Chemical Composition of the Cell Walls of
Bacillus stearothermophilus

ALLAN B. SUTOW1 AND N. E. WELKER
Department of Biological Sciences, Northwestern University, Evanston, Illinois

Received for publication 17 December 1966

Cell walls were isolated by mechanical disruption of mid-log phase cells of Bacillus stearothermophilus NCA 1503-4R grown in Trypticase-yeast extract-fructose medium at 55 C. The cell walls were purified by treatment with sodium dodecyl sulfate (SDS) and incubation with deoxyribonuclease and trypsin. The cell wall peptidoglycan contained glucosamine, muramic acid, α,ε-diaminopimelic acid, and glutamic acid. Low amounts of glycine, galactosamine, serine, aspartic acid, lysine, and valine were also present. The relative mole ratios of glutamic acid–α,ε-diaminopimelic acid–glycine-alanine were 1.00:1.26:0.08:1.55. The cell walls were free from ribonucleic acid and deoxyribonucleic acid and contained less than 0.2% chloroform-methanol extractable lipid and 0.09 μmole of phosphorus per mg of cell wall. Teichoic acid was not detected in the cell walls of this organism. Cell walls isolated without treatment with SDS contained 7.5% chloroform-methanol extractable lipid, 0.24 μmole of phosphorus per mg of cell wall, and relatively high concentrations of all amino acids. These results suggest that the extracted lipid is not a cell wall component per se, but a contaminant from the lipoprotein cell membrane.

Electron microscopy of bacterial cell wall preparations (10) and the lysis of bacteria by enzymes (7) have established that the cell wall is the rigid mechanical structure responsible for the characteristic shape of the bacterial cell. The cell wall also functions by protecting the more labile and biochemically active constituents of the cell from the external environment. Because of the more extreme conditions under which they must function, cell walls of obligate thermophiles may have a chemical composition or structure different from that of cell walls of mesophiles. Forrester and Wicken (5) have determined the chemical composition of cell walls isolated from a facultative strain of Bacillus stearothermophilus (unclassified strain) and from a strain of B. coagulans (NRS T 2007) which were grown at 37 and 55 C. At the higher growth temperature, an increased amount of peptidoglycan and a decreased amount of teichoic acid were found in the walls of both organisms. Salton and Pavlik (11) determined the mole ratios of the principal cell wall amino acids of an obligately thermophlic strain of B. stearothermophilus (NCA 2184). Their studies showed that the gross chemical composition of the cell wall did not differ from that found for mesophilic bacilli.

Marsh and Larson (9) demonstrated that strain NCA 2184 failed to hydrolyze either starch or gelatin, whereas all the strains of B. stearothermophilus studied by Gordon and Smith (6) hydrolyzed starch and 94% of the strains hydrolyzed gelatin. Welker and Campbell (unpublished data) have found that obligately thermophilic strains of B. stearothermophilus can be differentiated into two groups on the basis of morphology and biochemical characteristics. These findings prompted us to determine the chemical composition of cell walls of a strain of B. stearothermophilus which differs from strain NCA 2184 in vegetative-cell length, ability to hydrolyze starch, and the production of relatively high amounts of lactic acid.

In this paper we describe the isolation and chemical composition of cell walls of an obligately thermophilic strain of B. stearothermophilus (NCA 1503-4R).

MATERIALS AND METHODS

The organism used in this investigation was an obligately thermophilic strain of B. stearothermophilus (NCA 1503-4R). This strain, employed previously by Welker and Campbell (15, 16), was selected by Manning and Campbell (8) for its high α-amylase-produc-

1 Present address: Student Residence Hall, 818 South Wolcott, Box 329, Chicago, Ill.
ing capabilities by repeated passage of *B. steatorosothermophilus* NCA 1303 through nutrient starch broth followed by plating on nutrient starch-agar plates. Subsequently, two types of colonies, "smooth" (S) and "rough" (R), were observed when this organism was grown on Trypticase (BBL)-agar plates at 55 C (15). Cells giving rise to rough colonies were used in this investigation. According to the species characteristics of Smith, Gordon, and Clark (13), no taxonomic biochemical differences were observed among *B. steatorosothermophilus* strains 1503-4R, ATCC 7954 (NCA 1503), and neotype strain ATCC 12980. Stock cultures of this organism were maintained on 2% Trypticase-2% agar slants at ambient room temperature.

