MURAMIC ACID PHOSPHATE AND THE LINKAGE OF TEICHOIC ACID TO PEPTIDOGLYCAN IN BACILLUS STEAROTHERMOPHILUS CELL WALLS

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It is now recognised that, in the cell walls of a number of Gram-positive bacteria, polysaccharide and teichoic acid components are linked covalently to the basal peptidoglycan through phosphate groups. Muramic acid phosphate has been implicated as the cross-link between polysaccharide and peptidoglycan in a number of cell walls (Heymann et al., 1967; Liu and Gotschlich, 1967; Montague and Moulds, 1967; Knox and Holmwood, 1968). Phosphodiester linkage of teichoic acid to peptidoglycan in Staphylococcus aureus Copenhagen was proposed by Strominger and Ghuysen (1963) whereas in Staph. aureus H, Bacillus subtilis and L. arabinosus evidence for a phosphoramidate bond in the linkage of the two polymer types has been obtained (Hay et al., 1965). More recently an atypical teichoic acid in the cell walls of Staph. lactis has been shown to be linked to peptidoglycan through muramic acid phosphate (Button et al., 1966).

The cell walls of B. stearothermophilus B65 contain a glycerol teichoic acid (Wicken, 1966) and evidence is reported here which suggests that it is covalently linked to peptidoglycan through muramic acid phosphate.

METHODS

Cell Walls: Cell walls were prepared from B. stearothermophilus B65 grown at 55^{o} as described previously (Wicken, 1966; Forrester and Wicken, 1966).

Enzymic and Analytical Methods: Lysozyme digestion of cell walls was carried out at 37° in 0.06 M potassium phosphate buffer, pH 6.2, containing 0.1% NaCl and 30 µg/ml lysozyme (Sigma). Autolysis of cell walls was found to be optimal in 0.02 M sodium phosphate buffer, pH 7.5, at 55° and these conditions were used routinely. Phosphomonoesters were hydrolysed with alkaline phosphomonoesterase (Sigma) and the configuration of alanine was determined with D-amino acid oxidase as described previously (Wicken and Baddiley, 1963). Glucose, in acid hydrolysates, was estimated with glucose oxidase (Huggett and Nixon, 1957); phosphorus by the method of Ames (1966); muramic acid by the modified Elson-Morgan procedure of Gatt and Berman (1966); free amino groups by ninhydrin (Rosen, 1957) and FDNB (Ghuysen et al., 1965); reducing groups by the method of Park and Johnson (1949). Paper chromatographic and electrophoretic procedures, unless mentioned in the text, were as described previously (Wicken, 1966; Forrester and Wicken, 1966).

RESULTS

Peptidoglycan-Teichoic Acid Complexes: Autolysis of cell walls resulted in the solubilisation of 96% of their mass in 120 min. Concomitant with a decrease in optical density at 650 mµ, autolysing wall suspensions showed a net increase in ninhydrin reacting material and free amino groups but no significant increase in reducing groups could be detected. Dialysis of autolysates gave 15 - 20% of the original mass of walls as a diffusible fraction which contained large amounts of D-alanine[1], traces of other cell wall amino acids and a mixture of peptides. The latter was resolved by chromatography and paper electrophoresis into twelve peptide fractions and partial characterisation of the major fractions showed them to be composed of alanine, diaminopimelic acid and glutamic acid. L-Alanine was N-terminal in

[1]The labile D-alanyl esters of the teichoic acid would be readily hydrolysed at the slightly alkaline pH at which autolysis was conducted.

each case. A detailed analysis of the diffusible fraction forms the basis of a further study to be reported elsewhere but it is evident from the above that the autolytic activity in these cell walls is due, at least in part, to a N-acylmuramyl-L-alanine amidase.

ECTEOLA-cellulose (Cl^-) chromatography of the non-diffusible fraction separated a phosphorus-free peptidoglycan fraction from the phosphorus containing material which was eluted with 0.2 - 0.4 M NaCl. Rechromatography of the latter on DEAE-cellulose (Cl^-) with a linear gradient of 0.0 - 0.5 M NaCl gave, as sharp peaks, two components which accounted for 88% (Complex I, 0.20 - 0.26 M NaCl) and 12% (Complex II, 0.27 - 0.31 M NaCl) of the total wall phosphorus respectively. Analysis showed these fractions to contain constituents typical of both peptidoglycan and teichoic acid. Cell walls, from which some 70% of the teichoic acid had been removed by prolonged trichloracetic acid (TCA) extraction (Wicken, 1966), were solubilised by lysozyme and fractionated as above to yield a single complex (III) which was qualitatively similar to Complexes I and II.

High voltage paper electrophoresis of these complexes in buffers of pH 2.2, 3.7, 5.8, 7.8 and 9.1 failed to separate peptidoglycan from teichoic acid. Treatment of the complexes with phenylhydrazine, used previously (Hay et al., 1965) to break phosphoramidate bonds in other cell walls, did not result in a separation of the two polymer types. Mole ratios of glucose: phosphorus and phosphodiester:phosphomonoester groups in these complexes are shown in Table 1.

Muramic acid phosphate: Chromatography of a 6 N HCl hydrolysate (100° for 60 min) of whole cell walls on a column (20 x 1 cm) of Dowex 50 x 8 (H^+) resin (Liu and Gotschlich, 1967) gave a peak of ninhydrin-reacting material between 25 and 42 ml of eluant (H_2O). This material was contaminated with teichoic acid degradation products and was further fractionated on a column (19 x 1.3 cm) of DEAE-cellulose with a linear gradient of 0-0.2 M $(NH_4)_2CO_3$.

