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Concomitant Synthesis and Attachment of Cell Wall Polymers by a Membrane Preparation from *Micrococcus varians* ATCC 29750[1]

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Membranes from *Micrococcus varians* catalyse the *de novo* synthesis of poly(*N*-acetylglucosamine 1-phosphate) attached to noncrosslinked peptidoglycan through a linkage unit of *N*-acetylglucosamine phosphate-tri(glycerol phosphate). The absence of CDP-glycerol, one of the precursors of linkage unit, precludes the attachment of the sugar 1-phosphate polymer. This report is the first of such polymer attachment performed by membranes which are completely free of cell walls.

INTRODUCTION

Chemical evidence has established that teichoic acids and related bacterial wall polymers containing sugar 1-phosphate linkages are covalently attached to peptidoglycan. Attachment of the teichoic acid or sugar 1-phosphate polymer is through a linkage unit comprising *N*-acetylglucosamine 1-phosphate and three glycerol phosphate residues. A phosphodiester bond thus joins the 1-position of the *N*-acetylglucosamine and the 6-hydroxyl group of a muramic acid residue in the peptidoglycan, while the main teichoic acid chain is attached through a phosphodiester bond to the terminal glycerol phosphate residue of the linkage unit (reviewed in Ref. (*1*)).

The mechanism of peptidoglycan biosynthesis has been well established with membrane preparations from several microorganisms (*2–4*), while the synthesis of teichoic acid and sugar 1-phosphate polymers has been achieved with similar preparations (*5–9*) and can be resolved into two stages: (i) the formation of the main chain from its appropriate precursor on an uncharacterized amphiphilic acceptor called lipoteichoic acid carrier (*7–9*); (ii) the sequential synthesis of linkage unit from UDP-GlcNAc and CDP-glycerol which employs polyisoprenyl pyrophosphate intermediates (*10–15*). The second but not the first stage is inhibited by the antibiotic tunicamycin (*11–13, 15*) which specifically prevents the transfer of *N*-acetylglucosamine 1-phosphate from the corresponding uridine nucleotide to polyisoprenyl monophosphate (*16–18*), the initial intermediate in the synthesis of linkage unit. Similar intermediates are involved in the synthesis of *Micrococcus lysodeikticus* teichuronic acid linkage unit (*19*) and the "core" region of eukaryotic cell glycoprotein (*20*).

[1] This paper is dedicated to the late George Kenner.

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Although membrane preparations alone have hitherto been unable to carry out the synthesis and linkage of teichoic acid and peptidoglycan, this has been achieved with crude cell walls containing a strongly associated membrane fraction, termed wall + membrane preparations, and such systems have been used to assist the study of the incorporation of newly synthesized peptidoglycan and teichoic acid into the cell wall (*21–28*).

This communication describes for the first time a membrane preparation from *Micrococcus varians* ATCC 29750 (described previously as *Micrococcus* sp.2102), free of preexisting cell wall, which accomplishes the *de novo* synthesis of peptidoglycan and poly(*N*-acetylglucosamine 1-phosphate) and their subsequent linkage.

METHODS

Micrococcus varians membranes were prepared and kept as previously described (*13*). CDP-glycerol and CDP-[2-^{3}H]glycerol (580 μCi/μmol) were prepared as in Ref. (*29*). UDP-*N*-acetyl[U-^{14}C]glucosamine (300 μCi/μmol) and UDP-GlcNAc were obtained from the Radiochemical Centre (Amersham, England) and Sigma (St. Louis, Mo.), respectively.

