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Lipoteichoic Acid as a New Target for Activity of Antibiotics: Mode of Action of Daptomycin (LY146032)

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Daptomycin at the MIC allowed the cell mass increase of enterococcal strains and *Bacillus subtilis* to continue for 2 to 3 h at rates comparable to those of the controls. During this time the cell shape of the former changed to a rod configuration and that of the latter changed to long rods. In these bacteria, in which cell mass continued to increase, the MIC of daptomycin inhibited peptidoglycan synthesis by no more than 20% after 20 min of incubation and by roughly 50% after 2 h of incubation. Other macromolecules, such as DNA, RNA, and proteins, were only slightly affected. In contrast, incorporation of [^{14}C]acetate into lipids was reduced by about 50% in the various strains after 20 min of treatment with daptomycin at the MIC. When the effect of the major lipid-containing polymers on synthesis was evaluated in detail, it was found that under conditions in which peptidoglycan and the other macromolecules mentioned above were inhibited only slightly (20%) and total lipid synthesis was inhibited by 50%, synthesis of teichoic and lipoteichoic acids was inhibited by 50 and 93%, respectively. Daptomycin was not found to enter the cytoplasm of either bacterial or mammalian cells. It bound, in the presence of calcium ions only, to whole bacterial cells, cell walls (both those that contained and those that did not contain membranes), and isolated membranes of bacterial and mammalian cells. Washing with EDTA removed daptomycin from all cells mentioned above and cell fractions except the bacterial membrane. It is concluded that lipoteichoic acid is most likely the primary target of daptomycin.

Daptomycin (LY146032) is an acidic lipopeptide antibiotic that is active against gram-positive bacteria (F. T. Counter, P. J. Baker, L. D. Boeck, M. Debono, P. W. Ensminger, R. L. Hamill, V. M. Krupinski, R. M. Molloy, and J. L. Ott, Program Abstr. 24th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 1078, 1984). Its activity is strictly dependent on the presence of calcium ions (50 mg/liter) in the growth medium (11, 13, 16, 18, 21, 25, 42, 43).

In a previous report (1) it has been suggested that this antibiotic inhibits a cytoplasmic stage of the formation of the nucleotide-linked sugar-peptide precursors involved in peptidoglycan biosynthesis. This conclusion was mainly drawn from the observation that (i) daptomycin can inhibit the accumulation of UDP-*N*-acetylmuramylpentapeptide caused by vancomycin, and (ii) at concentrations as high as 100 μg/ml, daptomycin is capable of inhibiting the enzymes that catalyze the polymerization of nucleotide-linked sugar precursors.

All these observations, on which the conclusions regarding the mode of action of daptomycin were based, were made by using antibiotic concentrations which were chosen without reference to the MICs for bacterial growth.

In this study we examined the effect of the MIC of daptomycin on the synthesis of various polymers of several bacterial strains, in some of which cell mass increase continued for several hours after antibiotic addition. We show that the polymer which is inhibited most in the presence of the daptomycin MIC is lipoteichoic acid and suggest that lipoteichoic acid synthesis may well be the primary target of this antibiotic. Moreover, studies with labeled daptomycin showed that the antibiotic does not penetrate the bacterial or mammalian cell cytoplasm and, in the presence of calcium ions, binds several bacterial polymers and structures and mammalian cell membranes, but binds irreversibly only to the bacterial cytoplasmic membranes.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study were *Enterococcus faecium* PS (7, 8, 27), *Enterococcus faecalis* E6 (clinical isolate, our collection), *Staphylococcus aureus* A8 (9), and *Bacillus cereus* T (22). *E. faecium* and *E. faecalis* strains were grown in the chemically defined medium (CDM) described by Shockman (36), while *S. aureus* and *B. cereus* strains were grown in a modified CDM with a 10-fold reduction in salt concentration. Both media were supplemented with Ca^{2+} (50 mg/liter), as suggested by Eliopoulos et al. (16). Enterococci and *S. aureus* strains were grown at 37°C without shaking, while the *B. cereus* strain was grown at 30°C with vigorous shaking. Cell growth was monitored at a wavelength of 650 nm with a spectrophotometer (model DU6; Beckman Instruments, Inc., Fullerton, Calif.).

