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Glyceride-Cysteine Lipoproteins and Secretion by Gram-Positive Bacteria

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The membrane penicillinases of *Bacillus licheniformis* and *Bacillus cereus* are lipoproteins with N-terminal glyceride thioether modification identical to that of the *Escherichia coli* outer membrane lipoprotein. They are readily labeled with [^{3}H]palmitate present during exponential growth. At the same time, a few other proteins in each organism become labeled and can be detected by fluorography after sodium dodecyl sulfate-polyacrylamide gel electrophoresis of total membrane proteins. We distinguish these proteins from the O-acyl proteolipids by demonstrating the formation of glyceryl cysteine sulfone after performic acid oxidation and hydrolysis of the protein. By this criterion, *B. licheniformis* and *B. cereus* contain sets of lipoproteins larger in average molecular weight than that of *E. coli*. Members of the sets probably are under a variety of physiological controls, as indicated by widely differing relative labeling intensity in different media. The set in *B. licheniformis* shares with membrane penicillinase a sensitivity to release from protoplasts by mild trypsin treatment, which suggests similar orientation on the outside of the membrane. At least one protein is the membrane-bound partner of an extracellular hydrophilic protein, the pair being related as membrane and exopenicillinases are. We propose that the lipoproteins of gram-positive organisms are the functional equivalent of periplasmic proteins in *E. coli* and other gram-negative bacteria, prevented from release by anchorage to the membrane rather than by a selectively impermeable outer membrane.

Covalent modification of a cysteine residue with a glyceride thioether group was first described by Hantke and Braun (11) in 1973 for the major outer membrane protein of *Escherichia coli*. It is responsible for the hydrophobic properties of the mature lipoprotein, ensuring its anchorage in the outer membrane. It is now evident that *E. coli* possesses a class of such proteins, about 10 in number, that are less abundant than the Braun lipoprotein, but similar in structure, as shown by radioisotopic labeling with glycerol and long-chain fatty acids and by similar responses to the cyclic peptide antibiotic globomycin (12). Lai et al. (19) noted that the apparently identical modification could be found on *Bacillus licheniformis* membrane penicillinase when expressed in *E. coli* after cloning on a gamma vector. Simultaneously, we reported the same finding (20) and, furthermore, that the same glyceride thioether modification was present in the membrane-bound form of *B. licheniformis* penicillinase in the gram-positive organism itself, accounting for the hydrophobic properties of the cell-bound form. We have recently extended this observation (21) to the membrane penicillinases of two other class A penicillinases (1), those of *Bacillus cereus* (type I) and *Staphylococcus aureus* (types A and C). In all cases so far described, a cysteine residue near the junction of the hydrophobic membrane-spanning (10) stretch of the signal peptide and a hydrophilic bridge of conserved amino acids (21) become modified by an acyl glyceride residue. The signal peptidase of *B. licheniformis* cleaves the in vitro translation product of the *penP* gene just before this cysteine residue (5) and, by analogy with the well-characterized *E. coli* lipoprotein, functions at the same site in vivo to render this cysteine N-terminal in the mature membrane protein. In *E. coli*, the membrane form appears to be the final destination of most lipoproteins. In the three gram-positive organisms, however, the membrane-bound penicillinase is a stage of the secretory process in that the modified protein is largely cleaved from its site on the outside of the membrane late in the growth cycle and released as a hydrophilic product into the medium. Several lines of evidence point to the membrane-attached form not being an obligatory intermediate, but on an alternative pathway to secretion (7, 20).

In examining the penicillinases of *B. licheniformis*, *B. cereus*, and *S. aureus*, we noticed that similarly to *E. coli*, each of these organisms

possesses a set of apparent lipoproteins. In this paper, we describe the properties of these sets in the two bacilli.

MATERIALS AND METHODS

Bacterial strains. *B. licheniformis* 749 (penicillinase inducible) and 749/C (ATCC 25972; penicillinase constitutive) were obtained from M. R. Pollock. *B. cereus* 569 (ATCC 27348; type I, penicillinase inducible) was obtained from N. Citri.

