The Teichuronic Acid of Cell Walls of *Bacillus subtilis* W23 Grown in a Chemostat under Phosphate Limitation

By JOHN WRIGHT and JOHN E. HECKELS*

Microbiological Chemistry Research Laboratory, University of Newcastle upon Tyne,
Newcastle upon Tyne NE1 7RU, U.K.

(Received 20 December 1974)

Cell walls of *Bacillus subtilis* W23 contain teichuronic acid when grown in a chemostat under phosphate limitation at a low dilution rate, but teichoic acid at a higher dilution rate. The teichuronic acid was purified and shown to be a polymer of glucuronic acid and *N*-acetylglactosamine.

The cell walls of the bacilli, like those of other Gram-positive bacteria, are among those structures of the cell that are most susceptible to phenotypic variation (Ellwood & Tempest, 1969). One transition that has been studied in detail is the replacement of teichoic acid by teichuronic acid when cells are grown with phosphate as the growth-limiting nutrient (Tempest *et al.*, 1968). Thus walls of *Bacillus subtilis* species var. *niger*, W23 and 168 contain teichoic acids of differing structures when grown in batch culture or in a chemostat with Mg^{2+} , K^+ or glucose as the growth-limiting nutrient. On growth under phosphate limitation, each organism replaces teichoic acid with a teichuronic acid containing galactosamine and glucuronic acid (Ellwood & Tempest, 1972). This change has been used to monitor the assembly of newly formed cell-wall polymers (Mauk & Glaser, 1972) and to follow synthesis of bacteriophage-receptor sites in cell walls of *B. subtilis* W23 (Archibald & Coapes, 1971). The teichoic acid in this organism is a polymer of glucosylribitol phosphate (Chin *et al.*, 1966), but little detailed evidence is available on the structure of the teichuronic acid. However, cell walls of *Bacillus licheniformis* N.C.T.C. 6346 grown in batch culture contain both types of polymer (Janczura *et al.*, 1961), and the structure of the teichuronic acid has been established (Hughes & Thurman, 1970). Since the occurrence of the teichoic acid-teichuronic acid changeover has been utilized in other studies with *B. subtilis*, the aim of the present work has been to investigate the structure of the teichuronic acid of walls of *B. subtilis* W23 and to compare the results with those reported for the *B. licheniformis* polymer.

B. subtilis W23 was grown at 37°C and pH 7.0 in a 500 ml chemostat in the phosphate-limited medium described by Tempest *et al.* (1968) at

dilution rates of 0.1 and 0.3 h⁻¹. Cells were collected in a chilled reservoir, harvested by centrifugation at 2000g for 40 min and freeze-dried. The yield of cells was 4.0 mg/ml at a dilution rate of 0.1 h⁻¹ and 1.7 mg/ml at a dilution rate of 0.3 h⁻¹. Organisms were disrupted by shaking with glass beads in a Braun homogenizer and the cell-wall fraction was purified by digestion with trypsin followed by extraction with phenol (Archibald & Stafford, 1972).

Analysis of walls (Table 1) showed that those grown at a dilution rate of 0.1 h⁻¹ contained galactosamine, glucuronic acid but little phosphorus, characteristics of teichuronic acid, whereas walls grown at a dilution rate of 0.3 h⁻¹ contained phosphorus but little galactosamine or uronic acid, suggesting that teichoic acid was present but not teichuronic acid. This was confirmed by hydrolysis of walls in 4M-HCl at 100°C for 4 h followed by paper chromatography of the products in pyridine-ethyl acetate-acetic acid-water (5:5:1:3, by vol.). Walls grown at the lower dilution rate produced, in addition to peptidoglycan components, galactosamine, glucuronic acid and its lactone with traces of ribitol and glucose; those grown at the higher rate produced ribitol, anhydrosorbitol, phosphate and glucose but no uronic acid.

Cells grown under phosphate limitation at a dilution rate of 0.1 h⁻¹ thus produce a cell wall containing teichuronic acid and only traces of teichoic acid, whereas those grown at a dilution rate of 0.3 h⁻¹ contain a ribitol teichoic acid and no teichuronic acid. This phenomenon was not observed with *B. subtilis* var. *niger*, which still produced teichuronic acid at the higher dilution rate (Ellwood, 1970). A similar process, however, has been observed in growth of *Staphylococcus aureus* H, when teichoic acid reappeared in cell walls grown at higher dilution rates, and it was suggested that near to the maximum dilution rate the extracellular phosphate concentration would be close to a non-limiting value and hence teichoic acid synthesis could occur (Ellwood & Tempest, 1972).

