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Identification of Daptomycin-Binding Proteins in the Membrane of *Enterococcus hirae*

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Daptomycin, a lipopeptide antibiotic active against gram-positive bacteria, was preliminarily shown to inhibit lipoteichoic acid (LTA) synthesis as a consequence of membrane binding in the presence of Ca^{2+} (P. Canepari, M. Boaretti, M. M. Lleó, and G. Satta, Antimicrob. Agents Chemother. 34:1220–1226, 1990). In the present study, it is shown that, along with binding bacterial-membrane components, daptomycin binds the protein fraction with a noncovalent bond, as suggested by the instability of the bond in the presence of ionic detergents such as sodium dodecyl sulfate. Analysis of membrane proteins by isoelectric focusing electrophoresis reveals that five bands with isoelectric points ranging from 5.9 to 6.2 bind radioactive daptomycin. These proteins are therefore called daptomycin-binding proteins. In an attempt to correlate these proteins to the main inhibition observed during LTA synthesis, two-dimensional thin-layer chromatography of lipids synthesized during daptomycin treatment was performed. A threefold increase in diglucosyl diacylglycerol is demonstrated, while the compounds phosphatidyl-α-kojibiosyldiacylglycerol, glycerophospho-phosphatidyl-α-kojibiosyldiacylglycerol, and glycerophospho-kojibiosyldiacylglycerol, which follow diglucosyl diacylglycerol in LTA synthesis, decrease progressively with time during the course of daptomycin treatment.

Daptomycin is a member of the lipopeptide antibiotic class, whose members are active against gram-positive bacteria. This antibiotic also needs a certain Ca^{2+} concentration (50 mg/liter) to exert its antibacterial activity (8, 16, 22). As regards its mode of action, we proved for the first time that the specific inhibition of lipoteichoic acid (LTA) synthesis is a consequence of daptomycin binding to the bacterial membrane mediated by the presence of Ca^{2+} (6). Indirect support for this particular mode of action also comes from the findings that daptomycin is active against *Enterococcus hirae* protoplasts (thus excluding the possibility that it acts exclusively on peptidoglycan synthesis, as previously suggested by others [1]) and that LTA synthesis is the function that is most deeply and dramatically inhibited in these cells (4). This thus represented the first description of an antibiotic that specifically inhibits LTA synthesis.

The availability of new antibiotics active against new bacterial targets may represent a further and essential step towards solving the urgent problem of antibiotic resistance. Commonly, the search for new antibiotics which are active against resistant bacteria is based on the use of classical and thus well-known targets. The availability of new bacterial structures that act as targets for antimicrobial action is also of great potential clinical importance, since, in general, bacteria are not cross resistant to antibiotics that recognize different targets. Moreover, the availability of a new cell wall target (such as LTA synthesis) will probably allow selection of bactericidal antibiotics, as may be expected of all wall targets.

LTAs are among the main amphiphilic molecules found in practically all gram-positive microorganisms, and their synthesis is considered to be less dependent on growth condition than is the synthesis of other amphiphilic molecules (9, 23). This is a distinctive feature for a potential candidate to act as a target for an antibiotic, since bacteria that cause infections grow under very different conditions from those growing in vitro. LTAs are anchored in the cytoplasmic membrane by hydrophobic interaction. From a chemical point of view, these molecules are generally composed of a polyglycerophosphate chain which is covalently linked to a membrane glycolipid by a phosphodiester bond. In particular, the LTA of *Enterococcus faecalis* is composed of 20 glycerol phosphate units which are linked at one end via a phosphodiester bond to phosphatidylkojibiosyl diacylglycerol (11, 15). The synthesis of this molecule, despite many years of study, is not entirely clear, and few enzymes involved in its control are known (11, 19). What is most sure is that all enzymes involved in LTA synthesis are localized in the membrane, as suggested by the fact that a particulate membrane preparation can catalyze LTA synthesis in vitro, as with the terminal stages of glycolipid anchor synthesis, the elongation of the polyglycerolphosphate chain, and the attachment of D-alanine to the polyglycerolphosphate chain via an ester bond (5, 11, 15, 19). The availability of inhibitors of such synthesis will allow further detection and study of the enzymes involved, and these enzymes, in turn, could be used as new molecular targets for selection of new inhibitors.

