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Inhibition of Peptidoglycan Biosynthesis in Gram-Positive Bacteria by LY146032

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LY146032, a cyclic lipopeptide antibiotic, is an inhibitor of cell wall peptidoglycan biosynthesis in gram-positive bacteria. Although LY146032 at relatively high concentrations inhibited the in vitro polymerization of UDP-linked sugar precursors, inhibition of cell wall formation in intact *Staphylococcus aureus* and *Bacillus megaterium* cells did not lead to the accumulation of UDP-*N*-acetyl-muramyl (MurNAc)-peptide(s). Experiments that measured formation of UDP-MurNAc-peptides revealed that LY146032 inhibited the formation of these nucleotide-linked intermediates. This antibiotic had a disruptive effect on membrane permeability as evidenced by the loss of intracellular potassium immediately after exposure to the drug. The lack of any major disruption of the phosphoenolpyruvate:sugar phosphotransferase system indicated that the membrane is not likely a lethal target for this antibiotic. The findings are consistent with a mechanism by which LY146032 inhibits the formation of precursor molecules utilized in peptidoglycan biosynthesis. The observed membrane effects likely result from transit of the inhibitor to its lethal target site.

LY146032 is a new cyclic lipopeptide antibiotic containing an *n*-decanoyl fatty acid side chain at the amino terminus of the peptide moiety (Fig. 1) (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). This compound is related to A21978C, a complex of cyclic lipopeptide antibiotics produced by *Streptomyces roseosporus* (F. T. Counter, P. W. Ensminger, and L. C. Howard, 20th ICAAC, abstr. no. 69, 1980). LY146032 and A21978C are bactericidal for a wide variety of gram-positive bacteria including group D streptococci and methicillin-resistant staphylococci; in vitro activity is dramatically stimulated by calcium (4; F. T. Counter, manuscript in preparation). Evidence has also been reported that the target of LY146032 is cell wall peptidoglycan biosynthesis (N. Allen, W. Alborn, Jr., J. Hobbs, Jr., and H. Percifield, 24th ICAAC, abstr. no. 1081, 1984). This study investigates the mechanism by which LY146032 interferes with the biosynthesis of peptidoglycan.

MATERIALS AND METHODS

Bacteria. All experiments were performed with *Staphylococcus aureus* FDA 209P (ATCC 6538P) or *Bacillus megaterium* X67 (ATCC 8245).

Protein, DNA, RNA, and lipid biosynthesis in *S. aureus*. Trichloroacetic acid-insoluble incorporation of 0.05 μCi of [^{14}C]leucine (333 μCi/μmol), [^{14}C]thymidine (59 μCi/μmol), and [^{14}C]uracil (59 μCi/μmol) into protein, DNA, and RNA, respectively, was measured in cells suspended in phosphate-buffered saline (1) containing 0.1 μM glucose. Ten-minute reactions containing the appropriate radioisotope were run as described previously (1) except that 1.25 mM $CaCl_2$ was added to all reactions.

Incorporation of 0.5 μCi of [^{14}C]sodium acetate (54.7 μCi/μmol) into lipid was measured in cells suspended in synthetic growth medium (1) containing 0.5% glycerol as the carbon source plus 1.25 mM $CaCl_2$. Reactions were run as above, and radioactivity was extracted into chloroform-methanol (2:1, vol/vol), dried, and counted.

Biosynthesis of peptidoglycan. Incorporation of radiolabeled L-alanine into peptidoglycan of *S. aureus* was measured by the method of Lugtenberg and DeHaan (13). Cells were grown in CGPY broth to the mid-log phase, harvested, and suspended in cell wall synthesis medium (CWSM) containing 0.25 μCi of L-[^{14}C]alanine (18.6 μCi/μmol), 50 μg of L-lysine per ml in place of 2,6-diaminopimelic acid (DAP), 100 μg of chloramphenicol per ml, and 1.25 mM $CaCl_2$. Reaction mixtures (0.25 ml) containing 0.1 optical density units (measured at 600 nm; ca 10^7 CFU) were incubated for 15 min at 37°C.

Incorporation of 0.25 μCi of [^{14}C]DAP (50 μCi/μmol) into peptidoglycan of *B. megaterium* was measured by the same methodology but omitting the [^{14}C]alanine, increasing L-alanine to 50 μg/ml, and increasing the concentration of L-lysine to 500 μg/ml. Reaction mixtures were incubated for 30 min at 37°C.

