S]methionine (1.127 Ci/nmol) at 20 μCi/ml for 2 min. When necessary, globomycin dissolved in dimethyl sulfoxide (10 mg/ml) was added to the cultures to a final concentration of 100 μg/ml 30 min before labeling. Labeling was stopped by the addition of trichloroacetic acid to a final concentration of 10%. Precipitates were collected by centrifugation, washed with acetone and chloroform-methanol (2:1, vol/vol), dried, and dissolved in 1% SDS in 50 mM Tris hydrochloride buffer (pH 8.0) by heating at 100°C for 4 min. Subsequently, immunoprecipitations were carried out as described by Hayashi and Wu (13) with an antiserum against β-lactamase, and samples were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

RESULTS

BRP-β-lactamase hybrid gene construction. Plasmid pTG2 was used as the cloning vector for the construction of a hybrid gene encoding the amino-terminal signal sequence and a large portion of the pCloDF13-encoded BRP fused to the mature portion of β-lactamase. Plasmid pTG2 is a pBR322 derivative in which the nucleotide sequence encoding the amino-terminal signal sequence of the RETM β-lactamase is flanked at its 5' end by a unique EcoRI site and at its 3' end by a unique BstEII site. This DNA fragment of pTG2 was removed and replaced by a DNA fragment encoding the BRP signal sequence and 26 of the 28 amino acid residues of mature BRP. The latter DNA fragment was derived from the pCloDF13 derivative plasmid pYE67 and was cloned into pTG2 using a shotgun cloning procedure. The strategy used for the construction of the hybrid gene is shown in Fig. 1A. Cells of *E. coli* N3406 were transformed with the constructed plasmids, and transformants were selected for ampicillin resistance, assuming that the BRP portion would be able to direct the BRP-β-lactamase hybrid protein into the periplasmic space without affecting the enzymatic functioning of β-lactamase. Cells harboring deletion mutants of pTG2 lacking the β-lactamase signal sequence accumulate β-lactamase in the cytoplasm and are sensitive to ampicillin (16).

Of 14 ampicillin-resistant transformants, 2 harbored a portion of the expected size, expressed a protein which migrated more slowly than did β-lactamase on an SDS-polyacrylamide gel, and reacted with antiserum against β-lactamase in an immunoblotting experiment (data not shown). DNA sequence analysis of the small *XmnI* DNA fragment of the selected plasmids showed that the pEV67-derived DNA fragment was inserted in the correct orientation and reading frame (Fig. 1B). Unexpectedly, in both plasmids the Smal-*FnuDII* insert was preceded by the small *FnuDII* fragment containing terminator T2 of the cloacin operon in the orientation opposite to that of the *bla* promoter (Fig. 1A). Several attempts to remove this additional terminator, T2, by subcloning the hybrid gene in an expression vector downstream of a strong, controllable promoter were not successful. These results strongly suggested that a high level of expression of the hybrid gene is deleterious to the cells.

One of the constructed plasmids, designated pJL12, was selected for further studies.

Detection and subcellular localization of the hybrid protein. The expression of the hybrid gene was analyzed with *E. coli* minicells (Fig. 2). Two protein bands with apparent molecular weights of 32,500 and 34,000, respectively, were detected in minicells harboring pJL12. These bands were identified as the hybrid protein and its precursor. The apparent molecular weights of these bands were in good agreement with the calculated molecular weights of 31,588...
and 34,171, respectively. The processing of the precursor of the hybrid protein could be completely inhibited by the addition of 8.5% ethanol to the label mixture. Control minicells harboring pTG2 expressed β-lactamase (molecular weight, 27,895) and its precursor (molecular weight, 31,502) (Fig. 2; 16). The processing of this precursor was also completely hampered by the addition of 8.5% ethanol. Although the calculated molecular weights of the β-lactamase precursor and the mature hybrid protein hardly differ, the latter protein clearly migrated more slowly, suggesting a possible modification of the hybrid protein. In some experiments a double band was detected at the position of the hybrid protein precursor, suggesting incomplete modification (data not shown).

The β-lactamase activity and subcellular localization of the hybrid protein were studied with whole E. coli cells. The 50% lethal dose of ampicillin for cells harboring pJL12 was four times lower than that for cells harboring pTG2. Radioactive labeling experiments revealed that this difference was due to a four-times-lower expression of the hybrid gene, rather than to a loss in specific β-lactamase activity (data not shown).