The aim of the present study was to achieve a more-detailed characterization of the mode of action of daptomycin in *E. hirae* to allow the identification of five membrane proteins which bind daptomycin and a more-detailed characterization of the precise step in LTA synthesis that is inhibited by this antibiotic.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. hirae* (formerly *Streptococcus faecalis* ATCC 9790) strain used in this study has been previously described (6). This strain was grown in brain heart infusion medium supplemented with Ca^{2+} (50 mg/liter) at 37°C.

Antimicrobial agents, chemicals, and radiochemicals. Daptomycin was obtained from Eli Lilly & Co. (Indianapolis, Ind.). An aqueous solution of the antibiotic was freshly prepared immediately before use. In all experiments, 8 μg of daptomycin per ml were used. This dose corresponded to the MIC for the *E. hirae* strain used (4, 6). The [^{14}C]daptomycin (4 μCi/mg) was a gift from Eli Lilly & Co. The other radiochemical, sodium [1(2)-^{14}C]acetate (specific activity, 56

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TABLE 1. Counts per minute of [^{14}C]daptomycin extracted from *E. hirae* membrane protoplasts

Extraction by:	Cellular component extracted	cpm (%) of [^{14}C]daptomycin present in:	
		Upper phase	Lower phase
Chloroform-methanol[a]	Lipids	1,060 (4.7)	21,600 (95.3) (sediment)
45% Phenol at 65°C[b]	LTA	1,185 (4.0) (aqueous)	28,500 (96.0) (phenol)
1% Triton X-100	Protein	15,900 (93.3)	1,140 (6.7) (sediment)

[a] [^{14}C]daptomycin, at least in the concentration used for this experiment, was soluble in chloroform-methanol (2:1, vol/vol).
[b] [^{14}C]daptomycin, at the concentration used for this experiment, was found entirely in the aqueous phase when melted in 45% phenol at 65°C.

mCi/mmol), was from Amersham International (Amersham, England). Other chemicals were commercially purchased and were of the highest grade available.

Protoplast preparation and selective solubilization of various membrane components. *E. hirae* protoplasts were prepared by lysozyme treatment of exponentially growing cells as described in a previous work (6). In some experiments, protoplasts were treated with [^{14}C]daptomycin (8 μg/ml) for 10 min at 37°C in the presence of Ca^{2+} (50 mg/liter) and then collected at 4°C and disrupted with glass beads in an MSK Braun cell homogenizer under flowing nitrogen. Membranes were obtained by centrifugation at 30,000 rpm for 60 min at 4°C in a Beckman centrifuge with a type 40 rotor. Lipid extraction was performed by treatment with chloroform-methanol (2:1, vol/vol) as described by Bligh and Dyer (3). LTA was extracted by treatment with 45% aqueous phenol at 68°C as described by Kessler and Shockman (18). Proteins were extracted by treatment for 30 min at room temperature with 1% Triton X-100 in 10 mM phosphate buffer, pH 7.2. For each extraction, the radioactivity bound to daptomycin was evaluated in all phases.

Column chromatography of membrane proteins solubilized by Triton X-100. After solubilization of the [^{14}C]daptomycin-treated protoplast membranes with 1% Triton X-100, the material was chromatographed on a Bio-Gel P-10 column (1.5 by 30 cm) (Bio-Rad Laboratories) equilibrated with 10 mM phosphate, pH 7.2, containing 0.1% Triton X-100. The proteins were eluted with the same buffer at a flow rate of 0.5 ml/min. For each fraction (1.0 ml) collected, both the optical density at 280 nm and the level of radioactivity were evaluated.

