Proc. Natl. Acad. Sci. USA
Vol. 78, No. 6, pp. 3511–3515, June 1981
Biochemistry

Lipoprotein nature of *Bacillus licheniformis* membrane penicillinase

(β-lactamase/globomycin/enzyme secretion/protein processing/*Escherichia coli* lipoprotein)

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Communicated by P. Roy Vagelos, March 23, 1981

ABSTRACT **Membrane penicillinase (penicillin amido-β-lactamhydrolase, EC 3.5.2.6) from *Bacillus licheniformis* bears a striking resemblance to the major outer membrane lipoprotein of *Escherichia coli*. It can be specifically labeled *in vivo* with [^{3}H]glycerol, [^{35}S]cysteine, or [^{3}H]palmitate but not by [^{32}P]orthophosphate. The labeled residues are located at or near the NH_2 terminus of the membrane penicillinase because they can be completely removed by trypsin which cleaves a hydrophobic peptide(s) from the NH_2 terminus, thereby rendering the enzyme hydrophilic. The membrane penicillinase produced by the 749/C gene carried in *E. coli* on phage λ is similar to the enzyme formed in strain 749/C itself. The peptide antibiotic globomycin, which prevents processing of the *E. coli* prolipoprotein, severely inhibited the attachment of [^{3}H]palmitate or [^{3}H]glycerol to the 749/C enzyme (either in *B. licheniformis* 749/C or in *E. coli*), blocked the accumulation of penicillinase in the plasma membrane, and enhanced the formation of exoenzyme. Under the same conditions, globomycin does not prevent the attachment of palmitate or glycerol to the *E. coli* prolipoprotein but inhibits processing of the modified precursor to the mature lipoprotein. These results are in contrast with the lack of effect of globomycin on the RTEM-β-lactamase of *E. coli* which has no detectable hydrophobic membrane form and was not labeled with palmitate or glycerol.**

In a culture of *Bacillus licheniformis* 749/C, about half of the total penicillinase (penicillin amido-β-lactamhydrolase, EC 3.5.2.6) exists on the outer surface of the plasma membrane as a strongly hydrophobic form. Anchorage in the membrane has been reported (1) to be due to the presence of a phospholipopeptide segment attached to the NH_2-terminal residue of the usual exopenicillinase. This structure was proven to be incorrect by Simons *et al.* (2) who showed that no phosphorus was covalently attached to membrane penicillinase. They also presented the sequence of a hydrophilic breakdown product of membrane penicillinase that was 8 residues larger than the exoform and had an NH_2-terminal sequence differing from that of the reported NH_2-terminal phospholipopeptide. Simons' group, however, could offer no explanation of the strong hydrophobicity of the membrane enzyme other than that conferred by a putative stretch of hydrophobic amino acid residues. We (3) confirmed their partial sequence and noted that the phospholipopeptide was not part of the covalent structure of membrane penicillinase but probably was a contaminant from the cell wall or membrane.

We report here on the structure of membrane penicillinase. This report describes its similarities to the major outer membrane lipoprotein of *Escherichia coli* (4, 5) as revealed by *in vivo* radioisotopic labeling and by the effects of the peptide antibiotic globomycin on its formation. The effects on synthesis in *B. licheniformis* and in an *E. coli* strain carrying the cloned *Bacillus* penicillinase gene are compared with the effect of globomycin on synthesis and processing of the *E. coli* lipoprotein and the *E. coli* RTEM-β-lactamase.

MATERIALS AND METHODS

Bacterial Strains. *B. licheniformis* 749, inducible for penicillinase production, the penicillinase-constitutive mutant 749/C (ATCC 25972), and mutants 25 and 72 from 749/C were obtained from M. R. Pollock (6). The structural gene mutant 72 produces an inactive, immunologically crossreacting gene product; mutant 25 is a regulatory mutant and synthesizes about 0.1% as much penicillinase as does 749/C (7). The penicillinase-inducible *B. licheniformis* strain 6346 and the constitutive mutant 6346/C were from J. F. Collins. *E. coli* L956 [W3350 (λ pen att$^+$ srIλe° c1857 Qam73) from W. J. Brammar] is a K-12 derivative that carries phage L369 which contains the structural gene for 749/C penicillinase (8). This heat-inducible lysogen gives good β-lactamase production after 15 min incubation at 42°C. *E. coli* PRW-1 [strain C600 carrying the RTEM-β-lactamase on pBR322 (9)] was provided by C. E. Yeh.

