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The Chemical Composition of the Cell Walls of Some Thermophilic Bacilli

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SUMMARY

The chemical compositions of the cell walls of one strain each of *Bacillus stearothermophilus* and *B. coagulans* prepared from organisms grown at 37° and 55° are compared. The higher temperature of cultivation resulted in an increased proportion of mucopeptide and a decreased proportion of teichoic acid in the walls of both organisms. A higher lipid content than is usual in Gram-positive organisms was found in these walls. The teichoic acid from *B. stearothermophilus* walls was a glycerophosphate polymer substituted with glucose and alanine; evidence suggesting the presence of 2,3-phosphodiester linkages in this polymer is presented. The teichoic acid from *B. coagulans* walls was also a glycerophosphate polymer, substituted with two neutral sugars, glucose and galactose, but lacking amino acid substituents hitherto reported as characteristic of teichoic acids.

INTRODUCTION

The average size of the bacterial cell appears to be a direct function of the growth rate. The relationship between cell-wall composition and growth rate has been studied in *Bacillus megaterium* (Sud & Schaechter, 1964), where the content of wall hexosamine and diaminopimelic acid, and of membrane lipid phosphorus, on a whole organism dry-weight basis, was found to be inversely proportional to the rate of growth. Nothing appears to be known of the effect of temperature of growth on cell-wall composition. With the exception of the determination of the mole ratios of wall amino acids in a strain of *Bacillus stearothermophilus* (Salton & Pavlik, 1960) the chemical composition of the walls of thermophilic bacilli is unknown. In the present study we have analysed the walls of two facultatively thermophilic bacilli prepared from organisms grown at 37° and 55°, respectively.

METHODS

Organisms. The following two organisms were used. *Bacillus stearothermophilus* (numbered B 65 in our own collection and isolated originally from a 'Kilit Autoclave Control' spore suspension of *B. stearothermophilus* obtained from Baltimore Biological Laboratories, Inc., Baltimore 18, Maryland, U.S.A.) and *B. coagulans* (NRS no. T 2007). Cultures were maintained on nutrient agar slopes. Both organisms were facultatively thermophilic in broth culture under the conditions used.

Preparation of cell walls. Both organisms were grown in a broth medium based on that of Campbell & Williams (1953) and composed of 1% (w/v) trypticase

(BBL), 0·5% (w/v) yeast extract (BBL), 0·25% (w/v) K_2HPO_4, 0·2% (w/v) glucose, 0·1% (v/v) salts solution (0·5 g. of each of $MgSO_4.5H_2O$, $FeCl_3$, $CaCl_2$/100 ml. water). 2-l. batches of medium in 4-l. flasks were inoculated with 20 ml. of an 18 hr culture, grown in the same medium at 37° or 55° as required in a Gallenkamp shaking water-bath and incubated at either 37° or 55°, respectively, with sterile air sparging at a rate of 1 l. air/l. culture/min. Excessive foaming was controlled by periodic additions of 1 ml. of a 10% (v/v) silicone antifoam solution (Dow–Corning formula B). Growth was stopped near the end of the exponential phase of growth ($E^{1cm.}_{650m\mu}=1·00$) by adding chipped ice directly to the culture vessels. The organisms (no spores were seen) were collected by centrifugation in the cold, washed twice with cold 0·9% (w/v) saline and finally resuspended (20% w/v) in 0·9% saline. Mean generation times in the exponential phase of growth of 32 and 20 min. were obtained for *Bacillus stearothermophilus* and 60 and 18 min. for *B. coagulans* at 37° and 55°, respectively.

