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Chemical Analysis of Cell Walls and Autolytic Digests of *Bacillus psychrophilus*

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The amino acid composition of isolated cell walls of *Bacillus psychrophilus* has been determined before and after extraction of protein with ethylenediaminetetraacetic acid at 45 C. This revealed that the peptidoglycan consists of Ala, Lys, and Glu in a molar ratio of 3:1:2. By using autolytic digests of log-phase cell walls, it was possible to detect 14 ninhydrin-positive degradation products. Chemical analyses of the seven major bands from these digests indicated that the amino acid sequence of the peptide subunit in the murein of this organism consists of muramyl-L-alanyl-γ-D-glutamyl-L-lysyl-D-alanine, and the linkage between adjacent peptides is supplied by a second D-glutamic acid which is bound to the ε-amino group of lysine and the carboxyl group of the D-alanine through its amino group. The nature of the solubilized wall fragments indicates that each of the peptide bonds in the murein is hydrolyzed by autolysins except the L-alanyl-γ-D-glutamyl linkage.

Bacillus psychrophilus is a gram-positive, motile organism which forms round terminal to subterminal spores. Physiologically, the organism is considered a facultative psychrophile in that its growth range is from below 0 C to 28 C, with an optimum of 23 to 24 C. Previous studies with this organism revealed unusual lysis behavior by whole cells at elevated temperatures, since no maximal or optimal rate of lysis could be established with increasing temperatures (9). Subsequent work with isolated cell walls of *B. psychrophilus* established that different lytic mechanisms are involved at permissive and restrictive growth temperatures (10). At temperatures which will support growth (below 30 C), native cell walls lysed autolytically. At higher temperatures, the autolysins were rapidly inactivated but lysis proceeded via a progressive release of cell wall protein (9, 10). This temperature-mediated wall dissociation was shown to be reversible in that the soluble and insoluble wall components reaggregated at low temperatures (10).

The effects of pH, Ca^{2+}, and ethylenediaminetetraacetic acid (EDTA) on wall lysis at elevated temperatures suggested that ionic bonds contribute to the integrity of this unusual gram-positive wall by linking the protein to the peptidoglycan (10). Support for this proposal has been presented by Farzadegan and Roth (Abstr. Annu. Meet. Amer. Soc. Microbiol., 1972) and by Alsobrook et al. (1). Both groups found, with electron microscopy, that the wall of *B. psychrophilus* is not only extensively layered, but at elevated temperatures the external layers "peel off" from the electron-dense peptidoglycan. The present investigation was thus undertaken to determine whether the peptidoglycan composition or structure might reveal unusual properties which could account for the distinctive association of proteins with the cell wall of this psychrophile.

MATERIALS AND METHODS

B. psychrophilus ATCC 23304 was isolated and kindly supplied by J. L. Stokes, Washington State University, Pullman, Wash. The cells were grown in a medium containing yeast extract (Difco), 5.0 g; KH_2PO_4, 6.9 g; K_2HPO_4, 14.0 g; $(NH_4)_2SO_4$, 2.0 g; and distilled water to 1 liter. This medium (YEM) had a final pH of 7.0 after autoclaving. One-liter flasks containing 500 ml of YEM were inoculated with washed cell suspensions from 24-h slants of YEM plus 1.5% agar (wt/vol). The cultures were routinely grown at 20 C on a reciprocal shaker (100 strokes per min). All cells used in this study were harvested by centrifugation during the exponential phase of growth; absorbancy at 540 nm was 0.40 to 0.70 (0.3-0.6 mg dry weight per ml).

Cell walls of *B. psychrophilus* were isolated by differential centrifugation after sonic disruption of thick cell suspensions. A Branson sonifier at maxi-

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mum power was used to disrupt the cells. An ice bath was used to prevent excessive heating during this treatment. Usually two 45-s bursts were sufficient to obtain 80 to 90% cell disruption. The isolated walls were washed repeatedly with cold distilled water (by centrifugation) to remove cytoplasmic contaminants. The purity of the cell wall preparations was confirmed microscopically. All lysis experiments were conducted by using freshly isolated cell walls.