Formation of UDP-MurNAc-pentapeptide and UDP-MurNAc-tripeptide precursors. Antibiotic-induced accumulation of radioactive UDP-*N*-acetyl-muramyl (MurNAc)-peptides was detected in *S. aureus* and *B. megaterium* whole cells by labeling with [^{14}C]alanine and [^{14}C]DAP, respectively, as described above. Reactions included antibiotics as described in the appropriate figure legends and table footnotes. After incubation, reaction mixtures were centrifuged and suspended in isobutyric acid–1 M NH_4OH (5:3, vol/vol). This material was spotted on Whatman 3MM paper and chromatographed by descending chromatography in the same solvent (17). Radioactivity in muramyl peptides was measured with a radiochromatogram scanner.

Pentapeptide and tripeptide precursors were separable by using this chromatographic procedure. Identification of precursors was by chromatographic comparison with authentic samples isolated and identified in this laboratory by chromatogram elution followed by amino acid analysis.

Preparation of particulate fraction. A cell-free particulate fraction catalyzing the polymerization of peptidoglycan from UDP-linked precursors was isolated from *B. megaterium* by

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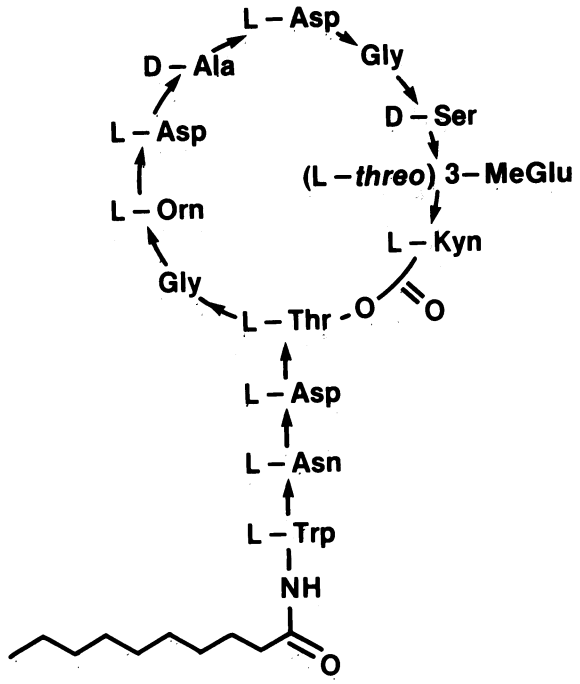

FIG. 1. Structure of LY146032. Orn, Ornithine; MeGlu, methylglutamic acid; Kyn, kynurenine.

the methods of Oka (16). Cells were broken by sonication or by grinding with alumina.

Isolation of UDP-MurNAc-pentapeptide (UDP-MurNAc-L-Ala-D-Glu-*meso*-DAP-D-Ala-D-Ala). Precursor was prepared from *Bacillus cereus* by the methods of Gorecki et al. (7), except that chromatography on Dowex was omitted and final purification was achieved by paper chromatography (Whatman 3MM; isobutyric acid–1 M NH_4OH [5:3, vol/vol]). Chromatograms were developed for 72 h to achieve maximum separation. Preparation of the radiolabeled precursor containing [^{14}C]DAP was essentially by the methods of Oka (16). The incubation medium used was CWSM [13] containing 0.125 μCi of [^{14}C]DAP per ml (118 μCi/μmol) plus vancomycin (100 μg/ml). Incubation was for 30 min at 37°C. Isolation and purification were as described above for the unlabeled precursor.

Polymerization of UDP-linked peptidoglycan precursors. Reaction mixtures contained (200 μL): 125 mM Tris hydrochloride (pH 8), 15 mM $MgCl_2$, 1.25 mM $CaCl_2$, 300 μg of particulate fraction protein, and either 0.1 mM UDP-MurNAc-[^{14}C]DAP-pentapeptide (2.9 Ci/μmol) and 0.5 mM UDP–*N*-acetylglucosamine (UDP-GlcNAc) or 0.05 mM UDP-[^{14}C]GlcNAc (10 μCi/μmol) and 0.25 mM UDP-MurNAc-pentapeptide. Reaction mixtures were incubated for 60 min at 30°C. Trichloroacetic acid-insoluble radioactivity was collected on glass fiber filters for counting. Radioactivity in lipid intermediates was extracted into *n*-butanol–6 M pyridinium acetate (pH 4.2; 4:1, vol/vol), dried, and counted. Modifications in this procedure are explained in the footnotes to the appropriate tables.