Volumes of suspension (45 ml.) were mixed with an equal volume of ballotini beads (English Glass Co., grade 11) and disintegrated at 0–5° at 14,000 rev./min. in a M.S.E. homogenizer until 90–95% of the organisms were broken, as judged by phase-contrast microscopy (25–35 min.). The cell-wall suspension was separated from glass beads by filtration through a No. 1 sintered glass filter and the beads washed twice with minimal volumes of cold saline. All subsequent operations were performed on an International Refrigerated Centrifuge (model HR 1 angle head no. 856). Unbroken organisms were separated from cell walls at 1000*g* for 10 min. (3 times). Walls were precipitated at 10,000*g* for 10 min. and washed by resuspension and recentrifugation in ice-cold M-KCl (3 times) and ice-cold distilled water (12 times). Final suspensions were freeze-dried and stored over P_2O_5. This procedure yielded 35–50 mg. dry-wt. cell wall/l. original culture. Electron-microscope examination of the isolated walls showed them to be free from cytoplasmic material. The absence of nucleic acids and proteins from these preparations was established by spectrophotometric examination at 260 mμ (Mitchell, 1950) and paper chromatography of acid hydrolysates as described in the text, respectively.

Paper chromatography. The following solvent systems were used: A, propan-1-ol + aq. ammonia (sp.gr. 0·88) + water (6+3+1, by vol.; Hanes & Isherwood, 1949), Whatman no. 4, ascending; B, pyridine + ethylacetate + water + acetic acid (5+5+3+1, by vol.; Fischer & Nebel, 1956), Whatman no. 1, descending; C, butan-1-ol + acetic acid + water (4+1+5, by vol.), upper layer (Partridge, 1948), Whatman 3MM, descending; D, butan-1-ol + pyridine + water (6+4+3, by vol.; Jeanes, Wise & Dimler, 1951), Whatman no. 4, descending; E, butan-2-ol + formic acid + water (7+1+2, by vol.; Roberts *et al.* 1955), Whatman no. 1, descending; F, phenol + water + aq. ammonia (sp.gr. 0·88) (80 g. + 20 ml. + 0·3 ml.; Roberts *et al.* 1955), Whatman no. 1, descending. Whatman no. 4 paper was washed before use with 2N-acetic acid containing EDTA (0·1%, w/v) and then water.

The following spray reagents were used where appropriate: periodate + Schiff reagent for polyols (Baddiley, Buchanan, Handschumacher & Prescott, 1956); perchloric acid + molybdate reagent for phosphates (Hanes & Isherwood, 1949); alkaline silver nitrate (Trevelyan, Procter & Harrison, 1950) and aniline phthalate (Partridge, 1949) for sugars; ninhydrin (Consden & Gordon, 1948) for amino acids; Elson–Morgan reagent for amino sugars (Partridge, 1949).

Hydrolysis conditions and analytical methods. For the analysis of amino acids 2 mg. dry-wt. cell wall were heated with 200 μl. 6N-HCl under N_2 in sealed tubes at 105° for 16 hr. After cooling, humin was removed by centrifugation and washed twice with water, the washing being pooled with the supernatant fluid. HCl was removed by repeated evaporation from water in vacuum over P_2O_5 and NaOH pellets. Two-dimensional chromatography in solvents E and F, by the technique of running standard substances and unknown mixture on the same paper (Saxena & Gandhi, 1962) was used to identify amino acids. For the quantitative estimation of diaminopimelic acid, glutamic acid, glycine and alanine, hydrolysates were dissolved in 100 μl. water and duplicate chromatograms were developed from 25 μl. portions in solvent D. Known concentrations of the four amino acids were chromatographed under identical conditions. The cadmium acetate + ninhydrin method of Heilmann, Barollier & Watzke (1958) was used to estimate amino acids on the developed chromatograms.

For the analysis of neutral sugars, 3 mg. dry-wt. cell wall were heated with 200 μl. 2N-H_2SO_4, under N_2 in sealed tubes at 100° for 2 hr. Hydrolysates were neutralized with 0·2N-$Ba(OH)_2$, the precipitate of $BaSO_4$ removed by centrifugation and washed twice with water, and supernatant fluid + washings passed through a short column of Dowex 50 (H^+) resin (1–2 ml.). The resin was washed with 5 column-volumes of water and the combined eluates concentrated by rotary evaporation. Paper chromatography in solvent B was used to identify the sugars present. For quantitative analysis eluates from the resin columns were made to 5 ml. with water and suitable portions used for analysis of total reducing sugar by the methods of Dubois *et al.* (1956) and Park & Johnson (1949). Glucose was estimated by the glucose oxidase method of Huggett & Nixon (1957) and galactose by the method of Park & Johnson (1949) after destruction of glucose by glucose oxidase.