* Present address: Department of Microbiology (Faculty of Medicine), University of Southampton, Medical and Biological Sciences Building, Bassett Crescent East, Southampton SO9 3TU, U.K.

Table 1. *Composition of walls of B. subtilis W23 from cells grown under phosphate limitation*

Walls were hydrolysed in 6M-HCl and analysed for amino compounds on a Jeol 6 AH autoanalyser. Phosphate was determined by the method of Chen *et al.* (1956) and uronic acid by the method of Bitter & Muir (1962).

Component	Composition ($\mu\text{mol/g}$ of cell wall)	
	Dilution rate 0.1 h^{-1}	Dilution rate 0.3 h^{-1}
Muramic acid	170	193
Glutamic acid	227	384
Alanine	364	639
Diaminopimelic acid	210	353
Glucosamine	207	296
Galactosamine	583	25
Phosphate	280	1935
Uronic acid	1040	—

This appears to happen in the present study, since analysis of the spent medium showed no detectable phosphate from growth at a dilution rate of 0.1 h^{-1} , but 25% of the phosphate was not utilized when cells were grown at dilution rate of 0.3 h^{-1} . Cells were therefore grown at the lower dilution rate to provide material for structural studies on the teichuronic acid.

Walls (520 mg) were stirred with 5% (w/v) trichloroacetic acid (50 ml) at 37°C for 16 h. The suspension was centrifuged at $30\,000g$ for 20 min and the supernatant solution was extracted five times with ether to remove trichloroacetic acid. The aqueous layer was applied to a column (20 mm $\times$ 400 mm) of DEAE-cellulose (acetate form). The column was eluted with a linear gradient of 0–1.0M-pyridine acetate buffer, pH 5.3 (1 litre). Two peaks were obtained. A minor peak was eluted in 0.2–0.33M buffer containing phosphate and was characterized as glucosyl ribitol teichoic acid by acid and alkali hydrolysis. The major peak was eluted in 0.45–0.75M buffer and contained uronic acid but no phosphate. Buffer was removed by evaporation *in vacuo* and the purified teichuronic acid was converted into the free acid form by passage through a column of Dowex 50 (H^+ form) resin. The yield was 176 mg.

The optical rotation of the polymer was measured and $[\alpha]_D^{20}$ found to be $+34.5^\circ$, a value similar to that obtained by Janczura *et al.* (1961) for the teichuronic acid from *B. licheniformis*, and indicates a high proportion of α -glycosidic linkages. Acid hydrolysis in 4M-HCl at 100°C for 4 h produced galactosamine, glucuronic acid, glururonolactone and an oligosaccharide with R_{GAIN} 0.18 in butanol-pyridine-water (6:4:3, by vol.).

The oligosaccharide that was isolated by preparative paper chromatography was neutral on electrophoresis in pyridine acetate buffer, pH 5.3, and on acid hydrolysis produced galactosamine, glucuronic acid and its lactone. The oligosaccharide was *N*-acetylated with acetic anhydride in aqueous methanol. When the *N*-acetylated product was reduced with NaBH_4 and hydrolysed and the products were examined by paper chromatography, glucuronic acid was still present but the galactosamine had been converted into galactosaminitol. The oligosaccharide was analysed by the Elson-Morgan reaction and produced the chromogen characteristic of 3-*O*-substituted hexosamines. The oligosaccharide produced thus has properties identical with those of the disaccharide, 3-*O*-glucuronosyl-D-galactosamine, obtained from partial hydrolysis of the *B. licheniformis* teichuronic acid (Hughes & Thurman, 1970), and arises because of the relative stability of glucuronosyl glycosidic bonds to acid hydrolysis.

The position of substitution of the glucuronic acid residues in the polymer was determined by Smith degradation. Preliminary experiments on teichuronic acid showed that the galactosamine residues were resistant to oxidation, whereas glucuronic acid was destroyed. Quantitative results were, however, difficult to interpret, since overoxidation occurred, presumably because of the presence of free carboxyl groups. The glucuronic acid residues were thus first reduced to glucose by using the method of Taylor & Conrad (1972). A solution containing 100 $\mu\text{equiv.}$ of uronic acid was treated with 3-cyclohexyl-3-(2-morpholinoethyl)carbodiimidetoluene-*p*-sulphonate (1 mmol). As the reaction proceeded the pH of the solution was maintained at 4.75, by automatic titration with 0.1M-HCl, for 2 h, when all H^+ uptake had ceased. The reaction mixture was heated to 50°C , freshly prepared 2M- NaBH_4 solution (20 ml) was added dropwise and reaction was allowed to proceed for 30 min. The product was dialysed overnight against running tap water, then against three changes of distilled water, and freeze-dried. Samples (2 mg) of the reduced polymer were hydrolysed in 2M-HCl (0.5 ml) at 100°C for 3 h. Chromatographic examination of the hydrolysate showed glucose and galactosamine to be the only products. Analysis of the reduced material showed galactosamine and glucose to be present in the relative molar proportions 1.0:1.02.