UDP-[^{14}C]MurNAc-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala (UDP-[^{14}C]MurNAc pentapeptide) was prepared by accumulation in *S. aureus* 3528. Cells grown overnight at 37°C in 0.5% peptone (Oxoid, London), 0.5% yeast extract (Oxoid), and 0.1% K_2HPO_4 were transferred (2% inoculum) to 50 ml of fresh, prewarmed medium and grown to midexponential phase ($E_{600} \approx 2.0$). Vancomycin (25 μg/ml) and chloramphenicol (50 μg/ml) were added and after 5 min [U-^{14}C]glucose (50 μCi, 377 μCi/μmol) was added. Incubation was continued for a further 90 min. Thereafter the accumulated nucleotide was extracted with ice-cold trichloroacetic acid (10% w/v) and the trichloroacetic acid was removed from the supernatant with diethyl ether according to well-defined procedures (*30*). The radioactive nucleotide was then purified by column chromatography on Sephadex G-25 and paper chromatography in solvents A (isobutyric acid–0.5 *M* ammonia, 5 : 3, v/v) and B (ethanol–0.5 *M* ammonium acetate, 7.5 : 3, v/v). Following hydrolysis of a sample in 4 *M* HCl at 100°C for 3 hr, 85% of the radioactive material cochromatographed with authentic muramic acid in solvent C (propan-1-ol–aq. NH_3, 0.88 sp. gr–water, 6 : 3 : 1, by vol); only muramic acid, alanine, glutamic acid, and lysine, in the expected ratios of 1 : 3 : 1 : 1, were detected by an amino acid analyser. The specific activity of the UDP-[^{14}C]MurNAc pentapeptide varied from 20 to 25 μCi/μmol. Unlabelled UDP-MurNAc pentapeptide was prepared in the same way with the exception that the growth medium contained 0.1% glucose.

Small-scale reaction mixtures contained 100 μl of membrane suspension and 50 m*M* (final concentration) magnesium acetate. Additions were made, where required, to the following concentrations in a final volume of 15 μl: CDP-[^{3}H]glycerol, 0.025 m*M* (10^6 dpm); UDP-[^{14}C]GlcNAc, 0.33 m*M* (2.0×10^5 dpm); UDP-[^{14}C]MurNAc pentapeptide, 0.1 m*M* (1.5×10^5 dpm); CDP-glycerol, 0.5 m*M*; UDP-GlcNAc, 2.0 m*M*; UDP-MurNAc pentapeptide, 1.0 m*M*. Incubation was at

25°C for 45 min. The reaction was terminated by immersion in boiling water for 2 min and the products were separated and analysed as previously described (*13*), using Whatman 3MM paper, developed in solvent A.

Polymeric material was isolated from larger-scale (×10) reaction mixtures as follows: after the termination of the reaction, particulate material was sedimented by centrifugation at 5000 *g* for 5 min. The pellet was washed three times by suspension in 1.0 ml of butan-1-ol for 15 min at 20°C followed by centrifugation. Polymeric material was then extracted from the pellet into 1.0 ml of 0.06 *M* ammonium acetate, pH 4.2, containing 1% (w/v) sodium dodecyl sulphate at 100°C for 15 min: this was repeated twice. Samples of the extracts were examined by chromatography in solvent A. The reaction mixture supernatant contained only substrates. The butan-1-ol extract contained polyprenyl pyrophosphate lipid intermediates for the synthesis of the peptidoglycan or linkage unit; the polymers were present in the sodium dodecyl sulphate extract, together with small amounts of labelled substrates. Less than 2% of the polymeric material remained in the detergent-extracted pellet. The polymers (eluted at the void volume) were separated from remaining substrate on a column (34 × 3 cm) of Sepharose 6B.

Paper chromatography was also carried out on Whatman No. 4 paper in solvent C and Whatman No. 1 paper in solvent D(pyridine–ethyl acetate–acetic acid–water, 5:5:1:3, by vol). Paper electrophoresis was carried out at 35 V/cm in pyridine–acetic acid–water (25:1:225, by vol) at pH 6.5 for 1 hr.

Authentic samples of glucosamine, glucosamine 6-phosphate, DL-glycerol 1-phosphate, and muramic acid were purchased from Sigma. Lysozyme and lysostaphin were obtained from Sigma and Schwarz/Mann (Orangeburg, N.Y.), respectively. Vancomycin hydrochloride was from Eli Lilly (Basingstoke, England) and chloramphenicol from Sigma. D-[U-^{14}C]Glucose and [2-^{3}H]glycerol were purchased from the Radiochemical Centre.

