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Regulation of the Bacterial Cell Wall: Effect of Antibiotics on Lipid Biosynthesis

BRUCE H. HEBELER, ANADI N. CHATTERJEE, AND FRANK E. YOUNG

Department of Microbiology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

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Antibiotics that inhibit the biosynthesis of the cell wall, such as vancomycin, penicillin, D-cycloserine, and bacitracin, stimulate the incorporation of lysine into lipids that are extractable with *n*-butanol-6 M pyridinium acetate. Approximately 93% of this lysine is in lysylphosphatidylglycerol (LPG). The remaining lysine is incorporated in another as yet uncharacterized lipid. Because the lysine in the latter lipid is released by mild alkaline hydrolysis, it is not the C_{55}-isoprenyl-pyrophospho-*N*-acetyl-muramyl pentapeptide. Vancomycin and penicillin stimulate the incorporation of lysine into both LPG and the minor lipid fractions, whereas treatment with D-cycloserine results in an increase only in LPG. Antibiotics that inhibit protein synthesis do not influence the incorporation of lysine into the lipid fractions. Analysis of the extracted lipids indicate that the incorporation of radioactive lysine into LPG is due to an enhancement in synthesis of LPG from phospholipids in the cytoplasmic membrane.

The occurrence of lipoamino acid complexes in bacteria has been studied extensively. MacFarlane (18) first demonstrated the presence of aminoacyl derivatives of phosphatidylglycerol (PG) in *Clostridium welchii*. These complexes were subsequently isolated from several other gram-positive bacteria such as *Staphylococcus aureus* (11), *Bacillus megaterium* (24), *Bacillus cereus* (11), and *Streptococcus faecalis* (12). Many amino acids (alanine, ornithine, glycine, or lysine) have been shown to be bound to PG by an *o*-acyl ester bond (10, 11, 24). Of these complexes, lysylphosphatidylglycerol (LPG) occurs most frequently. In *S. aureus*, LPG comprises about 14% of the total phospholipids present in the cytoplasmic membrane (27). Although the chemical structure and biosynthesis of LPG have been established (12, 16), its physiological role remains to be elucidated. These amino acid derivatives of PG have been postulated to be involved in the following processes: amino acid transport (5), regulation of ion permeability (7, 12), and as a carrier involved in protein or cell wall synthesis, or both (8, 13, 22).

Another class of lipids has been described recently. The C_{55}-isoprenyl phosphate, located in the cytoplasmic membrane, acts as a carrier in the synthesis of many polymers including *O*-antigen (35), peptidoglycan (9), and teichoic acid (4). The role of this phospholipid in the biosynthesis of peptidoglycan is now well established (14, 21). This lipid cycle has been shown to be interrupted by several antibiotics. For example, vancomycin inhibits the transfer of the disaccharide-decapeptide amide to the wall acceptor (1), whereas bacitracin blocks the dephosphorylation of the C_{55}-isoprenyl pyrophosphate (29). As a result, treatment with vancomycin causes an accumulation of lipid-linked peptidoglycan precursors (in *S. aureus* this would contain lysine), whereas bacitracin induces the accumulation of lipid pyrophosphate. Antibiotics like penicillin or D-cycloserine inhibit other steps of the biosynthetic pathway which are not directly associated with this lipid cycle (23, 30, 30a, 31-33, 35).

In an attempt to isolate mutants defective in the reactions mediated by the C_{55}-isoprenyl phosphate coenzyme, we developed techniques to rapidly extract lipids. During in vivo studies with *S. aureus* H, we observed an enhanced incorporation of lysine into a lipid fraction when the parental cells were treated with antibiotics that specifically inhibit peptidoglycan synthesis. This stimulation was observed when growing cells were treated not only with vancomycin but also with penicillin, D-cycloserine, and bacitracin. The last three antibiotics would not be expected to and, indeed, have not been shown previously to cause any accumulation of lipids

containing lysine (either the LPG or the lipid intermediate in peptidoglycan synthesis).