Antimicrobial agents, chemicals, and radiochemicals. Daptomycin and vancomycin were obtained from Eli Lilly & Co. (Indianapolis, Ind.). An aqueous solution of the antibiotic was freshly prepared immediately before use. [^{14}C]daptomycin (10 μCi/mg) was a generous gift from Eli Lilly & Co. All the other radiochemicals ([^{3}H]leucine, specific activity, 146 Ci/mmol; [^{3}H]lysine, specific activity, 85 Ci/mmol; [^{3}H]diaminopimelic acid, specific activity, 281 mCi/mmol; [^{3}H]thymidine, specific activity, 25 Ci/mmol; [^{3}H]uridine, specific activity, 35 Ci/mmol; [^{14}C]glycerol, specific activity, 20 mCi/mmol; [^{14}C]sodium acetate, specific activity, 56 mCi/mmol; and [^{32}P]sodium phosphate, specific activity, 1.66 mCi/mg of PO_4) were from The Radiochemical Centre (Amersham, England). Other chemicals were purchased from commercial sources at the highest grade available.

Evaluation of the MIC. The MICs of all the antibiotics were determined by the standard broth dilution technique (7,

27). Each tube was inoculated with bacteria that were in balanced exponential growth in order to obtain a starting density of 10^6 cells per ml. The MIC was defined as the lowest antibiotic concentration that prevented visible growth after 18 h of incubation.

Evaluation of the killing kinetics. Exponentially growing cultures were diluted into fresh medium containing the desired antibiotic concentration in order to obtain a starting population of 2×10^6 CFU/ml. Cultures were then incubated at the appropriate temperatures, and samples were withdrawn at suitable time intervals to determine viable counts (27).

Evaluation of macromolecular synthesis. Exponentially growing cultures in the appropriate medium were diluted into fresh prewarmed medium containing the labeled precursors ([^{3}H]thymidine, 4 μCi/ml; [^{3}H]leucine, 1 μCi/ml; [^{3}H]lysine, 6 μCi/ml; [^{3}H]diaminopimelic acid, 4 μCi/ml; [^{14}C]glycerol, 0.5 μCi/ml). At intervals, 1-ml samples were withdrawn in duplicate from the cultures and put into 10 ml of cold 10% trichloroacetic acid (TCA) (8). Before the radioactivity was counted (model LS7000 counter; Beckman), samples for peptidoglycan synthesis were incubated with pronase as described previously by Boothby et al. (5).

Evaluation of glycerol incorporation into lipids, lipoteichoic acid, and TCA-precipitable fraction. Exponentially growing cells were pulse-labeled for 5 min with [^{14}C]glycerol at different times after the addition of the antibiotic. Cells were immediately centrifuged in a microfuge (Microfuge B; Beckman) for 30 s at 4°C and washed twice with cold distilled water. Lipids were extracted with chloroform-methanol (2:1) as described by Bligh and Dyer (4). For evaluation of lipoteichoic acid, cells were extracted with 45% aqueous phenol at 68°C as described by Kessler and Shockman (24).

For evaluation of radioactivity in TCA-precipitable material, at the end of the pulse-labeling, cold TCA (final concentration, 10%) was immediately added to the cultures. Precipitated material was collected on filter membranes (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.), and the radioactivity was counted in a standard scintillation solution.

Evaluation of sodium acetate incorporation into lipids. Experiments for the evaluation of sodium acetate incorporation into lipids were performed exactly as described above for the incorporation of [^{14}C]glycerol, but [^{14}C]sodium acetate (0.5 μCi/ml) was used.

Evaluation of [^{32}P]phosphate incorporation into teichoic acid. Pulse-labeling (10 μCi/ml) of exponentially growing cells was performed as described above. Cells were immediately centrifuged at 4°C, washed twice, and suspended in cold 5% TCA. Teichoic acids were extracted at 90°C for 5 min as described by Wong et al. (44).

Evaluation of the accumulation of peptidoglycan precursors. Amino sugars were evaluated by using the reaction described by Elson and Morgan (17).

