A Survey for the Presence of Teichuronic Acid in Walls of *Bacillus megaterium* and *Bacillus cereus*

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INTRODUCTION

Teichuronic acids are heteropolymers containing uronic acid and other carbohydrate residues but little (if any) phosphorus; they are present in walls of some Gram-positive bacteria. White & Gilvarg (1977) isolated a teichuronic acid containing glucuronic acid, rhamnose and glucose from walls of *Bacillus megaterium* M46. No teichoic acid was present in these walls, whereas Ghuysen, Leyh-Bouille & Dierickx (1962) found a teichoic acid as a major component of walls of *B. megaterium* KM. Ellwood & Tempest (1972) showed that this polymer was replaced by a teichuronic acid when strain KM was grown with phosphorus-limitation in a chemostat. Neither teichoic acid (Baddiley, 1968) nor teichuronic acid (see below) are present in walls of *B. megaterium* NCIB7581 after growth in batch culture in a simple chemically-defined medium.

In this survey, walls of 10 strains of *B. megaterium* and two strains of *Bacillus cereus* have been assayed for uronic acids, rhamnose and phosphorus. Only strain M46 contained teichuronic acid, and walls of several strains contained neither teichoic nor teichuronic acids.

METHODS

Organisms. The origins of *B. cereus* NCIB6349 and NCIB7464, and of *B. megaterium* KM, 216, NCIB4821, NCIB7581, NCIB8508, NCIB9323, NCIB9376 and ATCC13632 were given by White (1972); *B. megaterium* KM and ATCC13632 are supposedly identical, but are clearly distinct strains (White, 1972). *Bacillus megaterium* M was from the Czechoslovak Collection of Micro-organisms, and *B. megaterium* M46 (a diaminopimelate- and lysine-requiring mutant, not derived from strain M) was described by Pitel & Gilvarg (1970). All were maintained on nutrient agar slopes (plus 100 µg diaminopimelate ml⁻¹ for M46) that were incubated overnight at 37 °C (30 °C for strain 13632), and stored at 2 °C.

Media. Minimal medium A (White, 1972) was used for *B. megaterium* M, KM, 216, 8508 and 9323. Supplements for *B. megaterium* 7581 and 9376, and for *B. cereus* 6349 and 7464 are listed by Day & White (1977). Supplements for other strains of *B. megaterium* were (in mg l⁻¹): L-glutamic acid (120) and L-leucine (60) for 13632; L-asparagine (400), L-glutamic acid (200) and L-proline (100) for 4821; and diaminopimelic acid (100) and L-lysine hydrochloride (50) for M46. Medium A1 contained 1% (w/v) glycerol and medium A2 contained 1% (w/v) glucose.

To restrict growth of *B. megaterium* 7581 by limitation of inorganic orthophosphate, the basal solution of medium A was modified to contain (l⁻¹): N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), 10 mmol; 3-(N-morpholino)propanesulphonic acid (MOPS), 10 mmol; piperazine-N,N'-bis(2-ethanesulphonic acid) (PIPES), 10 mmol; (NH₄)₂SO₄, 2 g; KCl, 20 mmol, KH₂PO₄, 0.5 mmol; trisodium citrate. 2H₂O, 20 mg; biotin, 0.2 µg; the pH value was adjusted to 7.2 with NaOH. These ingredients were autoclaved together (at about 1.25 times working strength); after cooling, glucose and salts of Mg²⁺, Fe²⁺ and Mn²⁺ were added, as for medium A2 (White, 1972).

Growth of organisms and isolation of walls. Batch cultures were grown in conical flasks and walls were isolated after breaking the organisms in the pressure cell of Milner, Lawrence & French (1950), as described by Day & White (1977).

Chromatography. Walls were hydrolysed in sealed tubes with 2 M-HCl at 100 °C for 1 h (to release monosaccharides) or with 6 M-HCl at 105 °C for 18 h (to release diaminopimelate). Hydrochloric acid was removed by repeatedly drying the hydrolysates in a desiccator. Descending chromatograms were run on

Whatman no. 1 paper at room temperature. Solvent 1 was ethyl acetate/acetic acid/water (9:2:2, by vol.); solvent 2 was butan-1-ol/pyridine/water (6:4:3, by vol.); and solvent 3 (Rhuland *et al.*, 1955) was methanol/pyridine/10 M-HCl/water (32:4:1:7, by vol.). Carbohydrates were detected with *p*-anisidine reagent (Bailey, 1969) and amino acids with ninhydrin, as described by Day & White (1977).