Identification of daptomycin-binding proteins (DBPs). The protein material, extracted with Triton X-100 as described above, was separated by isoelectric focusing electrophoresis. The buffers and solutions used were the ones described by Ames Ferro-Luzzi and Nikaido (2) with the following modifications: Triton X-100 instead of Nonidet P-40 was used, and the ampholines were in the pH range from 3 to 10, with a final concentration of 2%. The gels were 16 by 18 cm and were 1 mm thick. The electrophoresis was performed at 300 V for 16 to 18 h followed by 800 V for 1.5 to 2 h. At the end of the run, one half of the slab gel was fixed in 10% trichloroacetic acid for 2 h, stained in Coomassie Blue (1 mg/ml) in methanol-acetic acid-water (9:2:9, vol/vol/vol) for 2 h, and then destained in methanol-acetic acid-water (3:1:6, vol/vol/vol). The second half of the gel was subjected to fluorography by soaking the gel in En^{3}Hance solution (Dupont) as specified by the manufacturer. The gel was then dried under vacuum and exposed for 30 days to a temperature of −80°C on Hyperfilm-MP (Amersham).

Lipid analysis in TLC. Exponentially growing *E. hirae* cells were treated with daptomycin (8 μg/ml) and, at various times after starting the treatment, were pulse-labelled for 5 min with sodium [^{14}C]acetate. At the end of this time, the cells were immediately chilled and collected by centrifugation at 4°C. Cells were then disrupted with glass beads in the homogenizer as described above and were pelleted at 30,000 rpm for 30 min in a Beckman centrifuge with a type 40 rotor. Lipids were extracted with methanol-chloroform (2:1, vol/vol) and then with methanol-chloroform-water (2:1:0.8, vol/vol/vol) according to a modification of Bligh and Dyer's (3) procedure, as described by Kates (17). The volumes of the solutions were normalized to obtain the same amount of radioactivity per microliter. Two-dimensional thin-layer chromatography (TLC) was performed on precoated silica gel plates (Kieselgel 60; Merck). The first direction was run with chloroform-methanol-water (65:20:4, vol/vol/vol), and the second dimension was run with chloroform-acetic acid-methanol-water (80:14:10:2, vol/vol/vol/vol). The plates were then sprayed with En^{3}Hance and exposed for 30 days to a temperature of −80°C on Hyperfilm-MP. After the film was developed, labelled spot areas were marked off on the chromatographic plates, and the silica gel contained in these areas was scraped (7, 10). Radioactive material was then extracted from the scraped silica gel with chloroform-methanol (2:1, vol/vol), and radioactivity was counted as specified below.

Radioactivity evaluation. Samples (50 μl each) were dispersed in 5 ml of Insta-gel solution (Packard), and radioactivity was evaluated in a Beckman LS7000 scintillator.

RESULTS

Effects of selective solubilization of membrane components after daptomycin binding. Previously, we have shown that in the presence of Ca^{2+} daptomycin irreversibly binds only the cytoplasmic membrane of gram-positive bacteria (6), although a weak binding to other cellular polymers was observed (i.e., binding made reversible by treatment with EDTA, a bivalent-cation remover). In an attempt to identify the real target in the cytoplasmic membrane, we first tried to selectively solubilize the main membrane components (lipids, LTA, and proteins) and then tried to evaluate which of them daptomycin was bound to. In these experiments, we used cytoplasmic membrane from *E. hirae* protoplasts, to which [^{14}C]daptomycin had bound, instead of whole cells, to eliminate in advance those cell fractions capable of binding daptomycin aspecifically through labile Ca^{2+} bridges (4).