Materials. [9,10-^{3}H]Palmitic acid (17.6 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels), [2-^{3}H]glycerol (9.5 Ci/mmol), and L-[^{35}S]cysteine (113.5 Ci/mmol) were purchased from New England Nuclear; [^{32}P]orthophosphate (carrier free) and L-[^{35}S]methionine (1350 Ci/mmol) were from Amersham. Globomycin was a gift of M. Arai (Sankyo, Tokyo).

Labeling of *B. licheniformis* Strains. Cultures were labeled with [^{3}H]palmitate (60 μCi/ml) and [^{3}H]glycerol (20 μCi/ml) during growth in CH/S medium at pH 6.5 as described by Izui *et al.* (3). Penicillinase was assayed by the method of Sargent (10). For phosphate labeling, the medium contained 25 mM Tris·HCl, 1% peptone (Difco), 1% sodium lactate, 0.05 M NaCl, 0.2 mM $MgSO_4$ adjusted to pH 6.5, and [^{32}P]orthophosphate at 0.5 mCi/ml. Just before use, 1 ml of 12 mM $MgSO_4$/9 mM $CaCl_2$ was added per liter. For incorporation of [^{35}S]cysteine and [^{35}S]methionine, cultures were grown in minimal medium at pH 7.1 containing (per liter): $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 6 g; K_2HPO_4, 14 g; sodium citrate, 1 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; thiamin, 1 mg; and 1 ml of Pollock's salts (11). Sodium glutamate and glucose (10% solutions) were autoclaved separately and were added to give final concentrations of 1% glutamate and 0.5% glucose.

At the end of the labeling period, cells were collected by centrifugation, resuspended in protoplasting buffer [60% (wt/vol) sucrose/0.02 M potassium phosphate, pH 6.5/10 mM $MgCl_2$] containing lysozyme at 250 μg/ml. Protoplast formation at 30°C was monitored by microscope. The protoplasts were harvested by centrifugation and disrupted by the addition of a small volume of 1% Triton X-100/50 mM Tris·HCl, pH 7.5/5

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Abbreviation: kDal, kilodalton(s).

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mM EDTA/0.15 M NaCl. The solution was cleared of particulate material by centrifugation at 100,000 × *g* for 45 min prior to antibody addition. Assay, immunoprecipitation, and Na-DodSO_4 gel electrophoresis were performed as described (3). When the isotope was ^3H, gels were enhanced prior to drying by soaking for 30 min in 1 M sodium salicylate (12).

Phenylethyl alcohol treatment was performed by a modification of the toluene method of Halegoua *et al.* (13, 14). *B. licheniformis* 749/C was grown in minimal medium as above to a cell density of 0.7 mg (dry weight)/ml which corresponds to the period of maximal synthesis of cell-bound penicillinase. The cells were spun down and resuspended in 0.1 vol of chilled 10 mM Tris·HCl, pH 8.0/60 mM NH_4Cl/10 mM $MgCl_2$/7 mM mercaptoethanol. Phenylethyl alcohol was added to 0.7% and the cells were kept 10 min in ice with occasional shaking. They were then added to an equal volume of double concentration reaction mixture at 30°C. The final reaction mixture was 2 mM ATP/0.2 mM GTP/10 mM $MgCl_2$/50 mM NH_4Cl/50 mM Tris·HCl, pH 8.0/5 mM dithiothreitol containing 19 amino acids, excluding methionine, at 2.5 mM each and [^{35}S]methionine at 15 μCi/ml. After 20 min at 30°C with occasional shaking, the cells were collected by centrifugation and the cell-bound material was extracted and immunoprecipitated as described for *in vivo* labeling.

Labeling of *E. coli* Strains. For measurement of penicillinase production and for labeling with palmitate or glycerol, *E. coli* L956 (λ pen) was grown in modified L broth containing (in g/liter): tryptone (Difco), 10; yeast extract (Difco), 5; NaCl, 5; maltose, 2; the medium was adjusted to pH 7.2 with NaOH (15). When globomycin was to be added, the level of NaCl was increased to 0.2 M to provide osmotic support. Cultures were grown at 30°C until a cell density of 2–3 × 10^8/ml was reached. The temperature was raised to 42°C for 15 min and then lowered to 37°C. Globomycin was added at the end of the 42°C induction period, and labeled palmitate or glycerol was added 30 min later when enzyme formation was well under way. *E. coli* PRW-1 (RTEM) was grown in nutrient broth (Difco)/0.2 M NaCl medium, pH 6.8.