Alkaline phosphatase treatment was carried out in 100 μl 0.1 *M* ammonium carbonate, pH 10, at 20°C overnight, using 10 units of calf intestinal alkaline phosphatase (Boehringer Corp., Mannheim, FRG).

RESULTS

The biosynthetic capabilities of the membrane preparation are shown in Table 1. The synthesis of peptidoglycan was entirely dependent upon the presence of UDP-GlcNAc. When CDP-glycerol was included, peptidoglycan synthesis was unaffected. No components of the interpeptide bridge of this peptidoglycan (glycine, L-serine (*31*)) were included, and thus the peptidoglycan was not crosslinked. Indeed, further experiments showed that [^{14}C]glycine was not incorporated into polymer; such incorporation would presumably require the addition of a system to synthesize glycyl- and L-seryl-tRNA (*32*).

The membranes also synthesized poly(*N*-acetylglucosamine 1-phosphate) from UDP-[^{14}C]GlcNAc. In the presence of CDP-glycerol, required for the synthesis of linkage unit, a slight increase in polymer was obtained, as had been observed previously with toluene-treated cells of *M. varians* (*10*). When peptidoglycan

TABLE 1

POLYMER SYNTHESIS BY *M. varians* MEMBRANES

Radioactive substrate	Additional substrates	Polymer count/min
UDP-[^{14}C]MurNAc pentapeptide	—	170
	UDP-GlcNAc	7,210
	UDP-GlcNAc,CDP-glycerol	7,400
UDP-[^{14}C]GlcNAc	—	75,670
	CDP-glycerol	81,440
	UDP-MurNAc pentapeptide	57,100
CDP-[^{3}H]glycerol	—	850
	UDP-GlcNAc	12,300
	UDP-GlcNAc,UDP-MurNAc MurHAc	7,650

synthesis occurred through the inclusion of UDP-MurNAc pentapeptide, the total incorporation of radioactivity into the two polymers decreased.

As UDP-GlcNAc is a precursor of both poly(*N*-acetylglucosamine 1-phosphate) and peptidoglycan, the independent study of the synthesis of sugar 1-phosphate polymer by incorporation of label from this nucleotide was not possible. This could be overcome, however, by following the production of poly(*N*-acetylglucosamine 1-phosphate) attached to linkage unit. The incorporation of radioactivity from CDP-[^{3}H]glycerol into polymer-associated linkage unit was dependent upon UDP-GlcNAc, not only for the synthesis of the polymer itself, but also for the formation of the first lipid intermediate of linkage unit (*13*). When UDP-MurNAc pentapeptide was also present, the synthesis of polymer-associated linkage unit decreased considerably.

Polymeric material isolated from large-scale reaction mixtures after incubation in the presence of CDP-[^{3}H]glycerol and unlabelled UDP-GlcNAc was hydrolysed with 4 *M* HCl at 100°C for 60 min. After chromatography in solvent C, radioactivity was recovered as glycerol monophosphate(s) and glycerol diphosphate(s), together with a small amount of glycerol; these are the observed hydrolysis products of linkage unit obtained from cell walls (*33*). When unlabelled CDP-glycerol was incubated with UDP-[^{14}C]GlcNAc, after similar hydrolysis and chromatography, radioactivity was recovered as glucosamine 6-phosphate together with a trace of glucosamine; these are the observed hydrolysis products of the sugar 1-phosphate polymer (*5*). If unlabelled UDP-MurNAc pentapeptide was also included in this incubation, the same radioactive products were obtained, although the proportion of glucosamine rose to 30% of the total, indicating that peptidoglycan synthesis had also occurred.