Loss of intracellular potassium. Antibiotic-induced membrane disruption in *S. aureus* and *B. megaterium* was measured by following the release of intracellular potassium. Cells were suspended to an optical density at 600 nm of 0.2 to 0.5 in synthetic growth medium (1) prepared with sodium phosphate in place of potassium phosphate. In some experiments, cells were suspended in 50 μM Tris hydrochloride (pH 7.5). $CaCl_2$ (1.25 mM) was included in all experiments. Release of intracellular potassium into the extracellular medium was measured with an Orion Ionalyzer/901 equipped with an Orion ion-specific potassium electrode.

Uptake of α-methylglucoside. Uptake of α-methylglucoside was measured in exponentially growing *S. aureus* cells in synthetic growth medium (1) containing 0.2% glycerol as the carbon source and 1.25 mM $CaCl_2$. Methyl-α-D-[^{14}C]glucopyranoside was added to give a final concentration of 0.1 mM (0.05 μCi/ml). Samples (0.5 ml) were removed at intervals and collected on filters (0.45-μm pore size; Millipore Corp., Bedford, Mass.) for measuring cell-associated radioactivity.

RESULTS

Effects on macromolecular biosynthesis. LY146032 at 100 μg/ml inhibited by 93% the incorporation of [^{14}C]alanine by *S. aureus* into peptidoglycan (Table 1). Inhibition was 76% at 10 μg/ml. LY146032 had a negligible effect on the incorporation of labeled precursors into protein, RNA, or DNA, and there was only minor inhibition (less than 25%) of [^{14}C]acetate incorporation into lipid.

Inhibition of peptidoglycan biosynthesis in *S. aureus* was compared with that in *B. megaterium* (Fig. 2). In this experiment, approximately 25 μg of LY146032 per ml was required for 50% inhibition in *S. aureus*. Peptidoglycan biosynthesis in *B. megaterium*, measured by [^{14}C]DAP incorporation, was more sensitive, requiring only 2.5 μg/ml for 50% inhibition.

Effects on membrane-associated peptidoglycan-synthesizing enzymes. Incorporation of radiolabeled UDP-linked sugar precursors into trichloroacetic acid-insoluble peptidoglycan by a particulate fraction from *B. megaterium* was inhibited by LY146032 (Table 2). Although inhibition required 100 μg/ml, the same level of inhibition was observed regardless of whether the radioactive substrate was UDP-MurNAc-pentapeptide or UDP-GlcNAc. As anticipated, fosfomycin

TABLE 1. Effects of LY146032 and other antibiotics on macromolecular biosynthesis in *S. aureus*[a]

Antibiotic	% Inhibition at antibiotic concn[b] of:														
	Protein			DNA			RNA			Lipid			Cell wall		
	100	10	1	100	10	1	100	10	1	100	10	1	100	10	1
LY146032	<0	2	7	<0	<0	6	<0	<0	<0	20	17	10	93	76	<0
Erythromycin	60	58	35												
Actinomycin D				98	88	<0									
Rifampin							86	83	83						
Cerulenin										91	43	6			
Vancomycin													74	72	59

[a] Macromolecular biosynthesis was measured as described in Materials and Methods.
[b] Antibiotics were tested at 100, 10, and 1 μg/ml.

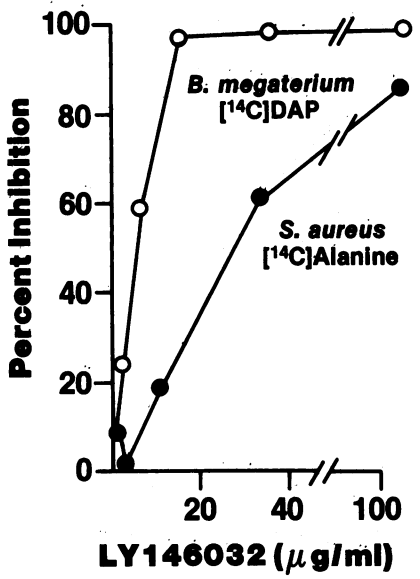

FIG. 2. Inhibition of [^{14}C]DAP and [^{14}C]alanine incorporation into acid-insoluble peptidoglycan of *B. megaterium* and *S. aureus*, respectively, by LY146032.

had no effect on these reactions, and vancomycin was inhibitory (6).