Penicillinase was assayed after release from the cells. Unlike the situation in Gram-positive organisms, the Gram-negative β-lactamases, such as RTEM, are cryptic because the substrate does not penetrate the outer membrane. Total penicillinase was assayed in sonicated cells. For the separation of periplasmic and membrane-bound penicillinases, spheroplasts were prepared with lysozyme/sucrose/EDTA (16). Cells were resuspended in 0.2 vol of 0.5 M sucrose/10 mM Tris·HCl, pH 8.0. Lysozyme was added at 500 μg/ml and cells were incubated for 10 min in ice. An equal volume of 20 mM EDTA/10 mM Tris·HCl, pH 8.0, was added, and incubation on ice was continued until spheroplast formation was complete. The spheroplasts were pelleted, lysed by suspension in buffer without sucrose, and assayed for bound enzyme. The supernatant contained the periplasmic penicillinase.

RESULTS

Isotopic Labeling of Membrane Penicillinases. The strongly hydrophobic nature of membrane penicillinase led us to determine if lipophilic modification of the polypeptide chain occurs. Cells of *E. coli* (14) or *B. licheniformis* 749/C (unpublished results) treated briefly with phenylethyl alcohol are deficient in processing of precursor forms. Lane A in Fig. 1, a radioautograph of penicillinase forms from phenylethyl alcohol-treated cells incubated with [^{35}S]methionine, demonstrates the mobilities of the moderately hydrophobic precursor [34 kilodaltons (kDal)] and the more strongly hydrophobic membrane enzyme (32 kDal).

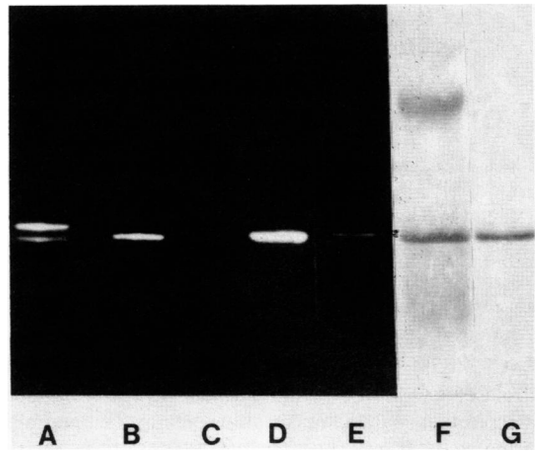

FIG. 1. Labeling of membrane penicillinase in *B. licheniformis* 749/C. A cell suspension incubated with phenylethyl alcohol (final concentration, 0.7%) prior to exposure to [^{35}S]methionine (50 μCi/ml) was used for lane A. Growing cultures were supplemented with [^3H]glycerol (lane B), [^{32}P]orthophosphate (lane C), [^3H]palmitate (lane D), or [^{35}S]cysteine (lane E). Cell-bound material was isolated, clarified by high-speed centrifugation after Triton solubilization, and immunoprecipitated. The immunoprecipitates were extracted twice at pH 9.0 with $CHCl_3/CH_3OH$, 2:1 (vol/vol), and boiled in 2% Na-DodSO_4 prior to electrophoresis on 10% gels. The gels were dried, stained (and enhanced when [^3H]glycerol or [^3H]palmitate was used), and radioautographed. Lanes F and G show Coomassie blue staining of immunoprecipitates of cell-bound penicillinase and purified membrane penicillinase, respectively.

When growing 749/C cultures were supplemented with [^3H]glycerol, [^3H]palmitate, or [^{35}S]cysteine, the 32-kDal band corresponding to membrane penicillinase became labeled. Exopenicillinase, which lacks cysteine residues (17), was devoid of radioactivity in all three cases. When [^{32}P]orthophosphate was present during growth, phospholipids became heavily labeled but there was no radioactivity coincident with membrane penicillinase after repeated extraction with $CHCl_3/CH_3OH$ (18) at pH 9.0 and subsequent electrophoresis.