For the detection of hexosamines, 2 mg. dry-wt. cell walls were heated in 200 μl. 4N-HCl in sealed tubes for 4 hr at 100°. Excess HCl was removed in vacuum over P_2O_5 and NaOH and the residue taken up in water. Neutral sugars were removed by passage of the hydrolysate through short columns of Dowex 50 (H^+) resin (1–2 ml.). After washing the resin with water (5 column-volumes) hexosamines were eluted with M-NH_4OH and evaporated to dryness in vacuum. Paper chromatography in solvent C was used to identify the hexosamines. Glucosamine was distinguished from galactosamine by ninhydrin oxidation and chromatography in solvent D (Ellwood, Kelemen & Baddiley, 1963). Quantitative estimations of amino sugar were made on hydrolysates prepared as above from 5 mg. dry wt. cell wall. After cooling, the hydrolysates were diluted quantitatively to 5 ml. with water. Portions (2 ml.) were neutralized with a slight excess of NH_4OH, freeze-dried and finally dissolved in 1 ml. water. Samples (50 μl.) were used to estimate hexosamines, as glucosamine, essentially by the procedure of Strominger, Park & Thompson (1959). The values obtained were corrected for acid destruction of hexosamines under the hydrolytic conditions used, by the method of Czerkawski, Perkins & Rogers (1963), ammonia in the diluted acid hydrolysates being estimated by Conway microdiffusion (Jacobs, 1956, 1960).

Phosphorus was estimated by the method of Fiske & SubbaRow (1925).

The lipid content of cell walls was determined, after heating with methanol by exhaustive extraction, under reflux, with ether, and then chloroform + methanol (1 + 1 by vol.).

The configuration of diaminopimelic acid in wall hydrolystes was determined by a combination of the chromatographic methods of Rhuland, Work, Denman & Hoard (1955) and Jusic, Roy, Schocker & Watson (1963).

Extraction of teichoic acids. Teichoic acids were extracted from 25 mg. portions of wall preparations with 10% (w/v) trichloracetic acid at 0–5° during 48 hr. After sedimentation by centrifugation, teichoic acid was precipitated from the supernatant solution by the addition of 5 vol. cold ethanol, and the precipitate washed with ethanol, ether, then dried in vacuum. The chemical composition of the teichoic acids was determined by hydrolysis with 2N-HCl or N-NaOH salution for 3 hr at 100°, followed by examination of the products by chromatography in solvents A and B. Alanine ester residues were detected by reaction with aqueous ammonia and chromatography of the resulting alanine and its amide in solvent A. Alkaline phosphomonoesterase (Sigma, type II) was used to degrade further the products of alkaline hydrolysis (see Wicken & Baddiley, 1963).

RESULTS

Acid hydrolysates from all of the cell-wall preparations of both organisms contained alanine, $\alpha\epsilon$-diaminopimelic acid, glutamic acid, glycine, muramic acid, glucosamine, glucose, glycerol and glycerol mono- and diphosphates; hydrolysates of *Bacillus coagulans* walls contained in addition galactose. The configuration of $\alpha\epsilon$-diaminopimelic acid in these walls was *meso-* as shown by paper chromatography of the isolated acid and its dinitrophenyl derivatives.

Table 1. *Composition of the cell walls of Bacillus stearothermophilus and B. coagulans grown at 37° and 55°*

	B. stearothermophilus		*B. coagulans*	
	37°	55°	37°	55°
	μmoles/mg. dry wt. cell wall			
Diaminopimelic acid	0·24	0·35	0·25	0·34
Glutamic acid	0·32	0·45	0·35	0·37
Alanine	1·14	0·96	0·50	0·57
Glycine	0·04	0·11	0·06	0·06
Hexosamine*	0·60	0·70	0·63	0·81
Glucose	0·98	0·88	0·67	0·53
Galactose	0·00	0·00	0·67	0·53
Phosphorus	1·27	1·28	0·83	0·71

* Calculated as glucosamine and corrected for destruction under the conditions of hydrolysis used as described in text.