**Medium and growth conditions.** The Trypticase-yeast extract-fructose (TYF) medium of Welker and Campbell (16) was used and contained the following: Trypticase, 20 g; yeast extract, 5 g; fructose, 5 g; FeCl₃·6H₂O, 7 mg; MnCl₂·4H₂O, 1 mg; MgSO₄·7H₂O, 15 mg; and deionized water, 1 liter. After sterilization by autoclaving at 121 C for 15 to 20 min, the medium was adjusted to pH 7.3 with 10% KOH. Fermentor vessels containing 12 liters of TYF medium were agitated at 1,000 rpm and aerated at 100 L/min for 1 hr at 121 C.

A Fernbach flask containing 1 liter of TYF medium was inoculated with 12- to 15-hr cells from a Trypticase-agar plate and shaken on a New Brunswick model G-25 gyrotry incubator shaker for 2 hr at 55 C. The cell inoculum was aseptically poured into a 14-liter fermentor vessel containing 12 liters of TYF medium, and the culture was agitated (300 rev/min) and aerated (5,000 cc of filter-sterilized air per min) at 55 C in a New Brunswick fermentor (model FS-314). Growth was measured in a Bausch & Lomb Spectronic-20 colorimeter at 525 mμ.

**Isolation of cell walls.** Mid-log phase cells (optical density at 525 mμ, 0.45 to 0.55) were harvested by centrifugation in a Sorvall RC2-B refrigerated centrifuge equipped with a KSB-R continuous flow system. The cells were washed three times with distilled water and frozen at -30 C until needed.

Portions (8 to 10 g) of cells were thawed slowly and transferred to a 40-ml Duran flask containing an amount of acid-washed glass beads (0.2 mm, Minneapolis Glass & Manufacturing Co., St. Paul, Minn.) that equaled three times the wet weight of the cells. Cold distilled water was added to produce a 50% suspension of bacteria (w/v), and the mixture was shaken for three 1-min intervals at 2,000 rev/min in a Braun model MSK mechanical homogenizer (Braun Co., Melsunger, Germany). The suspension was cooled with intermittent jets of liquid CO₂. The resulting slurry was diluted with 100 ml of cold distilled water, and the glass beads and intact bacteria were removed by centrifugation at 1,000 × g for 10 min. Disruption of the remaining bacteria in the residue was achieved by a second treatment in the Braun cell homogenizer. After centrifugation at 1,000 × g for 10 min, the cell wall suspensions were combined, diluted to 300 ml with distilled water, and treated with 10 μg of deoxyribonuclease per ml (Mann Research Laboratories, New York, N.Y.) for 30 min at 37 C. The cell wall suspension was again centrifuged at 1,000 × g for 10 min. The cell walls were sedimented by centrifugation at 10,000 × g for 15 min, suspended in 60 ml of 1% sodium dodecyl sulfate (SDS), transferred to a 250-ml Erlenmeyer flask, and shaken on a rotary shaker for 1 hr at 37 C. After the cell walls were sedimented by centrifugation at 27,000 × g for 10 min, the isolation procedure was interrupted and the cell wall pellet was stored overnight at 5 C. The cell walls were suspended in 40 ml of distilled water, centrifuged at 1,000 × g for 10 min, and then sedimented by centrifugation at 27,000 × g for 10 min. The walls were suspended once more in 40 ml of distilled water and sedimented by centrifugation at 27,000 × g for 10 min. The cell walls were suspended in 60 ml of 0.01 M sodium phosphate buffer (pH 7.0), treated with 100 μg of trypsin per ml (Mann Research Laboratories) for 2 hr at 37 C, and centrifuged at 1,000 × g for 10 min. The cell walls were sedimented by centrifugation at 27,000 × g for 10 min and suspended in 40 ml of distilled water. After this washing procedure was repeated three more times, the cell walls were suspended in 15 to 20 ml of distilled water, lyophilized, and stored over P₂O₅ in a vacuum desiccator.

**Amino acid and amino sugar analysis of cell walls.** Cell walls (10 to 13 mg) were placed in acid-washed Pyrex ampoules and suspended in 2 ml of 6 N HCl. The ampoules were flushed with nitrogen, evacuated, and sealed, followed by heating at 105 C for 8, 16, and 24 hr. The contents of each ampoule were filtered through a sintered-glass filter (fine porosity), transferred to beakers with distilled water, and evaporated to dryness over P₂O₅ in a vacuum desiccator. The hydrolysates were taken up in 10 ml of 0.2 N sodium citrate buffer (pH 2.2, sample dilute), and 2-ml samples were subjected to chromatographic analysis on a Beckman/Spinco model 120B amino acid analyzer. On 150-cm columns, the change from pH 3.28 to pH 4.25 buffer was made before the elution of valine.

