Evidence for the Presence of a New Class of Teichoic Acid in the Cell Wall of Bacterium NCTC 9742

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The composition of the cell wall of bacterium NCTC 9742, variously known as Chromobacterium iodinum or Pseudomonas iodinum and considered by some to be a species of Brevibacterium or Arthrobacter, has been studied. Walls isolated from cells grown in nutrient broth consisted predominantly of peptidoglycan and a phosphorylated polymer containing D-mannitol, glycerol, pyruvic acid, D-glucose and D-galactosamine. This phosphorus-rich polymer, which was slowly released on treatment of the walls with cold aqueous trichloroacetic acid, is considered to be a new type of teichoic acid. Quantitative analysis and the results of preliminary degradative studies indicated that the main backbone was a poly(mannitol phosphate) chain to which \( \beta \)-glucopyranosyl substituents and pyruvic acid residues (acetal-linked) were attached. It is suggested that the minor components, glycerophosphate and galactosamine, could form a linkage unit for the covalent attachment of the poly(mannitol phosphate) chain to peptidoglycan. In the walls of cells grown on nutrient agar, it appeared that the phosphorus-rich polymer was partly replaced by another polymer containing galactose and an unidentified component which reacted like a 2-keto-3-deoxyaldonic acid in the periodate/thiobarbituric acid test. The taxonomic implications of the cell-wall composition are discussed.

INTRODUCTION

In bacterial taxonomy, the organism represented by strain NCTC 9742 is a vagrant species. The original isolate (Davis, 1939) was identified as a new species of Chromobacterium and named Chromobacterium iodinum, because of its production of a dark purple, crystalline pigment (iodin). The characterization of iodin as a phenazine derivative (eventually shown to be 1,6-dihydroxyphenazine 5,10-dioxide) led to suggestions (Tobie, 1945; Gilman, 1953) that the organism should be transferred to the genus Pseudomonas, some typical members of which produce chemically related pigments (e.g. pyocyanin and aeruginosins from Pseudomonas aeruginosa). However, neither genus provides a satisfactory home for an organism that is both non-motile and Gram-positive (though with a tendency to be Gram-variable, particularly in young cultures). These and other properties of the organism (including its pleomorphism and poor fermentative powers) led Sneath (1960) to suggest that it might belong either to the genus Corynebacterium or to the genus Brevibacterium. The latter suggestion has been taken up by other workers, e.g. Herbert et al. (1972) and Gerber (1973). The coryneform connection was accepted by Colwell et al. (1969), but the ultrastructure of the organism and values for the mol % GC of its DNA [60-2 %, De Ley et al. (1966); 60-9 to 63 %, Colwell et al. (1969)] were considered to point to the genus Arthrobacter. Not surprisingly, the organism is still listed in the current edition of Bergey’s Manual of Determinative Bacteriology (Sneath, 1974) as Chromobacterium iodinum but as a species incertae sedis.

The organism first came to our attention during a survey of pseudomonads for sensitivity
to the lytic, bactericidal action of EDTA that characterizes *P. aeruginosa* and closely related species (Wilkinson, 1967). The atypical resistance of *P. iodinum* NCTC 9742 led us to examine the composition of isolated cell walls (Wilkinson, 1968). In the virtual absence of lipid and protein, in the high content of peptidoglycan (43% in walls from one batch of cells grown on nutrient agar) and in peptidoglycan composition (components and their molar ratios were: glutamic acid, 1; alanine, 2; 2,6-diaminopimelic acid, 1; glucosamine, 1; muramic acid, 1), the walls were similar to those of many other Gram-positive bacteria. Thus, the chemical data are in accord with the results of the ultrastructural studies carried out by Colwell *et al.* (1969) which revealed a typically Gram-positive type of cell wall. Additional results on wall composition from our earlier study included a variation in monosaccharide composition with growth conditions, and the presence of a phosphorus-rich polymer in the walls. Whereas glucose was the sole neutral monosaccharide in walls from cells grown in nutrient broth, galactose and an unidentified sugar reactive in the thiobarbituric acid test for 2-keto-3-deoxyaldonic acids were also present in the walls. The phosphorus-rich polymer was not a conventional teichoic acid, but appeared to be derived from a hexitol. We now report further on the chemical composition of the material and evidence that it constitutes a new class of teichoic acid.