Lysis of cell walls was followed by measuring the absorbance decrease at 540 nm with a Gilford model 240 spectrophotometer. Cell walls were suspended in 0.01 M carbonate buffer, pH 9.2, which was previously adjusted to the desired temperature.

In experiments where dissociation of autolytic enzyme activity was desired, the walls were suspended in 10 M LiCl at 0 C for 10 min and then washed in cold distilled water until the supernatant solution yielded a negative test for chloride ions.

Lysis was followed chemically by measuring the solubilization of N-terminal amino groups by using 1-fluoro-2,4-dinitrobenzene (DNFB) by the procedure of Ghuysen et al. (5). In addition, reducing groups liberated during lysis were assayed by using the ferricyanide procedure of Park and Johnson (13). For these analyses, samples of the cell wall suspensions were removed periodically during lysis and immediately frozen in an acetone-dry ice bath. These samples were later thawed at 0 to 4 C and centrifuged at 27,000 $\times$ g for 5 min. The supernatant solutions containing soluble cell wall fragments were then analyzed for N-terminal amino and reducing groups.

LiCl-treated cell walls (50 mg dry weight) were suspended in 0.01 M carbonate buffer (pH 9.2) containing 10^{-3} M EDTA, and the wall preparation was incubated at 45 C as described previously (10). The absorbance was monitored at 540 nm until no additional decrease occurred (2 min). The lysed wall suspension was then centrifuged at 45 C (27,000 $\times$ g, 10 min), and the resulting translucent pellet (peptidoglycan) was washed several times with distilled water and lyophilized.

Native cell walls (approximately 80 mg dry weight) were suspended in 0.01 M carbonate buffer, pH 9.2, and incubated at 20 C until no additional absorbance decrease occurred. Usually the absorbance decreased by 85%. The autolysed wall suspension was centrifuged (27,000 $\times$ g, 15 min) and the insoluble wall material was discarded.

The soluble autolysate (2–4 ml) was applied to a Sephadex G-50 column (1.5 by 100 cm) which had been equilibrated with 0.01 M carbonate buffer (pH 9.2). Fractions (4 ml) were collected and assayed for material absorbing at 280 nm and reacting with ninhydrin (15).

Samples were applied as a single band to prewashed Whatman 3 MM filter paper and subjected to high-voltage electrophoresis in a pyridine-acetic acid-water (11:8:981, vol/vol/vol) buffer system at pH 5.0. Guide strips were cut and developed with ninhydrin to locate the bands. The remainder of the bands were eluted with water and lyophilized. Two bands migrating to the cathode were located by electrophoresis at 45 V/cm (4 mA/cm) for 30 min. Electrophoresis of the slower moving band at 70 V/cm (5 mA/cm) for 120 min yielded five additional bands. Eight bands were detected migrating toward the anode after electrophoresis at 70 V/cm (5 mA/cm) for 90 min.

Both the cell wall and the peptidoglycan compositions were determined by using a Beckman-Spinco model 120 B automatic amino acid analyzer. By using a 60-cm column, the change from pH 3.25 to pH 4.25 buffer was made before the elution of valine. Under these conditions, muramic acid elutes between serine and glutamic acid but is not completely separated from the latter. Glucosamine elutes from the 60-cm column after phenylalanine. Amino acids were measured after hydrolysis with 6 N HCl (105 C) for 24 h, and amino sugars were determined after hydrolysis with 6 N HCl (105 C) for 4 h.

Since muramic and glutamic acids were not completely separated by the amino acid program used, the analyses were checked for accuracy by using peptidoglycan which was hydrolyzed for amino sugars. A total hexosamine analysis (5) was performed, glucosamine from the same sample was quantitated from the 15-cm column of the analyzer, and muramic acid was calculated by difference. There were no other amino sugars present in these cell walls.

