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Purification and Quantitative Chemical Analysis of Cell Wall Peptidoglycans of *Leptotrichia buccalis*

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Peptidoglycans of *Leptotrichia buccalis* ATCC 14201 and ATCC 19616 were isolated by extraction with sodium dodecyl sulfate and subsequent digestion of the sodium dodecyl sulfate-insoluble residue with proteases and α-amylase. Cell wall fractions obtained by sodium dodecyl sulfate extraction and protease digestion were highly contaminated by a glucose polymer. The polyglucose was removed by α-amylase treatment, and the peptidoglycans were left behind. Analyses with amino acids and amino sugars of the cell wall fractions and peptidoglycan specimens revealed that D-glutamic acid, D-alanine, L-alanine, *meso*-2,6-diaminopimelic acid (A_2pm), muramic acid, and glucosamine were the principal components. The dinitrophenylation method revealed that about half of the A_2pm residue had a free amino group, and analysis by hydrazinolysis showed that a small part of alanine and A_2pm was located at the C terminal. The above results indicate that one of the amino groups of the A_2pm residue at one strand of the stem peptide subunit cross-linked to the carboxyl group of alanine of the neighboring strand. It was thus revealed that the peptidoglycans of *L. buccalis* belong to the A1γ type of the classification by Schleifer and Kandler (Bacteriol. Rev. **36**:407–477).

The genus *Leptotrichia*, which had not been recognized as such for a long time, has secured a position as a valid genus in the eighth edition of Bergey's *Manual of Determinative Bacteriology* (10). The genus *Leptotrichia* is included in the family *Bacteroidaceae* and so far consists of a single species, *Leptotrichia buccalis*.

The chemical and molecular structures of the *L. buccalis* cell wall seem to be worthy of careful examination, because it is known that younger cells that have been in culture less than 6 h show gram-positiveness (7), whereas the cell surface structures revealed by electron microscopy (9) and the presence of endotoxic lipopolysaccharide indicate that *L. buccalis* is a typical gram-negative organism (6).

The cell wall composition of this organism was analyzed qualitatively (semi-quantitatively) by the paper chromatographic method of Hofstad (8) for strains L11 and L66 and by that of Baboolal (2) for 23 strains from various sources. Both investigators found alanine, glutamic acid, 2,6-diaminopimelic acid (A_2pm), and glycine as the major components. Baboolal claimed that aspartic acid was an additional major amino acid. However, no quantitative evidence has so far been available. We therefore attempted to isolate the cell wall peptidoglycans of this organism in as pure a state as possible and to analyze them quantitatively with regard to constituent amino sugars and amino acids.

MATERIALS AND METHODS

Organisms. *L. buccalis* ATCC 14201 and ATCC 19616 were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) containing 0.2% yeast extract (Difco) and 0.5% glucose under an anaerobic gaseous environment consisting of nitrogen, hydrogen, and carbon dioxide (80:10:10, vol/vol). After 3 to 5 days of culture at 37°C, growing cells were harvested by centrifugation at 12,000 × *g* for 30 min, washed three times with saline and once with distilled water, and then lyophilized.

Isolation of cell wall peptidoglycans. A peptidoglycan was prepared by the method of Yanai et al. (27) with minor modifications. Lyophilized cells were boiled in 4% sodium dodecyl sulfate (SDS) solution for 1 h, and the boiled cell suspension was centrifuged at 110,000 × *g* for 90 min. The pellet was washed four times with distilled water and submitted to successive treatments (at 37°C for 18 h each) with 20 mg of pronase per g as a substrate (pronase P [750 U/mg]; Kaken Kagaku Ltd., Tokyo, Japan) and 20 mg of crystalline trypsin per g as a substrate (Trypsilin, [2,500 U/mg]; Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) in 10 mM Tris-hydrochloric acid buffer (pH 7.4 and 8.2, respective-

TABLE 1. Chemical composition of cell wall fractions and peptidoglycans of *L. buccalis*

Strain	Amino acids and amino sugars[a]	Hexose[a,b]	(Glucose)[a,c]	Pentose[a]	Methylpentose[a,d]	Phosphorus[a,e]	KDO[a,f]
L. buccalis ATCC 14201							
Cell wall fraction	83	970	(913)	—[g]	16	—	0.6
Peptidoglycan	682	38		—	12	6	—
L. buccalis ATCC 19616							
Cell wall fraction	207	774	(574)	—	15	10	0.6
Peptidoglycan	514	55		—	9	17	0.5

[a] Micrograms per milligram of test specimen (dry weight).
[b] As glucose.
[c] Determined by gas chromatography.
[d] As rhamnose.
[e] As KH_2PO_4.
[f] 2-Keto-3-deoxyoctonate.
[g] —, None.

ly). A cell wall fraction was collected as a pellet by the centrifugation of the reaction mixture at 110,000 × *g* for 90 min, washed three times with distilled water, and lyophilized.