The particulate membrane fraction from *B. megaterium* catalyzes the transfer of MurNAc-pentapeptide from its nucleotide carrier to an endogenous lipid carrier (undecaprenylphosphate). This is followed by the addition of GlcNAc (from UDP-GlcNAc) via a transglycosylation reaction. The resulting disaccharide-pentapeptide is subsequently transferred from its lipid carrier to a nascent polymer of disaccharide-pentapeptide subunits. Although LY146032 inhibited the incorporation of the UDP-linked precursors into acid-insoluble peptidoglycan, it did not affect the amphomycin-sensitive transfer of phospho-MurNAc-pentapeptide to the lipid carrier (Table 3). Nor did LY146032 appear to block the disaccharide-pentapeptide transfer reaction, since accumulation of butanol–6 M pyridinium acetate-extractable radioactivity typical of inhibition by vancomycin was not detected (Table 4).

TABLE 2. Effect of antibiotics on the biosynthesis of peptidoglycan by a cell-free particulate fraction[a]

Radiolabeled precursor	Antibiotic (μg/ml)	dpm	% Inhibition
UDP-MurNAc-pentapeptide	None	3,863	
	LY146032		
	100	1,128	71
	10	4,835	<0
	Fosfomycin		
	300	4,082	<0
	30	3,990	<0
	Vancomycin		
	100	588	85
	10	2,942	24
UDP-GlcNAc	None	22,377	
	LY146032		
	100	7,182	68
	10	26,471	<0
	Fosfomycin		
	300	24,056	<0
	30	23,855	<0
	Vancomycin		
	100	3,275	85
	10	16,548	26

[a] Polymerization of UDP-linked precursors was measured as described in Materials and Methods.

TABLE 3. Effect of antibiotics on phospho-MurNAc-pentapeptide translocase[a]

Antibiotic (μg/ml)	Radioactivity (dpm) in lipid intermediate	% Inhibition
None	633	
LY146032		
500	666	<0
100	609	4
Amphomycin		
30	15	98
5	339	46
Vancomycin		
100	942	<0
10	603	5

[a] Incorporation of radioactivity into the lipid-diphospho-MurNAc-pentapeptide intermediate was determined by using the particulate fraction and the procedure for measuring polymerization of UDP-linked precursors as described in Materials and Methods. The radiolabeled substrate was UDP-MurNAc-[^{14}C]DAP-pentapeptide, and UDP-GlcNAc was omitted.

Accumulation of UDP-MurNAc-pentapeptide in intact cells. Inhibition of the membrane-associated enzymatic steps of peptidoglycan biosynthesis in intact cells may lead to precursor accumulation. When this cycle of reactions is inhibited in vivo, the availability of the monophosphorylated lipid carrier becomes limiting. If peptidoglycan biosynthesis in these cells occurs in the presence of [^{14}C]alanine or [^{14}C]DAP as a precursor, radioactivity can accumulate in UDP-MurNAc-peptides since the formation of the nucleotide-linked precursors continues. Inhibition of peptidoglycan biosynthesis in *S. aureus* by LY146032, however, did not result in the accumulation of any detectable UDP-MurNAc-peptide (Fig. 3). Vancomycin induced an accumulation of UDP-MurNAc-pentapeptide. In addition to vancomycin, D-cycloserine and amphomycin also induced a measurable accumulation of UDP-MurNAc-peptides (see Table 5). Unlike these antibiotics, LY146032 behaved more like fosfomycin (8). Identical results with LY146032 were obtained in separate experiments with *B. megaterium* and [^{14}C]DAP incorporation (data not shown). These results suggested that in intact bacteria, LY146032 interferes with an early step in biosynthesis leading to formation of the precursors.

TABLE 4. Effect of antibiotics on transfer of lipid-bound disaccharide-pentapeptide to nascent peptidoglycan[a]

Antibiotic (μg/ml)	Radioactivity (dpm) in peptidoglycan	Radioactivity (dpm) in lipid-bound intermediate
None	14,257	2,614
LY146032		
500	269	1,281
100	13,300	2,558
Vancomycin (100)	723	4,501
Amphomycin (30)	602	430

[a] Accumulation of radioactivity in the lipid-diphospho-disaccharide intermediate was determined by using the particulate fraction and the procedure for measuring polymerization of UDP-linked precursors as described in Materials and Methods.