**METHODS**

**Materials.** 1,4-Anhydro-α-mannitol was prepared by the method of Foster & Overend (1951) and 1,5-anhydro-α-mannitol by the method of Fletcher & Diehl (1952). Mannitol dehydrogenase (EC 1.1.1.67) isolated from *Agaricus bisporus* was a gift from Dr R. J. Sturgeon. Alkaline phosphatase (EC 3.1.3.1) was type 1 from calf intestinal mucosa (Sigma). Galactose oxidase (EC 1.1.3.9) was used as the Galactostat reagent (Worthington). Lactate dehydrogenase (EC 1.1.1.27), glycero kinase (EC 2.7.1.30), pyruvate kinase (EC 2.7.1.40), hexokinase (EC 2.7.1.1), glucose oxidase (EC 1.1.3.4), peroxidase (EC 1.11.1.7) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) were Boehringer products.

**Growth of organisms and preparation of cell walls.** During initial studies, bacterium NCTC 9742 (obtained as *Pseudomonas iodinum*) was grown for 24 h at 37 °C on nutrient agar (Oxoid). Subsequently, batch cultures (21) were grown for 24 h at 30 or 35 °C in nutrient broth (Oxoid CM67) with aeration at 20 l min⁻¹ and stirring at 300 rev. min⁻¹. Yields of cells were about 90 g wet wt. Walls were isolated from both types of cell using methods described previously (Wilkinson, 1968).

**Extraction and purification of the phosphorus-rich polymer.** Several standard treatments for the extraction of phosphorylated polymers from bacteria were applied to the isolated cell walls of bacterium NCTC 9742. These comprised treatment with (a) 40% (w/v) aqueous phenol for 40 min at 4 or 80 °C (Coley *et al.*, 1975), (b) dimethyl sulphoxide for 4 h at 60 °C (Adams, 1967), (c) 2% (v/v) N,N-dimethylhydrazine for 2 h at 80 °C (Anderson *et al.*, 1969), (d) 10% (w/v) aqueous trichloroacetic acid for various periods at 4 °C (Wilkinson, 1968). When treatment (d) was used, trichloroacetic acid was subsequently removed by repeated extraction with diethyl ether or n-butyl acetate (Young, 1964), and the wall polymer was purified by dialysis or gel permeation chromatography. In the procedure finally adopted, the walls were treated repeatedly (5 × 1 d, 1 × 3 d) with aqueous trichloroacetic acid (about 80 ml per g walls). After each treatment, the wall residues were collected by centrifugation at 4 °C and 20000 g for 30 min. Each aqueous supernatant was extracted six times with equal volumes of diethyl ether, carefully neutralized with dilute aqueous ammonia, and freeze-dried. The individual products were separately purified by chromatography on a column of Sephadex G-25 with pyridine/acetic acid/water (10:4:986, by vol.) pH 5.4 as the eluant.

**Paper chromatography and electrophoresis.** Separations were carried out on Whatman no. 1 or 3 MM paper (water-washed for preparative work). Solvent systems used for chromatography were as follows: A, the upper phase of ethyl acetate/pyridine/water (5:2:5, by vol.); B, the upper phase of ethyl acetate/acetic acid/water (3:1:3, by vol.); C, the upper phase of butan-1-ol/ethanol/water (18:2:1, by vol.); D, propan-1-ol/18 M-ammonia/water (6:3:1, by vol.); E, propan-2-ol/5% (w/v) aqueous boric acid (7:1, by vol). Buffer systems used for high-voltage electrophoresis were as follows: F, pyridine/acetic acid/water (5:2:43, by vol.) pH 5.3; G, pyridine/formic acid/acetic acid/water (2:3:20:180, by vol.) pH 2.8; H, pyridine/acetic acid/water (1:10:89, by vol.) adjusted to pH 2.7 with formic acid (Kosakai & Yosizawa, 1975); I, 0.114 M-molybdate buffer pH 5.0 (Mayer & Westphal, 1968). Reagents used for the detection of amino compounds, phosphate esters, neutral sugars and polyols were those described previously (Wilkinson, 1968).