The cell wall fraction was further purified by incubation with α-amylase (80,000 U/mg; Kishida Chemicals Co., Ltd., Osaka, Japan) in 20 mM phosphate buffer (pH 6.0) added with 7.5 mM sodium chloride at 37°C for 14 h to remove a glucose polymer (described below). The ratio of the enzyme to the substrate was adjusted to 1:50. By the centrifugation of the incubation mixture at 110,000 × *g* for 90 min, a peptidoglycan was recovered as a pellet, washed with distilled water, and lyophilized. The yields are described below.

Chemical analyses. Total hexose, methylpentose, and pentose were measured by the anthrone method (22), the cysteine-sulfate method (4), and the orcin-hydrochloric acid method (1), respectively. Phosphorus was determined by the method of Lowry et al. (18), and 2-keto-3-deoxyoctonate was estimated by the description of Weissbach and Hurwitz (26). For the colorimetric analyses, a spectrophotometer (type 124; Hitachi Seisakusho, Tokyo, Japan) was used.

Qualitative and quantitative analyses of amino acids and amino sugars were performed with an amino acid analyzer (KLA-5; Hitachi Seisakusho). Specimens were hydrolyzed in a sealed tube with 6 M hydrochloric acid by being heated in a boiling water bath for 14 h. Hydrolysates were dried in a vacuum-desiccator containing solid sodium hydroxide to remove hydrochloric acid as completely as possible and then redissolved in distilled water before analysis. No corrections were made for the destruction of amino sugars and amino acids during the acid hydrolysis.

The optical configuration of alanine and glutamic acid residues was determined by the use of D-amino acid oxidase (15 U/mg of protein; Sigma Chemical Co., St. Louis, Mo.) and L-glutamate decarboxylase (0.15 U/mg; Wako Pure Chemical Industries Ltd., Osaka, Japan) by the method described by Kotani et al. (16): one portion of hydrolyzed peptidoglycans was digested with D-amino acid oxidase, and the other was digested with L-glutamate decarboxylase, and the residual alanine and glutamic acid were estimated as L-alanine and D-glutamic acid, respectively, by the amino acid analyzer.

The configuration of A_2pm was determined by paper chromatography with Whatman filter paper no. 3 as follows. Methanol–pyridine–10 M hydrochloric acid–water (80:10:2.5:17.5, vol/vol) was used as a developing solvent, by the method of Rhuland et al. (20), to differentiate L,L-A_2pm holding a higher R_f value from D,D- or *meso*-type. The thin-layer chromatography of dinitrophenylated A_2pm, on the other hand, was performed to distinguish D,D-A_2pm from *meso*-A_2pm by the method of Bricas et al. (3): hydrolysates of peptidoglycan specimens were dinitrophenylated, and the formed di-2,4-dinitrophenol (DNP)-A_2pm was extracted into ether, dried, dissolved in 0.04 M ammonia, and analyzed with a silica gel (Kieselgel 60; Merck, Darmstadt, West Germany) plate and a solvent composed of benzyl alcohol, chloroform, methanol, water and 15 M ammonia (30:30:30:6:2, vol/vol).

The terminal amino acids of a peptide portion of test peptidoglycans were quantitatively determined by the method of Ghuysen et al. (5). Test specimens were dinitrophenylated before hydrolysis, and a decreased amount of amino acid by dinitrophenylation was regarded as the amount of amino acid which had a free amino group. The amino acid released by hydrazinolysis was determined by an amino acid analyzer and was taken as C-terminal amino acid.

Gas chromatographic analyses of monosaccharide were performed by the dithioacetal method (12). One milligram of specimen was hydrolyzed in 2 M trifluoroacetic acid at 100°C for 5 h, dried, and suspended in 0.5 ml of distilled water. A 250-μl sample of the suspension was dried in a test tube with 250 μg of an internal standard (inositol), mercaptalated, and trimethylsilylated. After centrifugation of the reaction mixture, 1.0 μl each of the supernatant was applied to a gas chromatograph (type 163; Hitachi Seisakusho) equipped with a glass capillary column coated with SF-96 (SCOT; Gasukuro Kogyo Co., Ltd., Tokyo, Japan; 50 m long by 0.28-mm inner diameter). The temperatures of the injection port and the column were 300 and 225°C, respectively. The flow rate of the carrier gas (nitrogen) was 2 ml/min.