Table 1 shows that when lipids were selectively extracted with chloroform-methanol from cytoplasmic membrane, daptomycin remained bound to cell debris. Similarly, when LTA was extracted with hot 45% phenol, daptomycin remained associated with that part of the cells that was found in the phenolic phase (protein component). The finding of daptomycin associated with protein component seems specific to this experiment, since daptomycin alone was found in the aqueous phase after phase separation of 45% phenol (data not shown). On the contrary, when a mild detergent such as Triton X-100 was used to solubilize proteins, apparently almost all the radioactivity bound to daptomycin was solubilized (Table 1).

Analysis of daptomycin binding to membrane proteins. The experiments reported above indicated that daptomycin was solubilized from membranes when extracted with a mild detergent (Triton X-100), but no evidence was found to establish whether or not daptomycin was really bound to proteins, nor was any evidence as to the type and strength of such a bond found. To solve this question, in a further experiment we precipitated the above-described proteins, solubilized from cytoplasmic membranes, with cold 5% trichloracetic acid to evaluate whether daptomycin would be coprecipitated, after demonstrating that daptomycin alone was soluble in 5% trichloracetic acid. Table 2 clearly shows that when proteins were

TABLE 2. Stability of [^{14}C]daptomycin binding[a]

Membrane treatment (temp)	% of TCA-precipitable radioactivity[b]
None	99
1% Triton X-100 (RT)[c]	97
Nonidet P-40 (RT)	95
2% SDS (100°C)	3
1% SDS (37°C)	3

[a] The stability of [^{14}C]daptomycin binding to the protein fraction from the membranes of *E. hirae* protoplasts was evaluated on the basis of the ability of [^{14}C]daptomycin to coprecipitate with the protein fraction in 5% trichloroacetic acid.
[b] [^{14}C]daptomycin was soluble in 5% trichloroacetic acid (TCA).
[c] RT, room temperature.

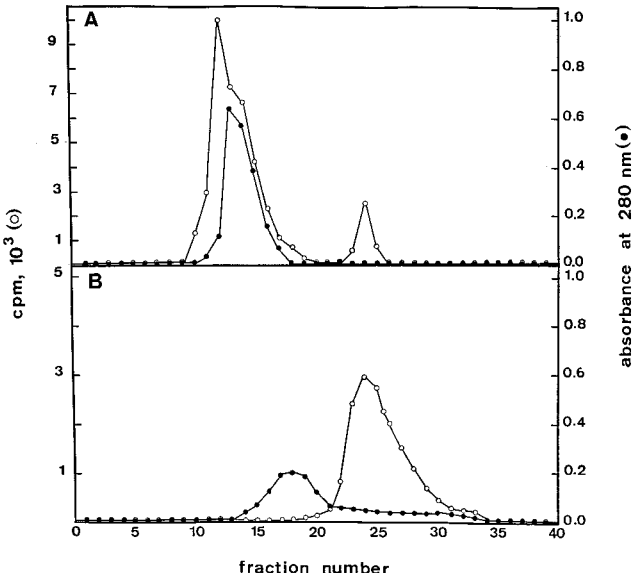

FIG. 1. Elution patterns of *E. hirae* solubilized membrane proteins to which [^{14}C]daptomycin was bound (A) and of [^{14}C]daptomycin mixed with bovine serum albumin (0.5 mg/ml) (B). Triton X-100-solubilized membrane proteins and bovine serum albumin were applied to Bio-Gel P-10 columns (1.5 by 30 cm), and the chromatographs were developed as described in Materials and Methods. Fractions (1 ml each) were collected at a flow rate of 0.5 ml/min and analyzed for A_{280} and for radioactivity.