Binding of radioactive daptomycin to *E. faecium* cells, protoplasts, and SDS walls. An exponentially growing culture (250 ml) of *E. faecium* (optical density, 0.5 units) was collected by centrifugation at 4°C. The pellet was washed twice with sodium phosphate buffer (0.01 M, pH 7.2) and suspended in 20 ml of the same buffer. A total of 10 ml of [^{14}C]daptomycin at 1× the MIC was added to a tube with Ca^{2+} (50 μg/ml), while to another tube 10 ml of [^{14}C]daptomycin was added without Ca^{2+}. After 10 min of incubation at 37°C, both tubes were centrifuged at 4°C and pellets were washed twice with cold sodium phosphate buffer to remove the unbound antibiotic. Cells were disrupted by sonication and then centrifuged at 30,000 rpm for 60 min in an ultracentrifuge (Beckman) with a type 40 rotor. Supernatants (cytoplasmic fractions) were collected to evaluate the radioactivity as described above, and the pellets were washed with phosphate buffer. After resuspension in 10 ml, 5 ml was treated twice for 10 min at room temperature with 50 mM EDTA and then centrifuged as described above. The radioactivity in all samples was then evaluated.

Detection of the binding of radioactive daptomycin to *E. faecium* protoplasts was performed as follows. Protoplasts were obtained by lysozyme treatment as described by Roth et al. (31). To 5 ml of protoplast suspension (10^9 cells), 50 μg of [^{14}C]daptomycin was added for 15 min. Cells were then centrifuged in a microfuge (Microfuge B) for 30 s, washed twice, and then suspended in 5 ml of the radioactivity counting solution.

Sodium dodecyl sulfate (SDS) walls (whole cells boiled with SDS) from *E. faecium* cells were prepared as described by Shockman et al. (37).

Binding of radioactive daptomycin to human epithelial cell membranes. Human epithelial cell membranes were prepared from a culture of HEp-2 cells (generously provided by M. Tognon, Institute of Biological Science, University of Verona) as described previously (12). Membranes were treated in 0.01 M sodium phosphate buffer at 37°C with 10 μg of [^{14}C]daptomycin per ml in the presence or absence of Ca^{2+} (50 μg/ml) for 15 min. Membranes were pelleted by ultracentrifugation, and part of the membranes were counted for radioactivity and part of them were treated for 30 min with 0.01 M phosphate buffer containing 50 mM EDTA. Membranes were collected by ultracentrifugation, and bound radioactivity was determined.

RESULTS

Effect of daptomycin MIC on macromolecular synthesis and cell shape of some gram-positive strains. The MICs of daptomycin for the strains used in this study were as follows: *E. faecium* PS, 8 μg/ml; *E. faecalis* E6, 8 μg/ml; *S. aureus* A8, 4 μg/ml; and *B. cereus* T, 4 μg/ml. In the initial studies we compared the effects of the MICs of vancomycin and daptomycin on cell mass increase and macromolecular synthesis. Vancomycin caused an almost complete inhibition of increase of cell mass and DNA, RNA, and protein synthesis in all strains tested (Fig. 1). In contrast, daptomycin at the MIC caused an immediate inhibition of cell mass increase in *S. aureus* A8 (Fig. 1), but not in any of the other strains tested, i.e., *E. faecium* PS (Fig. 1), *E. faecalis* E6, or *B. cereus* T (data not shown), in which the optical density increase continued at rates that were not much lower than those of the untreated control for 120 to 180 min. During this time period, synthesis of DNA, RNA, and proteins in *E. faecalis* (Fig. 1) and in *E. faecium* and *B. cereus* (data not shown) strains continued at rates that were not much lower than those in the untreated controls, while such polymers were all virtually completely inhibited in *S. aureus* (Fig. 1).

Neither daptomycin nor vancomycin caused cell shape alterations in *S. aureus* A8 at the MIC (Fig. 2). In all other strains, daptomycin, but not vancomycin (which had no apparent effect at concentrations above the MIC; data not shown), caused changes in cell shape, which consisted of the formation of rods (*E. faecium*) or long rods (*B. cereus*).

Effect of daptomycin on peptidoglycan and lipid synthesis. The observation that daptomycin at the MIC for strains other than staphylococci did not block a cell mass increase for hours provides a tool for identifying the biochemical