The orientation opposite to that of the promoter of β-lactamase. (B) Nucleotide sequence of part of the pJL12-derived hybrid gene, containing the fusion point between the genes encoding the BRP and β-lactamase. The sequence which corresponds to the consensus prolipoprotein modification and cleavage tetrapeptide is presented in the boxed area.

FIG. 1. (A) Construction of pJL12. Arrows indicate the direction of transcription. Only essential restriction endonuclease restriction sites are indicated. Abbreviations: bla, gene encoding β-lactamase; Tc, tetracycline resistance marker; imm, part of immunity protein gene; clo, part of cloacin gene; T1 and T2, terminators of transcription derived from the cloacin operon; PIM, β-lactamase promoter; SD, Shine-Dalgarno sequence preceding BRP gene; Kb, kilobase. Terminator T1 of the cloacin operon which is located upstream of the hybrid BRP–β-lactamase gene in pJL12 has been cloned in the

FIG. 2. Autoradiogram of polypeptides synthesized in minicells harboring pJL12 or pTG2 in the absence or presence of 8.5% ethanol or globomycin (40 μg/ml). Lane A, pJL12; lane B, pJL12 plus ethanol; lane C, pJL12 plus globomycin; lane D, pTG2; lane E, pTG2 plus ethanol; lane F, pTG2 plus globomycin. The positions of β-lactamase (L) and its precursor (PL), the hybrid protein (HP) and its precursor (PHP), and marker proteins (molecular masses in kilodaltons) are indicated.
shown). Immunoblotting of subcellular fractions showed that the hybrid protein was located mainly in the total membrane fraction (Fig. 3). Only minor amounts were found in the periplasmic and cytoplasmic fractions, and the protein could not be detected in the culture supernatant fraction. Most of the hybrid protein was solubilized when the total membrane fraction was treated with either Triton X-100 or Sarkosyl at room temperature, which suggested that the hybrid protein was not an integral outer membrane protein. The total membrane fraction was also separated into cytoplasmic and outer membrane fractions, and the β-lactamase activity was determined. About 70% of the total activity was found in association with the outer membranes, and the remainder was found in the cytoplasmic membrane fraction.

To verify the localization of the hybrid protein, attempts were made to localize the hybrid protein in whole cells by using immunoelectron microscopy. Several different technical procedures were used, but unfortunately, the hybrid protein could not be localized properly because of its low abundance.

**Posttranslational modification of the BRP-β-lactamase hybrid protein.** The antibiotic globomycin is a specific inhibitor of the signal peptidase SPase II, which specifically cleaves lipid-modified prolipoproteins (8, 14). To obtain information on the possible lipoprotein nature of the pCloDF13-encoded BRP, the effect of globomycin on the processing of the precursor of the BRP-β-lactamase hybrid protein was studied (Fig. 4). Cells of *E. coli* N3406 harboring pJL12 accumulated the precursor of the hybrid protein when they were treated with globomycin before being labeled with [35S]methionine. In the absence of globomycin, the mature form of the hybrid protein, as well as its precursor, was detected on the gel. This showed that the processing of the precursor of the hybrid protein was not complete in cells in the absence of inhibitor, probably because of the short labeling period. Globomycin did not affect the expression of β-lactamase or the processing of its precursor (Fig. 4; 24). Comparable results were obtained when the effect of globomycin in minicells was studied (Fig. 2). These results indicated that the precursor of the hybrid protein is processed by SPase II and is, consequently, a prolipoprotein.

To obtain further evidence that the BRP is a lipid-modified protein, the incorporation of [3H]glycerol and [3H]palmitate into the hybrid protein was studied (Fig. 4). The hybrid protein was detected after cells harboring pJL12 were labeled with [3H]glycerol or [3H]palmitate, β-Lactamase expressed in cells harboring pTG2 could be labeled not at all with [3H]palmitate and only weakly with [3H]glycerol, probably because of nonspecific binding (24). The precursor form of the hybrid protein was not detected after labeling with these precursors, because the cells were labeled for a long period.

Preliminary experiments on the nature of the lipid modification indicated that about 75% of the label was lost after a mild alkali treatment of [3H]palmitate-labeled hybrid protein. This result suggested that palmitate was attached by both ester and amide linkages (1).