All three labels were completely removed from the membrane penicillinase by treatment with trypsin (data not shown). This enzyme cleaves a small peptide or peptides from the NH_2 terminus, resulting in the loss of all hydrophobic properties (1–3). Thus, the labeled residues (Fig. 1) were specifically attached to the NH_2-terminal region or to a structure tightly associated to it and unseparable by Na-DodSO_4 gel electrophoresis after boiling in 2% Na-DodSO_4 and subsequent organic extraction, a procedure which removes all detectable phospholipid and teichoic acid.

Further evidence that the incorporated fatty acid was specifically associated with penicillinase is shown in Fig. 2. Upon induction, the inducible strain 749 forms immunoprecipitable penicillinase detectable by enzymatic activity and a protein band at 32 kDal. Palmitate was incorporated into a 32-kDal band in the presence but not in the absence of inducer. The same is true of the related inducible strain 6346 (6) which produces the other main type of penicillinase characteristic of *B. licheniformis*. This enzyme is antigenically cross-reactive with the 749/C form but readily distinguishable in substrate specificity and, as with strain 749, approximately 50% of the total enzyme activity is bound to the membrane. Structural gene mutant 72 of *B. licheniformis* 749/C (7), known to produce an inactive but crossreacting penicillinase protein, incorporated palmitate into the immunoprecipitable band at 32 kDal. Regulatory mutant 25, which forms essentially no penicillinase protein (7), showed no labeling at 32 kDal.

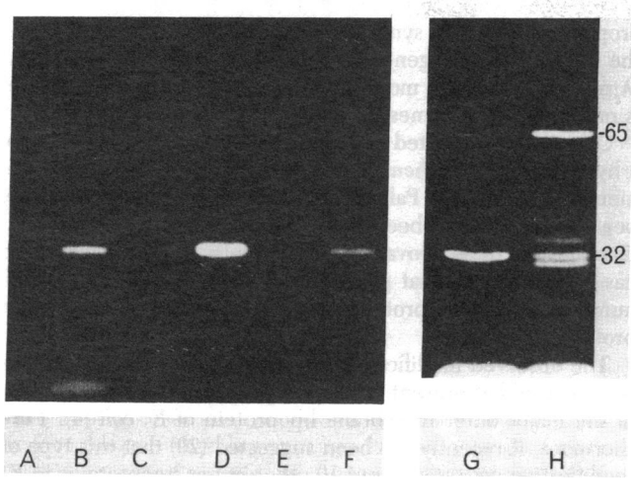

FIG. 2. Specificity of [^{3}H]palmitate labeling of membrane, penicillinases in various *B. licheniformis* strains (lanes A–F). Equal volumes of cultures at similar cell densities were protoplasted; cell-bound material was extracted and immunoprecipitated with antibody to 749/C penicillinase. The precipitates were extracted with $CHCl_3/CH_3OH$ at pH 9.0, electrophoresed, and fluorographed. Lanes A, immunoprecipitate from 749 cells grown without inducer; B, 749 cells with inducer; C, strain 6346 without inducer; D, 6346 with inducer; E, mutant 25; and F, mutant 72. The gratuitous inducer 2(2'-carboxylphenyl)benzoyl-6-aminopenicillanic acid (5 μM) was used. Lanes G (control) and H (globomycin) show the effect of globomycin (50 μg/ml, conditions as in Fig. 3) on palmitate labeling of total (not immunoprecipitated) cell-bound proteins in strain 749/C. The samples were taken at 4 hr in the experiment shown in Fig. 3. Numerals at right are size in kDal.

The patterns of labeling with [^{3}H]palmitate obtained by using immunoprecipitation (Fig. 2) were also evident when total cell extracts were subjected to $NaDodSO_4$ gel electrophoresis (data not shown) because only a few proteins of *B. licheniformis* contain a substantial amount of radioactivity from palmitate. Under our usual culture conditions, membrane penicillinase is the most prominently labeled protein in strains 749/C and 6346/C and in induced cells of strains 749 and 6346.