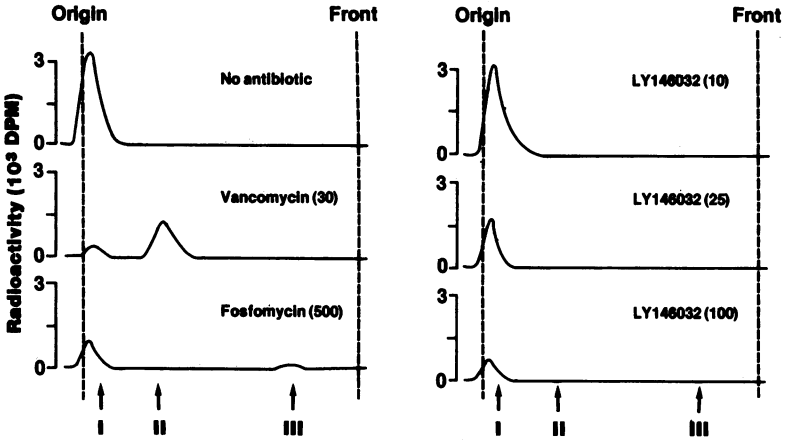

FIG. 3. Antibiotic-induced accumulation of radioactivity in UDP-MurNAc-peptides by *S. aureus*. Whole cells of *S. aureus* were fed [^{14}C]alanine to radiolabel peptidoglycan as described in Materials and Methods. Antibiotics were added 5 min before the isotope at the concentrations indicated (micrograms per milliliter). UDP-MurNAc-pentapeptide was detected chromatographically as described in Materials and Methods. I, Peptidoglycan; II, UDP-MurNAc-pentapeptide; III, alanine.

Inhibition of precursor formation. The formation of UDP-MurNAc-peptides in *S. aureus* was measured by exposing cells to amphomycin, vancomycin, or D-cycloserine to induce accumulation of radiolabeled nucleotide-linked sugar-peptide precursors. LY146032 and fosfomycin were tested for inhibition of precursor accumulation. Both LY146032 and fosfomycin inhibited the accumulation of radioactivity from [^{14}C]alanine in UDP-MurNAc-pentapeptide and UDP-MurNAc-tripeptide in *S. aureus* (Table 5). An experiment with lower concentrations of LY146032 showed that 12.5 μg/ml inhibited accumulation of radioactivity in the pentapeptide precursor (Fig. 4A). Very similar results were obtained when this experiment was performed in *B. megaterium*, using [^{14}C]DAP to label the precursor (Fig. 4B). In *B. megaterium*, 5 μg of LY146032 per ml was inhibitory and 20 μg/ml completely blocked accumulation of radioactivity in this precursor. These findings are consistent with the data in Fig. 2 and 3 and the suggestion that LY146032 interferes with the formation of cell wall peptidoglycan precursors.

TABLE 5. Effect of antibiotics on the formation of UDP-MurNAc-peptides in *S. aureus*

Expt	Inhibitor	Concn (μg/ml)	Radioactivity (dpm) in UDP-MurNAc-peptides[a]	% Inhibition
1[b]	None		17,242	
	LY146032	100	244	99
	Fosfomycin	500	1,024	94
2[c]	None		28,848	
	LY146032	100	1,059	96
	Fosfomycin	500	1,972	93
3[d]	None		24,219	
	LY146032	100	161	99
	Fosfomycin	500	320	99

[a] In the absence of added antibiotic to induce accumulation of precursor, 467 dpm of radioactivity was detected in UDP-MurNAc-peptides.

[b] *S. aureus* was incubated for 15 min in CWSM containing [^{14}C]alanine and 100 μg of amphomycin per ml to induce accumulation of UDP-MurNAc-[^{14}C]pentapeptide. Reactions were run in the absence or presence of other antibiotics as indicated. Radioactivity in the precursor was determined by paper chromatography (see Materials and Methods) followed by radiochromatogram scanning and cutting, eluting, and counting of the appropriate peak areas.

[c] As above except that 100 μg of vancomycin per ml was used to induce accumulation of UDP-MurNAc-[^{14}C]pentapeptide.

[d] As above except that 100 μg of D-cycloserine per ml was used to induce accumulation of UDP-MurNAc-[^{14}C]tripeptide.