The present paper demonstrates that all peptidoglycan inhibitors which act at different points of the biosynthetic cycle cause a rapid incorporation of lysine into lipid fractions. The major incorporation of lysine is shown to be in lysylphosphatidylglycerol. A preliminary account of this work has already been published (Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 43, 1972).

MATERIALS AND METHODS

Organisms. The isolation and characterization of the streptomycin-resistant parent strain *S. aureus* H (*str*) has been previously described (2). *S. aureus* U71 was obtained from D. C. White (University of Kentucky Medical Center, Lexington).

Media. The growth medium (PYK medium) consisted of 0.5% Phytone (BBL), 0.5% yeast extract (Difco), and 0.3% K_2HPO_4 adjusted to pH 7.2 with 4 N HCl. After autoclaving, glucose was added to yield a final concentration of 0.2%.

Growth conditions. Overnight cultures were shaken at 100 rpm in 50 ml of PYK medium at 36 C in 250-ml Erlenmeyer flasks. After addition of a 5% inoculum of the overnight culture to fresh PYK medium, experimental cultures were grown at 180 rpm at 37 C. Growth was monitored turbidimetrically at 585 nm with a Gilford 300N spectrophotometer. Dry-weight determinations were approximated from standardized calculations based on the optical density (OD) of the culture. At 585 nm an OD of 1.0 is equivalent to 0.218 mg (dry weight) per ml in cells not treated with antibiotics.

Isolation of lipids. Cells were harvested by centrifugation at 6,000 $\times$ *g* for 6 min at 4 C. The lipids were extracted according to the chloroform-methanol (C-M) (2:1, vol/vol) method described by Gould and Lennarz (6) or by a modification of the *n*-butanol-6 M pyridinium acetate (2:1, vol/vol; BUOH-PYR ACE) procedure of Anderson et al. (1). For the latter method, the packed cells were washed three times with 5 to 10 vol of cold 5% trichloroacetic acid; the lipids were subsequently extracted with BUOH-PYR ACE (pH 4.1), and the polar compounds were removed by washing the lipid extract three times with distilled water. The washed extract was evaporated to dryness on a Buchler flash-evaporator and resuspended in chloroform.

Chromatographic procedures. Paper chromatography was carried out on a silica gel-impregnated paper (Whatman SG-81; W & R Balston, Ltd., England) by using chloroform-methanol-water (65:25:4, vol/vol/vol). The various lipid fractions were stained with rhodamine 6G and ninhydrin (19, 20). The lipids from unstained chromatograms were recovered quantitatively by cutting out the spots and soaking them in 3 ml of chloroform-methanol-18 mM aqueous ammonium hydroxide (20:20:1, vol/vol/vol) for 1 h as described by Lillich and White (17).

A column of Sephadex G-25 (particle size 100 to 300 μm, Sigma Chemical Co., St. Louis, Mo.) was used to remove polar impurities (28). Silicic acid columns (100 to 200 mesh, Unisil, Clarkson Chemical Co., Williamsport, Pa.) were used to separate the glycolipids and the phospholipids (15).

A 40-ml volume of the following concentrations of C-M (vol/vol) were used as the elution scheme: (i) 100:0; (ii) 98:2; (iii) 96:4; (iv) 94:6; (v) 92:8; (vi) 90:10; (vii) 87:13; (viii) 85:15; (ix) 83:17; (x) 80:20; (xi) 78:12; (xii) 75:25; (xiii) 70:30; (xiv) 50:50; (xv) 0:100. The various fractions were taken to dryness and resuspended in chloroform.