Several preliminary analyses showed that the hydrolysis conditions used for amino acid determination resulted in an increase in the apparent glutamic acid content of about 15% due to muramic acid overlap. The glutamic acid values reported here have been reduced by this amount. Phosphate was assayed by the phosphomolybdate procedure of Chen et al. (3). N-terminal amino acids and the configuration of alanine were determined as previously described (5). The proportion of L- and D-isomers of glutamic acid with L-glutamic decarboxylase (Sigma) in 0.3 M pyridine acetate buffer (pH 5.0) for 3 h at 37 C as described by Warth and Strominger (19). γ-Aminobutyric acid and glutamic acid were separated by high-voltage electrophoresis at 70 V/cm (5 mA/cm) for 30 min in a pyridine-acetic acid-water (11:8:981, vol/vol/vol) buffer system (pH 5.0). Relative amounts of glutamic acid were then determined with ninhydrin by the procedure of Kay et al. (7). The configuration of lysine was not determined in this investigation.

RESULTS

Chemical analysis of B. psychrophilus cell walls. The unusual lytic responses of whole cells and isolated cell walls of *B. psychrophilus* (9, 10) prompted this analysis of the chemical composition of native and heat-dissociated cell walls. The first analyses were made by using acid hydrolysates of wall preparations which were obtained by sonic disruption, washed several times with distilled water, and shown to be free of whole cell contamination. No further purification procedures were employed. Table 1 shows the constituents detected in these preparations. The expected amino sugars were present as were numerous amino acids. This con-

TABLE 1. *Chemical analysis of whole cell walls of B. psychrophilus*

Wall component	μmol/mg[a] (dry weight)
Muramic acid	0.60 ± 0.05
Glucosamine	0.66 ± 0.06
Alanine	0.80 ± 0.05
Glutamic acid	0.63 ± 0.07
Lysine	0.52 ± 0.02
Aspartic acid	0.54 ± 0.03
Serine	0.22 ± 0.02
Threonine	0.47 ± 0.02
Glycine	0.26 ± 0.02
Valine	0.41 ± 0.02
Isoleucine	0.19 ± 0.01
Leucine	0.25 ± 0.02
Tyrosine	0.09 ± 0.01
Phenylalanine	0.16 ± 0.01
Phosphate	<0.10

[a] Major constituents detected in acid-hydrolyzed cell walls are expressed here as the average of two determinations. Other amino acids such as arginine, histidine, and methionine were present at levels of 0.05 μmol/mg (dry weight) or less and are not included. See Materials and Methods for a description of the analytical procedures.

firmed our previous, indirect evidence that considerable amounts of protein are normally associated with the wall of this organism (10). The very low level of phosphate evident after acid hydrolysis indicates that teichoic acid, a common constituent in the cell wall of other *Bacillus* species (14), is not present in the wall of *B. psychrophilus*. In addition, the low level of phosphate supports the conclusion that the protein is of wall origin rather than membrane contamination.

In our previous investigation of the lytic behavior of *B. psychrophilus* cell walls at elevated temperatures, we concluded that most of the absorbance decrease exhibited by autolysin-deficient cell walls results from the dissociation of protein from the wall at temperatures above 30 C. Since the rate of this wall dissociation was greatly increased by EDTA (1 mM), the insoluble wall residue which remained after incubation of the native walls at 45 C in EDTA was analyzed after acid hydrolysis. The results are shown in Table 2. The most obvious difference between native and temperature-dissociated cell walls is the absence of the so-called non-peptidoglycan amino acids. Each of these was reduced to less than 10% of their original value. The proportion of amino sugars relative to wall (by weight) increased after heat dissociation, and the levels of the peptidoglycan amino acids relative to wall mass decreased. These results would be expected if protein were removed from the wall by incubation at elevated temperatures.

The composition of the wall residue remaining after the temperature and EDTA-induced dissociation indicates that it is essentially pure peptidoglycan (Table 2). However, the composition is not typical of the genus *Bacillus*. The following distinguishing features are evident from these data: (i) of the various peptidoglycans from other *Bacillus* species which have been analyzed, only *B. sphericus* (6) has been reported to contain lysine rather than diaminopimelic acid. (ii) The observed ratio of Glu to Lys (2:1) has not previously been reported from any other *Bacillus* species. (iii) Assuming each Lys residue represents a tetrapeptide subunit (4, 6), only about 17% of the muramic acid residues in the glycan are substituted.