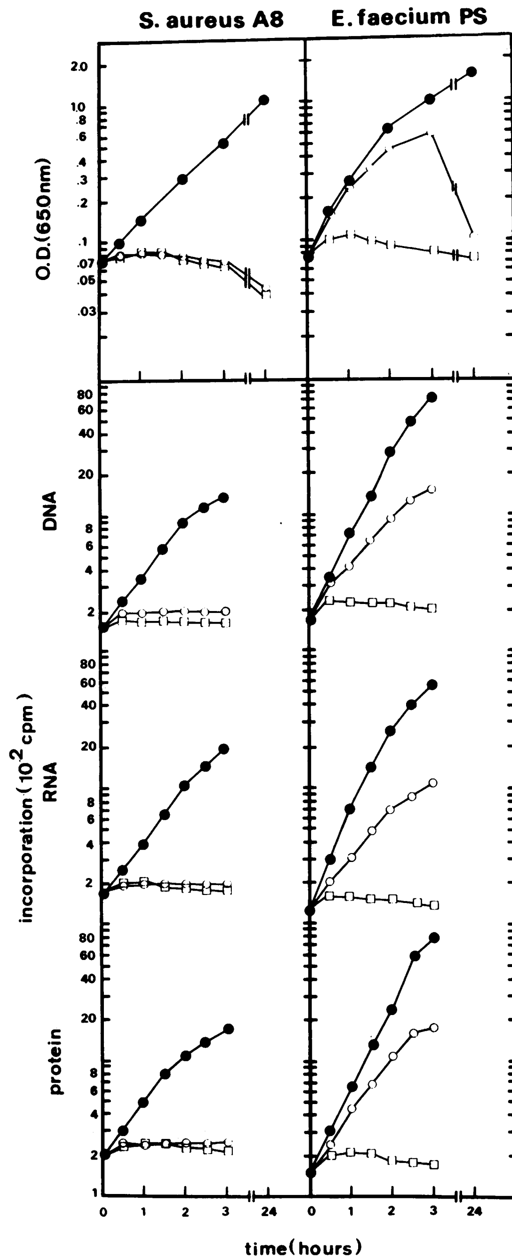

FIG. 1. Effects of daptomycin and vancomycin on optical density (OD) increase and DNA, RNA, and protein synthesis of two gram-positive bacteria grown in CDM. ●, Control; ○, daptomycin at 1× the MIC; □, vancomycin at 1× the MIC.

pathway, alteration of which is likely to be responsible for growth inhibition. Figure 3 shows the effect of concentrations equal to or above the MIC on peptidoglycan synthesis in *S. aureus* A8 and *E. faecium* PS. In contrast to *S. aureus* for which the MIC of the antibiotic completely inhibited peptidoglycan synthesis and synthesis of the other macromolecules that were evaluated, in all other strains peptidoglycan synthesis continued in the presence of the daptomycin MIC at a rate similar to those of the other macromolecules (see also Fig. 1). Doses above the MIC, however, rapidly proved inhibitory for peptidoglycan synthesis (Fig. 3). *E. faecalis* E6 and *B. cereus* T behaved exactly like *E. faecium* PS did (data not shown).

Unlike daptomycin, vancomycin at the MIC inhibited peptidoglycan synthesis immediately when it was added to all of the strains described above (data not shown).

When the effects of daptomycin both on the amount of intracellular UDP-*N*-acetylmuramylpentapeptide and on the accumulation of this same precursor caused by vancomycin were evaluated, it was found that daptomycin did not cause accumulation of intracellular UDP-*N*-acetylmuramylpentapeptide even at concentrations that were much higher than the MIC, in full accordance with previous observations (1). Concentrations exceeding the MIC by fivefold or more prevented the accumulation of peptidoglycan precursors caused by vancomycin, as has also been shown by other investigators (1). However, the daptomycin MIC, which was not evaluated in the previous study (1), was virtually completely devoid of effects on such synthesis in all strains tested, including *S. aureus* (data not shown). In contrast, this same concentration of antibiotic blocked the incorporation of [^{14}C]glycerol into TCA-precipitable material almost immediately in *S. aureus* and within 30 min in *E. faecium* (Fig. 3) and caused a clear reduction of [^{14}C]acetate incorporation into lipids in all strains tested as early as 10 min after treatment (Table 1). Such inhibition proved highly significant, exceeding or being close to 50%, 20 min after the beginning of treatment, when peptidoglycan synthesis was, in contrast, only slightly reduced in *E. faecium* PS (Fig. 3).

Effect of daptomycin on teichoic acid and lipoteichoic acid synthesis. When the effect of daptomycin on teichoic acid synthesis was evaluated on the basis of incorporation of [^{32}P]phosphate, it was found that the MIC of daptomycin in all of the strains tested inhibited this polymer by 45 and 57% after 10 and 20 min of incubation, respectively (Table 2).