**Thin-layer chromatography (t.l.c.).** Chromatography on silica gel 60 F₂₅₄ (Merck) in solvent system J,
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Cell-wall composition

In the original studies (Wilkinson, 1968), walls were isolated from two batches of cells grown on nutrient agar. The two batches were qualitatively similar in composition. Both were rich in peptidoglycan but contained little or no protein or lipid. They differed in their contents of phosphorus and carbohydrate. In the first batch (phosphorus 3.07\%\(^\text{a}\)), glucose predominated over galactose, whereas in the second batch (phosphorus 1.67\%\(^\text{a}\)) the proportions of the monosaccharides were reversed. Analyses of a third batch of walls from broth-grown cells (phosphorus 1.71\%\(^\text{a}\)) were rich in peptidoglycan but contained little or no protein or lipid. They differed in their phosphorus and carbohydrate content from the first batch (phosphorus 3.07\%\(^\text{a}\)). Glucose was the only neutral monosaccharide component, and no reaction was obtained in the periodate/thiobarbituric acid test. Thus, the walls apparently contained...
Fig. 1. Gel permeation chromatography of the phosphorus-rich wall polymer. Materials extracted from isolated walls by treatments with 10% (w/v) trichloroacetic acid at 4 °C were applied to a column (2 × 90 cm) of Sephadex G-25 (fine). Elution was carried out with pyridine/acetic acid/water (10:4:96, by vol.) pH 5.4 at a flow rate of 20 ml h⁻¹. Fractions (4 ml) were analysed for phosphorus (○, $A_{360}$) and carbohydrate (●, $A_{400}$). Results are shown for the materials from (a) the second of five 1 d treatments, (b) a final 3 d treatment.

only one of the carbohydrate polymers found in the walls of agar-grown cells. Both types of wall had a similar peptidoglycan content (32 to 46%), and an identical peptidoglycan composition (glutamic acid/alanine/2,6-diaminopimelic acid/glucosamine/muramic acid in the molar ratio 1:2:1:1:1). Galactosamine was a minor component of both types of wall (content about 1%).

Isolation of the phosphorus-rich wall polymer

Because the walls from cells grown in liquid culture were available in relatively large amounts, and lacked the galactose-containing carbohydrate, they were used for the isolation and study of the phosphorus-rich polymer. The earlier studies (Wilkinson, 1968) had shown that this polymer was slowly released on treatment of the walls with cold 10% (w/v) trichloroacetic acid. In the present work, the extent of the release varied from 52 to 86% of the available phosphorus in treatments lasting 7 or 8 d. In the most efficient case, in which the walls were extracted four times (2 × 1 d, 2 × 3 d), the accumulated release of phosphorus was as follows: 1 d, 33%; 2 d, 54%; 5 d, 78%; 8 d, 86%.

Various methods for recovering the phosphorus-rich polymer from the extracts were tried. Because the polymer was not precipitated by the addition of acetone (4 vol.) or ethanol (4 vol.) to the aqueous solutions, trichloroacetic acid was removed by repeated extraction with n-butyl acetate (no phosphorus was lost in the process). The aqueous solutions were then neutralized, dialysed and freeze-dried. However, variable and sometimes serious losses of phosphorus (12 to 72%) occurred during dialysis. To minimize the hydrolytic formation and subsequent loss of low molecular weight products, individual treatments with trichloroacetic acid were limited to 1 d (except for the final treatment), diethyl ether was used in place of n-butyl acetate (with which separation of the two phases was slow) and dialysis was replaced by gel permeation chromatography.