Inhibition of Processing by Globomycin. Fig. 3 shows the time course of penicillinase production in 749/C cultures in relation to growth and palmitate incorporation in the absence and in the presence of globomycin at 50 μg/ml. Growth and total penicillinase were unchanged in the presence of globomycin, but there was a sharp reduction in the amount of cell-bound enzyme produced and a corresponding increase in the penicillinase secreted into the medium. The incorporation of palmitate into the membrane enzyme was sharply reduced by globomycin; labeling of other, minor lipoproteins was increased (Fig. 2, lanes G and H). The sizes of both bound and released forms were not detectably altered in the presence of globomycin. The secreted enzyme is the exo-large form (3) which has a molecular weight, based on sequence, of 30,500 but runs on $NaDodSO_4$ gels as 32,000, indistinguishable from membrane penicillinase.

Sensitivity of 749/C Penicillinase Synthesis in *E. coli* to Globomycin. The effect of globomycin on the formation of periplasmic and bound penicillinases in a heterologous setting—i.e., with the *B. licheniformis* 749/C gene in λ phage-infected *E. coli*—is shown in Fig. 4. During the 3-hr period of message expression possible in a λ Qamber mutant, nearly all the penicillinase remained membrane bound (not released on spheroplast formation with lysozyme/EDTA) and was modified as in the homologous system. Glycerol and palmitate were incorporated into a penicillinase band indistinguishable from the

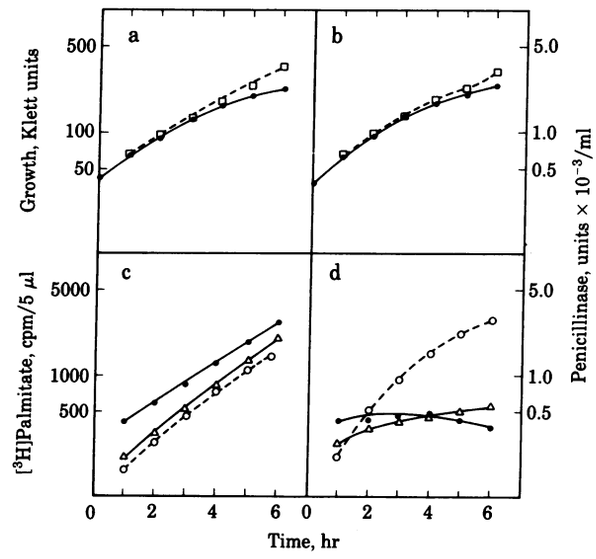

FIG. 3. Growth, penicillinase production, and [^{3}H]palmitate incorporation into hot trichloroacetic acid-insoluble material in cultures of *B. licheniformis* 749/C. (*a* and *c*) No globomycin, (*b* and *d*) Globomycin at 50 μg/ml added at 0 hr. [^{3}H]Palmitate in 2% Tween-20 was added at 0.5 hr to 60 μCi/ml. (*a* and *b*) Growth (●——●, Klett units) and total penicillinase (□– – –□, units/ml). (*c* and *d*) Bound penicillinase (●——●, units/ml), exopenicillinase (○– – –○, units/ml), and palmitate incorporation (△——△, cpm/5 μl).

Bacillus product in mobility on $NaDodSO_4$ gel electrophoresis, in hydrophobicity on octyl-Sepharose, and in cleavage by trypsin to a hydrophilic 29.5-kDal product lacking glycerol or palmitate (data not shown).

Globomycin at 25 μg/ml caused an increase in the release of *hydrophilic* penicillinase into the periplasm and a decrease