Radioactive peptidoglycan from large-scale incubations was treated with lysozyme (100 μg/ml) or lysostaphin (20 μg/ml) in 0.05 *M* Tris–HCl, pH 7.5, at 37°C for 3 hr. The reaction mixtures were transferred to Whatman 3MM paper and chromatograms were developed in solvent A. Lysozyme and lysostaphin digestion decreased the amount of material that remained at the origin of the

chromatogram by 48 and 62%, respectively. Several radioactive compounds were produced, two of which had properties consistent with those expected for disaccharide pentapeptide (R_f 0.4) and tetrasaccharide-bis(pentapeptide) (R_f 0.16).

Peptidoglycan synthesized from UDP-[^{14}C]MurNAc pentapeptide in the presence and absence of unlabelled CDP-glycerol was hydrolysed with 6 *M* HCl at 100°C for 1 hr, and chromatographed in solvent C. In the absence of CDP-glycerol the only radioactive material obtained ($R_{\text{glycerol 1-phosphate}}$ 2.2) had the same chromatographic mobility as authentic muramic acid (Fig. 1a). In the presence of CDP-glycerol, an additional radioactive product ($R_{\text{glycerol 1-phosphate}}$ 0.6) was detected; this accounted for between 5 and 8% of the total radioactivity (Fig. 1b). This material was, however, absent if the hydrolysis products had been treated with phosphatase before chromatography (Fig. 1b). Elution of the minor product, rechromatography in solvents C and D, and electrophoresis at pH 6.5 before and after phosphatase treatment confirmed its conversion from muramic acid phosphate to muramic acid. Similar results were obtained from doubly labelled polymeric material, produced when CDP-[^{3}H]glycerol was included in incubation mixtures. As previously observed [^{3}H]glycerol and its mono- and diphosphate(s) were obtained.

DISCUSSION

Membrane preparations from *M. varians* can synthesize concomitantly, noncrosslinked peptidoglycan, and poly(*N*-acetylglucosamine 1-phosphate) attached to linkage unit. A considerable reduction in the amount of linkage unit attached to sugar 1-phosphate polymer was observed on the introduction of UDP-MurNAc pentapeptide into incubation mixtures (Table 1). This is a further indication of the involvement of polyisoprenyl phosphate as a common intermediate in the synthesis of different cell wall polymers (*29, 34*). Thus the presence of UDP-MurNAc pentapeptide additionally permits the participation of the lipid in peptidoglycan synthesis as well as in that of linkage unit. The reciprocal effect of CDP-glycerol on peptidoglycan synthesis was not however observed, but in this instance the requirement for UDP-GlcNAc for the synthesis of peptidoglycan also allows the formation of the first intermediate of linkage unit, polyisoprenyl pyrophosphate *N*-acetylglucosamine (*13*).

Under conditions where the syntheses of poly(*N*-acetylglucosamine 1-phosphate), linkage unit, and peptidoglycan occur, muramic acid phosphate is detected, indicative that the sugar 1-phosphate polymer is attached through linkage unit to the peptidoglycan (*1, 27*). Substitution occurs at about 1 in 12 of the muramic acid residues which is similar to values obtained from vegetative cell walls (*35*). Although the phosphate moiety of the muramic acid phosphate (at least in *B. licheniformis*) is derived from UDP-GlcNAc (*27*), if synthesis of the linkage unit is disrupted by omission of its other precursor CDP-glycerol, no evidence of the attachment of the sugar 1-phosphate polymer can be found.