Assays. The phenol/sulphuric acid method of Dubois *et al.* (1956) and the modified anthrone reagent method of Herbert, Phipps & Strange (1971) were used to measure carbohydrate in walls. In the former assay, 1 ml of 5 % (w/v) phenol was always used per tube, and glucose was the standard for both methods. Uronic acids were determined by the assay of Bitter & Muir (1962), with glucuronic acid as standard. Rhamnose was measured by the method of Dische & Shettles (1948). Phosphorus was measured as described by Ames (1966); all samples (test and standard) were ashed with magnesium nitrate before assay to convert organic phosphorus to inorganic orthophosphate. Diaminopimelate was assayed by the method of Work (1957) in which acidic ninhydrin is used at 37 °C.

Bacteriological tests. *Bacillus megaterium* M46 was examined using the methods listed by White (1972), except that the procedure of Conn, Jennison & Weeks (1957) with 0.3 % (w/v) creatine in the KOH solution was used for the Voges-Proskauer test. Diaminopimelic acid (100 µg ml⁻¹) was added to all the crude media. Incubations were at 37 °C except for stab cultures in nutrient broth (Oxoid) containing 5 % (w/v) gelatine (and diaminopimelate), which were incubated at room temperature. Spores of strain M46 were produced at 30 °C in medium A containing 0.1 % (w/v) glucose, diaminopimelate (100 µg ml⁻¹) and L-lysine hydrochloride (20 µg ml⁻¹), by addition of dipicolinic acid and CaCl₂ (both to 1 mM final concentration) at the end of exponential growth (Pitel & Gilvarg, 1970).

Chemicals. Diaminopimelic acid was a mixture of all three isomers, synthesized as described by Saleh & White (1976). HEPES, MOPS and PIPES were from Sigma.

RESULTS AND DISCUSSION

Of the organisms grown in batch culture in medium A, only *B. megaterium* M46 contained more than traces of uronic acid and rhamnose in its wall (Table 1). However, this strain does seem, in other respects, to have the properties expected of the species *B. megaterium* (Buchanan & Gibbons, 1974; White, 1972). The organisms were Gram-variable rods, about 1.5 µm wide and 3 to 5 µm long, growing in chains of a few organisms, and very sluggishly motile in medium A at 30 °C. They were catalase-positive and formed terminal or sub-terminal, oval to spherical spores; the sporangia were not swollen by the spores. Gelatine was liquefied in 2 to 3 days, and starch was hydrolysed. There was no anaerobic growth [in N₂/CO₂ (95:5, v/v)] within 3 days at 37 °C in glucose/nutrient broth or on glucose/nutrient agar, though aerobically these media allowed heavy rapid growth of highly vacuolated organisms. The lecithinase and Voges-Proskauer tests were negative. Acid but no gas was produced from glucose, mannitol and arabinose, but not from xylose. No growth factors were required other than diaminopimelate and lysine, the requirements for which were deliberately imposed by mutation (Pitel & Gilvarg, 1970). Unfortunately, the wild-type parent strain of M46 was not available, hence the inclusion of teichuronic acid by M46 could be the result of an additional mutation that occurred while the blocks in the biosynthesis of diaminopimelate and lysine were being imposed.

The teichuronic acid of strain M46 was first recognized in organisms grown at 32 °C with glucose as main carbon source, and then treated with detergent and enzymes (White & Gilvarg, 1977). The walls of other strains now examined were isolated mostly from organisms grown at 37 °C with glycerol, and broken by shearing forces. However, walls of strain M46 still contained teichuronic acid after growth with glycerol at 37 °C or at 30 °C, followed by breakage of the bacteria in the pressure cell. Furthermore, strain 7581 did not form a teichuronic acid when grown with glucose at 30 or 37 °C (Table 1).

In the modified medium A2 with limited phosphate, growth of strain 7581 stopped when the yield of organisms was about 1 mg dry wt ml⁻¹. With 1 mM-KH₂PO₄ (or higher concentrations), growth in the modified medium was as heavy and rapid as in ordinary medium A2. After growth with restricted phosphate, walls of strain 7581 contained more anthrone-positive material than was found after growth with glucose and excess phosphate, and some uronic acid was found by assay, though no uronic acid spot was identified on chromato-

