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Isolation of the Lipid Intermediate in Peptidoglycan Biosynthesis from *Escherichia coli*

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The lipid intermediate in peptidoglycan biosynthesis was isolated from *Escherichia coli* strain W and characterized as C_{55}-isoprenyl-pyrophosphoryl *N*-acetylmuramyl(-pentapeptide)-*N*-acetyl-glucosamine.

Isoprenyl alcohols have been identified as components of lipid intermediates in the biosynthesis of bacterial cell wall peptidoglycan in the gram-positive bacteria *Micrococcus luteus* (4) and *Staphylococcus aureus* (5). Similar lipids have been shown to be involved in the synthesis of extracellular mannan in *M. luteus* (9) and *Mycobacterium tuberculosis* (12). In the gram-negative bacterium, *Salmonella typhimurium*, isoprenyl alcohols were demonstrated to participate in the synthesis of O-antigens (15). To determine if the same kind of lipid was involved in cell wall peptidoglycan biosynthesis in gram-negative organisms as in gram-positive organisms, and to compare the lipid involved in peptidoglycan biosynthesis more directly to those involved in O-antigen biosynthesis, the C_{55}-isoprenyl-pyrophosphoryl *N* - acetylmuramyl(- pentapeptide) - *N* - acetylglucosamine was isolated from *Escherichia coli* and characterized. This material has previously been shown to function in peptidoglycan synthesis (6).

MATERIALS AND METHODS

Preparation of lipid pyrophosphoryl N-acetylmuramyl(-pentapeptide)-N-acetylglucosamine. A membrane fraction was prepared from 316 g of frozen early log-phase cells of *E. coli* strain W obtained from Grain Processing Corp. (Muscateen, Iowa) by use of lysozyme and ethylenediaminetetraacetic acid (8). The membrane fraction was suspended in 300 ml of 100 mM tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride, *p*H 7.5, to a protein concentration of 6.4 mg/ml as determined by the method of Lowry et al. (7). To this preparation was added 40 ml of 1 M Tris-hydrochloride (*p*H 7.5), 80 ml of 100 mM $MgCl_2$, 80 ml of 0.9 mM uridine diphosphate (UDP)-*N*-acetylglucosamine, 1 ml of 12 mM UDP-*N*-acetylmuramyl-pentapeptide, 80 ml of a 50 μg/ml penicillin G solution (to inhibit the D-alanine carboxypeptidase [6]), and 1.5 ml of ^{14}C-UDP-*N*-acetylmuramyl-pentapeptide (3.5×10^7 counts/min, 0.35 μmole) labeled in both terminal D-alanines (6). After incubation at 24 C for 5 min, the mixture was extracted three times with butanol-pyridinium acetate (*p*H 4.2, 2:1) (2). The extract was taken to dryness in vacuo and resuspended in chloroform-methanol (1:1). A yield of 1.2 μmoles of lipid intermediate was obtained.

The extract was applied to a 36 by 6 cm column of diethylaminoethyl (DEAE)-cellulose and eluted as described previously (4). The *E. coli* lipid intermediate contained one negative charge more than those from *M. luteus* or *S. aureus* because the *E. coli* pentapeptide moiety contained diaminopimelic acid rather than lysine. This lipid intermediate was not eluted with 6 M pyridinium acetate (*p*H 4.2)-methanol (1:1) but was eluted with 3.2 M ammonium acetate in 4 M acetic acid in 50% methanol.

The pooled fractions from the DEAE-cellulose column were concentrated in vacuo and applied to a silicic acid column (Unisil, Clarkson Chemical Co., Williamsport, Pa.; 56 by 2 cm) equilibrated in chloroform-methanol (3:1) and eluted with a gradient from 400 ml of chloroform-methanol (3:1) to 400 ml of methanol. The pooled fractions from this column were then rechromatographed on a silicic acid column (90 by 1.5 cm), eluted with a gradient from 350 ml of chloroform-methanol (3:1) to 500 ml of methanol. The eluted lipid showed a coincidence of phosphate and radioactivity in a molar ratio of 1:1. The pooled fractions contained 275 nmoles of lipid intermediate.