Before this modified procedure was adopted, alternative and potentially milder methods of extraction were tried. No phosphorus was extracted by treatment of the walls with either hot or cold aqueous phenol, and only 10% by treatment with hot dimethyl sulph-
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Although the solubilization of wall phosphorus with aqueous N,N-dimethylhydrazine was extensive (81%), chromatography of the extract on DEAE-cellulose gave two overlapping peaks for phosphorus-containing materials. Careful treatment of the walls with cold aqueous trichloroacetic acid was therefore the extraction method of choice. The materials present in the extracts from successive treatments had similar chromatographic properties on Sephadex G-25. The bulk of the material was only slightly retarded by the gel, and the elution profiles for phosphorus and carbohydrate were in good agreement. The peaks for polymeric material showed little evidence of tailing, and even in late extracts the amount of low molecular weight material was relatively small (Fig. 1).

Composition of the phosphorus-rich wall polymer

The phosphorus content of different preparations was generally in the range 6 to 7%, and the carbohydrate content in the range 15 to 21% (expressed as glucose). On the assumption that the polymer was the sole source of phosphorus and carbohydrate, it accounted for 30 to 40% of the dry weight of the wall.

D-Glucose (identified by paper chromatography with solvent systems A, B and C, and by positive reactions in the glucose oxidase and hexokinase assays) was the sole neutral monosaccharide present in the polymer, but it accounted for only about one-half of the total carbohydrate as determined by the phenol/H₂SO₄ method with unhydrolysed polymer. However, the reaction products from the latter estimation had λ_max 480 nm, compared with 486 nm for a glucose standard. With hydrolysates, a discrepancy between the values for glucose and total carbohydrate was found when neutralization was carried out with dilute alkali, but not when Dowex 1 resin (HCO₃⁻ form) was used. These observations, and the appearance of a carbonyl band at 1720 cm⁻¹ in the infrared spectrum of polymer that had been passed down a column of Dowex 50 resin (H⁺ form) then redried, suggested the presence of a sugar acid. Although the carbazole assay suggested an uronic acid content of about 2%, the colour produced was a different shade from that given by glucuronic acid, and no uronic acid was detected by paper electrophoresis of hydrolysates (buffer systems F and H). Further studies of alkali-neutralized hydrolysates showed that the above results could be attributed to the presence of pyruvic acid. The product isolated by preparative paper electrophoresis had properties identical with those of the reference compound in the following tests: (a) the colour (λ_max 475 nm) produced in the phenol/H₂SO₄ reaction; (b) the reduction of alkaline AgNO₃ and the production of a light yellow colour (with white fluorescence under ultraviolet light) on heating an electrophoretogram treated with aniline hydrogen oxalate; (c) electrophoretic mobility at pH 5.3 (mₑleₐkA 2.0) and pH 2.8 (mₑleₐkA 3.1); (d) a positive reaction in the lactate dehydrogenase assay; (e) t.l.c. of the 2,4-dinitrophenylhydrazone in solvent system J; (f) g.l.c. of the O-(trimethylsilyl)quinoxalinol derivative on column VIII.

The proton magnetic resonance spectrum of the polymer contained a prominent, sharp singlet at δ 1.62 p.p.m., as expected for an acetal-linked pyruvate residue (e.g. Bebault et al., 1973; Lindberg et al., 1979). Further evidence for an acetal rather than an ester linkage was the absence of a carbonyl absorption at 1720 cm⁻¹ from the infrared spectrum of polymer that had been passed down a column of Dowex 50 resin (Na⁺ form), and the marked difference in complexity between the mixture of phosphate esters produced on acid hydrolysis of the polymer and that produced on alkaline hydrolysis (see below). The results of enzymic assays for pyruvic acid and glucose in one batch of polymer (Table 1) indicated that these components accounted for about 90% of the phenol/H₂SO₄ reactivity and were present in a molar ratio of 2:1. This was supported by the relative intensity (about 6:1) of the signals in the proton magnetic resonance spectrum for the pyruvate methyl group (δ 1.62 p.p.m.) and the anomeric proton(s) (δ 4.68 p.p.m., J₁,₂ 9 Hz, attributable to a β-D-glucopyranosyl group).
Table 1. Quantitative analysis of the phosphorus-rich wall polymer