**DISCUSSION**

The pCloDF13-encoded BRP is essential for the release of bacteriocin cloacin DF13 and causes lysis of host cells when expressed in large amounts. The following observations led to the conclusion that this BRP and other BRPs encoded by Col plasmids are posttranslationally lipid modified and processed by the lipoprotein signal peptidase SPase II. (i) All
BRPs for which the primary structures have been deduced contain the tetrapeptide sequence Leu-X-Y-Cys at their putative signal peptide recognition site. In the consensus sequence of this tetrapeptide, X and Y are small neutral amino acids. This tetrapeptide is present in all lipoprotein precursors (42). (ii) Processing of the pColEl-P9-encoded BRP could be inhibited by globomycin (6). (iii) In E. coli, the pColEl-P9 and pColDF13-encoded BRPs have been localized in the membrane fraction of whole cells and minicells, respectively, despite the absence of large hydrophobic or amphophilic domains in the mature protein (6, 28).

In this paper we present evidence for the lipoprotein nature of the pClODF13-encoded BRP. Incorporation of [3H]glycerol and [3H]palmitate was found in a hybrid protein consisting of the almost complete pClODF13-encoded BRP and the mature portion of β-lactamase. Furthermore, processing of the BRP signal peptide from the hybrid protein precursor could be inhibited by globomycin. RTEM β-lactamase itself, encoded by pTG2, could not be labeled with radioactive lipid precursors, and the processing of its precursor could not be inhibited by globomycin, which is in agreement with results described previously (24).

Subcellular localization experiments revealed that the mature hybrid protein is located in both the cytoplasmic and outer membranes of E. coli cells. However, about 75% of the protein was found in the outer membrane. This finding corresponds well with the localization of the pClODF13-encoded BRP in E. coli minicells (28). Apparently, the cloned BRP portion is capable of directing β-lactamase, deprived of its own signal peptide, to the cell envelope and of anchoring the hybrid protein in the membranes. RTEM β-lactamase itself is located in the periplasm. The fact that the hybrid protein is fully active against ampicillin suggests that the β-lactamase portion of the hybrid protein protrudes into the periplasm and is in the correct conformation for catalytic activity. It is conceivable that the association of the hybrid protein with the outer membranes is due to the presence of the lipid moiety in the BRP portion of the hybrid protein, as is the case with Braun’s lipoprotein (4). The nature of the association of the hybrid protein to the inner membranes is unknown. Possibly, information within the structure of the mature portion of the BRP is involved in this association, or these hybrid proteins are not yet completely modified and processed and are therefore still bound to the cytoplasmic membranes.

Ghavreab and Inouye (11) constructed a fusion in which the signal sequence plus nine amino acids of the mature portion of the outer membrane lipoprotein were fused to the mature portion of the normally periplasmic β-lactamase. This hybrid protein was localized exclusively in the outer membrane of E. coli cells, and the β-lactamase portion of the hybrid protein was fully active. Assuming that the posttranslational modifications of the BRP and the lipoprotein are identical, the difference in the localization of the two hybrid proteins must be explained by the difference in the primary structures of the BRP and the murein lipoprotein.

All known BRPs possess strong sequence homology. Thus, posttranslational modification of BRPs with a glyceride fatty acid may be a general phenomenon. Yamada et al. (43) described the inhibition of release of colicin E1 by globomycin. They concluded that colicin E1, which contains an internal signal-like sequence, and lipoprotein use a common site for their translocation across the cytoplasmic membrane. However, our results suggest that globomycin inhibits the proper modification and processing of the pColEl-encoded BRP, which is required for colicin E1 export (34, 44). Excretion experiments (Oudega, unpublished data) also showed that suboptimal concentrations of globomycin which had no direct effect on cell lysis inhibited the release of colicin DF13 by induced cells and thus the functioning of the BRP.

Vernet et al. (39) constructed a fusion between the almost complete BRP of pColE3-CA38 (hic gene product) and human proinsulin. Attempts to characterize the hybrid protein were not successful, and cell lysis was observed after 60 to 90 min of induction. Results described in this paper strongly suggest that a higher level of expression of the BRP-β-lactamase hybrid protein than that observed in strain N3406 harboring pJLl2 results in cell death. Possibly, the BRP part of both hybrid proteins is responsible for this deleterious effect.

The experiments described in this paper indicate that the targeting and the functioning of the pClODF13-encoded BRP is aided by lipid moieties. Currently, we are constructing, by site-directed in vitro mutagenesis, several BRP mutants to further investigate the role of posttranslational lipid modification in the localization and functioning of the BRP. Analysis of a constructed mutant showed that the amino-terminal cysteine residue of the mature BRP is essential, since a replacement of this cysteine by a glycine residue resulted in a nonfunctional release protein.

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LITERATURE CITED