Chemical Composition of Cell Walls of Alkalophilic Strains of Bacillus

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Cell walls of 10 alkalophilic Bacillus strains were prepared by inactivation of autolytic enzymes with SDS, disruption with a sonic oscillator, trypsin digestion and washing with SDS. The walls were composed of peptidoglycan and acidic compounds. The peptidoglycan was similar in composition to that of B. subtilis. After hydrolysis, the acidic compounds detected were glucuronic acid, glutamic acid and aspartic acid. The strains tested could be divided into three groups as follows. Group 1, the glucuronic acid and hexosamine contents were high; no growth was observed at pH 7-0; Na⁺ was essential for their growth. Group 2, large amounts of glutamic acid, aspartic acid and glucuronic acid were detected. Growth at higher pH values increased the content of acidic compounds; growth was observed at pH 7-0; Na⁺ was essential for their growth. Group 3, no remarkable difference was detected in the chemical components in comparison with B. subtilis; growth was observed in the presence of Na⁺ or K⁺ at pH 7-0 and 10-2.

INTRODUCTION

Previously, we have isolated many alkalophilic Bacillus strains which produce extracellular enzymes such as alkaline proteases and alkaline amylases (Horikoshi, 1971a, b). Several Bacillus strains were studied both physiologically and biochemically. It was found that some alkalophilic Bacillus strains had an absolute requirement for Na⁺ for their growth. Such a requirement was supported by results with membrane vesicles (Kitada & Horikoshi, 1982). The intracellular pH of these alkalophilic bacteria was neutral (Horikoshi & Akiba, 1982), therefore, the pH difference must be due to surface components. However, no analytical data have been reported for the walls of alkalophilic Bacillus strains. This paper presents data for the compositions of walls prepared from alkalophilic Bacillus strains and the effect of pH on their chemical compositions.

METHODS

Organisms. Strains of alkalophilic Bacillus used in this paper were as follows: Bacillus no. A-40-2 and no. A-59, alkaline amylase producers; Bacillus no. 2B-2, C-3, C-11 and C-125, rayon waste utilizing bacteria; Bacillus no. Y-25, a host of Al-K-1 phage; Bacillus no. C-59-2, xylanase producer; Bacillus no. M-29, deoxyribonuclease producer; Bacillus no. 57-1, alkaline protease producer (Horikoshi & Akiba, 1982). All were isolated from soils and maintained in our laboratory. Bacillus subtilis GSY 1026 from T. Shibata was analysed as reference strain.

Cultivation and harvest of cells. The culture medium used contained, per litre of deionized water: K₂HPO₄, 13-7 g; KH₂PO₄, 5-9 g; citric acid, 0-34 g; MgSO₄, 7H₂O, 0-05 g; glucose, 5 g; peptone, 5 g; yeast extract, 0-5 g; Na₂CO₃, 11-7 g. Glucose and Na₂CO₃ were sterilized separately and then added to the medium. The pH of this medium was 10. The same medium containing 10-6 g NaCl instead of Na₂CO₃ was used to cultivate the organisms which could grow at neutral pH. This medium was adjusted to pH 7-0. Each strain was grown aerobically overnight at 30 °C. This culture was diluted with fresh medium of the same pH and again grown aerobically at 30 °C. At the late-exponential phase of growth (OD₅₅₀ 0-8–1-2), cells were harvested by centrifugation at 7000 g for 10 min at 5 °C, washed once with 0-2 M-NaH₂PO₄/NaOH buffer (pH 7-0) and resuspended in 2% (w/v) SDS.

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containing 0.1 M-NaCl. The suspension was incubated at 60 °C for 2 h with gentle shaking. The cells were collected by centrifugation at 16000 g for 15 min at 20 °C and stored at −20 °C.

Requirement for Na+ and K+ was determined in the same medium containing 2 g (NH₄)₂SO₄ instead of peptone. The medium containing only Na+ contained equal molar amounts of Na₂HPO₄, 12H₂O and NaH₂PO₄, 2H₂O instead of K₂HPO₄ and KH₂PO₄. On the other hand, Na₂CO₃ was replaced by K₂CO₃ in medium containing only K+.