Effects on membrane function. Using intracellular potassium leakage as an indicator of membrane disruption in *S. aureus*, we compared LY146032 with the cationic, surface-active antibiotic gramicidin S (Fig. 5). Both antibiotics were disruptive, but the initial rate of release caused by 50 μg of LY146032 per ml was at least 1 order of magnitude lower than that caused by 50 μg of gramicidin S per ml (estimated from Fig. 5A). Moreover, LY146032 had no effect on cells suspended in Tris hydrochloride buffer, whereas gramicidin

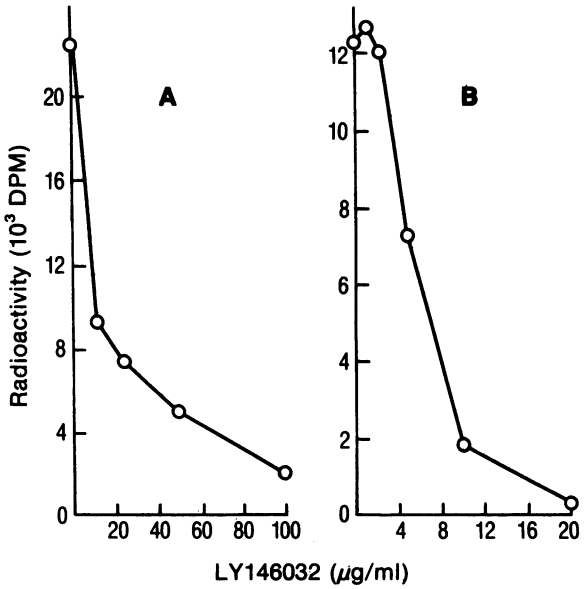

FIG. 4. Inhibition of formation of UDP-MurNAc-[^{14}C]pentapeptide in *S. aureus* (A) and *B. megaterium* (B) by LY146032. This experiment was run essentially as described in Table 5. Accumulation of radioactive precursor was facilitated by incubating cells in the presence of [^{14}C]alanine (*S. aureus*) or [^{14}C]DAP (*B. megaterium*) in the presence of 100 μg of vancomycin per ml. The effect of LY146032 on the accumulation of radioactivity was measured at the concentrations indicated. In the absence of vancomycin, radioactivity detected in the pentapeptide precursor was 938 dpm for *S. aureus* and 211 dpm for *B. megaterium*.

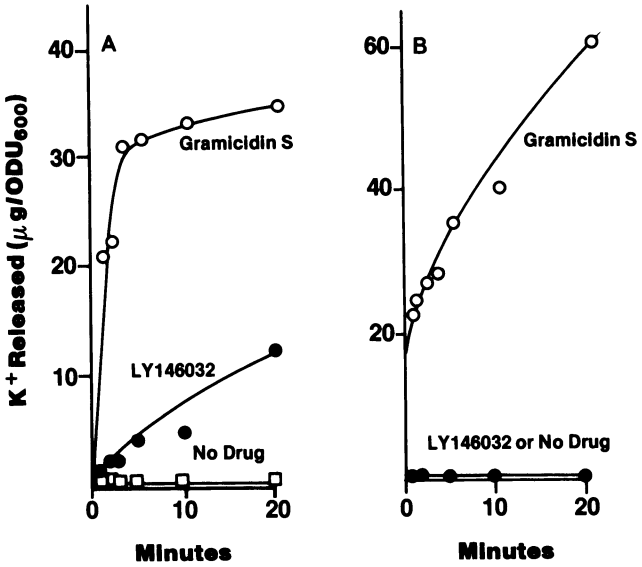

FIG. 5. Antibiotic-induced loss of intracellular potassium in *S. aureus*. Cells were suspended at 37°C in synthetic growth medium (A) or in Tris hydrochloride (B). Antibiotics (50 μg/ml) were added at zero time, and potassium released into the extracellular medium was measured as described in Materials and Methods. ODU_{600}, Optical density units at 600 nm.

S did (Fig. 5B). The membrane-disruptive effects of LY146032 were also lost when the temperature was lowered to 2°C; gramicidin S retained activity under these conditions (data not shown).