RESULTS

Analyses of the lipid pyrophosphoryl-N-acetylmuramyl - (pentapeptide) - N - acetyl - glucosamine. Thin-layer chromatography of the purified lipid on silica gel showed a single radioactive peak with R_F corresponding to the

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crude lipid intermediate in isobutyric acid-1 N NH_4OH (5:3) and diisobutyl ketone-acetic acid-water (40:25:5). The purified lipid contained a ratio of muramic acid, glucosamine, amino acids, and phosphate expected from the proposed structure (Table 1). Pyrophosphate was present as demonstrated by an increase of inorganic orthophosphate (from 0.51 nmole to 1.0 nmole) after treatment of an acid-hydrolyzed sample (20 min, 0.01 M ammonium acetate, pH 4.2, 100 C) with inorganic pyrophosphatase (3, 4).

Presence of C_{55}-isoprenol. After hydrolysis of the lipid intermediate at pH 4.2, thin-layer chromatography with 15% ethyl acetate in heptane or 2% methanol in benzene on silica gel, or by reversed-phase chromatography with acetone-water (100:8) on paraffin-impregnated silica gel, revealed spots corresponding to ficaprenol and the expected acid-catalyzed beta-elimination derivatives of ficaprenol (10). The hydrolyzed lipid also served as a substrate for C_{55}-isoprenyl alcohol phosphokinase from *S. aureus* (4).

Mass spectroscopy was performed as previously described (4). The spectrum (Fig. 1) showed a pattern of peaks closely resembling that obtained with ficaprenols, consisting of a mixture of which greater than 90% was a C_{55}-isoprenol (M^+ = 766 *m/e*, $M^+ - H_2O$ = 748 *m/e*) which fragmented in units of *m/e* = 68. A smaller amount of C_{60}-isoprenol (M^+ = 836 *m/e*, $M^+ - H_2O$ = 818 *m/e*) was also observed. These latter values are two mass units higher than expected and suggest that a single saturated double bond could be present in this compound. However, the small amounts of material available precluded attempts to confirm the presence of such an unusual C_{60}-isoprenol.

TABLE 1. *Analysis of isolated E. coli lipid intermediate*[a]

Amino acid	Ratio to glutamic acid
Glutamic acid	1.00
Alanine	3.07
Diaminopimelic acid	0.86
Muramic acid[b]	0.84
N-Acetylglucosamine	0.86

[a] From the pooled fractions, 5 nmoles (calculated from the radioactive label) of lipid intermediate were dried in a 1-ml test tube, and to this was added 50 μliters of 6 N HCl. This was heated at 105 C for 10 hr, and the hydrolyzed samples were analyzed on a Beckman Spinco amino acid analyzer. The organic phosphate content was 2.1 moles per mole of lipid intermediate.

[b] Corrected for hydrolytic loss.

