THE SYNTHESIS OF COVALENTLY-LINKED TEICHOIC ACID AND PEPTIDOGLYCAN BY CELL-FREE PREPARATIONS OF *BACILLUS LICHENIFORMIS*

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Summary: The synthesis of 1,-3 poly (glycerol phosphate) covalently linked to peptidoglycan has been studied in membrane + wall preparations of *Bacillus licheniformis*. The attachment of the majority (approximately 80%) of the teichoic acid required the synthesis of cross-linked peptidoglycan, whereas the attachment of a minor fraction (approximately 20%) of the teichoic acid occurred in the absence of peptidoglycan synthesis. Evidence was also found for the participation of a membrane-bound precursor in the synthesis of the major fraction of covalently-linked teichoic acid.

INTRODUCTION

The walls of many Gram-positive bacteria contain teichoic acids which in *Staphylococcus aureus* (11) and in both *Bacillus licheniformis* and *B. subtilis* (14) are covalently linked to peptidoglycan. More recently, Mauck and Glaser (16) have shown that in *B. subtilis* newly synthesised teichoic and teichuronic acids are linked only to peptidoglycan which has been synthesised concomitantly. The cell-free synthesis of the teichoic acids poly (glycerol phosphate) and poly (glycerol phosphate glucose) by membrane preparations of *B. licheniformis* and *B. subtilis* has been described (1,4,5,6, 13), but these were in a soluble form not attached to peptidoglycan. The present investigation was carried out to study the synthesis of peptidoglycan and teichoic acid and the formation of the linkage between the two polymers in membrane + wall preparations of *B. licheniformis*.

MATERIALS AND METHODS

The organism used was a penicillinase and autolysin-deficient mutant of B. licheniformis 6346 designated B. licheniformis 94 (20). Growth of the organism and isolation of the membrane + wall preparations were carried out as described previously (20), except that the buffer used for washing and for resuspending the final membrane + wall preparation was 50mM-Tris-HCl buffer, pH 7.8 containing 60mM-$MgCl_2$ and 1mM-dithroerythritol. L-α-[U-^{14}C]glycerol-3-phosphate, synthesised from [U-^{14}C]glycerol (The Radiochemical Centre, Amersham, Bucks, U.K.) with ATP and glycerokinase (3), was used for the enzymic synthesis of CDP-[U-^{14}C]glycerol (18). UDP-N-acetylmuramyl-L-alanyl-D-isoglutamyl-meso-diaminopimelyl-D-alanyl-D-alanine (UDP-N-acetylmuramyl-pentapeptide) labelled with diamino-[^{3}H]pimelic acid (300mCi/mmol) was prepared as described previously (21). Unlabelled UDP-N-acetylmuramyl-pentapeptide was prepared from B. licheniformis by accumulation in a medium lacking Mg^{2+} (10).

A typical reaction mixture for synthesis of teichoic acid and peptidoglycan contained 2mM-CDP-[U-^{14}C]glycerol (5mCi/mmol), 2mM-UDP-N-acetylglucosamine and 10mM-UDP-N-acetylmuramyl pentapeptide in a total volume of 200µl. After incubation at 28° for the appropriate time reactions were terminated by the addition of an equal volume of 10% sodium dodecyl sulfate (SDS) and heating at 60° for 5 min. The walls were recovered by centrifuging at 12,000g for 5 min and then re-extracted with 0.5ml of 5% SDS with heating at 60° for 5 min. After centrifuging as before, the two supernatants were combined as the SDS-soluble fraction. The walls were then washed four times with water (1ml), resuspended in water (1ml) and heated at 100° for 15 min, recovered by centrifuging and finally resuspended in water (0.4ml) to give the SDS-insoluble fraction.

RESULTS AND DISCUSSION

The membrane + wall preparations of B. licheniformis synthesised polymeric material from CDP-glycerol in addition to the synthesis of peptidoglycan from UDP-N-acetylmuramyl-pentapeptide and UDP-N-acetylglucosamine described previously (20). A fraction (15 - 25%) of this polymeric glycerol was associated with the pre-existing wall since it was not removed by treatment of the walls with 5%-SDS or by additional extraction with 80% phenol. Resistance to such extraction procedures has been taken as indicative of a covalent linkage between newly synthesised material and the pre-existing wall.