Table 1. Mole ratios of glucose:phosphorus and phosphodiester:phosphomonoester groups in Complexes I, II and III and TCA-extracted teichoic acid.

Material	Source[1]	Glc:P (P = 1.00)	$P_D:P_M$[2] (P_M = 1.00)
Complex I	Wall autolysate	0.58	> 1,000
Complex II	Wall autolysate	0.67	> 1,000
Complex III	Lysozyme digest of TCA-extracted cell walls	0.27	23
Teichoic acid	TCA extract of cell walls	0.79	18 - 19

[1] All preparations were from the same batch of isolated cell walls.

[2] Ratio of total phosphorus to phosphorus released by phosphomonoesterase. Inhibition of colour development in inorganic phosphate determinations by peptidoglycan was corrected by the use of internal standards.

Abbreviations used: Glc, glucose; P, phosphorus; P_D, phosphodiester groups; P_M, phosphomonoester groups.

Two phosphate containing peaks were eluted with 0.02 - 0.05 M and 0.05 - 0.10 M $(NH_4)_2CO_3$ respectively. Paper electrophoresis of material from the second peak on washed Whatman 3MM paper in pyridine/acetic acid/water (135:7:900, pH 6.3) at 16 volts/cm for 4 hr gave a component which migrated 7.2 - 10.0 cm towards the anode and which was both reactive to ninhydrin and contained phosphorus. It had an $R_{\alpha\text{-glycerophosphate}}$ = 0.70 in ethanol/1.0 M ammonium acetate (7:3) in comparison to a value of 0.72 reported for muramic acid-6-phosphate (Heymann et al., 1967). Treatment with alkaline phosphomonoesterase gave inorganic phosphate and a single amino sugar component with identical chromatographic mobility to muramic acid in several solvent systems. Quantitative analysis showed a mole ratio of muramic acid: phosphorus of 1.00:98. The spectrum of the Elson-Morgan chromophore of this material was identical to that of muramic acid with a peak at 507 mμ. Periodate oxidation under the conditions described by Heymann et al. (1967)

and chromatography in n-propanol/ammonia/water (6:3:1) gave a strongly reducing spot which contained phosphorus and corresponded in mobility to glycolaldehyde phosphate. This suggests that, in common with previously reported isolates of muramic acid phosphate, the phosphate group is attached to C6 of muramic acid. Phosphoryl migration from C4 during acid hydrolysis is not however precluded (cf., Button et al., 1966).

Muramic acid phosphate was similarly isolated and characterised from 6N HCl hydrolysates of Complexes I, II and III. Partial acid hydrolysis of these complexes in 60% HCOOH at 100° for 30 min followed by treatment with phosphomonoesterase yielded fragments of teichoic acid still covalently linked to peptidoglycan material.

DISCUSSION

Peptidoglycan-teichoic acid complexes, involving a presumed phosphodiester linkage between the two polymer types, have been isolated from B. subtilis (Young, 1966) and Staph. aureus Copenhagen (Ghuysen et al., 1965). In the latter case, teichoic acid extracted from cell walls by TCA had a lower degree of polymerisation than 'native' teichoic acid in the complex suggesting that splitting of both inter-ribitol phosphodiester bonds of the teichoic acid and the phosphate linkage to the peptidoglycan had occurred during TCA extraction. In contrast, the elegant studies of Hay et al., (1965) have shown that TCA extraction of the teichoic acids from the cell walls of Staph. aureus H, B. subtilis and L. arabinosus 17-5 did not involve polymer degradation and evidence has been produced for a phosphoramidate bond between the terminal phosphate group of the teichoic acid and a hexosamine amino group of the peptidoglycan in these cell walls; such a bond would be more acid labile than the phosphodiester bonds of the teichoic acid.

In B. stearothermophilus cell walls the lack of phosphomonoester groups in Complexes I and II (Table 1) is indicative of the involvement of the terminal phosphate of the teichoic acid in linkage with peptidoglycan and such

linkage is unlikely to involve a phosphoramidate bond as phenylhydrazine failed to achieve a separation of the two polymer types. A phosphodiester linkage of teichoic acid to muramic acid, particularly if to C6 of the amino sugar, would be more resistant to acid hydrolysis and it is likely that prolonged treatment with TCA would cause preferential splitting of phosphodiester bonds within the teichoic acid chains. The isolation of muramic acid phosphate from whole cell walls and peptidoglycan-teichoic acid complexes of B. stearothermophilus is considered presumptive evidence of such an interpolymer linkage in these walls. The resistance of this teichoic acid towards TCA extraction has already been noted (Wicken, 1966) and the presence of phosphomonoester groups in Complex III, derived from TCA extracted walls, is further suggestive of inter-glycerol phosphodiester bond breakage during extraction. Hill (1967) has shown that sodium deoxycholate at pH 11 and 0° will rapidly release teichoic acid and polysaccharide components from the cell walls of some organisms. Similar treatment of B. stearothermophilus cell walls over 48 hr solubilised < 10% of the total wall phosphorus. It would therefore appear likely that two different modes of attachment of teichoic acid and, perhaps, polysaccharide to peptidoglycan are possible.

Heterogeneity of side-chain glycosidic substitution of the B. stearothermophilus teichoic acid is apparent from the Glc:P ratios (Table 1) of the various complexes and TCA extracted polymer. Similar heterogeneity has been noted in B. licheniformis (Hughes, 1965) and B. subtilis (Young, 1966). Whether this reflects a mixture of fully substituted and unsubstituted glycerophosphate polymers or a variation in glycosidic substitution along growing teichoic acid chains has yet to be determined.

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