Media. CH/S medium contained 10 g of acid-hydrolyzed Casamino Acids per liter (Difco Laboratories, Detroit, Mich.), 20 mM KH_2PO_4, 10 mM $MgCl_2$, 0.1% Pollock's salts (23), pH 6.5. Low-phosphate Casitone medium was prepared by precipitation with magnesia, using a modification of the method of Takeda and Tsugita (26). Magnesia was prepared by adding concentrated NH_4OH to a solution of 10 g of $MgCl_2 \cdot 6H_2O$ and 20 g of NH_4Cl in 100 ml of water until alkaline. After standing at 4°C overnight, the solution was filtered, adjusted to pH 6.0 with HCl, and diluted to 200 ml. For 1 liter of phosphate-free medium, 20 ml of this solution was added to 10 g of Casitone (Difco) in 10 ml of water, stored at 4°C for 2 days, and centrifuged at 25,000 × *g* for 1 h. The supernatant was carefully decanted, 6 g of Tris and 3 g of NaCl were added, and the solution was centrifuged again as described before. 1-ml salt solution containing (in 100 ml) $FeSO_4 \cdot H_2O$ (100 mg), $ZnSO_4 \cdot H_2O$ (100 mg), $MnSO_4 \cdot 4H_2O$ (10 mg), K_2CrO_7 (0.2 mg), and $CuSO_4 \cdot H_2O$ (5 mg), was added. The pH was adjusted to 7.0 and the volume was adjusted to 1 liter before autoclaving. Separately autoclaved $MgSO_4$ (1.2 mM), $CaCl_2$ (0.8 mM), and glucose (0.1%) were added. The inoculum was grown in the same medium as above, except that glucose was omitted and 2 mM KH_2PO_4 was added. The cells were pelleted and washed before use for inoculation. Without this treatment, the phosphate level in Casamino Acid medium is about 0.5 mM, sufficient for substantial repression; after treatment, it is less than 0.05 mM and allows the production of more than 200 U of alkaline phosphatase per mg (dry weight) of cells. The assay was that described by Ghosh et al. (9). In this low-phosphate medium, penicillinase production was about 30% lower than in CH/S medium containing 20 mM phosphate.

L-broth contained (per liter) 10 g of tryptone, 5 g of yeast extract (Difco), and 5 g of NaCl, pH 7.2. Brain heart infusion broth contained 37 g/liter of the BBL product.

Materials. [9,10-^{3}H]palmitic acid (11.8 Ci/mmol), and L-[^{35}S]cysteine (1,108.5 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass., and L-[^{35}S]methionine (1,100 Ci/mmol) was purchased from Amersham Corp., Arlington Heights, Ill. Globomycin was a gift of M. Arai (Sankyo, Tokyo, Japan), antibody to *B. licheniformis* 749/C alkaline phosphatase was a gift of B. K. Ghosh (Rutgers Medical School). Penicillin-binding proteins from *B. licheniformis* 749 labeled with [^{14}C]benzylpenicillin were prepared at the Squibb Institute for Medical Research, Lawrenceville, N.J., by N. Georgopapadakou (8).

Antibodies. Antibody to purified exopenicillinase was prepared as previously described (14). The 63-kilodalton (kd) lipoprotein was obtained from partially purified membrane penicillinase preparations (through DEAE-Sephadex chromatography [25]) that were subjected to sodium dodecyl sulfate-gel electrophoresis. Unstained bands were excised with a stained 63-kd band on the edge of the gel as a guide. About 100 μg of the protein could be isolated from one 3-mm gel. A total of 400 μg was used to prepare antibodies by injecting the macerated gel intradermally (27).