RESULTS

Glucose polymer as a contaminant of *L. buccalis* cell wall fraction. The chemical composition

TABLE 2. Amino acid and amino sugar composition of peptidoglycans of *L. buccalis* ATCC 14201 and ATCC 19616

Strain	Muramic acid (nmol/mg)[a]	Glucosamine (nmol/mg)	D-Glutamic acid (nmol/mg)[b]	L-Alanine[c] (nmol/mg)	D-Alanine[d] (nmol/mg)	A_2pm (nmol/mg)
L. buccalis ATCC 14201	582 (0.70)	628 (0.75)	826 (1.00)	719 (0.87)	806 (0.98)	873 (1.07)
L. buccalis ATCC 19616	327 (0.49)	622 (0.94)	664 (1.00)	544 (0.82)	544 (0.82)	663 (1.00)

[a] Molar ratio is given in parentheses.
[b] None of the glutamic acid residue disappeared by L-glutamate decarboxylase treatment.
[c] Alanine residue resistant to D-amino acid oxidase treatment.
[d] Alanine residue disappeared by D-amino acid oxidase treatment.

of cell wall fractions and peptidoglycans obtained from ATCC 14201 and 19616 is shown in Table 1. Although 3.29 and 1.74 g of cell wall fractions were obtained from 10 g of whole cells of ATCC 14201 and ATCC 19616, respectively, they were characterized by a very high content of hexose, which was identified as glucose by gas chromatographic determination. By α-amylase digestion, the hexose content was markedly reduced from 97 and 77% in the cell wall fractions to 3.8 and 5.5% in the peptidoglycans in ATCC 14201 and ATCC 19616, respectively. This finding strongly suggests that *L. buccalis* contains a large amount of polyglucose susceptible to α-amylase digestion as a contaminant of cell surface structures.

As constituents other than amino sugars and amino acids, a small amount of methylpentose and a trace of 2-keto-3-deoxyoctonate were detected in the cell wall fractions of both strains.

The yields of peptidoglycans obtained by α-amylase treatment of the cell wall fractions of ATCC 14201 and 19616 were about 39 and 65 mg per g of cells, respectively.

Amino acid and amino sugar composition of *L. buccalis* peptidoglycans. Table 2 indicates that both ATCC 14201 and 19616 peptidoglycan specimens contained muramic acid, glucosamine, glutamic acid, alanine, and A_2pm as the principal amino sugars and amino acids. Aspartic acid, glycine, and other amino acids were not detected in appreciable amounts. Although there were some deviations from an integral number, the analytical data obtained with the peptidoglycans and cell wall fractions seem to permit the following statement: with both test strains, molar ratios of glutamic acid, alanine, and A_2pm were roughly 1:1.75:1; about half of the alanine residue was abolished by treatment with D-amino acid oxidase; and no glutamic acid disappeared by L-glutamate decarboxylase treatment. In the paper chromatogram, A_2pm in the hydrolysates of test specimens ran the same distance as authentic *meso*- or D,D-A_2pm and was obviously separated from the L,L-type. The thin-layer chromatography of dinitrophenylated hydrolysates of test peptidoglycans showed that di-DNP-A_2pm in the specimens had the same R_f value (0.42) as an authentic di-DNP-*meso*-A_2pm. It was therefore proved that the A_2pm residue in the peptidoglycans of *L. buccalis* was the *meso*-type. No significant differences in amino acid composition were observed between the peptidoglycans of ATCC 14201 and those of 19616.

Determination of terminal amino acids of peptidoglycans. We determined terminal amino acids to find a clue to the molecular structures of a peptide portion of *L. buccalis* peptidoglycans (Table 3). The amount of glutamic acid and alanine was unchanged by dinitrophenylation, whereas about half of the A_2pm disappeared. Hydrazinolysis, on the other hand, released about 13% of alanine and 5% of A_2pm in ATCC 14201 peptidoglycan and 5% of alanine and 4% of A_2pm in the ATCC 19616 specimen as C-terminal amino acids.