Measurement of radioactivity. Lipid extracts were evaporated to dryness and resuspended in 5 ml of Triton X-100 scintillation fluid (TXS). The radioactivity was monitored in a Beckman LS 230 liquid scintillation counter. Paper chromatograms were assayed for radioactive compounds by cutting zones from the paper and counting them in 5 ml of toluene scintillation fluid. The radioactivity of whole cells was assayed by counting a sample of washed cells in TXS fluid. TXS consisted of 2 liters of toluene, 1 liter of Triton X-100, 15.2 g of 2,5-diphenyloxazole (PPO), and 380 mg of 1,4-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP). Toluene scintillation fluid contained 2 liters of toluene, 1 liter of ethylene-glycol monoethyl ether, 12 g of 2,5-diphenyloxazole, and 300 mg of POPOP.

Analytical methods. Free lysine in the cell pool was removed by cold 5% trichloroacetic acid. The distribution of radioactive lysine in the cell wall, protein, and lipid fractions was determined by the Park-Hancock fractionation procedure (25) and by BUOH-PYR ACE extraction (1). Acid hydrolysis of the lipid fraction was accomplished in sealed tubes containing 6 N HCl at 105 C for 24 h. After hydrolysis the HCl was removed by flash evaporation. The residue was washed twice by resuspension in distilled water followed by evaporation. The hydrolysate was finally resuspended in 1 ml each of distilled water and chloroform. The aqueous and organic layers were separated for further analysis. Alkaline hydrolysis was performed by heating the lipid extract in 1 N methanolic NaOH at 37 C for 20 min. After hydrolysis an equal volume of water and chloroform was added, and the resultant layers were separated for further analysis. The presence of *o*-acyl groups was determined by a modification of the hydroxylamine procedure of Sjöholm et al. (30). In this method, 100 μliters of lipid extract suspended in chloroform was added to 300 μliters of freshly prepared 2 M NH_2OH-HCl adjusted to pH 7.5. After heating for 30 min at 37 C, 2 ml of C-M (50:50, vol/vol) was added and the aqueous layer was separated. The amount of radioactive lysine released by hydrolysis was determined and extracted in the aqueous layer in TXS. The phosphate content of the lipid extracts was measured by the method of Chen et al. (3).

RESULTS

One of the major aims of this study was to isolate mutants of *S. aureus* H which were

defective in the C_{55}-isoprenyl phosphate cycle. The control used in screening for such mutants was the parental strain grown in the presence of either vancomycin, penicillin, or D-cycloserine. The lipids from radioactively labeled parental cells (^{3}H-lysine) were extracted by BUOH-PYR ACE which had been previously used in the isolation of the lipid intermediate (1). It was noted that not only vancomycin but also penicillin and D-cycloserine showed a large accumulation of a radioactive lipid fraction. It was decided, therefore, to investigate this phenomenon further.

Accumulation as a function of growth phase. Quite early in our studies, we observed a striking variation in the pattern of incorporation of lysine in the lipid fractions when *S. aureus* H was treated with antibiotics that inhibit the biosynthesis of the cell wall. To determine the effect of growth on the incorporation of lysine into lipids, samples of logarithmically growing cells were removed at 30-min intervals and incubated with radioactive lysine with and without vancomycin for a period of 70 min at 37 C on a gyratory water bath at 180 rpm. The cells were then harvested by centrifugation and the incorporation of lysine into lipid was determined as described in Materials and Methods. Figure 1 shows the incorporation of radioactive lysine into the lipid fraction from control and vancomycin-treated cells. The upper portion of the figure is a typical growth curve of *S. aureus* H (in the absence of antibiotics). The lower half of the figure represents BUOH-PYR ACE extracts taken from control and vancomycin-treated cultures at 30-min intervals. The observed stimulation by vancomycin was due to a progressive decrease of incorporation of lysine in control cultures with age and to a corresponding increase in cells treated with vancomycin. Because the BUOH-PYR ACE extraction of the cell pellet used in the above experiments is most effective for the isolation of the lipid-linked peptidoglycan intermediate (1), it was necessary to compare the efficiency of extraction of the lysine-labeled lipids by other solvents. BUOH-PYR ACE is the most efficient solvent for the extraction of lysine-containing lipids (Table 1). The prior acidification of the cell pellet by acetate buffer does elevate the amount of extractable counts in all cases except with C-M. The amount of phosphate in the lipid extract was about 50 μmol/g of cells (dry weight). This value is similar to the amount of phospholipids in *S. aureus* reported by other investigators (27).