Isotopic labeling. Palmitate labeling was performed in 2.5-ml cultures by adding isotope at 60 μCi/ml. Palmitic acid in benzene was dried in a nitrogen stream and dissolved in 2% Tween 20. In phosphate-free medium, the usual level of Tween (0.016%) caused lysis and had to be reduced to 0.004%. Several rinses with the culture were made to ensure complete transfer of the palmitate. Different media were used as noted in the figure legends. [^{35}S]cysteine labeling for the subsequent isolation of glyceryl cysteine sulfone was performed in CH/S medium containing acid-hydrolyzed casein, because acid hydrolysis yields an amino acid mixture virtually free of cysteine. After 2 generation times, labeling in this case was terminated by the addition of 20 mM cysteine, which was present also in all subsequent steps of the isolation. Cells were harvested and converted to protoplasts as previously described for *B. licheniformis* and *B. cereus* (20). The protoplasts were disrupted by boiling in 2× electrophoresis buffer, and the extracts were electrophoresed on 10% sodium dodecyl sulfate gels (17). Extracts containing [^{3}H]palmitate and those with [^{35}S]cysteine were never analyzed in the same gel, because [^{35}S]cysteine binds nonspecifically to proteins in low amounts. The slightest contamination with ^{35}S overburdens the weaker ^{3}H signal in autoradiography. [^{3}H]palmitate-containing gels were enhanced with sodium salicylate (4) before being dried, autoradiographed, and scanned on a Schoeffel Spectrodensitometer SD3000. Gels with [^{35}S]cysteine were dried without enhancement and were autoradiographed overnight. The dried lanes were cut into 0.5-cm strips, the protein was eluted, and sodium dodecyl sulfate was removed. The protein was then oxidized with performic acid, hydrolyzed with 6 N HCl, and subjected to high-voltage electrophoresis as previously described (21). Six 1-cm strips were cut from the paper in the regions of glyceryl cysteine sulfone and of cysteic acid for assay of ^{35}S as an indicator of the presence of the substituted sulfone.

RESULTS

Labeling of membrane proteins with palmitate. When [^{3}H]palmitate is present during exponential growth of *B. licheniformis* or *B. cereus*, several proteins become covalently labeled with fatty acid. Autoradiograms of sodium dodecyl sulfate gels of membrane proteins from these organisms (grown in CH/S medium) are shown in Fig. 1. With *B. licheniformis* 749 induced to form penicillinase, the labeled band corresponding to the membrane-bound enzyme can be seen at 32 kd (Fig. 1, lane A; compare with extract of uninduced cells in lane B); mutant 749/C, magnoconstitutive for penicillinase synthesis, produces more of this band (Fig. 1, lane D). Globomycin, a peptide antibiotic (13) whose effect appears limited to those proteins bearing a glyc-

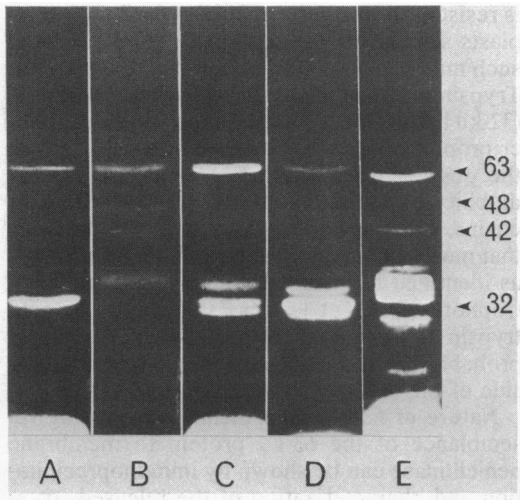

FIG. 1. Labeling of membrane proteins in *B. licheniformis* and *B. cereus* cells with [^{3}H]palmitate. Cultures (2.5 ml) in CH/S medium were incubated with [^{3}H]palmitate at 60 μCi/ml for two generations. Cells were harvested, and membrane extracts were electrophoresed and fluorographed as described in Materials and Methods. Lane A, membrane proteins from *B. licheniformis* 749 induced for penicillinase synthesis by 5 μM 2-(2'-carboxyphenyl)benzoyl-6-aminopenicillanic acid; lane B: same as lane A but without inducer; lane C, extract of *B. licheniformis* 749/C cells incubated with globomycin (50 μg/ml); lane D, same as lane C without globomycin; and lane E, extract, of *B. cereus* 569 cells grown without inducer. Sizes are shown in kilodaltons deduced from Coomassie blue-stained standards and from membrane penicillinase at 32 kd.