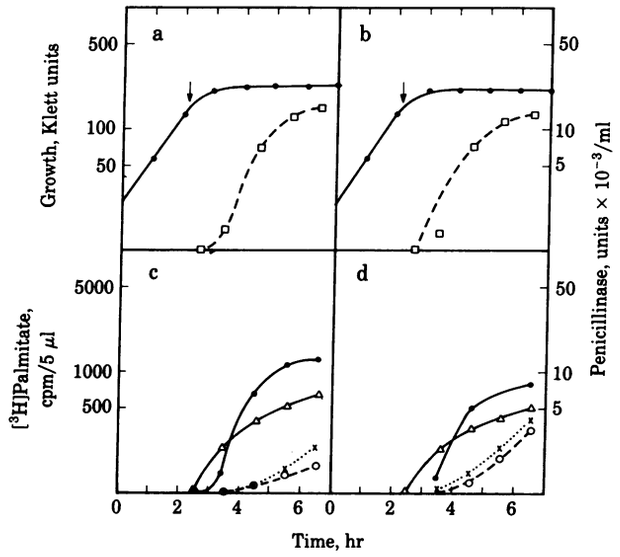

FIG. 4. Growth, penicillinase production, and [^{3}H]palmitate incorporation in cultures of *E. coli* L956. (*a* and *c*) No globomycin. (*b* and *d*) Globomycin at 25 μg/ml. Heat induction (42°C) of the β-lactamase-carrying lysogen was started at 2 hr. At the end of the induction period, the cells were returned to 37°C and then globomycin was added (↓). Fifteen minutes later, at 2.5 hr, [^{3}H]palmitate was added at 60 μCi/ml. (*a* and *b*) Growth (●——●, Klett units) and total penicillinase (□– – –□, units/ml). (*c* and *d*) Bound penicillinase (●——●, units/ml), periplasmic penicillinase (○– – –○, units/ml), penicillinase released into the medium by cell lysis (×·····×, units/ml), and palmitate incorporation (△——△, cpm/5 μl).

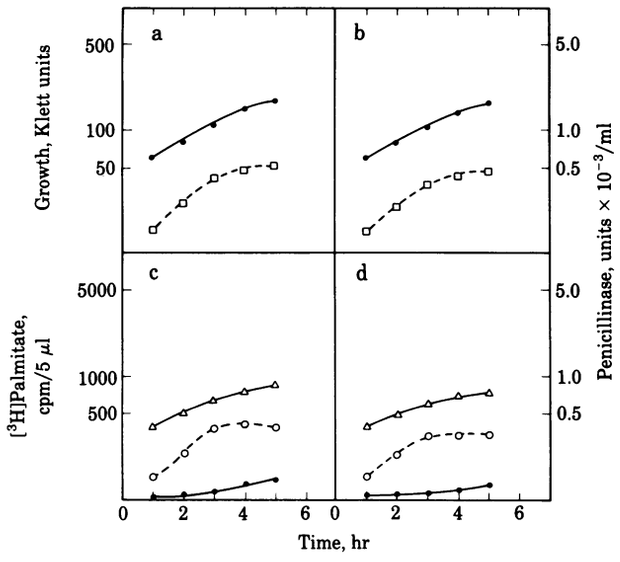

FIG. 5. Growth, penicillinase production, and [^{3}H]palmitate labeling in cultures of *E. coli* PRW-1 carrying RTEM-β-lactamase. (*a* and *c*) No globomycin. (*b* and *d*) Globomycin at 150 μg/ml added at $t = 0$. [^{3}H]Palmitate at 60 μCi/ml was added at 0.5 hr. (*a* and *b*) Growth (●——●, Klett units) and total penicillinase (□– – –□, units/ml). (*c* and *d*) Bound penicillinase (●——●, units/ml), periplasmic penicillinase (○– – –○, units/ml), and palmitate incorporation (△——△, cpm/5 μl).

in the accumulation of membrane penicillinase (Fig. 4). The *E. coli* host, a K-12 derivative, is relatively insensitive to globomycin, and 100–150 μg/ml is required for substantial inhibition of the processing of the various outer membrane lipoproteins (19, 20). However, globomycin does cause membrane fragility at concentrations down to 50 μg/ml and, when the cells are further stressed by induction of the lysis-defective phage, substantial lysis occurs. As a result there is no ideal concentration at which to measure the effect of globomycin on processing and export of penicillinase, but with antibiotic levels giving partial inhibition (Fig. 4) the effect in this heterologous system is the same as in the *Bacillus:* specifically, modification of penicillinase and insertion into the membrane are depressed.

Absence of Lipoprotein Form of RTEM-β-Lactamase. The formation and secretion of RTEM-β-lactamase in *E. coli* were examined because little of this enzyme is retained on the plasma membrane. RTEM-β-lactamase has a signal sequence that contains a cysteinyl residue (9) but not the -Leu-Ala-Gly-Cys- segment at which modification and cleavage of the outer membrane prolipoprotein occur (5). Globomycin at 150 μg/ml did not noticeably affect release of the RTEM enzyme into the periplasm (Fig. 5). In contrast to its enhancement of release of the bacillus enzyme in bacilli and in *E. coli*, globomycin caused a slight depression of the release of hydrophilic RTEM enzyme and this corresponded to the slight depression of total synthesis. No palmitate label could be detected in immunoprecipitates of the small amount of enzyme (<5%) that remained cell bound after spheroplast formation.