In contrast, daptomycin at the MIC inhibited incorporation of [^{14}C]glycerol into lipoteichoic acid by 82 to 85% and up to 93% at 10 and 20 min after treatment, respectively (Table 2). After 20 min this same concentration of daptomycin inhibited the incorporation of [^{14}C]glycerol into total lipids by only 50%.

Binding of [^{14}C]daptomycin to different bacterial structures and different cells and effect of EDTA on binding stability. It is known that the synthesis of complex lipids in bacteria occurs in the cytoplasmic membrane (19). Therefore, an antibiotic that is likely to have lipoteichoic acid as its target should not need to enter the cytoplasm to perform this binding. Table 3 shows that [^{14}C]daptomycin did not enter the cytoplasms of either whole cells or protoplasts at significant levels, even in the presence of a calcium concentration that was optimal for the antimicrobial activity of the antibiotic. For daptomycin binding, it was observed that daptomycin interacted with whole cells and their fractions only in the presence of calcium ions. Under these conditions the drug bound both whole cells and cell walls that did or did not contain membranes, and most importantly, it bound the cytoplasmic membrane very efficiently (see discussion above of binding to protoplasts).

The observation that, in addition to membranes, daptomycin also bound other bacterial structures prompted us to evaluate the possibility that the nature of the bond established with the bacterial membranes might be different from that established with the other structures. Confirmation of this possibility would provide further support for the conclusion that lipoteichoic acid synthesis is likely to be the target of daptomycin. In order to evaluate this possibility, we studied the effect of EDTA washing on bound daptomycin. Table 4 shows that EDTA almost completely removed the radioactivity bound to walls that did not contain mem-