LY146032 also induced leakage of intracellular potassium in *B. megaterium*. The data in Fig. 6 compare the effect of concentration of LY146032 on potassium leakage in *S. aureus* and *B. megaterium*. Concentrations needed to induce leakage were very close to those required to inhibit peptidoglycan biosynthesis (Fig. 2) and formation of the UDP-MurNAc-pentapeptide (Fig. 4). Moreover, *B. megaterium* was more sensitive to the disruptive effects of LY146032 than was *S. aureus*. This same relationship was seen for

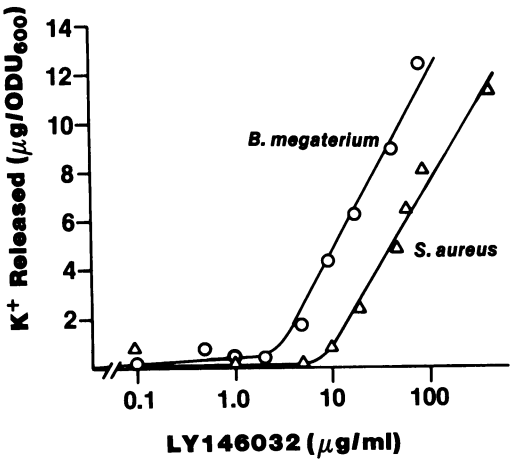

FIG. 6. Effect of concentrations of LY146032 on release of intracellular potassium in *B. megaterium* and *S. aureus*. Potassium released into the extracellular medium was measured after a 20-min exposure to various concentrations of LY146032. ODU_{600}, Optical density units at 600 nm.

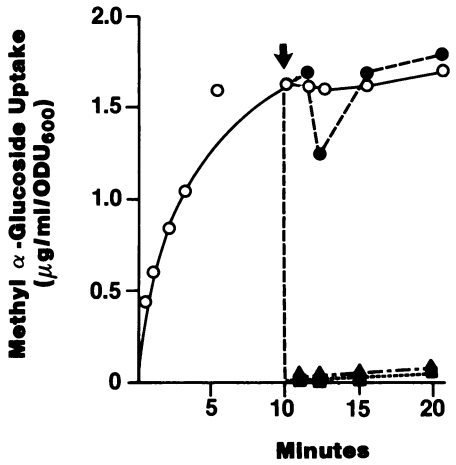

FIG. 7. Effect of antibiotics on accumulation of α-methylglucoside by *S. aureus*. Compounds were added at the time indicated by the arrow. Symbols: ○, no drug; ●, LY146032 (50 μg/ml); ▲, cetylpyridinium chloride (50 μg/ml); ■, gramicidin S (50 μg/ml).

inhibition of peptidoglycan biosynthesis (Fig. 2) and inhibition of precursor accumulation (Fig. 4).

Despite the effects on intracellular potassium, LY146032 had little effect on the accumulation of radiolabeled α-methylglucoside by *S. aureus* (Fig. 7). Accumulation of the nonmetabolizable substrate was completely destroyed and over 90% of the cell-associated radioactivity was lost within 1 min after exposure to either gramicidin S or cetylpyridinium chloride. In contrast, addition of LY146032 caused a slight perturbation of sugar uptake, but accumulation was essentially restored within 5 min, and there was no permanent loss of cell-associated radioactivity.

DISCUSSION

As a cyclic lipopeptide, LY146032 shares structural similarities with the polymyxin-type compounds (22) and, to a lesser extent, with amphomycin (23), bacitracin (6), and gramicidin S (6). Although these antibiotics all share some degree of structural relatedness, they have diverse modes of action (6). LY146032 appears to have yet another mechanism of action. The study reported here confirms that LY146032 is an inhibitor of cell wall peptidoglycan biosynthesis in gram-positive bacteria. The antibiotic inhibited the incorporation of [^{14}C]alanine and [^{14}C]DAP into peptidoglycan of *S. aureus* and *B. megaterium*, respectively. No significant inhibition of protein, RNA, DNA, or lipid biosynthesis by LY146032 was observed. Eliopoulous et al. (4) reported that the structurally similar antibiotic A21978C_1 specifically inhibited peptidoglycan biosynthesis in both *S. aureus* and *Streptococcus faecalis*.

The incorporation of UDP-[^{14}C]GlcNAc and UDP-MurNAc-[^{14}C]pentapeptide into peptidoglycan by a particulate membrane fraction from *B. megaterium* was inhibited by 100 μg of LY146032 per ml. A concentration of 10 μg/ml had no effect. The particulate fraction from *B. megaterium* and from other bacteria catalyzes the polymerization of nucleotide-linked sugar precursors (2, 16, 17), which is inhibited by amphomycin (23, 24), moenomycin (14, 25), azureomycin B (21), vancomycin (14, 25), bacitracin (20), and other antibiotics by a variety of mechanisms. Inhibition of polymerization by LY146032 was unlike that of the lipopeptide amphomycin (Table 3) or the glycopeptide antibiotic vanco-

mycin (Table 4). Thus, an effect on either the phospho-MurNAc-pentapeptide translocase or the disaccharide-pentapeptide transglycosylase reactions is not likely. The exact mechanism by which LY146032 inhibits the in vitro system remains to be determined, but in view of the high drug concentration required for inhibition along with the findings described below, the lethal target in intact bacteria may not be the polymerization of nucleotide-linked precursors.