eryl cysteine modification, depresses the accumulation of membrane penicillinase (20) and the labeling of the 32-kd band by palmitate (Fig. 1, lanes C and D). In constrast, its presence increased palmitate labeling of a 63-kd protein. The related *B. cereus* penicillinase, type I (1), behaves in an analogous manner (21). It is evident from the intensity of labeling in induced cells of *B. licheniformis* 749 and in 749/C that the most abundant fatty acid-substituted membrane protein is penicillinase, but that there are several other minor but discrete bands in addition to the phospholipid band which runs near the front in the gel system used. The [^{3}H]palmitate is not extractable from these bands (20 to 63 kd) by $CHCl_3/CH_3OH$ (3:1) at pH 9.0 (3). With both bacilli, several bands are clustered around 35 kd, more in *B. cereus* (Fig. 1, lane E) than in *B. licheniformis*, and with each organism there is a prominent band at 63 kd. In *B. licheniformis*, none of the minor protein bands above or below penicillinase (32 kd) is immunologically related to penicillinase (20). *S. aureus*, whose membrane penicillinase we have shown to be a lipoprotein, also yielded a cluster of minor bands around 35 kd (21), unrelated immunologically to penicillinase, but there were very few palmitate-labeled bands of higher molecular weights.

Thioether nature of lipoprotein bands. To determine whether the sets of palmitate-labeled bands represent a class of lipoproteins of the *E. coli* type, cultures of *B. licheniformis* 749 were separately labeled with [^{35}S]cysteine and [^{3}H]palmitate in the absence of an inducer for penicillinase. (Without the dominant penicillinase band, other bands in the 32-kd region of the gel may be examined more readily.) The extracted membrane proteins were subjected to electrophoresis. Serial segments of the ^{35}S-labeled gel were analyzed for [^{35}S]glyceryl cysteine sulfone after oxidation and acid hydrolysis. The recovery as the substituted sulfone, plotted against mobility in a 10% gel and superimposed on a densitometer scan of the ^{3}H-labeled proteins, is shown in Fig. 2 (upper panel). A similar experiment with an uninduced *B. cereus* 569 culture is shown in the lower panel. It can be seen that with every palmitate band from both bacilli, there is [^{35}S]glyceryl cysteine sulfone.

Factors affecting formation of lipoproteins. The wide variations in intensity of palmitate labeling (Fig. 2) suggest that the bands fall into at least several classes of physiological control. Figure 3 compares densitometer scans of palmitate radioactivity incorporated into membrane proteins of *B. licheniformis* 749 in three media providing increasing growth rates. Labeling of the bands increased in the series from CH/S medium to L-broth to brain heart infusion broth; the one exception (63-kd band) showed much less labeling in rich media than in CH/S. No new bands emerged in the rich media, except for the appearance of [^{3}H]palmitate labeling at 20 kd as a single broad peak in L-broth and a doublet in brain heart infusion broth. However, even in CH/S, this 20-kd protein(s) showed glycerol substitution of the cysteine despite the lack of labeling from the palmitate pool (Fig. 2).

We examined one specific growth medium with the possibility in mind that the membrane-bound alkaline phosphatase of *B. licheniformis* (9) or one of its accessory proteins (A. Ghosh and B. K. Ghosh, personal communication) may be a lipoprotein. Bacilli secrete a large portion of their total alkaline phosphatase activity into the medium and, as in the case of penicillinase, retain the rest as a membrane-bound form. This form differs, however, from penicillinase in being extractable by 1 M $MgSO_4$ rather than by detergents. Under conditions derepressive for alkaline phosphatase synthesis (Fig. 4), the palmitate labeling of most bands other than penicillinase (the prominent band at 32 kd; the constitutive penicillinase producer 749/C was used) is markedly reduced. The bands thus affected are