Table 1 lists the results obtained from quantitative analyses of acid hydrolysates of cell walls; the relative mole ratios of amino acids and hexosamine are shown in Table 2.

Ether extractable, chloroform+methanol extractable and total extractable lipid from the cell walls of *Bacillus stearothermophilus* grown at 55° were 4·3, 3·7 and 8·0% of the dry-wt. of wall, respectively. Corresponding figures for *B. coagulans* were 8·3, 4·3 and 12·6%. Insufficient material was available for comparable

analyses of walls from organisms grown at 37°. Chromatographic examination of acid hydrolysates of extracted lipid material in solvents B, E and F did not show neutral sugars, hexosamines or amino acids.

The chromatographically identified products of acid and alkali hydrolysis of teichoic acids extracted from the walls of both organisms are listed in Table 3. Ammonolysis of the teichoic acid from, and whole cell walls of, *Bacillus stearothermophilus* gave alanine and alanine amide, indicating the presence of alanyl ester linkages. The absence of similar residues from *B. coagulans* walls or extracted

Table 2. *Mole ratios* of amino acids and hexosamines in the cell walls of Bacillus stearothermophilus and B. coagulans grown at 37° and 55°*

	Hexosamines	Glutamic acid	DAP†	Alanine	Glycine
B. stearothermophilus, 37°	1·89	1·00	0·76	3·57	0·01
B. stearothermophilus, 55°	1·50	1·00	0·77	2·10	0·02
B. coagulans, 37°	1·80	1·00	0·70	1·43	0·02
B. coagulans, 55°	2·17	1·00	0·93	1·55	0·02

* Relative to glutamic acid = 1·00. † DAP = *meso*-αε-diaminopimelic acid.

Table 3. *Hydrolysis products of teichoic acids extracted from Bacillus stearothermophilus and B. coagulans*

	B. stearothermophilus			*B. coagulans*	
	Acid hydrolysis	Alkali hydrolysis	Alkali hydrolysis + alkaline phospho-mono-esterase	Acid hydrolysis	Alkali hydrolysis*
Glycerol	+	Trace	+	+	–
Glyceromonophosphates	+	+	–	+	Trace
Glucosylglycerophosphates	–	+	–	–	–
Glycerodiphosphates	+	+	–	+	Trace
Glucosylglycerol	–	Trace	+	–	–
Glucose	+	–	–	+	–
Galactose	–	–	–	+	–
Inorganic phosphate	+	Trace	+	+	–
Alanine	+	+	.	–	–

* Highly resistant to alkali hydrolysis.

teichoic acid was confirmed by ammonolysis. No ninhydrin-positive material was found in acid hydrolysates of the teichoic acid from *B. coagulans*; this acid was highly resistant to alkali hydrolysis.

The teichoic acid of *Bacillus stearothermophilus* on alkali hydrolysis followed by treatment with alkaline phosphomonoesterase gave glycerol and a compound that reacted rapidly with the periodate Schiff reagent and had an R_F value of glucosylglycerol in solvent A. Acid hydrolysis of this material gave glucose and glycerol as the sole products.

DISCUSSION

The cell walls of *Bacillus coagulans* and *B. stearothermophilus* appear to be composed of three main components: glycosaminopeptide (mucopeptide), teichoic acid, lipid. The constituents of the glycosaminopeptide fractions, (glucosamine, muramic acid, meso-$\alpha\varepsilon$-diaminopimelic acid, glutamic acid, alanine, small amounts of glycine) are typical of many Gram-positive bacteria (Salton, 1964). In both organisms a significantly greater proportion of glycosaminopeptide components to other wall constituents was found for cell walls prepared from organisms grown at 55° as compared with those grown at 37°. Variations in the molar proportions of hexosamines and wall amino acids with temperature of growth were seen and these may reflect differences in glycosaminopeptide structure within the same organism. A greater resistance to mechanical rupture during cell-wall preparation was noted with organisms grown at 37° as compared with those grown at 55°. Salton & Pavlik (1960) reported mole ratios of glutamic acid:$\alpha\varepsilon$-diaminopimelic acid:alanine:glycine of 1·0:0·50:1·9:0·0 for the cell walls of a strain of *B. stearothermophilus* but did not state the conditions of culture used. The higher content of alanine in *B. stearothermophilus* walls than in *B. coagulans* found in the present work can be accounted for by the presence of alanyl ester substitution of the teichoic acid in the former organism and the absence of such substitution in *B. coagulans*.