DISCUSSION

L. buccalis, a normal resident in dental plaque and gingival crevice debris, is now classified as a genus of the family *Bacteroidaceae* with the genera *Bacteroides* and *Fusobacterium*. This organism is a fusiform bacillus having either one or both ends pointed like *Fusobacterium* sp., but it is distinguishable from *Fusobacterium* sp. by the formation of lactic acid as the sole fermentation product from glucose.

In this investigation, cell wall peptidoglycans were successfully isolated from *L. buccalis* cells by boiling 4% SDS extraction and enzymatic treatments. The noteworthy finding is that an insoluble residue (a cell wall fraction) obtained by SDS extraction and protease digestion of whole cells was characterized by a very high content of a polysaccharide composed solely of glucose (77 to 97% in terms of hexose and 57 to 91% as glucose). It may be added here that high contents of glucose in *L. buccalis* cell wall fractions have been reported by previous investigators (2, 8). The presence of polyglucose in the cell wall or cell envelope fraction has so far been reported in *Escherichia coli* B by Leutgeb and Weidel (17) and in *Vibrio parahaemolyticus*

TABLE 3. Determination of NH_2- and C-terminal amino acids of peptidoglycans of *L. buccalis*

Strain	Glutamic acid[a]			Alanine			A_2 pm		
	Before treatment	After dinitrophenylation	Released by hydrazinolysis	Before treatment	After dinitrophenylation	Released by hydrazinolysis	Before treatment	After dinitrophenylation	Released by hydrazinolysis
L. buccalis ATCC 14201	1.00	1.00		1.85	1.84	0.24	1.07	0.52	0.05
L. buccalis ATCC 19616	1.00	1.00		1.64	1.49	0.08	1.00	0.48	0.04

[a] All data are expressed as molar ratios to the content of glutamic acid residue in each of the test peptidoglycans.

by Tamura et al. (23). The former authors stated that the glycogen-like polysaccharide neither contributed to the integrity of the cell walls nor had any structural association with the cell wall peptidoglycan. Holme and Palmstierna (11) observed that a glycogen-like polysaccharide of *E. coli* B was accumulated or consumed depending upon cultural conditions and concluded that this polysaccharide was not a structural component of the cell wall. The present finding, that the glucose polymer in the cell wall fraction of *L. buccalis* was easily removed by digestion with α-amylase without significant changes of amino acid and amino sugar composition, also strongly suggests that the polyglucose is not a compound inherent to the cell walls of this organism.

With the chemical structures of the peptidoglycans of *L. buccalis* ATCC 14201 and 19616, we could not detect an appreciable amount of aspartic acid or glycine, in contrast to analytical results reported by Hofstad (8) and Baboolal (2). The discrepancies might be due to differences in the test strains from which the peptidoglycan preparations were isolated. The analytical data reported here, particularly the finding that when the peptidoglycan was treated with 2,4-dinitrofluorobenzene, about half of the A_2pm residues were not dinitrophenylated, indicate that roughly 50% of the A_2pm residues are involved in the cross-link and that the peptidoglycans of *L. buccalis* belong to a simple, direct cross-linkage type (A1γ type in the classification of Schleifer and Kandler [21]), which has been found in a wide range of gram-negative bacteria. Analytical results on C-terminal amino acids in the peptidoglycan specimen of ATCC 14201 showed that a part of alanine residues were located on the C-terminal position. A very low recovery of alanine as a C-terminal amino acid in the peptidoglycan of ATCC 19616 suggests that A_2pm might be a true C terminal. However, if one or two carboxyl group(s) of A_2pm is amidated, just as in the case of the cell walls of *Lactobacillus plantarum* (19) and others (13, 16), it is impossible to recover A_2pm as a C-terminal amino acid by hydrazinolysis. It may deserve special emphasis, from the taxonomic point of view, that the chemical composition of the peptidoglycan of *L. buccalis* was found to be clearly different from that of *Fusobacterium* peptidoglycan, which contained lanthionine as a dibasic amino acid (14, 15, 24). This finding provides further support for the position that *Leptotrichia* sp. and *Fusobacterium* sp. should be classified as separate genera. During the preparation of this manuscript, we became acquainted with the latest paper of Vasstrand et al. (25). They reported that a peptide moiety of *L. buccalis* L11 peptidoglycan consisted almost exclusively of glutamic acid, alanine, A_2pm, and an unidentified amino acid in molar ratios of 1.0:1.8:1.2:1.5 (as histidine). Their finding seems to be in harmony with our analytic results, except that we could not detect an appreciable amount of amino acids other than glutamic acid, alanine, and *meso*-A_2pm.

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