The unusual wall and peptidoglycan composition revealed by the data in Tables 1 and 2 prompted an investigation of the structure of the peptidoglycan of this organism. This was pursued by using cell wall which had been digested with the autolysins which had previously been shown to be indigenous to these walls (10). An indication of the nature of the *B. psychrophilus* autolysins was initially obtained from the data shown in Fig. 1. As indicated, exponential-phase walls of *B. psychrophilus* autolyse rapidly at pH 9.2 (20 C), with a progressive release of soluble amino groups. Very few reducing groups are generated under these conditions. The wall fragments solubilized during autolysis under the conditions described in Fig. 1 were isolated and analyzed to obtain an indication of the peptidoglycan structure.

The large amounts of protein associated with the native *B. psychrophilus* cell walls made it necessary to pass the solubilized wall material through Sephadex G-50 to separate the peptidoglycan fragments from this protein (Fig. 2). The

TABLE 2. *Chemical analysis of the peptidoglycan of B. psychrophilus*[a]

Wall component	μmol/mg (dry weight)
Muramic acid	1.45 ± 0.05
Glucosamine	1.65 ± 0.06
Alanine	0.63 ± 0.06
Glutamic acid	0.41 ± 0.04
Lysine	0.24 ± 0.03
Phosphate	<0.10

[a] See Table 1 and Materials and Methods for a description of the procedures.

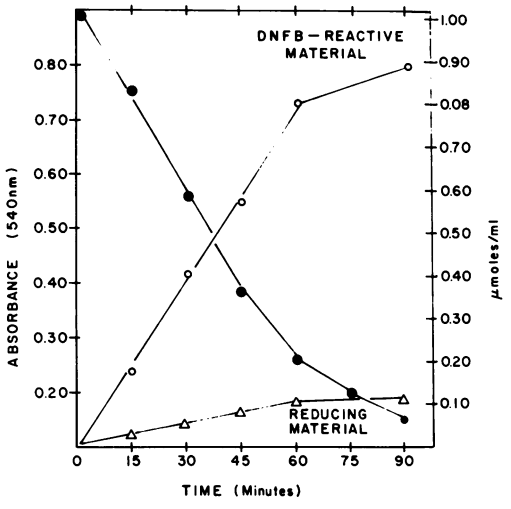

FIG. 1. *Autolysis of cell walls of B. psychrophilus. A cell wall preparation was adjusted to an initial density of 1.7 mg (dry weight) per ml and incubated at 20 C in 0.01 M sodium carbonate buffer, pH 9.2. Periodic samples were obtained throughout the incubation period and immediately frozen in a dry ice-acetone bath. The samples were thawed in an ice bath, centrifuged, and the supernatant solution was analyzed for soluble N-terminal amino groups and reducing groups.*

cell wall protein (designated P1) was excluded from the gel, and the lower-molecular-weight, ninhydrin-positive material, P2, was easily obtained by lyophilization. Reducing material was present in P1 but not in P2 (Mattingly and Best, unpublished data).

The digested wall material (P2) recovered from the Sephadex column, when subjected to high-voltage electrophoresis, yielded 14 ninhydrin-positive bands, of which 7 appeared to be major constituents. These seven bands were eluted from the paper in distilled water and analyzed chemically. The results are shown in Table 3.

The major peptide (based on the intensity of ninhydrin staining) which was released during autolysis of these walls was band E. Analyses with DNFB revealed free amino groups associated with both alanine and glutamic acid. Automatic amino acid analysis after acid hydrolysis showed that the peptide in band E contained two glutamic acid residues and one each of alanine and lysine. All of the glutamic acid was found to be in the D configuration and all of the alanine was in the L configuration. The actual configuration of the lysine was not determined but is also assumed to be L. Finally, it is apparent from the charge on this peptide that none of the free glutamyl carboxyl groups was amidated.

It seems likely that band E represents the major peptide subunit in the cell wall of *B. psychrophilus* even though no D-alanine is present. Band B5 was composed entirely of D-alanine (Table 3). This observation and the finding that none of the other peptides which were analyzed contained D-alanine suggest extensive carboxypeptidase II activity during autolysis.