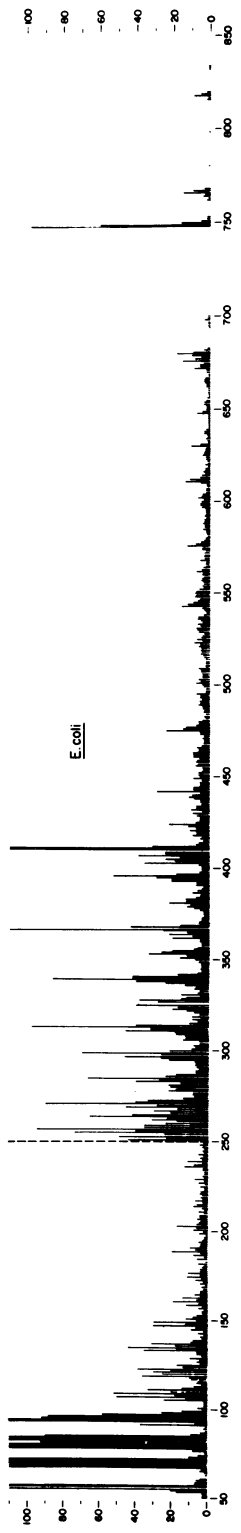

FIG. 1. *Mass spectrum of the lipid intermediate isolated from E. coli. For the mass spectral analysis, 53.7 nmoles of lipid were hydrolyzed by drying the sample in vacuo, adding 20 μliters of 10 mM ammonium acetate, pH 5.2, and heating at 100 C for 20 min. After cooling, 100 μliters of water, 10 μliters of 1 N HCl, and 200 μliters of ether were added, and the ether phase was separated. Extraction with ether was performed once again, and the ether phases were collected and concentrated. The sample was dried in vacuo and analyzed by direct probe. The ion current was normalized to m/e 748 from m/e 250 to m/e 850 and to ten times m/e 748 from m/e 50 to m/e 250.*

Gas-liquid chromatography performed by a slight modification of the method of Wellburn and Hemming (14) demonstrated a vast majority of C_{55}-isoprenol, with only traces of lower or higher homologues (Fig. 2). Unidentified peaks and shoulders accounted for about 5% of the total mass.

DISCUSSION

E. coli utilized as the lipid carrier in cell wall biosynthesis a C_{55}-isoprenol with a structure closely related to that found in O-antigen biosynthesis (15) in gram-negative bacteria, and cell wall peptidoglycan (4) and mannan biosynthesis (9) in gram-positive bacteria. Only the C_{50}-isoprenol from *M. tuberculosis* appears to be different (12). It has been suggested (13) that the same lipid pool was involved in the biosynthesis of cell wall peptidoglycan and teichoic acids, although it is not known if some reversible modification could be involved. The presence of a very similar lipid in gram-negative bacteria suggests the possibility that O-antigen and peptidoglycan are also synthesized by use of a common precursor lipid pool. It is possible that the biosynthesis of cell envelope polysaccharide is regulated by control of this isoprenoid lipid pool and its phosphate derivatives, either by compartmentalization of the lipid by the various enzyme complexes (1), or by the operation of enzymatic systems which catalyze the phosphorylation and dephosphorylation of C_{55}-isoprenyl phosphate (11), or both.

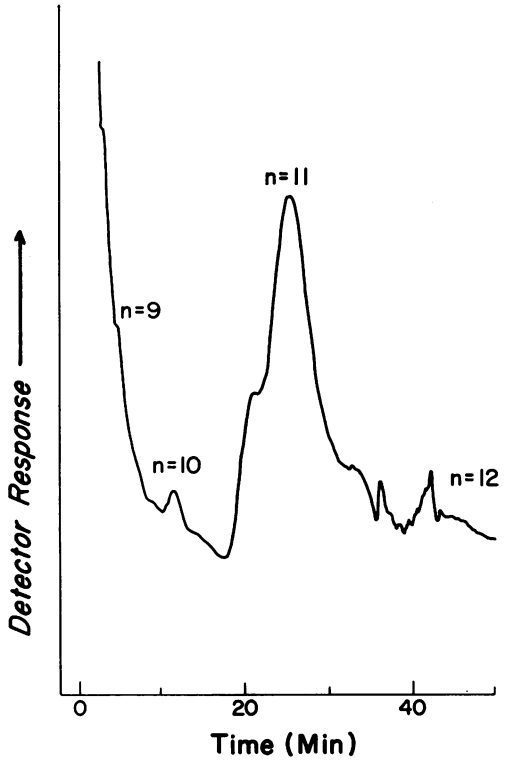

FIG. 2. *Gas-liquid chromatography of the hydrolyzed lipid intermediate from E. coli. Chromatography was performed in glass column (1 mm by 2 ft), packed with Chromosorb W, loaded with 1.5% SE-30 (Hewlett Packard, Medford, Mass.), and fitted with Covar seals and Swagelok fittings on the detector side of the column. A Packard series 9000 gas chromatograph with a flame ionization detector was used. The program ran isothermally for 35 min (sufficient time to allow the n = 11 to elute) at 289 C, and then the temperature was increased at 3 C/min to 320 C and held until n = 12 and any other peaks were eluted.*