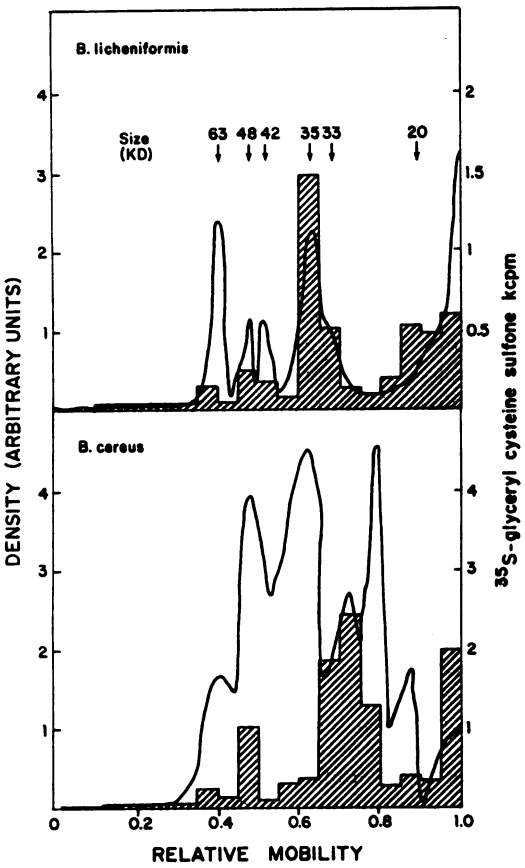

FIG. 2. Congruence of palmitate labeling and glyceryl cysteine thioether in membrane proteins from cultures of *B. licheniformis* and *B. cereus* uninduced for penicillinase. The techniques used are described in Materials and Methods. [^{3}H]palmitate labeling was carried out as described in the legend to Fig. 1 and [^{35}S]cysteine was added to 50 μCi/ml; both were in CH/S medium. The densitometer scan of ^{3}H activity is represented by the solid line, and ^{35}S activity is represented by the shaded bars.

not phospholipids because no covalently attached ^{32}P, was incorporated into any of these bands (results not shown). Cells grown in 0.05 mM phosphate medium did not release palmitate label during incubation with 1 M $MgSO_4$, although a complex of alkaline phosphatase and associated proteins was extracted from the membrane. Thus, none of the lipoproteins we observe in *B. licheniformis* appears to have any connection to the alkaline phosphatase system.

Cellular location of lipoproteins. Membrane penicillinase is located on the outside of the cell membrane and is readily released from protoplasts by trypsin as a soluble exoenzyme with the N-terminal, lipid-containing peptide cleaved off and presumably remaining attached to the membrane (2). The released soluble penicillinase is resistant to further proteolysis, and the protoplasts sustain very little damage. The effects of such mild trypsinolysis are illustrated in Fig. 5a. Trypsin released about 75% of the penicillinase (32-kd band) from the membrane and even greater proportions of all the other lipoproteins with the possible exception of a small peak at 0.8 mobility. The palmitate label, now on short chains, has migrated off the gel. It is obvious that most of the lipoprotein class of strain 749/C, as identified by palmitate labeling and gel electrophoresis, are at least as susceptible to mild trypsin treatment as is penicillinase and thus probably have a similar orientation on the outside of the cell membrane.

Nature of 63-kd lipoprotein. The striking resemblance of the 63-kd protein to membrane penicillinase can be shown by immunoprecipitation and characterization of the released, trypsin-stable core molecule (Fig. 5b). *B. licheniformis* 749/C cells were labeled in CH/S for one generation with [^{35}S]methionine, and a mem-

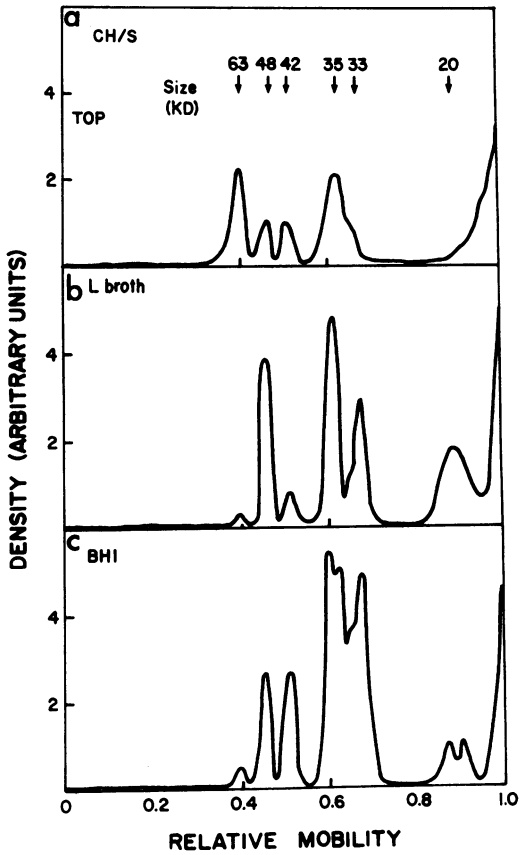

FIG. 3. Incorporation of [^{3}H]palmitate into membrane proteins of *B. licheniformis* 749 (uninduced) in three media. The experimental procedures are described in the legend to Fig. 1. Densitometer scans of fluorographed gels are shown.