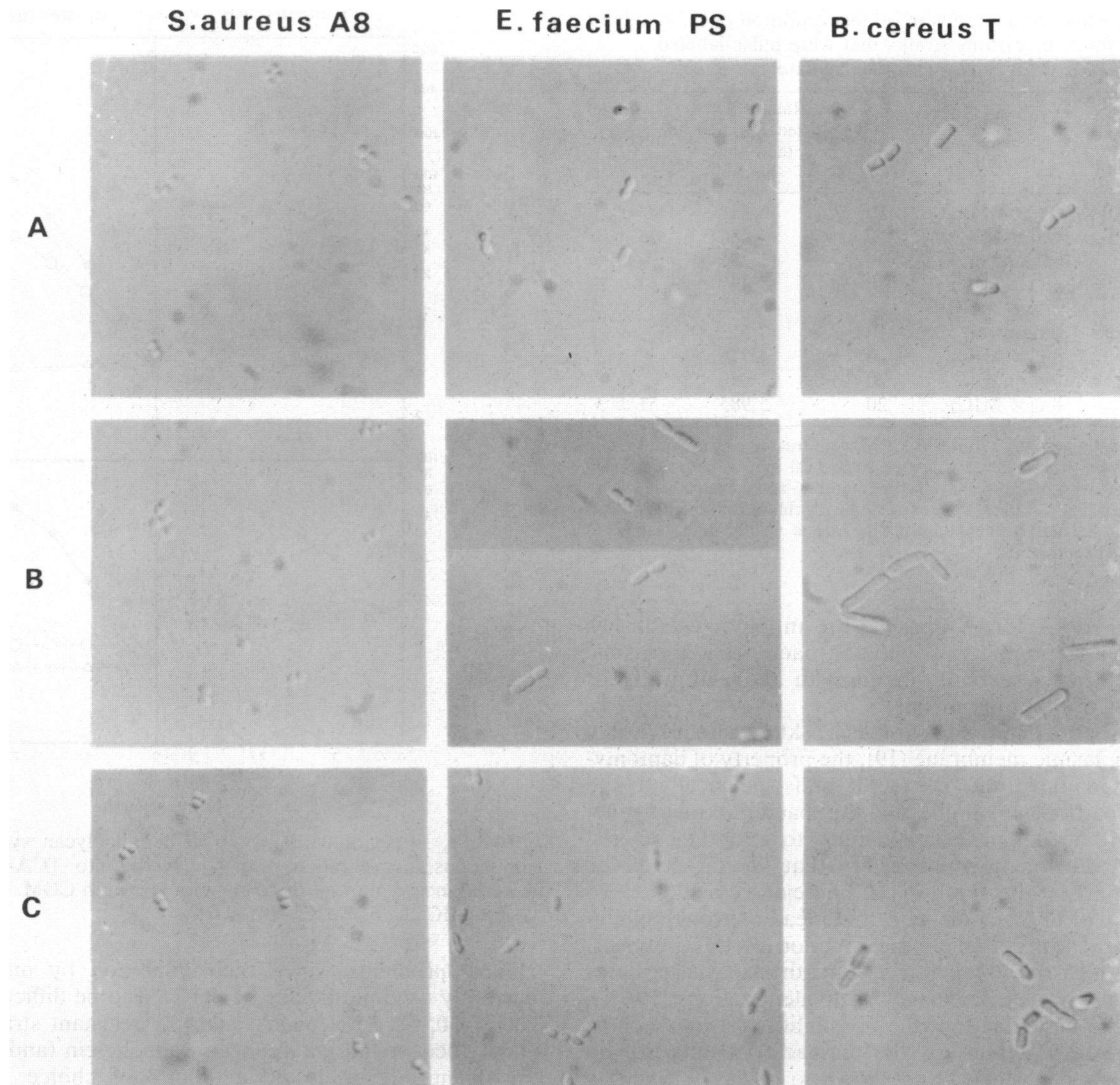

FIG. 2. Effects of daptomycin and vancomycin on the cell shapes of *S. aureus*, *E. faecium*, and *B. cereus*. Panels in row A, controls; panels in row B, daptomycin at 1× the MIC; panels in row C, vancomycin at 1× the MIC.

branes (namely, SDS walls), while it left at least 30% of the radioactivity in walls that contained membranes and released only 15% of the radioactivity that was bound to isolated membranes.

It is important that the irreversible binding by bacterial membranes appears to be a fairly specific phenomenon, since daptomycin also bound to membranes from HEp-2 human epithelial cells, again, only in the presence of calcium ions, but the bound antibiotic was almost completely removed by washing with EDTA (more than 95%; data not shown).

DISCUSSION

Daptomycin has been described previously as causing inhibition of bacterial growth by inhibiting peptidoglycan synthesis. More precisely, the target of daptomycin has been suggested as being a cytoplasmic reaction in the synthesis of precursors of this polymer (1). A similar mode of action has also been considered likely for one of these analogs (15). The data presented here showed that the MIC of daptomycin does not inhibit the cell mass increase of *E. faecalis*, *E. faecium*, or *B. cereus* cells for approximately 3 h. In such nondividing cells, among all the macromolecules assayed, lipoteichoic acid was the only one that was dramatically inhibited (over 80%) as early as 10 min after antibiotic treatment. This indicates that inhibition of lipoteichoic acid synthesis is likely to be the target of daptomycin. Results of this study confirmed the fact that daptomycin inhibits peptidoglycan synthesis (1), but a strong inhibition of the polymer was observed only at concentrations that exceeded the MIC.

Daptomycin was unable to enter the cytoplasm, but it was found to be capable of binding to cell walls and membranes. Although binding to both of these structures requires Ca^{2+}, binding to bacterial cytoplasmic membranes appears to be of a different, specific nature. EDTA removes daptomycin from cell walls and membranes of mammalian cells, but from cytoplasmic membranes of bacteria it removes daptomycin only to a very limited extent. These findings indicate that daptomycin is probably bridged by Ca^{2+} to cell membranes

TABLE 1. Effect of daptomycin on incorporation of radioactivity into lipids of various strains that were pulse-labeled for 5 min with [^{14}C]acetate

Strain	Daptomycin concn (μg/ml)	Incubation time (min)	Radioactivity incorporated (cpm) into lipids[a]	% Inhibition (±SD)
S. aureus A8	0 (control)	10	6,847	0
	4 (1× MIC)	10	4,485	35 ± 3
	0 (control)	20	11,216	0
	4 (1× MIC)	20	5,437	52 ± 2
E. faecium PS	0 (control)	10	7,513	0
	8 (1× MIC)	10	4,716	38 ± 1
	0 (control)	20	12,069	0
	8 (1× MIC)	20	5,985	51 ± 3

[a] Exponentially growing cultures in CDM were treated or were not treated with daptomycin at 1× the MIC. After 10 and 20 min of treatment, 1-ml samples were withdrawn from all the cultures and pulse-labeled with [^{14}C]acetate for 5 min. Lipids were extracted with chloroform-methanol (2:1) as described in the text. Values represent the means ± standard deviations of three separate experiments.

and walls. After it forms a bridge, the antibiotic establishes a bond of a different type with the bacterial cytoplasmic membrane, but it cannot do so with the cell walls or membranes of other organisms.