A sample of the reduced teichuronic acid (30 mg) was treated with 0.1M- NaIO_4 (3.2 ml) in the dark at 4°C . Oxidation was complete after 100 h, when 1.2 μmol of NaIO_4 had been reduced/ μmol of galactosamine. NaBH_4 was added and the mixture was incubated overnight. Excess of NaBH_4 was destroyed by the addition of acetic acid, and the

solution was passed through a column of Dowex 50 (H^+ form) resin. The eluate was dried *in vacuo* and borate was removed by repeated evaporation from methanol. Analysis of the Smith-degraded material showed that the galactosamine was resistant to oxidation, which is consistent with its 3-*O*-substitution, but that over 90% of the glucose had been destroyed. Together with the consumption of 1 mol of periodate/mol, the glucose is clearly substituted at C-2 or C-4. Hydrolysis of the material produced galactosamine, erythritol and a trace of glycerol. The glycerol presumably arose from a terminal non-reducing group. The proportion of erythritol, which can arise only from carbon atoms C-6-C-3, confirms position 4 as the point of substitution of glucose and hence of the glucuronic acid in the unmodified polymer. A similar conclusion was reached by methylation studies on the *B. licheniformis* teichuronic acid (Hughes & Thurman, 1970).

The teichuronic acid of cell walls of *B. subtilis* W23 grown under phosphate limitation is thus a polymer of alternating glucuronic acid and *N*-acetylgalactosamine residues in which the glucuronic acid is substituted on C-4 and the galactosamine on C-3. The majority at least of the glycosidic linkages have the α -configuration. This structure is identical with that proposed for the teichuronic acid found in cell walls of *B. licheniformis* N.C.T.C. 6346 grown in batch culture. Although in neither case has the strict alternation of the residues been proved, no evidence was obtained, by either partial hydrolysis or Smith degradation, of adjacent sugar moieties of the same type. The chain length of the *B. subtilis* teichuronic acid as measured by incorporation of 3H from NaB^3H_4 or chromogen formation was 19–21 disaccharide units, which is

again in agreement with the values obtained for the *B. licheniformis* polymer (Hughes & Thurman, 1970).

The similarity in cell-wall composition of *B. licheniformis* N.C.T.C. 6346, *B. subtilis* W23 and also probably *B. subtilis* var. *niger* and *B. subtilis* 168 (Ellwood & Tempest, 1972), when under conditions where teichuronic acid is present, is of some interest, since, when these organisms are grown under conditions where teichoic acid is synthesized instead, cell-wall polymers of differing structure are produced.

We thank Professor J. Baddiley, F.R.S., for his interest and the Science Research Council for an Advanced Course Studentship (to J. W.).

- Archibald, A. R. A. & Coapes, H. E. (1971) *Biochem. J.* **125**, 667–669
Archibald, A. R. A. & Stafford, G. H. (1972) *Biochem. J.* **130**, 681–690
Bitter, T. & Muir, H. M. (1962) *Anal. Biochem.* **4**, 330–334
Chen, P. S., Toribara, T. Y. & Warner, H. (1956) *Anal. Chem.* **28**, 1756–1758
Chin, T., Burger, M. M. & Glaser, L. (1966) *Arch. Biochem. Biophys.* **116**, 358–367
Ellwood, D. C. (1970) *Biochem. J.* **118**, 367–373
Ellwood, D. C. & Tempest, D. W. (1969) *Biochem. J.* **111**, 1–5
Ellwood, D. C. & Tempest, D. W. (1972) *Advan. Microbial Physiol.* **7**, 83–117
Hughes, R. C. & Thurman, P. F. (1970) *Biochem. J.* **117**, 441–449
Janczura, E., Perkins, H. R. & Rogers, H. J. (1961) *Biochem. J.* **80**, 82–93
Mauk, J. & Glaser, L. (1972) *J. Biol. Chem.* **247**, 1180–1187
Taylor, R. L. & Conrad, E. (1972) *Biochemistry* **11**, 1383–1388
Tempest, D. W., Dicks, J. W. & Ellwood, D. C. (1968) *Biochem. J.* **106**, 237–243