Effect of antibiotics on the incorporation of amino acids into a BUOH-PYR ACE extract.

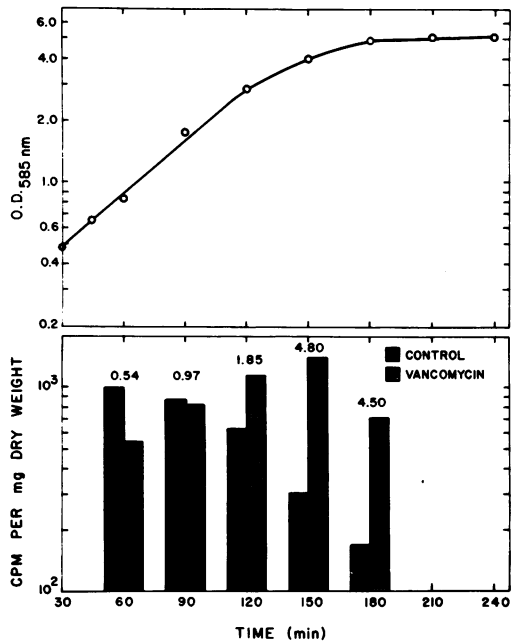

FIG. 1. *The upper figure represents a typical growth curve of S. aureus (str) grown in PYK broth shaken at 180 rpm at 37 C. The lower figure represents the extent of accumulation of lysyl lipid in control and vancomycin-treated cultures. At the times indicated, vancomycin (50 μg/ml) and ^{3}H-L-lysine (0.05 μCi/ml, specific activity 2.92 μCi/μmol) were added. After 70 min the lipids were extracted by BUOH-PYR ACE.*

TABLE 1. *Extraction of ^{3}H-lysyl lipid by various solvents*

Solvent	Incorporation of lysine[a]
BUOH-PYR ACE	4,410.0
BUOH-PYR ACE in ACE buffer	5,194.0
n-BUOH	128.0
n-BUOH in ACE buffer	408.0
C-M (2:1, vol/vol)	3,497.4
C-M (2:1, vol/vol) in ACE buffer	494.2

[a] Expressed as counts per minute per milligram (dry weight).

Since an increase in incorporation of lysine into lipid was caused by vancomycin, it was possible that the label was in the C_{55}-isoprenyl pyrophospho-*N*-acetyl-muramyl pentapeptide. In order to explore this possibility, other antibiotics were added to late log-phase cultures (OD at 585 nm = 3.0). The amount of radioactive lysine incorporated into lipid was measured after a 70-min incubation period by extraction of the lipids with BUOH-PYR ACE. It is clear from the data (Table 2) that the incorporation of lysine

into lipid is markedly enhanced when the cells are grown in the presence of antibiotics which inhibit cell wall biosynthesis. No accumulation was noted with chloramphenicol (Table 2) or puromycin (50 μg/ml, not shown). The percentage of radioactive lysine extracted by BUOH-PYR ACE from cold 5% trichloroacetic acid-precipitated cells varied in control cultures from 3 to 14%. This variation was not noted in any of the cultures treated with antibiotics. To investigate this phenomenon further, other amino acids known to occur in the peptidoglycan of *S. aureus*, D-glutamic acid, D-alanine, and glycine, were tested for incorporation into lipid in the presence of antibiotics. Phenylalanine, incorporated only into protein, was used as a control. The conditions of this experiment were identical to the previous ones. The percentage of ^{3}H-lysine extracted by BUOH-PYR ACE from 5% trichloroacetic acid-precipitated cells is shown in parenthesis. The data presented in Table 3 clearly show that only the amino acids found in cell walls are incorporated into the lipid fraction. However, this degree of incorporation in the presence of antibiotics of these amino acids does not approach the relative incorporation of ^{3}H-lysine as seen in Table 2. This suggested that lysine was being incorporated into some lipid fraction other than the C_{55}-isoprenyl pyrophospho-*N*-acetyl-muramyl pentapeptide.