<table>
<thead>
<tr>
<th>Component</th>
<th>Percentage of dry weight of polymer</th>
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<tbody>
<tr>
<td>Phosphorus</td>
<td>6.8</td>
</tr>
<tr>
<td>Total carbohydrate</td>
<td>21.4*</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>14.3</td>
</tr>
<tr>
<td>D-Galactosamine</td>
<td>1.5</td>
</tr>
<tr>
<td>Pyruvic acid</td>
<td>12.6</td>
</tr>
<tr>
<td>Glycerol</td>
<td>2.8†</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>23.8†</td>
</tr>
</tbody>
</table>

* Determined as glucose by the phenol/H₃SO₄ method.
† Determined by gas-liquid chromatography.
‡ Determined by enzymic assay.

Paper chromatography (solvent systems A, B, D and E) of deionized acid hydrolysates revealed the presence not only of glucose, but of components with the mobilities and reactions of glycerol, mannitol and 1,4-anhydromannitol (but no 1,5-anhydromannitol). 1,4-Anhydromannitol was readily detected by means of the strong yellow colour given with the periodate/Schiff reagents (Hardy & Buchanan, 1963). Much larger amounts of the polyols were obtained when an acid hydrolysate of the polymer (dried repeatedly over P₂O₅ and KOH) was treated with alkaline phosphatase. The acid hydrolysate was separated into monophosphate and diphosphate fractions by paper chromatography in solvent D. Phosphatase action on both of these fractions yielded glycerol and mannitol but only the monophosphate fraction also gave rise to 1,4-anhydromannitol. All three polyols were also released by alkaline phosphatase treatment of the mixture of phosphate esters produced by alkaline hydrolysis of the polymer. The following additional methods were used to confirm the identities of the polyols obtained by one means or another. Glycerol was identified by (a) paper electrophoresis (buffer system I), (b) g.l.c. of its acetate ester (column VII) and (c) enzymic assay. Mannitol was identified by (a) paper electrophoresis (buffer system I), (b) g.l.c. of its acetate ester (columns III, IV, V and VI), (c) g.l.c. of its trimethylsilyl ether (columns I and II) and (d) enzymic assay with mannitol dehydrogenase (which also confirmed the D configuration). 1,4-Anhydromannitol was also identified by g.l.c. of its acetate ester (columns V and VI).

Whereas the action of alkaline phosphatase on the mixtures of phosphate esters present in acid and alkaline hydrolysates of the polymer led to essentially complete release of inorganic orthophosphate, less than 1% of the phosphorus present in the intact polymer was released by such treatment. Gel permeation chromatography and paper electrophoresis confirmed that alkaline (as well as acid) hydrolysis caused depolymerization of the wall material, which indicated the involvement of phosphodiester linkages. Paper chromatography (solvent system D) and electrophoresis (buffer system F), as well as the results of alkaline phosphatase treatment discussed above, indicated that polyol monophosphates and diphosphates were the major components of acid hydrolysates. Such phosphate esters were, however, only minor products in alkaline hydrolysates. By means of DEAE-cellulose chromatography and preparative paper electrophoresis, some nine phosphorus-containing fractions were isolated from an alkaline hydrolysate. This proliferation of components can be attributed to the effects of glucosyl and pyruvate substitution on the course of hydrolysis and on the variable presence of the substituents in the final products. Analysis confirmed that all of the glucose was alkali-stable.

The last component of the polymer to be identified was galactosamine. As acid hydrolysates also gave a positive reaction with galactose oxidase but did not contain galactose, it appeared that the hexosamine had the D configuration (Sempere et al., 1965). Quantitative
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analyses for this and other components of the polymer, all obtained for the same batch, are summarized in Table 1.