In *M. varians* attachment of the sugar 1-phosphate polymer is to noncrosslinked

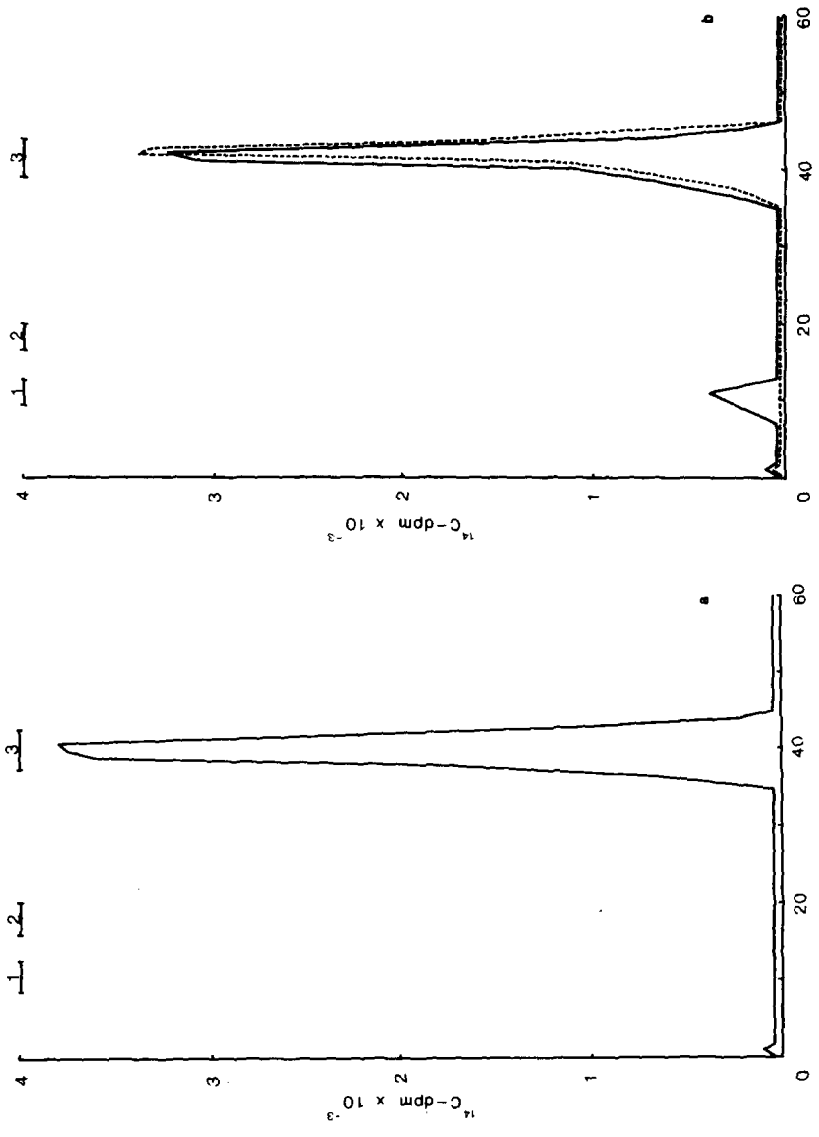

FIG. 1. Determination of radioactivity (1-cm strips) in acid-hydrolysed peptidoglycan (6 *M* HCl, 100°C, 1 hr) synthesized from UDP-[^{14}C]MurNAc pentapeptide in the absence (a) or presence (b) of CDP-glycerol after chromatography in solvent C. The effect of phosphatase treatment on the glycerol-containing material prior to chromatography is also shown (b) (– – –). The markers indicate the location of glucosamine 6-phosphate (1), glycerol 1-phosphate (2), and muramic acid (3).

peptidoglycan. In wall + membrane preparations from *Staphylococcus aureus* and *Bacillus subtilis* (*26, 28*) teichoic acid was attached to preexisting wall and to concomitantly synthesized peptidoglycan. However, in *B. licheniformis* (*25*) teichoic acid could be attached only to concomitantly synthesized, crosslinked peptidoglycan, a result also obtained with whole cells (*36, 37*).

The results of the experiments described here are in full agreement with the earlier conclusions on the structure and mechanism of biosynthesis of teichoic acid–linkage unit–peptidoglycan complexes in bacterial cell walls (*1, 10–15, 25–28*). Previous studies have however been carried out using either whole or toluene-treated cells or wall + membrane preparations.

The synthesis and linkage of poly(*N*-acetylglucosamine 1-phosphate) and peptidoglycan described here are therefore the first by totally wall-free membrane preparations. These offer great advantages over wall + membrane systems in that they permit the study of the synthesis of both polymers, and of linkage unit, independently of subsequent events in wall assembly thus providing greater insight into the proposed biosynthetic routes, the participation of intermediates, and the control of the system.

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