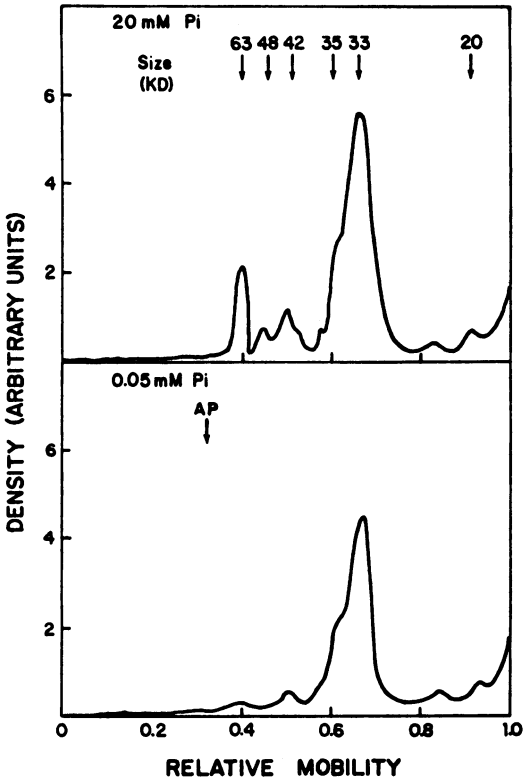

FIG. 4. Effect of phosphate concentration on palmitate labeling of *B. licheniformis* 749/C. As in Fig. 3, 2.5-ml cultures contained 60 μCi/ml [^{3}H]palmitate in CH/S medium (20 mM P_i) or in phosphate-free CH/S (see Materials and Methods) which is derepressive for alkaline phosphatase synthesis. AP, Alkaline phosphatase.

brane extract was prepared. One half was treated with a mixture of antibody to penicillinase and antibody to the 63-kd protein, and the remainder was treated with trypsin before immunoprecipitation with the mixed antibodies. It can be seen that for both molecules, the core resistant to further trypsin digestion (Fig. 5b, lane B) is 2 to 3 kd smaller than the intact molecule (Fig. 5b, lane A). A similar experiment using [^{3}H]palmitate labeling showed the total elimination of high-molecular-weight acyl groups from both molecules after trypsin treatment (data not shown). The 63-kd protein had attracted our attention during purification of membrane penicillinase because it is the last protein to be removed and is very similar to penicillinase in all the properties examined. On gel filtration in detergents, the two proteins, despite their size difference, copurify in a mixed micelle. They are not immunologically cross-reactive, and there is no evidence for the larger being a penicillinase dimer. Peptide patterns following digestion of the two proteins with *S. aureus* V8 protease (6) showed no obvious bands in common between the two proteins.

A hydrophilic protein of about 60-kd appearing in the medium toward the end of growth is precipitable by the antibody prepared to the 63-kd lipoprotein. These two proteins then are presumably the released and membrane-bound forms of a secreted enzyme, related just as exo- and membrane-bound penicillinase. Two dissimilarities in behavior between penicillinase and the 63-kd protein point to different mechanisms controlling their synthesis or fatty acid acylation. As mentioned earlier, whereas labeling of