A tentative structure of the peptidoglycan in the walls of *B. psychrophilus* is presented in Fig. 3. We assumed that the second mole of glutamic acid found in the hydrolysate of heat-dissociated walls and band E contributes to the cross-linkage between peptides. Even though all of the ninhydrin-reactive bands evident after paper electrophoresis were not analyzed, the major degradation products support this proposed structure. For example, peptide H is a dipeptide containing L-alanine (N-terminal) and D-glutamic acid, and band B2 is the expected tripeptide Ala-Glu-Lys.

The other ninhydrin-positive bands which were analyzed are not only consistent with the structure proposed, but also reflect on the variety of autolysins associated with the wall of this organism. The sites of hydrolysis of the peptidoglycan, as suggested from the autolysis products, are indicated in Fig. 3. Aside from carboxypeptidase (site 2) mentioned above, *N*-acetylmuramyl-L-alanine amidase activity (site 1) would release the N-terminal L-alanine which is found in peptides B2, E, and H (Table 3). This enzyme appears to be an autolysin common to many other bacterial species and recently was isolated and purified from *Staphylococcus aureus* (18). A more surprising observation was the free lysine and D-glutamic acid (bands A and D, respectively; Table 3). If

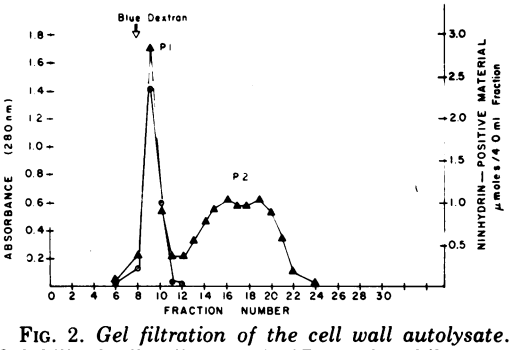

FIG. 2. *Gel filtration of the cell wall autolysate. Solubilized cell walls (80 mg) of B. psychrophilus were fractionated on a G-50 Sephadex column (1.5 by 100 cm). The column was equilibrated and eluted with 0.01 M sodium carbonate buffer, pH 9.21. Fractions (4.0 ml) were collected and analyzed for material absorbing ultraviolet light at 280 nm and reacting with ninhydrin.*

TABLE 3. *Chemical characterization of amino acids and peptides released by autolysis of cell walls*

Band	Electrophoretic[a] migration	Amino acid composition and ratios[b]		
		Ala	Glu	Lys
A	Lys (+1.0)			*1.0 (di-Dnp)
B2	Ala (+1.4)	*0.95 (L)	1.0 (D)	*0.89 (mono-Dnp)
B3	Ala (+1.3)		*1.0 (D)	*1.04 (mono-Dnp)
B5	Ala (+1.0)	*1.0 (D)		
D	Glu (−1.0)		*1.0 (D)	
E	Glu(−0.85)	*0.53 (L)	*1.0 (D)	0.52
H	Glu (−0.52)	*1.0 (L)	1.0 (D)	

[a] Electrophoretic mobility of the amino acids and peptides is represented according to their migration relative to a Lys, Ala, or Glu standard. The (+) or (−) indicates the charge on the amino acid or peptide. See the Materials and Methods section for a description of the electrophoresis conditions.

[b] These values were determined after acid hydrolysis (6 N HCl, 105 C, 20 h) by automatic amino acid analysis and are expressed relative to Glu or Lys. The 2,4-dinitrophenyl (Dnp) derivatives (before hydrolysis) are indicated with an asterisk. The configuration of the Ala and Glu residues is indicated in parentheses.

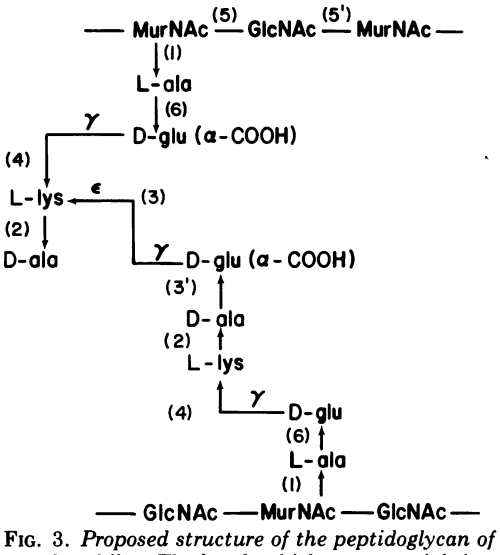

FIG. 3. *Proposed structure of the peptidoglycan of B. psychrophilus. The bonds which are potential sites of autolysin activity are designated by the numbers in parentheses.*