Because lipoteichoic acid synthesis is known to occur only in the cytoplasmic membrane (19), the property of daptomycin discussed above, i.e., the fact it binds specifically to and shows a particular affinity for the bacterial membrane, together with its demonstrated inability to enter the bacterial cytoplasm, adds to the probability that the target of the antibiotic may be the synthesis of lipoteichoic acid.

Inhibition of lipoteichoic acid could lead to growth inhibition through destruction of the function of this polymer, whether as a carrier in teichoic acid synthesis, as a regulator of autolysins, or as a scavenger of divalent cations (19).

This is the first time that the possibility that lipoteichoic acid may be the target for the antibacterial effect of an antibiotic has been raised and shown to be likely. Such an observation is important, particularly because daptomycin has been demonstrated to have bactericidal activity against staphylococci and enterococci (15, 16, 42), much higher than that demonstrated by the beta-lactams and also higher than that of vancomycin. Staphylococci (particularly those that are resistant to methicillin) and enterococci still raise serious

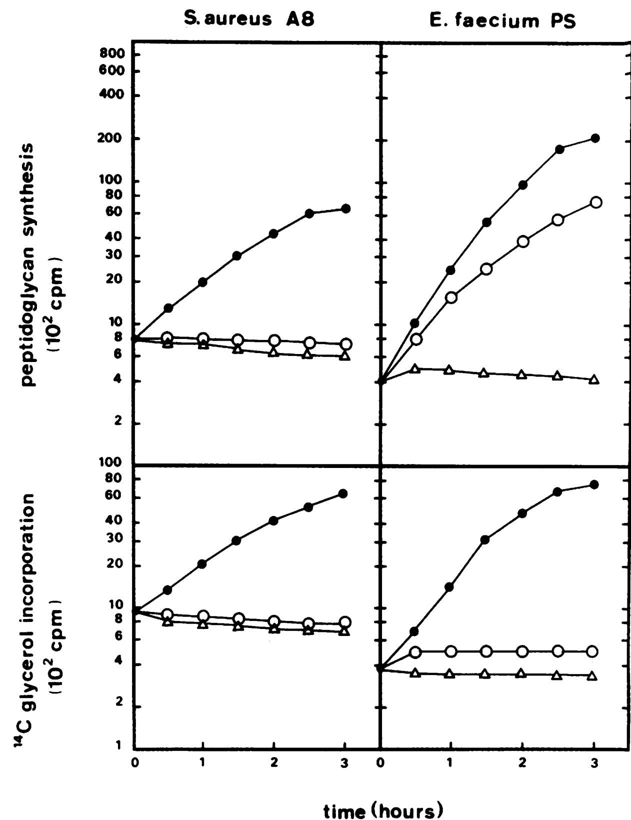

FIG. 3. Effect of daptomycin on peptidoglycan synthesis and continuous incorporation of [^{14}C]glycerol into TCA-precipitable material in two gram-positive bacteria grown in CDM. ●, Control; ○, 1× MIC; △, 5× MIC.

clinical problems, since their inhibition by most of the currently used antibiotics has proved to be difficult (14, 23, 26, 28–30, 38, 41). Against multiply resistant strains of the staphylococci and enterococci, vancomycin (and, recently, teicoplanin) is often the antibiotic of choice. However, particularly in some patients with serious infections, such as endocarditis, failures with this drug are by no means rare, and more importantly, the extended use of vancomycin has led to the selection of resistant strains (35). The recent description of strains that are resistant to vancomycin means that resistance to this antibiotic is likely to become a clinical

TABLE 2. Percent ± standard deviation inhibition of teichoic acids, lipoteichoic acid, and lipids by 1× the MIC of daptomycin in *S. aureus* and *E. faecium* cells

Strain	Daptomycin concn (μg/ml)	Incubation time (min)	% ± SD inhibition of ^{32}P into teichoic acids[a]	% ± SD inhibition of incorporation of [^{14}C]glycerol into[a]		
				Lipids	Liptoteichoic acid	TCA-precipitable material
S. aureus A8	0 (control)	10	0	0	0	0
	4 (1× MIC)	10	45 ± 4	48 ± 3	85 ± 4	49 ± 2
	0 (control)	20	0	0	0	0
	4 (1× MIC)	20	57 ± 3	51 ± 3	92 ± 4	57 ± 3
E. faecium PS	0 (control)	10	0	0	0	0
	8 (1× MIC)	10	46 ± 2	47 ± 2	82 ± 3	47 ± 2
	0 (control)	20	0	0	0	0
	8 (1× MIC)	20	56 ± 2	51 ± 3	93 ± 3	58 ± 2