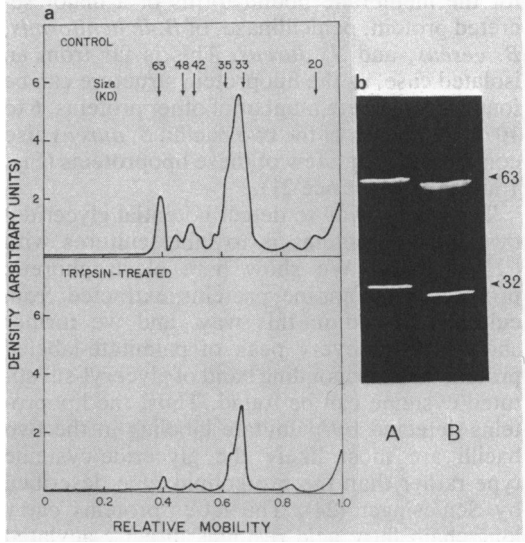

FIG. 5. (a) Cleavage of palmitate-labeled proteins on protoplasts of *B. licheniformis* 749/C by trypsin. Protoplasts prepared from 2.5-ml cultures, labeled as previously described, were suspended in osmotically supported medium (2) and incubated with and without 40 μg of trypsin per ml for 30 min at 37°C. Trypsin cleavage was terminated by the addition of 20 μl of 0.1 M diisopropyl fluorophosphate. Membrane extracts were prepared, electrophoresed, and fluorographed as described in the legend to Fig. 1. (b) The 63-kd lipoprotein has a trypsin-resistant core analogous to that of membrane penicillinase. A culture of *B. licheniformis* 749/C was incubated with [^{35}S]methionine (50 μCi/ml) for one generation, and membranes were prepared and solubilized in Triton X-100 as described previously (20). To one half of the culture, antibody to penicillinase plus antibody to the 63-kd protein were added. The other half was digested with trypsin at 100 μg/ml for 2 h at 37°C and treated with excess diisopropyl fluoro-phosphate before the addition of both antibodies. After 1 h at room temperature, excess *S. aureus* cells (Cowan strain) were added (15). After another 1 h at room temperature, the cells were washed, and antibody and antigen were extracted and electrophoresed. Lane A shows penicillinase (32 kd) and the 63-kd protein before trypsin treatment, and lane B, shows their trypsin-resistant cores at approximately 30 and 60 kd, respectively.

membrane penicillinase with palmitate was depressed by globomycin, that of the 63-kd proteins was enhanced. In addition, rich media, which increase penicillinase labeling by palmitate (data not shown), depress that of the 63-kd protein.

DISCUSSION

We have demonstrated that a class of lipoproteins with glyceride-cysteine thioether structure can be found in gram-positive bacteria. This structure, hitherto considered unique to a group of outer membrane proteins from gram-negative organisms, has recently been established (19–21) for the membrane bound forms of a major secreted protein, penicillinase, of *B. licheniformis*, *B. cereus*, and *S. aureus*. This is far from an isolated case, as the lipoprotein structure can be found in a discrete number of other proteins, 6 to 10 or so, in each of the two bacilli. *S. aureus* also contains at least a few of these lipoproteins (Fig. 1 and 3 of reference 21).

The easiest way to detect potential glyceride-cysteine liporpoteins is to label cultures with [^{3}H]palmitate. We show here electrophoretic profiles of membrane proteins extracted from cultures labeled in this way, and we further show that for every peak of palmitate-labeled protein, a corresponding band of glyceryl-substituted cysteine can be found. Thus, the lipoproteins detected by palmitate labeling in the two bacilli are most likely the glyceride-cysteine type rather than the proteolipid type described by Schlesinger (24). The latter proteins carry long-chain fatty acid residues directly acylated to amino acids, probably serine, in the membrane-binding region and have been found in several viruses and in mammalian cells in tissue culture. From our results, we cannot rule out the existence of proteolipids in bacilli. We can only say that there is no significant palmitate band for which we must invoke an O-acyl serine structure rather than an acyl glyceryl cysteine.

Palmitate labeling then is indicative of glyceryl substitution of a cysteine residue, but is only poorly related to quantitative ratios of glyceryl cysteine residues in individual members of the set. This can be seen in the widely varying ratio of [^{3}H]palmitate to [^{35}S]glyceryl cysteine sulfone for different proteins in Fig. 2. Variations in the intensity of palmitate labeling of individual bands are also obvious in different media (Fig. 3). We can only comment that for most proteins, labeling was greater in richer medium and might be related to greater abundance of this class of proteins at higher growth rates or to more efficient labeling of the phospholipid pool, the probable acyl donor to these lipoproteins, as is the case in fatty acid labeling of the major *E. coli* lipoproteins (18). Furthermore, only in rich medium could we label the amide-linked fatty acyl residue on the amino group of the N-terminal cysteine of *B. licheniformis* 749 penicillinase. (O-linked fatty acyl groups are released by treatment with 0.1 N NaOH at 37°C for 1 h [19], whereas N-linked residues are resistant to release.) Because of these factors and the complexities of the specific activities of pools contributing differentially to O- and N-linked fatty acids, labeling with [^{3}H]palmitate is not useful for quantitation of the abundance of these lipoproteins. Nevertheless, in conjunction with isolation of glyceryl cysteine sulfone, palmitate labeling qualitatively demonstrates sets of lipoproteins in *B. licheniformis* and *B. cereus*.