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LITERATURE CITED

1. Anderson, R. G., H. Hussey, and J. Baddiley. 1972. The mechanism of wall synthesis in bacteria: the organization of enzymes and isoprenoid phosphates in the membrane. Biochem. J. **127**:11-25.
2. Anderson, J. S., and J. L. Strominger. 1965. Isolation and utilization of phospholipid intermediates in cell wall glycopeptide synthesis. Biochem. Biophys. Res. Commun. **21**:516-521.
3. Goodman, S. D., and G. Popjak. 1960. Studies on the biosynthesis of cholesterol. XII. Synthesis of allyl pyrophosphates from mevalonate and their conversion into squalene with liver enzymes. J. Lipid Res. **1**:286-300.
4. Higashi, Y., J. L. Strominger, and C. C. Sweeley. 1967. Structure of a lipid intermediate in cell wall peptidoglycan synthesis: a derivative of a C_{55}-isoprenoid alcohol. Proc. Nat. Acad. Sci. U.S.A. **57**:1878-1884.
5. Higashi, Y., J. L. Strominger, and C. C. Sweeley. 1970. Biosynthesis of the peptidoglycan of bacterial cell walls. XXI. Isolation of free C_{55}-isoprenoid alcohol and of lipid intermediate in peptidoglycan synthesis from *Staphylococcus aureus*. J. Biol. Chem. **245**:3697-3702.
6. Izaki, K., M. Matsuhashi, and J. L. Strominger. 1968. Biosynthesis of the peptidoglycan of bacterial cell walls. XIII. Peptidoglycan transpeptidase and D-alanine carboxypeptidase: penicillin-sensitive enzymatic reactions in strains of *Escherichia coli*. J. Biol. Chem. **243**:3180-3192.
7. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
8. Repaske, R. 1956. Lysis of gram-negative bacteria by lysozyme. Biochim. Biophys. Acta **22**:189-191.
9. Scher, M., W. J. Lennarz, and C. C. Sweeley. 1968. The biosynthesis of mannosyl-1-phosphoryl-polyisoprenol in *Micrococcus lysodeikticus* and its role in mannan synthesis. Proc. Nat. Acad. Sci. U.S.A. **59**:1313-1320.
10. Stone, K. J., and J. L. Strominger. 1972. Biosynthesis of

the peptidoglycan of bacterial cell walls. XXIV. Isolation of C_{55}-isoprenyl pyrophosphate. J. Biol. Chem. **247**:5107–5112.

11. Strominger, J. L., Y. Higashi, H. Sandermann, K. J. Stone, and E. Willoughby. 1972. The role of polyisoprenyl alcohols in the biosynthesis of the peptidoglycan of bacterial cell wall and other complex polysaccharides, p. 135–154. *In* R. Piras and H. G. Pontis (ed.), Biochemistry of the glycosidic linkage, PAABS Symposium, vol. 2. Academic Press Inc., New York.
12. Takayama, K., and D. S. Goldman. 1970. Enzymatic synthesis of mannosyl-1-phosphoryl-decaprenol by a cell-free system of *Mycobacterium tuberculosis*. J. Biol. Chem. **245**:6251–6257.
13. Watkinson, R. J., H. Hussey, and J. Baddiley. 1971. Shared lipid phosphate carrier in the biosynthesis of teichoic acid and peptidoglycan. Nature N. Biol. **229**: 57–59.
14. Wellburn, A. R., and F. W. Hemming. 1966. Gas-liquid chromatography of derivatives of naturally occurring mixtures of long-chain polyisoprenoid alcohols. J. Chromatogr. **23**:51–60.
15. Wright, A., M. Dankert, P. Fennessey, and P. W. Robbins. 1967. Characterization of a polyisoprenoid compound functional in O-antigen biosynthesis. Proc. Nat. Acad. Sci. U.S.A. **57**:1798–1803.