Under these conditions, muramic acid elutes between serine and glutamic acid, diaminopimelic acid between valine and methionine, and glucosamine and galactosamine after phenylalanine. However, glucosamine and galactosamine were quantitated from the values obtained from the 15-cm column. All analyses were performed in duplicate.

**Lipid extraction.** Lipids were extracted from cell walls (10 to 20 mg) by use of a modification of the procedure described by Folch, Lees, and Sloane-Stanley (4). The extraction was performed with 10 to 15 ml of chloroform-methanol (2:1, v/v) in a Tenbrock tissue grinder at ambient room temperature. The fine wall precipitate was removed by filtration through Whatman no. 1 filter paper washed with chloroform-methanol. The extract was shaken with 0.2 volume of distilled water in a 100-ml separatory funnel and held at 5 C for 16 to 18 hr to allow separation of the phases. The upper aqueous phase was carefully removed with a pipette, and the lower solvent layer was overlayed with 0.2 volume of 0.05 M CaCl₂. After standing for 30 min, the CaCl₂ layer was discarded and the washing procedure was repeated once more with CaCl₂ and once with distilled water. The washed extract was evaporated in a stream of nitrogen, dissolved in 10
to 20 ml of chloroform-methanol, and quantitatively transferred to a tared weighing bottle. The solvent was evaporated as before, and the residue was dried over P₂O₅ in a vacuum desiccator. The weight of the extracted lipid was determined gravimetrically.

Chemical determinations. The phosphorus content of cell walls was determined by use of the procedure described by Chen, Toribara, and Warner (2). Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) were determined by the procedures described by Burton (1) and Dische (3), respectively.

Electron microscopy. A droplet of an aqueous suspension of cell walls was placed on a Formvar-coated copper grid, air-dried, and shadowed with nichrome. The grid was examined in a Hitachi HS-7 electron microscope at an initial magnification of 7,500 X.

Chemicals. Glucosamine and galactosamine were obtained from Mann Research Laboratories, and α,ε-diaminopimelic acid was obtained from Sigma Chemical Co., St. Louis, Mo. Muramic acid was obtained from H. Plaut of Cyclo Chemical Corp., Los Angeles, Calif. The amino acids used for the calibration of the Beckman/Spinco amino acid analyzer were obtained from the Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.

RESULTS AND DISCUSSION

Vegetative cells of B. stearothermophilus NCA 1503-4R are 1.0 to 1.3 μm wide and 2.1 to 3.4 μm in length. In contrast, cells of the obligately thermophilic strain (NCA 2184) used by Sulton and Pavlik (11) are 0.6 to 0.9 μm wide and 3.0 to 4.5 μm in length (Welker, unpublished data).

An electron micrograph of cell walls of B. stearothermophilus NCA 1503-4R is shown in Fig. 1. The cell walls appear free from electron-dense cytoplasmic material, retain the characteristic rod shape of the intact cell, and have a relatively homogenous appearance without any obvious fine structure.

The major components of these cell walls were alanine, α,ε-diaminopimelic acid, glutamic acid, muramic acid, and glucosamine (Table 1). A comparison of the analytical values from 8-, 16-, and 24-hr hydrolysates indicated that a complete liberation of alanine, glutamic acid, and diaminopimelic acid occurred within 8 hr. The decomposition of glucosamine and muramic acid between 8 and 16 hr of hydrolysis was 21.3 and 28.1%, respectively. The concentration of glucosamine and muramic acid was calculated by extrapolation to zero-time (Fig. 2). Only the 8- and 16-hr points were used in the calculation of the amino sugars. The cell walls contained relatively low amounts of lysine, aspartic acid, serine, glycine, valine, and galactosamine and no arginine, proline, histidine, or half-cystine (Table 2). The tryptophan content of the cell walls was not determined.