**DISCUSSION**

The present results and those of the earlier study (Wilkinson, 1968) show that the major components of the cell wall of bacterium NCTC 9742 are peptidoglycan and one or both of two carbohydrate-containing polymers, both of which can be extracted with cold 10\% (w/v) trichloroacetic acid. Whereas the phosphorus-rich polymer was present in the walls of cells from both surface and liquid cultures, the second polymer which apparently contained galactose and a 2-keto-3-deoxyaldonic acid was only present in the walls from surface cultures. It seems very likely that the second polymer is produced as a response to phosphorus limitation of growth. Such a response would be entirely analogous to the replacement of the wall teichoic acid by a teichuronic acid during the growth of other Gram-positive species under phosphorus-limited growth conditions (Ellwood & Tempest, 1972).

The main concern of the present study was to optimize conditions for the isolation of the phosphorus-rich polymer, and to identify and estimate its components. To the best of our knowledge, no wall polymer of similar composition has previously been obtained from a Gram-positive organism. Nevertheless, in a number of its compositional and structural features the material resembles a teichoic acid. Its novel features include the presence of D-mannitol and pyruvic acid, neither of which is found in conventional teichoic acids (Duckworth, 1977). Although detailed structural studies have yet to be completed, it seems likely that the main backbone of the polymer consists of a poly(mannitol phosphate) chain to which glucosyl substituents and acetal-linked pyruvic acid residues are attached. The approximate molar ratio for mannitol/glucose/pyruvic acid of 2:1:2 shows that not all mannitol residues could carry glucosyl substituents and indicates that the pyruvic acid is more likely to be attached to mannitol than to glucose.

The slow extraction of the polymer from the cell walls with trichloroacetic acid suggests that extraction involves the cleavage of a covalent linkage from the polymer to the insoluble peptidoglycan. It is therefore tempting to suggest that the glycerophosphate and galactosamine residues in the polymer constitute a linkage unit. In several Gram-positive organisms, the units that link the wall teichoic acid to peptidoglycan have been shown to contain tri(glycerol phosphate) and glucosamine residues (Coley et al., 1978).

Because the phosphorus-rich polymer isolated from bacterium NCTC 9742 appears to be unique, this study of the cell-wall composition of the organism does not immediately help to establish its taxonomic position or relationships. Nevertheless, cell-wall composition has been used extensively in the classification of coryneform bacteria (Keddie & Cure, 1978). Both we and Fiedler et al. (1970) have shown that the peptidoglycan of bacterium NCTC 9742 contains glutamic acid, alanine, 2,6-diaminopimelic acid, glucosamine and muramic acid in the molar ratio 1:2:1:1:1. Fiedler et al. (1970) also recorded that the diaminopimelic acid was the meso isomer, and studies of our preparation by Dr P. J. White have confirmed this finding. Thus, in peptidoglycan type the organism resembles Corynebacterium sensu stricto and is clearly differentiated from many coryneform organisms, including Arthrobacter globiformis, of which the peptidoglycan is different or more complex in composition (Schleifer & Kandler, 1972; Keddie & Cure, 1978). The neutral sugar composition of the cell wall has also proved to be useful in the classification of coryneform organisms. For example, Corynebacterium sensu stricto is considered to demand the presence of both arabinose and galactose. However, the available data (Keddie & Cure, 1978) and the phenotypic variation encountered in the present study do not help to determine the taxonomic position of ‘P. iodinum’. Little seems to be known about the possible occurrence of teichoic acids in coryneform organisms (Archibald, 1974; Duckworth, 1977). In several species, e.g. Corynebacterium poinsetiae (Diaz-Mauriño & Perkins, 1974) and Arthrobacter
globiformis (Duxbury et al., 1977), the presence of phosphorus in the cell wall was not indicative of a teichoic acid component. Thus, although the results of the present study leave ‘P. iodinum’ in taxonomic limbo, the discovery of the novel mannilot phosphate polymer could eventually prove to be useful in clarifying the situation. It may be relevant to note that mannilot 1-phosphate was isolated from Lactobacillus arabinosus during the course of a study that eventually led to the discovery of the ribitol and glycerol teichoic acids (Baddiley et al., 1956).

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preparation of 1,5-anhydro-β-mannitol (styracitol) from D-mannitol. Journal of the American Chemical Society 74, 3175–3176.