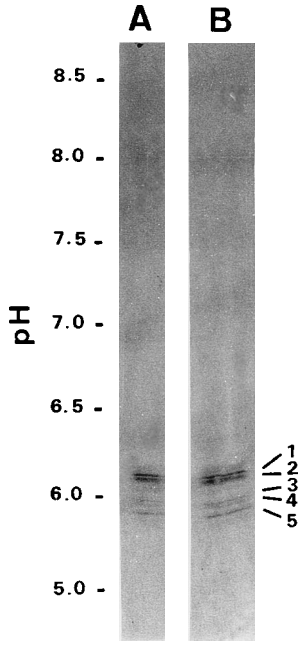

FIG. 2. Results of fluorography of DBPs in *E. hirae* membranes. *E. hirae* membranes were treated with [^{14}C]daptomycin, solubilized with 1% Triton X-100, and separated by isoelectric focusing gel electrophoresis as described in Materials and Methods. Fluorography was performed by exposing dried gel on radiographic film for 30 days to a temperature of −80°C. On the left, the resulting pH gradients after gel migration are shown. On the right, DBP numbers are indicated. (A and B) Results from two distinct experiments.

solubilized from cytoplasmic membranes with a mild detergent such as Triton X-100 or Nonidet P-40 virtually all the radioactivity bound to daptomycin was coprecipitated with membrane proteins. On the contrary, when membrane protein solubilization was performed in sodium dodecyl sulfate (SDS), whether at 37 or 100°C, in all cases the radioactivity remained in the supernatant when proteins were precipitated with trichloracetic acid, thus indicating a noncovalent bond between daptomycin and the membrane protein(s).

To further demonstrate the close association between daptomycin and the membrane protein(s), we chromatographed the proteins solubilized from membranes by Triton X-100 on a Bio-Gel P-10 column. Figure 1A shows that the eluted radioactivity bound to daptomycin peaked with the protein peak, while a small peak of eluted radioactivity occurred later and corresponded to free daptomycin (Fig. 1B). On the basis of the observation that the maximum radioactivity was eluted on fraction 12, which occurs earlier than the major protein fraction (fraction 13), the experiment whose results are reproduced in Fig. 1A also suggests that daptomycin prevalently bound a small protein fraction with a higher molecular weight.

Identification of the DBPs. As mentioned above, daptomycin binding to the protein fraction seems to be noncovalent. For this reason, the separation of membrane proteins previously placed in contact with [^{14}C]daptomycin in SDS-polyacrylamide gel electrophoresis followed by autoradiography as normally used to visualize penicillin-binding proteins (20) proved unreliable as a means of establishing whether labelled daptomycin would be linked to a specific protein(s). Owing to the noncovalent binding to proteins, we used a nondenaturating condition (isoelectric-focusing electrophoresis) to separate membrane proteins. Figure 2 shows a typical result of fluorography after electrofocusing of membrane proteins. Five radioactive bands (to which [^{14}C]daptomycin was bound), with pIs ranging from 5.9 to 6.2, are clearly detectable. We called these bands DBPs by analogy with the abbreviation for penicillin-binding proteins. As regards the reproducibility of these experiments, it should be stressed that these results were obtained in three of six different experiments but that results were similar with regard to the number of DBPs and their pIs.

Analysis of daptomycin's effect on lipid synthesis. Previously, we showed that daptomycin inhibited total lipid synthesis by no more than 50% when LTA synthesis already showed more than 90% inhibition (6). Since some lipids are known to figure in LTA synthesis, and particularly in glycolipid synthesis, we studied the effect of daptomycin on specific lipid synthesis in detail, paying particular attention to such lipids (11, 12, 14). Figure 3 shows a two-dimensional silica gel TLC autoradiogram of lipids synthesized during a 5-min pulse after 20 min of