Preparation of cell walls. The frozen cells were suspended in 10 times their volume of 0.1 M-NaCl and 0.1% (w/v) NaCl₃ and disrupted at 15 °C for 10 min in a 10 kHz sonic oscillator. Unbroken cells were removed by centrifugation at 10000 g for 15 min at 20 °C. An equal volume of 4% (w/v) SDS solution was added to the supernatant. The mixtures were incubated for 30 min in a boiling bath and then at 37 °C overnight. Cell walls were sedimented at 17000 g for 20 min at 20 °C and then washed with 0.1 M-NaCl containing 0.1% NaN₃. Washed cell walls were resuspended in 0.05 M-Tris/HCl buffer (pH 7.9) containing 0.1 M-CaCl₂, and digested with trypsin (0.5 mg ml⁻¹) at 37 °C overnight. The walls were subsequently washed several times with 0.01 M-EDTA/NaOH (pH 7.3), 1 M-NaCl plus 1% SDS, 0.1 M-NaCl plus 1% SDS, 0.1 M-NaCl plus 0.1% NaN₃, deionized water, ethanol, acetone, and finally diethyl ether, before being dried in vacuo.

Dry weight. The dry weight of the cell walls was measured by heating at 105 °C in vacuo until a constant weight was obtained.

Analysis. (i) Amino acids and hexosamines. The walls were hydrolysed in 4 M-HCl in sealed tubes at 105 °C for 15 h. HCl was removed in vacuo over NaOH. The hydrolysates were dissolved in water and analysed with an automatic amino acid analyser. L-Glutamic acid content was determined by using a glutamic acid assay kit from Boehringer, which contained l-glutamic acid dehydrogenase.

(ii) Neutral sugars. Neutral sugars were estimated by the anthrone reagent method without acid hydrolysis (Spiro, 1966). Glucose in the walls hydrolysed in 2 M-HCl at 100 °C for 1 h was assayed with hexokinase and glucose-6-phosphate dehydrogenase (Bergmeyer et al., 1974). Galactose in the hydrolysates (1 M-HCl, 100 °C, 2 h) was assayed by galactose dehydrogenase (Kurz & Wallenfels, 1974) before and after treatment with alkali phosphatase.

(iii) Uronic acid. Total uronic acids were directly determined by the carbazole reagent method (Davidson, 1966) and expressed as glucuronic acid. Glucuronate was estimated with the thioglycolic acid/mannose/sulphuric acid reagent (Dische, 1947).

(iv) Phosphorus. The walls were ashed with magnesium nitrate and then hydrolysed in 0.5 M-HCl to convert organic phosphates into inorganic phosphate. Total phosphorus was determined by the molybdate method (Ames, 1966).

(v) Glycerol. The cell walls were hydrolysed in 1 M-HCl at 100 °C for 3 h. After removal of HCl in vacuo, both free glycerol and L-α-glycerophosphate in the same sample were serially assayed with L-α-glycerophosphat dehydrogenase and glycerokinase (Wieland, 1974). The content of L-α-glycerophosphate was multiplied by 2.3 to obtain the total glycerol content (Burger & Glaser, 1966). The total glycerol content was obtained by subtracting the content of the total glycerophosphate and that of free glycerol.

TCA-soluble fraction. Walls (30 mg) were suspended in 3 ml water, and 1 ml 20% TCA was added to this suspension. The mixture was incubated at 37 °C overnight and centrifuged at 7000 g for 30 min. The supernatant fluid was collected. This extraction was repeated at 37 °C followed by two extractions at 60 °C. All the supernatants were pooled, treated with diethyl ether to remove TCA and then dried in vacuo.