TABLE 2. *Effects of antibiotics on the incorporation of lysine into the lipid fraction*

Expt	Antibiotic[a]	^{3}H-Lysine incorporation[b]
1	Control	509 (3.6%)
	Vancomycin	2,045 (33.0%)
	Penicillin	1,677 (39.2%)
	D-Cycloserine	1,200 (23.6%)
2	Control	500 (10.0%)
	Bacitracin	1,680 (39.9%)
	Chloramphenicol	115 (3.5%)

[a] Concentration of antibiotics (μg/ml): vancomycin, 50; penicillin, 10; D-cycloserine, 50; bacitracin, 70; chloramphenicol, 75.

[b] ^{3}H-L-lysine was added to a final concentration of 0.05 μCi/ml, with a specific activity of 0.05 μCi/μMol. Results are given as counts per minute per milligram (dry weight) of cells. The percentage of ^{3}H-lysine extracted by BUOH-PYR ACE from 5% trichloroacetic acid-precipitated cells is shown in parenthesis.

Kinetics of lysyl-lipid accumulation. The rate of incorporation of ^{3}H-lysine into the lipid fraction was determined by treating the late log-phase cells (OD at 585 nm = 3.0) simultaneously with radioactive label and antibiotics (time = 0 min). At various intervals, samples of cells were harvested and assayed for the incorporation of lysine into the lipid fraction by the BUOH-PYR ACE procedure (1). In the control culture there was an initial linear rise for the first 20 min followed by a plateau (Fig. 2). In

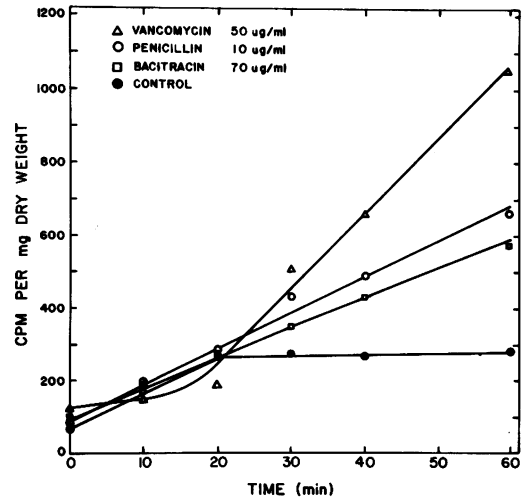

FIG. 2. *Kinetics of lysine incorporation into lipid. S. aureus (str) cells were grown to an OD of 3.0, and the antibiotic and label were added (time 0). At intervals indicated, samples were taken from the cultures and the lipid was extracted by BUOH-PYR ACE.*

TABLE 3. *Effect of antibiotics on amino acid incorporation into the lipid fraction*[a]

Antibiotic[b]	Glutamic acid[c]	Glycine	Alanine	Phenylalanine
Control	39.5 (1.1)	80.2 (1.8)	83.7 (1.6)	68.9 (1.0)
Vancomycin	64.9 (3.0)	712.1 (6.9)	337.6 (12.4)	82.4 (1.3)
Penicillin	63.9 (6.7)	574.4 (12.6)	293.4 (21.2)	104.5 (4.2)
D-Cycloserine	60.8 (2.6)	565.2 (11.8)	94.2 (1.8)	98.0 (1.8)

[a] Results are given as counts per minute per milligram (dry weight) of cells. The percentage of ^{3}H-lysine extracted by BUOH-PYR ACE from 5% trichloroacetic acid-precipitated cells is shown in parenthesis.