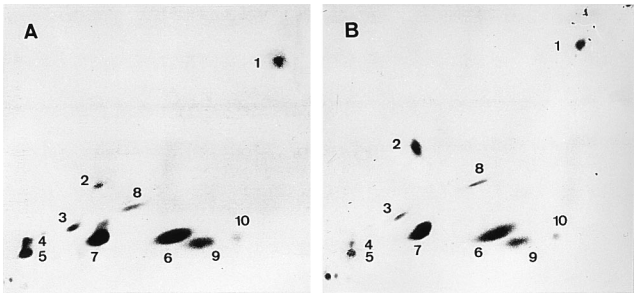

FIG. 3. Results of fluorography of two-dimensional TLCs of crude *E. hirae* lipids. TLCs were performed on silica gel plates (Kieselgel 60; Merck) with lipids which were pulse-labelled for 5 min with [^{14}C]acetate. The first direction (upward) was run with chloroform-methanol-water (65:20:4, vol/vol/vol), and the second direction was run with chloroform-acetic acid-methanol-water (80:14:10:2, vol/vol/vol/vol). Lipids of exponentially growing control cells (A) and lipids of cells after a 20-min treatment with 8 μg of daptomycin per ml (B) are shown. The compounds corresponding to the spot numbers are identified in Table 3.

TABLE 3. Percentage of lipids synthesized by *E. hirae* during daptomycin treatment[a]

Compound no.[b]	Compound[c]	% Synthesis at time (min):				
		0	5	10	15	20
1	Glc(α1-3) $acyl_2$Gro	7.2	6.9	7.5	7.5	7.7
2	Glc(α1-2)Glc(α1-3)$acyl_2$Gro	6.4	10.9	15.2	17.6	18.7
3	Glc(α1-2)Glc(α1-3)$acyl_2$Gro └Ptd	3.2	2.8	2.1	1.5	0.9
4	GroPGlc(α1-2)Glc(α1-3)$acyl_2$Gro └Ptd	9.0	7.9	6.2	4.5	3.3
5	GroPGlc(α1-2)Glc(α1-3)$acyl_2$Gro	11.8	9.3	7.4	5.5	3.6
6	Cardiolipin	15.1	15.5	15.7	16.1	16.3
7	Phosphatidylglycerol	32.5	31.3	31.6	32.3	34.1
8	Alanyl phosphatidylglycerol	5.4	5.6	5.2	5.4	5.6
9	Phosphatidic acid	7.8	8.0	7.5	7.7	8.0
10	Undecaprenol phosphate	1.6	1.8	1.6	1.9	1.8

[a] For details, see the legend to Fig. 3.
[b] Compound numbers correspond to those in Fig. 3.
[c] Abbreviations: $acyl_2$Gro, 1,2-di-*O*-acyl glycerol; Glc, D-glucopyranosyl; Gro, glycerol; GroP, SN-glycero-1-phosphate; Ptd, phosphatidyl residue.

daptomycin treatment and an autoradiogram of normally growing cells. In Table 3, all spots appearing on the radiographic films are identified on the basis of similarities in migration patterns (10, 13), and the percentage of the corresponding areas after silica gel scraping is indicated. Figure 3 and Table 3 clearly reveal an accumulation of compound number 2, identified as diglucosyl diacylglycerol. In this case, the compound in untreated cells amounts to 6.4% of the total lipids, while, during daptomycin treatment, it increases with time, rising to as much as 18.7% of the total after 20 min of treatment. Conversely, compounds 3, 4, and 5 (to be precise, phosphatidyl-α-kojibiosyldiacylglycerol, glycerolphosphophosphatidyl-α-kojibiosyldiacylglycerol, and glycerophosphokojibiosyldiacylglycerol, respectively) decreased with time and after 20 min of treatment went down to as low as one-third of the original value. Under the same conditions, the other lipids identified underwent no variations.