DISCUSSION

We have investigated the general nature of the NH_2-terminal region of *B. licheniformis* membrane penicillinase by examining the *in vivo* incorporation of potential precursors. The polypeptide chain contains a cysteinyl residue at or near the NH_2 terminus as well as glycerol and fatty acid residues. This derivatization occurs in growing *B. licheniformis* cells and leads to the retention, on the outer side of the plasma membrane, of about 50% of the total penicillinase of the culture. An identical hydrophobic product is synthesized in an *E. coli* host into which the *B. licheniformis* gene has been introduced by a λ lysogen (λ pen). In this case, more than 90% of the total penicillinase is modified and becomes membrane bound.

Others have reported (21) that glycerol is incorporated into a hydrophobic *B. licheniformis* penicillinase, presumably the membrane enzyme. Palmitate incorporation into membrane penicillinase has not been noted before; in fact, Simons *et al.* (2) could not detect covalently bound fatty acids. However, it has been reported that palmitate is incorporated into a small number of viral lipoproteins (22) and mammalian membrane proteins (23).

The observed modification of *B. licheniformis* penicillinase is similar to that present on the NH_2-terminal cysteine residue of the major outer membrane lipoprotein of *E. coli* (4). Furthermore, it recently has been suggested (20) that this type of modification occurs in about 10 other minor lipoproteins in *E. coli*, and we find that similar minor components are present in extracts of *B. licheniformis* strains (data not shown). It appears, then, that the addition of glycerol esterified with long-chain fatty acids may be a widespread phenomenon that confers upon polypeptide chains a hydrophobic domain sufficient to ensure attachment to the cell membrane. The present report demonstrates this phenomenon in Gram-positive organisms.

The initial translation product of *B. licheniformis* penicillinase mRNA is 34 residues longer than the largest detectable product secreted *in vivo* (summarized in ref. 24). This is unusually long for a simple signal sequence, generally 18–25 amino acids in length (for a review on signal sequences in prokaryotes see ref. 25). We propose that the 34 residues comprise both a standard signal sequence and a membrane-anchoring region. Removal of the signal segment may give rise to a new NH_2-terminal residue which has already been modified with diacyl glycerol or will be modified subsequently, concomitantly with anchorage to the membrane.

Ichihara *et al.* (20) proposed that the antibiotic globomycin interacts with the -Leu-Ala-Gly-Cys(diacylglycerol)- segment of *E. coli* prolipoprotein and of similar regions in about 10 other minor lipoproteins of *E. coli*, and they reported that precursors carrying the glyceride modification accumulate in the presence of globomycin. We find that globomycin prevents the net incorporation of both glycerol and palmitate into membrane penicillinase without affecting total penicillinase production. Furthermore, we find no evidence of glycerol or palmitate labeling in precursor forms detectably larger than membrane penicillinase in either *B. licheniformis* or *E. coli*. These differences may reflect differences in relative rates of the critical processing steps for the two proteins or perhaps differences in sensitivities of individual steps to globomycin. The simplest interpretation of our results is that globomycin blocks the derivatization of prepenicillinase (and subsequent membrane attachment) and renders the unmodified precursor more available to the peptidase that normally produces exopenicillinase. Earlier experiments (26) support the conclusion that the membrane form is not an obligatory intermediate in the secretion of penicillinase.

Lai *et al.* (27) reported that *B. licheniformis* membrane penicillinase produced in *E. coli* (λ pen) is a lipoprotein that contains cysteinyl glyceride thioether, in agreement with our results. The incorporation of [2-^{3}H]glycerol into the membrane penicillinase of their strain of *B. licheniformis* 749/C, however, was low. Consequently, we have also demonstrated, with our highly active strain, the presence of glyceryl cysteine (by isolation of glyceryl cysteine sulfone as in ref. 27) in the membrane enzyme of *B. licheniformis* 749/C. It is clear that the modification reactions are not restricted to Gram-negative bacteria.

We thank Dr. S. Mizushima for providing a manuscript in advance of publication and for helpful discussions, Dr. M. Arai for his generous gift of globomycin, and Ms. Julia Sohm for skilled technical assistance. This work was supported by grants from the National Institutes of Health (AI-04572), from Miles Laboratories, and from the Charles and Johanna Busch Fund.

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