In order to demonstrate that synthesis of covalently linked teichoic acid was dependent on the concomitant synthesis of cross-linked peptidoglycan incubations were carried out in the presence of benzylpenicillin (10µg/ml). This concentration of the antibiotic has

previously been shown to inhibit transpeptidation and thus the formation of cross-linked peptidoglycan in B. licheniformis (20). Addition of benzylpenicillin to the incubation mixtures resulted in a 81% decrease in the amount of covalently linked teichoic acid (Table 1). The amount of synthesis found in the presence of the antibiotic was almost identical to the formation of covalently-linked [^{14}C]glycerol occurring in the absence of added peptidoglycan precursors. Examination of the SDS-soluble material, by chromatography in isobutyric acid - 0.5M-

TABLE 1.

The effect of benzylpenicillin on the synthesis of teichoic acid linked to peptidoglycan.

	Assay			
	Complete		- peptidoglycan precursors	
Penicillin 10µg/ml	-	+	-	+
Linked teichoic acid (SDS-insoluble)	765	146	179	162
SDS-soluble	4520	4530	3820	3540

The results are given as pmoles of [^{14}C]glycerol incorporated per mg protein/hr. The isolation of covalently-linked teichoic acid and the SDS-soluble fraction were carried out as described in the text.

NH_3 (5.3 v/v), revealed that this concentration of benzylpenicillin had no apparent effect on the amount of this polymeric material synthesised (ie material remaining on the origin of the chromatogram) both in the presence and absence of peptidoglycan precursors. On the other hand, the presence of peptidoglycan precursors resulted in a small (15 - 22%) stimulation of the synthesis of soluble teichoic acid, the reason for which is currently not known. It seems likely that this soluble material is identical to that previously described as being synthesised by various membrane preparations of Bacilli (1, 4, 5, 13). Soluble peptidoglycan, synthesised by B. licheniformis under conditions where transpeptidation was inhibited, has been shown to lack associated phosphorus, a finding taken to indicate the absence of linked teichoic acid (19). Thus in the present cell-free system the formation of teichoic acid linked to peptidoglycan (SDS-insoluble) appears to require the concomitant synthesis of both teichoic acid and of cross-linked peptidoglycan. Such a finding is in agreement with the in vivo observations of Mauck and Glaser (16) that in B. subtilis both teichoic and teichuronic acids are linked only to peptidoglycan synthesised at the same time.

In a second experiment, membrane + wall preparations were incubated for 15 min with CDP-[^{14}C]glycerol as described in Methods. Excess substrate was then removed by washing (3 x 2ml buffer) and the membrane + wall preparation reincubated with non-radioactive CDP-glycerol with the results shown (Table 2). In the presence of peptidoglycan precursors the incorporation of covalently linked teichoic acid after 15 min incubation was 27% of that found after 1 hr. Subsequent incubation with non-radioactive CDP-glycerol, resulted in an increase in the incorporation to 76% of that found in the control when CDP-[^{14}C]glycerol was the sole substrate. In contrast preparations, incubated in the absence of peptidoglycan precursors, showed no increase in the small amount of covalently linked

TABLE 2.

The formation of a membrane-bound precursor of covalently-linked teichoic acid.

Peptidoglycan precursors	Time of incubation					
	60 min [a]		15 min [a]		15 min + 45 min [b]	
	+	-	+	-	+	-
Expt. I	765	146	206	78	579	67
II	633	153	393	111	655	97

The results are given as pmoles of [^{14}C]glycerol incorporated into the SDS-insoluble fraction per mg of protein per hr.

a) Incubated for 60 min and 15 min with CDP-[^{14}C]glycerol.

b) Incubated for 15 min with CDP-[^{14}C]glycerol, washed and reincubated for 45 min with CDP-glycerol as described in the text.