Table 1. Assays on walls of *B. megaterium* and *B. cereus*

Organism	Medium and growth temp. (°C)	Carbohydrate [$\mu\text{mol mg}^{-1}$]		Diaminopimelate [$\mu\text{mol mg}^{-1}$]	Uronic acid [$\mu\text{mol} (\mu\text{mol Dap})^{-1}$]	Rhamnose [$\mu\text{mol} (\mu\text{mol Dap})^{-1}$]	Sugars detected by chromatography†	Phosphorus [$\mu\text{mol} (\mu\text{mol Dap})^{-1}$]
		Phenol/H ₂ SO ₄ assay	Anthrone assay					
<i>B. megaterium</i>								
M	A1, 37	0.37	0.45	0.56	<0.02	0.05	ND	0.07
KM	A1, 37	0.57	0.60	0.09	<0.11	<0.11	ND	3.67
216	A1, 37	0.47	0.46	0.75	<0.01	<0.01	ND	0.25
4821	A1*, 30	0.39	0.34	0.23	<0.04	0.13	ND	4.17
8508	A1, 37	0.94	0.68	0.38	<0.03	<0.03	Glc	0.34
9323	A1, 37	0.31	0.43	0.64	<0.02	<0.02	ND	0.28
9376	A1*, 37	0.40	0.51	0.51	<0.02	0.06	Glc	0.18
13632	A2*, 30	0.64	0.74	0.29	<0.04	<0.04	Glc	0.90
7581	A1*, 37	0.43	0.47	0.41	<0.02	0.07	ND	0.12
7581	A2*, 37	0.59	0.57	0.11	<0.09	0.45	ND	0.36
7581	A2*, 30	0.40	0.50	0.42	<0.02	0.07	Glc	0.12
7581	Low phosphate, 30	0.72	0.78	0.22	0.41	0.32	Glc	0.14
7581	Nutrient broth, 37	0.31	0.42	0.45	<0.02	0.07	ND	0.07
M46	A1*, 30	1.44	1.08	0.46	0.65	2.30	Glc, GlcA, Rham	0.33
M46	A1*, 37	1.24	1.00	0.14	1.43	5.14	ND	1.36
<i>B. cereus</i>								
6349	A1*, 37	1.00	0.88	0.46	<0.02	0.46	Glc	0.24
7464	A1*, 37	0.38	0.49	0.20	<0.05	0.35	ND	0.50

ND, Not done.

* Supplements added (see Methods).

† Glc, glucose; GlcA, glucuronic acid; Rham, rhamnose.

grams (Table 1). In a batch culture, only the organisms that are still growing when the culture approaches the stationary phase will really be phosphate-limited, and so the walls isolated in the present study are not strictly comparable with walls of phosphate-limited organisms from a chemostat.

In no walls, except those of M46 (which are known to contain rhamnose and glucuronic acid) and 8508, was there a large excess of total carbohydrate (measured with phenol/sulphuric acid) over anthrone-positive material, which is largely hexose (Herbert *et al.*, 1971). Glucose was the only hexose that could be identified clearly in more than traces on paper chromatograms (solvents 1 and 2). Whether glucose is really part of the wall structure, or whether it is derived from trapped granules of a glycogen-like polymer (cf. White & Gilvarg, 1977) has not been established. Rhamnose and glucuronic acid (or its lactone) were not detected in hydrolysates of any wall except strain M46, where they were obvious.

Diaminopimelate was assayed in most of the walls, to assess roughly their content of peptidoglycan. If, for every 1 mol diaminopimelate, 2 mol *N*-acetylhexosamine, 2.5 mol alanine and 1 mol glutamate are present as peptidoglycan (total mol. wt about 950), then some of the walls (e.g. *B. megaterium* 216 and 9323) contain at least 60 % (w/w) peptidoglycan. Some of the other wall preparations (e.g. strain 7581 grown in medium A2 at 37 °C) contained trapped granules of poly- β -hydroxybutyrate, and hence seem to contain a lower proportion of peptidoglycan than is really present in the wall itself. In every case except *B. cereus* 7464, LL- as well as meso- and/or DD-diaminopimelate was found. Previously, the small proportion of LL-isomer present in peptidoglycan of strain M46 was not detected, perhaps because less diaminopimelate could be loaded on to chromatograms when hydrolysates of 'sacculi' were used (White & Gilvarg, 1977). Alanine and glutamate were the only other amino acids found (as more than traces) in any of the walls.

Since teichoic acids are, by definition, phosphorus-containing polymers, a wall with little phosphorus must also have little (if any) teichoic acid. Thus, after growth in medium A1, walls of *B. megaterium* strains M, 216, 7581, 9323 and 9376 must contain less than 0.1 g teichoic acid (assuming that 1 mol phosphate is equivalent to about 300 g teichoic acid) per 1 g peptidoglycan (assuming that 1 mol Dap is equivalent to 950 g peptidoglycan). Teichoic acid is present in walls of *B. megaterium* KM; whether there is teichoic acid in walls of some other strains of *B. megaterium* (e.g. 4821 and 13632) that contain more than 0.5 μ mol phosphate (μ mol Dap)⁻¹ is unknown.

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