[a] Exponentially growing cultures in CDM were treated or were not treated with daptomycin at 1× the MIC. After 10 and 20 min of treatment, four samples of 1 ml each were withdrawn from all the cultures and pulse-labelled for 5 min with [^{32}P]phosphate or [^{14}C]glycerol. Teichoic acid, lipoteichoic acid, and lipid synthesis and TCA-precipitable material were evaluated as described in the text. Values represent the means ± standard deviations of three separate experiments.

TABLE 3. [^{14}C]daptomycin penetration into cytoplasm and binding to *E. faecium* PS and its subfractions

Cell type	Cell subfraction	cpm (%) of [^{14}C]daptomycin[a]	
		$+Ca^{2+}$	$-Ca^{2+}$
Unaltered	Whole	6,456 (100)	98 (1.5)
	Cytoplasm	75 (1.1)	56 (0.8)
	Membrane and wall	6,182 (95.7)	69 (1.0)
Protoplast	Whole	4,467 (100)	102 (2.2)
	Cytoplasm	96 (2.1)	74 (1.6)
	Membrane	4,395 (98.3)	61 (1.3)
Whole cell boiled with SDS	SDS wall	11,767 (100)	250 (2.1)

[a] In all experiments [^{14}C]daptomycin was used at 10 μg/ml (final concentration).

problem in the near future. The finding that daptomycin acts on a different target from that of vancomycin is of great potential clinical importance, since, in general, bacteria are not cross-resistant to antibiotics that recognize different targets. In addition, antibiotics that interfere with different bacterial metabolic pathways, particularly when they are bactericidal, can often potentiate the effects of each other. The fact that daptomycin may demonstrate similar important properties is supported by the fact that some staphylococcal strains in our collection that have MICs of vancomycin that are fourfold higher than the MICs for 90% of strains tested that are generally reported for these species demonstrated an MIC of daptomycin that was the lowest that we found among the staphylococci (unpublished data).

In addition, it should also be noted that lipoteichoic acid could be a promising new target for antibiotics. In the gram-positive pathogenic bacteria in which adhesins have been better characterized, lipoteichoic acid has been shown to be the most important constituent of such structures (2, 3, 39, 40). Adhesins are known to be necessary for bacterial colonization of human mucosae (which is a prerequisite for the ability of microorganisms to trigger infections), and adherent bacteria have been described to be less responsive to antibiotics (20). Drugs that inhibit lipoteichoic acid should therefore act directly against one of the most important determinants of virulence in bacteria and (to a greater extent than other drugs) could prevent bacteria from proliferating in the adherent state, which may render them less susceptible to antibiotics. It is also important that lipoteichoic acid, like peptidoglycan, is not synthesized by mammalian cells.

TABLE 4. Effect of treatment with EDTA on binding stability of [^{14}C]daptomycin to various *E. faecium* PS subfractions

Cell type	Cell subfraction	Treatment[a]	cpm (%) of [^{14}C]daptomycin bound[b]
Unaltered	Wall	None	6,182 (100)
		Two washings with EDTA	1,839 (29.7)
Protoplast	Membrane	None	4,395 (100)
		Two washings with EDTA	3,751 (85.3)
Whole cell boiled with SDS	SDS wall	None	11,767 (100)
		Two washings with EDTA	873 (7.4)

[a] Washings with EDTA (50 mM) were performed at room temperature for 10 min.
[b] In all experiments [^{14}C]daptomycin (10 μg/ml) was used in the presence of Ca^{2+} (10 mg/liter).

The mechanism by which lipoteichoic acid is synthesized has yet to be clearly defined, and little is known about its role in microbial physiology. Identification of an antibiotic that inhibits its synthesis provides a useful tool for tackling this problem. In previous reports (6, 32–34), we have proposed that bacterial cell shape and division are regulated by the activity of two different reactions that compete with each other and that are responsible for lateral wall elongation and septum formation, respectively. The finding that daptomycin, at the MIC, blocks septum formation indicates that lipoteichoic acid may contribute to the balance between these different reactions.

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