Paper or thin-layer chromatography. Paper chromatography was done on Whatman no. 1 paper at room temperature using n-butanol/pyridine/water (6:4:3, by vol.) as the solvent system. Avicel cellulose thin-layer chromatograms were run in the following solvents: (A) n-butanol/acetic acid/water (4:1:1, by vol.); (B) phenol/water (3:1, by vol.); (C) ethyl acetate/pyridine/acetic acid/water (5:5:1:3, by vol.); (D) phenol/1% NH₄OH (2:1, by vol.). Spots were revealed with the following reagents: silver nitrate/NaOH; diphenylamine/amine; ninhydrin.

RESULTS AND DISCUSSION

The methods used gave comparatively reproducible preparations of cell walls from alkalophilic Bacillus strains even though the walls were liable to autolysis during preparation. Table 1 shows the amounts of components obtained by chemical or enzymic analyses of the total cell walls. Each component was determined without correction for destruction during hydrolysis. Considerable amounts of glucosamine, muramic acid, glutamic acid, alanine and diaminopimelic acid were detected in all samples. Thus the peptidoglycan of the alkalophilic Bacillus strains studied resembles that of neutrophilic Bacillus strains (Tipper & Wright, 1979). However, there are several interesting differences between B. subtilis and the alkalophilic Bacillus strains tested.
Table 1. Composition of cell walls of alkalophilic Bacillus strains

The assay method for each compound is detailed in the text. Determination is uncorrected for destruction during acid hydrolysis. Each blank space represents not detected. The following abbreviations are used: Asp, aspartic acid; Glu, glutamic acid; Gly, glycine; Ala, alanine; DAP, diaminopimelic acid; Mur, muramic acid; GlcN, glucosamine; GalN, galactosamine; P, phosphorus; Glyc, glycerol; Glc, glucose; Gal, galactose.

<table>
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<th>Group</th>
<th>Strain</th>
<th>Culture pH</th>
<th>Asp [pmol (mg cell wall)^{-1}]</th>
<th>Glu</th>
<th>l-Glu</th>
<th>Gly</th>
<th>Ala</th>
<th>DAP</th>
<th>Mur</th>
<th>GlcN</th>
<th>GalN</th>
<th>P</th>
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<td>0.36</td>
<td>0.67</td>
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<td>0.08</td>
<td>0.05</td>
<td>0.90</td>
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On the basis of the composition of the cell walls, the strains can be divided into three groups. In group 1 (strains A-40-2 and 2B-2), the contents of glucosamine, galactosamine and uronic acids were high. Identification of the hexosamines was confirmed by cellulose thin-layer chromatography in solvent (C). The uronic acids were thought to be mostly glucuronic acid by their reaction with thioglycolic acid/mannose/sulphuric acid. The amount of phosphorus was less than that in \textit{B. subtilis} and no polyl was detected. Small amounts of neutral sugars were detected by the anthrone reagent. A precise determination of neutral sugars was difficult because of the weak interaction of large amounts of uronic acids and the anthrone reagent. After hydrolysis in 2 M-HCl at 100 °C for 2 h, each hydrolysate was examined by paper chromatography and cellulose thin-layer chromatography in solvents (C) and (D). The hydrolysates were also treated with alkaline phosphatase before chromatography. Trace amounts of glucose were found in strain 2B-2. The molar ratios of diaminopimelic acid, glutamic acid, alanine, muramic acid and glucosamine were approximately 1 : 1 : 2 : 1 : 2. It was concluded that these walls were composed mainly of peptidoglycan and an acidic polymer, namely teichuronic acid. The peptidoglycan was composed of diaminopimelic acid, glutamic acid, alanine, muramic acid and glucosamine (1 : 1 : 2 : 1 : 1). The teichuronic acid contained glucosamine, galactosamine and glucuronic acid (1 : 1 : 1). Teichuronic acids which are composed of uronic acid and hexosamine are widely present in bacteria of the genus \textit{Bacillus} (Tipper & Wright, 1979), but their physiological function is unclear. Strains A-40-2 and 2B-2 did not grow at neutral pH. It is interesting to speculate whether the presence of teichuronic acid in the walls is associated with this response.