The lipid content of the walls of these thermophilic organisms was surprisingly high as compared with that of many mesophilic Gram-positive bacteria, which seldom exceed 1–2% (Salton, 1964). The absence of amino acids other than typical cell-wall amino acids in acid hydrolysates of these wall preparations suggests that the extracted lipid is a true wall component and not due to contamination with lipoprotein cell membrane. The nature of the lipid was not investigated other than to establish the absence, in acid hydrolysates of extracted lipid, of neutral sugars, amino sugars or amino acids.

The teichoic acids in these organisms show some unusual features. That from *Bacillus stearothermophilus* walls gave on acid hydrolysis products which may be considered typical of a glycerol teichoic acid substituted with glucose in glycosidic linkage to a polyol hydroxyl group and with alanine in ester linkage to the polymer (see Wicken & Baddiley, 1963). The course of alkali hydrolysis of glycerol teichoic acids was discussed by Kelemen & Baddiley (1961). It has been shown that increasing glycosidic substitution of a 1,3-phosphodiester-linked glycerophosphate polymer increases the resistance of the polymer to alkali degradation, substitution of the 2-hydroxyl group of the glycerol moieties with a sugar preventing the necessary intermediate formation of a cyclic phosphate (see Wicken & Baddiley, 1963). A 2,3-phosphodiester-linked glycerol teichoic acid, on the other hand, would be expected to be degraded in alkali, even if highly substituted with sugar, to a mixture of glyceromonophosphates, glycosylglyceromonophosphates and small amounts of glycerol and glycerol diphosphates. The glycerol teichoic acid extracted from *B. stearothermophilus* walls was completely degraded in alkali to such a mixture of phosphates. Treatment of an alkali hydrolysate with alkaline phosphomonoesterase gave glycerol as a minor product and a major component which had the R_F value of glucosylglycerol in solvent A. Acid hydrolysis of this material gave glucose and glycerol only. The fast reaction obtained between this material and the periodate

Schiff reagent is indicative of the presence of an α-glycol group within the molecule (Roberts, Buchanan & Baddiley, 1963), which in turn suggests that the glycosidic linkage is to a primary hydroxyl group of glycerol. It is therefore suggested that this teichoic acid contains 2,3-phosphodiester linkages rather than the more usual 1,3-phosphodiester linkages. The possible existence of a similarly linked glycerol teichoic acid from *B. licheniformis* was reported by Burger (1963).

The glycerol teichoic acid isolated from *Bacillus coagulans* showed two unusual features. No substitution by alanine or another amino acid was found and this was confirmed by ammonolysis of intact cell walls. The presence of both glucose and galactose in acid hydrolysates of this teichoic acid and the high degree of resistance of the polymer to alkali degradation suggests either a single 1,3-phosphodiester-linked glycerophosphate polymer substituted with both sugars, or a mixture of two polymers each substituted with a single sugar. More recent work has confirmed the former possibility (Forrester & Wicken, unpublished observations). The possible linkage of glycerophosphate moieties through sugar residues in this polymer is not precluded by these observations. Further investigations into the complete structures of these two teichoic acids is in progress. The increased glycosaminopeptide content of the walls of organisms when grown at the higher temperature was accompanied by a decrease in total neutral reducing sugars. This might be equated with a decrease in teichoic acid content at the higher growth temperature, or alternatively a decrease in the degree of glycosidic substitution of the polymers. Corresponding decreases in total phosphorus content were not as marked but it has been established that teichoic acid phosphorus does not necessarily represent the total cell-wall phosphorus (Ellwood *et al.* 1963). In *Bacillus stearothermophilus* walls a lower total alanine content and lower mole ratio of this amino acid to other wall constituents was noted for walls from 55°-grown organisms. This, too, would be consistent with a lower teichoic acid content of these walls.

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