radioactivity synthesised in the first 15 min. Clearly, replacement of the radioactive substrate abolishes the incorporation of [^{14}C] glycerol in the absence of peptidoglycan precursors, whereas in their presence additional incorporation occurred. Presumably this reflects the synthesis during the initial period of incubation of some precursor of teichoic acid in the membrane which, in subsequent incubation, transfers the [^{14}C]glycerol, either in total or in part, to the wall. The synthesis of the wall teichoic acid, poly (ribitol phosphate) in Staph. aureus H involves the participation of a membrane-bound lipoteichoic acid carrier (LTC) (8, 9). This has been characterised as a

polymer of glycerol phosphate with a hydrophobic terminus containing D-glucose and fatty acid residues (7). A similar LTC has been isolated from B. subtilis (17) but in this case the participation of the carrier in wall teichoic acid synthesis has not been established. One possibility would be the formation of radioactive teichoic acid as the LTC-linked precursor and its subsequent transfer from the precursor to the wall with the synthesis of a covalent link between the teichoic acid and peptidoglycan.

The [^{14}C]glycerol linked to the wall both in the presence and

TABLE 3.

The effect of preincubation of membrane + wall preparations with CDP-glycerol on the subsequent synthesis of covalently-linked teichoic acid.

Time of preincubation (mins)	Assay	
	Complete	- peptidoglycan precursors
0	633	153
60	317	184
120	268	185

The conditions of pre-treatment of the membrane + wall preparations with non-radioactive CDP-glycerol are given in the text. The results are expressed as pmoles of [^{14}C]glycerol incorporated into the SDS-soluble fraction per mg of protein per hr.

absence of peptidoglycan synthesis has been shown to be present as 1,-3 poly (glycerol phosphate) by identification of the products of alkaline hydrolysis. Peaks of radioactivity corresponding to glycerol, glycerol monophosphate, glycerol diphosphate and diglycerol triphosphate were obtained by chromatography on DEAE cellulose (HCO_3^-) (2) and their identity confirmed by paper chromatography with appropriate standards in propan-1-ol : NH_3 : H_2O (6: 3: 1, by vol.). The diglycerol triphosphate, which can only arise from alkaline hydrolysis of poly 1,-3 (glycerol phosphate) (15) was further characterised by dephosphorylation with alkaline phosphatase and chromatography of the resulting diglycerol phosphate as described above.

One possible explanation for the incorporation of [^{14}C]glycerol in the absence of added peptidoglycan precursors was the utilisation by the membrane + wall preparations of some endogenous membrane-bound intermediates allowing the synthesis of a small amount of peptidoglycan. Hammes and Neuhaus (12) have described the removal of one such intermediate, undecaprenyl-diphosphate-N-acetylmuramyl pentapeptide, from the membranes of Staph. aureus by incubation with UMP. A similar pre-treatment was carried out with the membrane + wall preparation of B. licheniformis (UMP final conc^n 9 x 10^{-5}M). Subsequent incubation of the treated preparation with CDP-glycerol gave synthesis of SDS-insoluble radioactivity both in the presence and absence of peptidoglycan precursors in amounts similar to that found in untreated preparations. Thus it seems unlikely that the incorporation of poly (glycerol phosphate), occurring in the absence of added peptidoglycan precursors, was by linkage to newly synthesised peptidoglycan. Another possibility was the addition of glycerol phosphate units to some material already present in the membrane + wall preparation. In an attempt to saturate these 'sites of addition', enzyme preparations were pretreated with non-radioactive CDP-glycerol (50nmol). Preincubation in this way for

periods of up to 2 hr were without effect on the incorporation of radioactivity on subsequent incubation with CDP-[^{14}C]glycerol alone. On the other hand, preincubation did affect the synthesis of polymeric material in the presence of peptidoglycan precursors. After 2 hr preincubation incorporation of [^{14}C]glycerol was reduced by 58%. However, under identical conditions peptidoglycan synthesis was also reduced by 64%, which presumably explains the observed reduction in synthesis of the linked teichoic acid.

Clearly the resistance of the 1,-3 poly (glycerol phosphate) attached in the absence of peptidoglycan synthesis to subsequent extraction of the walls with SDS and phenol is indicative of some covalent linkage between the polymer and the pre-existing wall. However, at this time the nature of this linkage and the place of the glycerol phosphate polymer in wall structure remains unknown.

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