The decomposition of standard glucosamine (1.04 μmoles) and muramic acid (0.53 μmole), hydrolyzed for 8 hr in 6 N HCl, was 35.2 and 50.7%, respectively. In contrast, after 8 hr of hydrolysis, the decomposition of cell wall glucosamine and muramic acid was 17.6 and 22.0%, respectively. The cell wall hydrolysates contained eight unidentified minor components, five of which corresponded to decomposition products of standard glucosamine and muramic acid. The release of ammonia was linear with hydrolysis time (Table 2), and extrapolation to zero-time gave a value of 0.31 μmole per mg of cell wall. These results suggest that the rate of destruction of glucosamine and muramic acid, during the

Fig. 1. Electron micrograph of cell walls of Bacillus stearothermophilus NCA 1503-4R. Shadow-cast with nichrome. × 21,900.
first 8 hr of hydrolysis, is probably greater than indicated in Fig. 2. If this interpretation is correct, the amino sugar content of the cell walls is somewhat higher than we have indicated in Table 1. Some of the ammonia in the hydrolysates, however, may result from the breakdown of unidentified compounds.

The mole ratios of the cell wall amino acids of *B. stearothermophilus* NCA 1503-4R are compared in Table 3 with those reported by Salton and Pavlik (11) and Forrester and Wicken (5) for the cell walls of *B. stearothermophilus* strain NCA 2184 and an unclassified strain, respectively. The high alanine content in the cell walls of the unclassified strain was accounted for by the presence of D-alanine in ester linkage with glycerol of the teichoic acid (17). Salton and Pavlik (11) did not determine the teichoic acid content in the cell walls of strain NCA 2184. The procedures for the extraction of teichoic acid described by Strominger and Ghuysen (14) and Shaw and Baddiley (12) were used in our study, but no teichoic acids were detected in the cell walls of strain NCA 1503-4R. Variations in the relative proportions of the cell wall amino acids observed in the three strains of *B. stearothermophilus* may be due to strain differences or to the different growth conditions and cell wall isolation procedures employed.

No detectable RNA or DNA was found in these cell wall preparations, and the chloroform-methanol extractable lipid amounted to less than 0.2% of the dry weight. The phosphorus content averaged 0.09 μmole per mg of cell wall. Forrester and Wicken (5) reported that the total extractable lipid in cell walls of *B. stearothermophilus* (unclassified strain) grown at 55 C amounted to 8% of the dry weight. They concluded that the lipid was a true cell wall compo-
nent. Cell walls of strain NCA 1503-4R, prepared without the SDS treatment, contained 7.5% chloroform-methanol extractable lipid and 0.24 μmole of phosphorus per mg of cell wall. In addition to the common cell wall amino acids, these cell walls also contained relatively high concentrations of many other amino acids (Table 4). Since Bodman and Welker (Bacteriol. Proc., p. 107, 1966) have reported that SDS completely disaggregates cytoplasmic membranes of this strain, we believe that the extracted lipid does not represent a true cell wall component but is a contaminant from the lipoprotein cell membrane.

The gross chemical composition of the cell wall peptidoglycan does not differ greatly from that reported for mesophilic bacilli (11). In the absence of an adequate assay system, it is impossible to determine whether the walls per se of thermophiles are relatively more heat-stable than the corresponding structures of mesophiles. For the present, therefore, this problem is best attacked through a detailed study of the cell wall structure, to determine whether any unique wall structures or components are present.

Because of our interest in the biochemical and physical nature of thermophily and in view of the strain differences among obligate thermophiles, it seems desirable that a complete study of the

### Table 4. Amino acid composition of cell walls not treated with sodium dodecyl sulfate

<table>
<thead>
<tr>
<th>Component</th>
<th>Amt (μmoles) present per mg of cell wall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>0.34</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.07</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.12</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.47</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.41</td>
</tr>
<tr>
<td>Serine</td>
<td>0.30</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.56</td>
</tr>
<tr>
<td>Proline</td>
<td>0.11</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.32</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.85</td>
</tr>
<tr>
<td>Valine</td>
<td>0.41</td>
</tr>
<tr>
<td>Diaminopimelic acid</td>
<td>0.25</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.03</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.18</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.33</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.14</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.13</td>
</tr>
</tbody>
</table>

* Cell walls of *Bacillus stearothermophilus* NCA 1503-4R were prepared as described in the text, except that the SDS treatment was eliminated. Cell walls (10 to 13 mg) were hydrolyzed for 24 hr in 6 N HCl at 105 C and analyzed on a Beckman/Spinco amino acid analyzer.

**B. stearothermophilus** group should be conducted with the objective of establishing further strain differentiation. In this connection, cell wall composition and structure may prove to be a useful taxonomic criterion.

**Acknowledgments**

We are grateful to Rita Bentley for the preparation of the electron micrographs.

This investigation was supported by Public Health Service research grant AI 06382 from the National Institute of Allergy and Infectious Diseases, and by the Undergraduate Research Participation Program of the National Science Foundation.

**Literature Cited**