[b] Concentration of antibiotics (μg/ml): vancomycin, 50; penicillin, 10; D-cycloserine, 50.

[c] Radioactive amino acids were added to yield a final concentration of 0.05 μCi/ml. Specific activities (μCi/μmol) of the amino acids were: ^{14}C-glutamic acid, 198; ^{14}C-glycine, 84; ^{14}C-alanine, 120; ^{14}C-phenylalanine, 486.

marked contrast to the control, the incorporation of labeled lysine into the lipid fraction in cultures treated with vancomycin, penicillin, D-cycloserine, and bacitracin was linear after 20 min. Chloramphenicol- and puromycin-treated cultures were similar to the controls. Chloramphenicol did not inhibit the incorporation of radioactive lysine in cultures treated with vancomycin.

Analysis of lipid. The extracted, labeled lipids from control, vancomycin, and D-cycloserine treated cultures were chromatographed on Sephadex G-25 to remove any nonlipid impurities (28). In this experiment the total increase of ^{3}H-lysine incorporation for the vancomycin- and D-cycloserine-treated cultures over the control was 1.80- and 1.29-fold, respectively. Free ^{14}C-alanine was added to the column as a measure of the effectiveness of this procedure. Elution from the column was accomplished by 100 ml of each of the following solvents: 1, C-M (19:1, vol/vol) saturated with water; 2, C-M (19:1, vol/vol; 5 vol) plus acetic acid (1 vol) saturated with water; and 3, methanol water (1:1, vol/vol). The ^{14}C-alanine was retarded by the Sephadex and not removed until elution with solvent 3. The lipids eluted by solvents 1 and 2 were concentrated and chromatographed on silica gel-impregnated paper (SG81) with chloroform-methanol-water (65:25:4, vol/vol/vol). Recoveries for the control, vancomycin-, and D-cycloserine-treated extractable lipids from the Sephadex G-25 column were 94, 77, and 98%, respectively.

The chromatogram was then cut into 1-cm strips and assayed for radioactivity. In all samples, control, vancomycin, and D-cycloserine, two fractions (A with an R_f of 0.38 and B with an R_f of 0.65) were eluted by solvent 1 (Table 4). Fraction A represented about 90% of the total recovered radioactivity, whereas fraction B contained 10%. Solvent 2 yielded only one major fraction which had the same R_f as fraction A and probably represented a small portion of label which was not removed by solvent 1. Fraction A co-chromatographed with LPG extracted from *S. aureus* U71 by C-M (2:1, vol/vol). Although the percentage of counts remained the same within each sample, the counts varied between samples. The vancomycin-treated sample demonstrated a 1.74-fold increase in counts in fraction A and 2.0-fold stimulation in fraction B over the control. The D-cycloserine sample, however, only yielded an increase in fraction A. Fractions A and B eluted from the Sephadex G-25 column by solvent 1 were further purified by column chromatography on Unisil. The results in Table 5 indicate that two peaks of radioactivity were present in the lipid extracted from control and vancomycin-treated samples. The greater amounts of radioactivity were eluted by solvents 7 and 10 in the control sample, whereas in the vancomycin-treated sample solvents 7 and 9 eluted higher levels of radioactive lipid. Although this procedure yields a complex elution pattern, it ensures maximal separation of the components. Further chromatography of these fractions on SG-81 paper developed in C-M-water (65:25:4, vol/vol/vol) yielded only one radioactive spot. Alkaline hydrolysis of the Unisil fractions (Table 6) by 1 N methanolic NaOH yielded 90% hydrolysis of the lipid eluted by solvent 7 in both the control and vancomycin-treated samples. Control fraction 10 and vancomycin fraction 9 yielded 97% hydrolysis under the same conditions. Chromatographs of the aqueous layer of the hydrolyzed fractions on Whatman 3MM strips developed in isobutyric-NH_3 (5:3, vol/vol) revealed only one area of radioactivity that

TABLE 4. *SG-81 chromatograph of ^{3}H-lysyl lipids*

Relative mobility	Radioactivity[a]		
	Control	Vancomycin	D-Cycloserine
0.38	51,150 (92.5)	89,000 (91.5)	72,500 (94.6)
0.65	4,150 (7.5)	8,300 (8.5)	4,100 (5.4)

[a] Expressed as counts per minute based on the total number of counts present in the sample. Numbers in parentheses indicate the percentage of counts which migrated to the R_f.