Inhibition by antibiotics that interfere with the polymerization reactions typically leads to an accumulation of the UDP-MurNAc-pentapeptide precursor in the cytoplasm of intact bacteria (12, 14). Surprisingly, however, inhibition by LY146032 in intact *S. aureus* as well as *B. megaterium* cells did not lead to detectable accumulation of any radiolabeled precursor. This finding suggested that rather than having an effect on precursor utilization, LY146032 interferes with precursor formation. Formation of nucleotide-linked sugar-peptide precursors by *S. aureus* and *B. megaterium* was measured by following the incorporation of radioactivity from labeled alanine or DAP into the tripeptide and pentapeptide intermediates. Chromatographic detection of these compounds was facilitated by adding amphomycin or vancomycin to induce accumulation of the pentapeptide or by adding D-cycloserine to induce accumulation of the tripeptide. (In the absence of antibiotics, neither intermediate could be detected chromatographically [Fig. 3].) The addition of LY146032 in these experiments with both *S. aureus* and *B. megaterium* inhibited the appearance of label in the precursors. Inhibition of this effect occurred at the same concentrations that inhibited incorporation of [^{14}C]alanine and [^{14}C]DAP, suggesting that inhibition of cell wall biosynthesis was due to an effect on precursor formation. If LY146032 blocks a specific step in precursor biosynthesis, the block must occur before formation of the tripeptide. Moreover, no accumulation of any [^{14}C]alanine-containing intermediate was detected in the experiments with *S. aureus*, suggesting that formation of UDP-MurNAc-L-Ala or an earlier step is inhibited.

Antibiotics that inhibit precursor formation are relatively low-molecular-weight compounds, often substrate analogs, and have specific mechanisms for crossing the cytoplasmic membrane (18). D-Cycloserine (15), alaphosphin (3), fosfomycin (8), and bacilysin (9, 10) have molecular weights between 102 and 270, share structural similarities with the substrates for the enzymes they each inhibit, and utilize very specific cytoplasmic membrane transport systems (19). LY146032, in contrast, has a molecular weight of 1,619, does not show an obvious structural similarity to any peptidoglycan substrate, and may or may not cross the cytoplasmic membrane. At this time we cannot rule out the possibility that precursors are synthesized in the presence of LY146032 and are lost into the medium owing to effects on membrane permeability. However, attempts to detect precursors excreted into the medium as a result of disruptive effects of LY146032 were negative (unpublished experiments). Further experimentation will be necessary to pinpoint the exact mechanism by which LY146032 affects precursor formation.

If LY146032 inhibits one or more of the reactions leading to the biosynthesis of the nucleotide-linked sugar-peptide precursors, it may have to cross the cytoplasmic membrane (18). The fact that LY146032 perturbs membrane function (as evidenced by leakage of an intracellular cation) at the same concentrations that inhibit peptidoglycan biosynthesis is consistent with this notion. At the same time, if the target is cytoplasmic, the perturbation of membrane function might be expected to be nonlethal and transitory. That this indeed is the case is supported by findings from the present study. LY146032 had only minor effects on the functioning of the phosphotransferase system (uptake of α-methylglucoside); and on a weight basis, the membrane effects of LY146032 were less disruptive than those of gramicidin S. In addition, gramicidin S disrupted cells at 2°C as well as cells suspended in Tris hydrochloride buffer, but LY146032 did not.

Lakey and Lea (11) have shown that A21978C analogs (including LY146032) significantly increase the conductivity of lipid planar bilayers in vitro. Although this finding indicated that membrane interactions are a fundamental part of the action of LY146032, Lakey and Lea believe that this action alone cannot account for the antimicrobial activity of the compound. There was no evidence for formation of transmembrane channels, nor for sufficient ionophore activity to account for the potassium leakage which we observed. Rather, the report of Lakey and Lea is consistent with the notion that the effect of LY146032 on membrane function is a result of transit of this antibiotic to its target site.

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