We have been unable to identify any of the lipoproteins other than penicillinase in these organisms. That the others are not rare proteins is clear when it is remembered that *B. licheniformis* 749 and *B. cereus* 569, and in particular their magnoconstitutive mutants, have been selected for high penicillinase production; 1 to 2% of their total protein and up to 50% of that secreted is penicillinase. The others, although less abundant, are labeled in substantial amounts (see Fig. 4 and 5, where labeling of 749/C, the constitutive penicillinase producer, is shown). It seemed possible that one or more of the observed lipoproteins was a penicillin-binding protein because amino acid homologies between the major penicillin-binding protein (45 kd) of *B. subtilis* and class A penicillinases suggest that they all derive from a common ancestral gene (30). However, there was no congruence between the lipoprotein profile (palmitate labeling) of *B. licheniformis* 749 (Fig. 3c) and that of the penicillin-binding proteins from comparable cells labeled in vitro with [^{14}C]benzylpenicillin (8). The penicillin-binding proteins, which usually remain cell bound, apparently developed a different mode of membrane attachment by means of a hydrophobic region near the carboxyl terminus (30).

As we have shown (Fig. 4 and text), neither the structural proteins for the membrane alkaline phosphatase of *B. licheniformis* 749 nor the binding proteins appear to be lipoproteins. α-Amylase, another major secretory product of *B. licheniformis* (28), does not have a true membrane-bound form (H. Kuhn and J. O. Lampen, unpublished results). Moreover, the closely homologous α-amylase of *B. amyloliquefaciens* (16), in which DNA was recently sequenced by Palva et al. (22), does not have a modifiable cysteine residue in its signal sequence.

Despite the fact that we have not identified an enzymatic or structural role for any of this group of lipoproteins other than penicillinase, we have demonstrated their release from *B. licheniformis* protoplasts by mild trypsin treatment. Such treatment causes very little damage to osmoti-

cally supported protoplasts. They continue synthesizing proteins for at least 3 h, replacing the released membrane penicillinase before resumption of release (2). The location of the 20% or so of penicillinase not readily released is not known; this enzyme is completely accessible to substrate. The 80% released is located on the outside of the cytoplasmic membrane and accessible to trypsin digestion. The other lipoproteins are similarly exposed to trypsin in protoplasts and resemble penicillinase in being largely but not entirely released by trypsin.

The 63-kd protein, one we have examined more closely because we were able to isolate enough of it to prepare antibodies, shares at least two further properties with penicillinase. It has a trypsin-resistant core that is 2 to 3 kd smaller than the lipoprotein form (25, 29) and a hydrophilic exo form that appears in the medium late in growth. The 63-kd protein, then, is most certainly the membrane-bound partner of a released protein. As far as we have been able to examine the other lipoproteins of the two bacilli, they also appear to be related to the secretory process. Their average size is about 35 kd, approximately the same as the average size of all protein subunits in bacteria and considerably larger than the mean size of *E. coli* lipoproteins, where the major outer membrane lipoprotein has a mass of 7 kd and the minor lipoproteins range up to 52 kd (12). Those identified, including the 25-kd product of the *traT* gene (N. B. Perumal and E. G. Minkley Jr., personal communication) are integral outer membrane proteins.

The lipoprotein modification offers the gram-positive bacterium an efficient way of retaining a variable portion of its secreted proteins in an active releasable form. Gram-negative organisms have a periplasmic space bounded by a selectively permeable outer membrane and are much less dependent on such a structure. Most of their lipoproteins appear to be small structural proteins associated mainly with the outer membrane, although small amounts of larger molecules are present (12). We propose that the sets of larger lipoproteins in *B. licheniformis* and *B. cereus* and to some extent in *S. aureus* are the functional equivalent of the free periplasmic proteins in *E. coli*. It will be interesting to determine whether a gram-negative organism such as *Pseudomonas aeruginosa*, which produces a variety of extracellular proteins, also possesses corresponding lipoproteins.

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