TABLE 5. *Unisil fractionation of ^{3}H-lysyl lipids*

Fraction no.	Solvent	Solvent concn (vol/vol)	Total counts per minute eluted	
			Control	Vancomycin
1	Chloroform	100%	90	80
2	C-M	98:2	20	
3	C-M	96:4	50	20
4	C-M	94:2	570	50
5	C-M	92:8	940	310
6	C-M	90:10	2,080	2,060
7	C-M	87:13	2,640	8,110
8	C-M	85:15	1,070	3,820
9	C-M	83:17	630	29,540
10	C-M	80:20	27,000	11,180
11	C-M	78:22	8,290	10,510
12	C-M	75:25	2,010	2,670
13	C-M	70:30	820	1,110
14	C-M	50:50	750	1,000
15	Methanol	100%	300	400

TABLE 6. *Hydrolysis of lipids purified by Unisil fractionation*

Unisil fraction[a]	Percent hydrolyzed by 1 N methanolic NaOH	Percent hydrolyzed by hydroxylamine
C-7	89.5	0.0
V-7	93.6	3.0
C-10	96.6	86.5
V-10	96.9	80.5

[a] C and V designate control and vancomycin fractions, respectively.

co-chromatographed with free lysine. Hydrolysis by hydroxylamine, which cleaves the *o*-acyl groups, yielded approximately 85% hydrolysis of control fraction 10 and vancomycin fraction 9. In marked contrast, no hydrolysis occurred in fraction 7 of the control and vancomycin samples. This indicated that the lysine in these fractions was not linked to the lipid by an *o*-acyl bond.

DISCUSSION

Temperature-sensitive mutants that are defective in the lipid cycle involved in peptidoglycan synthesis should accumulate lipid-bound peptidoglycan precursors at the nonpermissive temperature comparable to those induced by the parental strain by antibiotics such as vancomycin. The major evidence that vancomycin leads to the accumulation of lipid-bound disaccharide-peptide intermediates has come from in vitro studies (1, 14, 21). Surprisingly, with whole cells of *S. aureus* H we observed a striking stimulation of lysine incorporation in the lipid fraction(s) when peptidoglycan synthesis was inhibited by various antibiotics (e.g., vancomycin, penicillin G, bacitracin, and D-cycloserine).

At present there are only two known lipids in *S. aureus* that contain a lysine moeity: lysyl phosphatidylglycerol and the C_{55}-isoprenyl-pyrophospho-*N*-acetyl-muramyl pentapeptide. The first of these compounds, LPG, has been studied extensively. Due to the high-energy nature of the bond between lysine and phosphatidylglycerol, it was originally speculated that it played a role in amino acid transport (5) and protein or cell wall biosynthesis (8, 13, 22); however, Gould and Lennarz (6) have provided compelling arguments against such a role. An attractive, alternative role has recently been proposed by Haest et al. (7). These workers demonstrated a correlation between the permeability of cells with a high and low LPG-PG ratio. The spacing of lipid molecules such as LPG and PG was shown to regulate nonelectrolyte and cation permeability. The charges on the polar head-groups were thought to help the cell control its permeability during variations in the external concentrations of hydrogen ions. It is still not known, however, whether the increase in LPG-to-PG ratio in acidic environments (11) is directly due to an effort by the cell to control permeability or is a secondary effect due to an alteration in cellular metabolism.