DISCUSSION

In previous papers (4, 6), we suggested the hypothesis that daptomycin may act on gram-positive bacteria through specific inhibition of LTA synthesis as a result of membrane binding which is allowed only in the presence of Ca^{2+}. The data presented in this paper are consistent with the initial hypothesis, indicating that LTA is the target of daptomycin action after binding one or more protein components of the cytoplasmic membrane. The data presented here clearly indicate, in fact, that of the three possible main bacterial-membrane components (LTA, lipids, and proteins), daptomycin binds only to the protein fraction with a noncovalent bond, as demonstrated by the instability of the bond when membrane proteins are treated with a denaturing agent such as SDS. Daptomycin was also cochromatographed on a Bio-Gel P-10 column with the protein fraction solubilized with nonionic detergent (Triton X-100) from bacterial membranes, thus indicating that the bond, though noncovalent, is stable enough to persist over time. From the analysis of the chromatogram, daptomycin also seems to be bound to a small fraction of high-molecular-weight proteins. Among the membrane protein components, analysis of fluorography of isoelectrofocused membrane proteins, to which labelled daptomycin had previously bound, reveals that five bands, with pIs ranging from 5.9 to 6.2, could be detected with a certain frequency. We have called these bands DBPs. Even though this pattern was not detected in all experiments performed (i.e., only in three of six), it should be recalled that in the three positive experiments we obtained similar results with regard to the number of bands and their pIs. The negative results in the other three experiments may be explained by the fact that we used a labelled daptomycin with low specific activity and/or, alternatively, by the fact that isoelectric focusing electrophoresis needs to be performed with very thin gels, on which only a small amount of proteins should be loaded in order to achieve optimal band resolution.

A question regarding the role of these proteins in cell physiology arises. Two-dimensional TLC analysis of lipids synthesized during daptomycin treatment indicates that diglucosyl diacylglycerol (compound 2 in Fig. 3) accumulates proportionally with the time of treatment. This compound is known to be one of the lipid intermediates involved in LTA synthesis at the glycolipid synthesis level (11, 13). The observation that the compound is accumulated during daptomycin treatment may suggest the hypothesis that the blockade of LTA synthesis caused by the antibiotic occurs immediately after the synthesis of this intermediate, as specified in Fig. 4. This is confirmed by the fact that there is a decrease in compounds 3, 4, and 5, i.e., phosphatidyl-α-kojibiosyldiacylglycerol, glycerophosphophosphatidyl-α-kojibiosyldiacylglycerol, and glycerophosphokojibiosyldiacylglycerol, respectively, which are thought to follow compound 2 in LTA biosynthesis (7, 11) (Figure 4).

At present it is impossible to directly link these observations

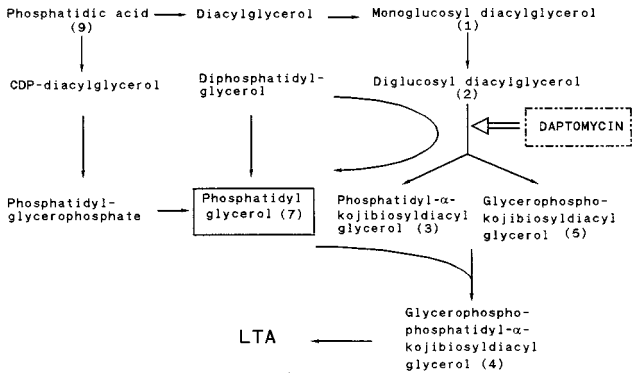

FIG. 4. Outline of lipid metabolism for *E. hirae*, with indication of the proposed site of action of daptomycin.

with the antimicrobial action of daptomycin. However, it would seem important to stress that another lipid synthesis inhibitor such as cerulenin blocks bacterial growth (21) and that, since mutants deficient in the glycolipid anchor of LTA have never been isolated, it is suggested that LTA may play an essential role in bacterial physiology and, consequently, may act as a target for antibiotic action.

It is also impossible to correlate the five proteins that bind daptomycin to the observed inhibition of the glycolipid precursor of LTA, among other things because not all the enzymes controlling each stage of LTA synthesis are known (11, 19); thus, at present it is impossible to purify these enzymes and analyze the ability of daptomycin to bind and inhibit enzymatic activity, and therefore it is also impossible to confirm this correlation immediately. However, though this study provides no conclusive data, it may be interesting to recall that all enzymes involved in LTA synthesis are known to be membrane located, as suggested by the finding that membrane preparations are capable of synthesizing LTAs in vitro in various microorganisms (5, 11, 15, 19).

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