In group 2 (strains C-11, C-125, Y-25, A-59 and C-3), the contents of glutamic acid and uronic acids were high. The major uronic acid was again glucuronic acid. Aspartic acid was found in the walls of strains A-59 and C-3, and glycine was present in strain C-3; l-glutamic acid was detected in the walls of all five strains. Aspartic acid, glycine and large amounts of glutamic acid, especially the L-isomer, are not usually present in the cell walls of neutrophilic \textit{Bacillus} strains (Tipper & Wright, 1979). To characterize these amino acids further, the TCA-soluble and insoluble fractions were analysed. Both fractions were hydrolysed in 6 M-HCl at 105 °C for 15 h, and then analysed enzymically and by cellulose thin-layer chromatography in solvents (A), (B) and (C). Aspartic acid, glycine and L-glutamic acid were found only in the TCA-soluble fraction, indicating that these amino acids were present in cell wall polymers other than peptidoglycan. All these strains except C-11 grew at a neutral pH. The contents of glutamic acid, aspartic acid, glycine and uronic acids were higher in walls prepared from cells grown in an alkaline medium than in walls from cells grown in a neutral medium. In contrast, the contents of other components, e.g. diaminopimelic acid, were lower in an alkaline medium. The acidic components of the walls might enable the bacteria of this group to grow at an alkaline pH.

In group 3 (strains C-59-2, M-29 and 57-1), phosphorus and neutral sugars were commonly present in large amounts. The molar ratios of diaminopimelic acid, glutamic acid, alanine and muramic acid were approximately 1 : 1 : 2 : 1. Some variations were found for the other compounds. Aspartic acid was found in strain C-59-2. Much glucosamine was present in the walls of strains C-59-2 and M-29. Acid hydrolysates (2 M-HCl, 100 °C, 1 h) of the cell walls were dried \textit{in vacuo}, dissolved in 0.1 M-Na$_2$CO$_3$ (pH 9-5) and treated with alkaline phosphatase at 37 °C for 3 h. The reaction mixture was deionized by passing it through a small column of Amberlite IRA400 (CO$_3^-$) and IR120 (H$^+$), concentrated \textit{in vacuo}, and then developed by paper chromatography. Large amounts of glycerol were found in the cell walls of strains C-59-2 and 57-1, and less in strain M-29. No other polyl was detected. Neutral sugars interfered slightly with the determination of uronic acids: colour yields of glucose were 10% of the glucuronic acid under the conditions used. Therefore, it was hard to give the precise contents of uronic acids in the samples of this group. Glucose and galactose were found in the hydrolysates mentioned above. No other neutral sugar was detected. From these results we presume that the walls of this group contain a teichoic acid. Several variations of teichoic acids are known in \textit{Bacillus}, although the acids are all composed of phosphate, polyl, hexose and hexosamine (Tipper & Wright, 1979). Strains M-29 and 57-1 grew at both pH 7 and pH 10; no difference in the composition of cell walls was found between organisms grown at the two pH values.
Cation requirement for growth was tested in culture medium containing only Na⁺ or K⁺. Growth was determined after 48 h incubation at 37 °C. All the strains tested, except for C-59-2, M-29 and 57-1, had an absolute requirement for Na⁺ for their growth. Organisms of group 3 did not require Na⁺ when K⁺ was present in the medium. Bacteria of this group seem to occupy a unique position among the alkalophilic Bacillus strains.

In all the strains tested, the peptidoglycan was similar in composition to that of B. subtilis. The variations in wall composition described above were found in the TCA-soluble fractions and not in the peptidoglycan. We have attempted to classify the alkalophilic Bacillus strains studied by the composition of their cell walls; further studies are required to determine whether these variations can be associated with the growth characteristics of the strains.

We thank Dr. T. Shibata of this Institute for kindly supplying B. subtilis GSY 1026. We are indebted to Mr. M. Chijimatsu of this Institute for excellent analyses of amino acids and amino sugars.

REFERENCES