In the present study we noted that antibiotics that inhibit peptidoglycan synthesis have a striking effect on the accumulation of LPG in *S. aureus* strains H and U71. One of the most important factors affecting the accumulation of LPG in cells grown in the presence of cell wall antibiotics was the age of the culture. As the age of the culture increased, there was a progressive decrease in the amount of lysine that was incorporated into the lipid fraction in the control cultures, whereas there was an increase of incorporation in cells treated with cell wall antibiotics. This phenomenon was not due to a pH shift, since the pH of the medium did not drop below 7.0 during the growth cycle. Lysine incorporation into lipids in control cultures demonstrated an initial linear rise followed by a plateau. In cultures treated with cell wall inhibitors there was a continuous rise in the level of lysine incorporation. Inhibitors of protein synthesis such as chloramphenicol and puromycin had no effect on the incorporation of labeled lysine into lipids either in control or in vancomycin-treated cultures (data not shown). These observations are consistent with our finding that the majority of the labeled lysine (85 to 90%) was incorporated into LPG. Lennarz et al. (16) demonstrated that inhibition of protein synthesis by chloramphenicol or puromycin did not affect the aminoacyl transferase system involved in the formation of LPG. There are several possible explanations which could result in the observed stimulation of lysine incorporation: (i) increased turnover, (ii) increased synthesis, and (iii) prevention of degradation of LPG to lysine and PG. Since the total phosphorus in lipids extracted from control and antibiotic-treated cultures was the same, it is likely that the LPG could have been derived directly from membrane phospholipids such as PG or cardiolipin. Turnover of phospholipids during the bacterial growth is by now well documented. Short and White (27) showed that *S. aureus* accumulated cardiolipin and lost PG during stationary phase of growth, whereas the LPG level remained constant. Houtsmuller and van Deenen (12) demonstrated earlier that as the growth media became acidic, LPG was

formed at the expense of PG. Whether such a mechanism is operational in our system remains to be elucidated.

The minor lipid which was observed in this study is extractable only by BUOH-PYR ACE and not by C-M. It is more hydrophobic than LPG, since it has a greater mobility on the SG-81 paper and also elutes earlier on the silicic acid column. This lipid fraction is present in cells grown in the presence of vancomycin and penicillin. Although D-cycloserine does stimulate lysine incorporation in the LPG fraction, it has no effect on its incorporation in the minor lipid. Lysine present in this fraction is not hydrolyzed by neutral hydroxylamine but is released as free lysine by methanolic NaOH. Since hydroxylamine splits *o*-acyl ester bonds and the treatment with methanolic NaOH is too mild to degrade the peptide bonds in the C_{55}-isoprenyl-pyrophospho-*N*-acetyl-muramyl pentapeptide, this lysine-containing lipid is apparently distinct from the known lipoamino acid derivatives present in *S. aureus*. The chemical nature of the lipid is presently under investigation.

It is possible that this increased level of LPG may have a profound effect on the activity of lipid-requiring enzymes. For instance, Pless et al. (D. D. Pless, M. M. Jonah, and F. C. Neuhaus, Fed. Proc. **31**:413, 1972) and Umbreit et al. (33) have demonstrated that the activity of phospho-N-acetyl-muramyl pentapeptide translocase required C_{55}-isoprenyl phosphate and a neutral lipid after solubilization by Triton X-100. The exchange reaction of uridine diphosphate-*N*-acetyl-muramyl pentapeptide required a polar lipid, tentatively identified as phosphatidylglycerol. Phosphatidyl serine, phosphatidyl ethanolamine, and phosphatidyl inositol could also restore the activity of the enzyme. These workers did not mention studying the effect of LPG on this reaction. In addition, other workers have described the requirement of phospholipid for the biosynthesis of O-antigen in *Salmonella* (26). Further investigations are required to determine the precise significance of this increased synthesis of LPG. It is clear from this study, however, that the incorporation of radioactive lysine into lipids cannot be used as a major screening device to detect mutants defective in the lipid-mediated reactions in cell wall biosynthesis.

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