the structure of the peptidoglycan which is proposed in Fig. 3 is correct, then these amino acids could be released from the peptide network only if hydrolysis occurs at sites 2, 3, 3′, and 4. The absence of free L-alanine in band B5 indicates that only the cross-linking glutamic acid is released and that there is no aminopeptidase or endopeptidase activity at site. 6.

At least one of the autolysins suggested by these results is apparently novel. Many autolytic endopeptidases with specificity toward various peptidoglycan cross-linkages have been reported (4), so hydrolysis at sites 3 and 3′ is not too unexpected. However, autolytic activity at site 4 has not been reported previously. Our evidence for hydrolysis of this bond is free lysine (band A), L-Ala-D-Glu (band H), and the D-Glu-L-Lys dipeptide (band B3). This latter peptide could arise from the proposed wall structure only by hydrolysis at sites 2, 3′, and 4. Additional studies with a known substrate and a purified enzyme will be required to firmly establish this, however.

DISCUSSION

Previous studies showed that, on a weight basis, the wall of this organism consists of 70% protein and 30% peptidoglycan (9). The evidence of Alsobrook et al. (1) that the cell wall of this organism exists as a multilayered structure with an electron-dense inner layer which corresponds to the peptidoglycan in other organisms suggests an unusual wall architecture in this organism. Although protein layers have been described in the cell walls of other organisms, such as *B. polymyxa* (12), it is unusual for protein to be the predominate polymer. *B. sphericus*, however, was reported by Hungerer and Tipper (6) to have only 23% peptidoglycan in the wall, with protein and polysaccharide comprising the remainder of the wall material in this organism.

Even though other organisms have been shown to have protein layers, there is essentially no information relating to the manner in which these proteins adhere to the peptidoglycan. A clue to the association between the two major polymers in the wall of *B. psychrophilus* was afforded from a previous observation that EDTA at temperatures above 30 C solubilized the wall protein (10). The completeness of this solubilization is apparent from the amino acid content of the walls before and after this treatment (Tables 1 and 2). These observations, and

the ability of Ca^{2+} to retard lysis specifically at elevated temperatures (9, 10), suggested that ionic linkages are involved in, if not completely responsible for, the interaction of the wall protein and the peptidoglycan.

If it is assumed that a metal such as Ca^{2+} normally provides the bridge between the wall layers, carboxyl groups on the two polymers could be involved as the anionic participants. A gram-positive wall structure dependent on ionic linkages has not been described previously, but Ca^{2+} is known to link lipopolysaccharide to the remainder of the wall in a variety of gram-negative organisms (2). For a peptidoglycan to participate in this manner, it must have certain features not commonly associated with this polymer. The most important of these could be free carboxyl groups. Most of the potential C-terminal residues in the peptidoglycans from other organisms which have been analyzed are not ionizable due to various substitutions or to their direct participation in peptide or amide bond formation (14). This probably is not the case with *B. psychrophilus*. Of the potential sources of free carboxyl groups, one would be the C-terminal D-Ala on those peptide strands which are not involved in a glutamyl cross-linkage to adjacent peptide strands. The relative amounts of the three major amino acids shown in Table 2 suggest that there could well be D-Ala-D-Ala C-terminal chains which are not crosslinked. In addition, each of the glutamyl residues in the peptidoglycan has a free carboxyl group. This conclusion is based on the electrophoretic mobility of the free glutamic acid and the glutamic acid-containing peptides (Table 3). A quantitatively greater source of free carboxyl groups, and another distinctive feature of *B. psychrophilus* cell walls was revealed by the data in Table 2. As indicated, there is five times as much *N*-acetylmuramic acid as lysine in the peptidoglycan of this organism. If lysine is presumed to represent a peptide unit, this means that only about 20% of the muramic acid is substituted with peptides. Therefore, the carboxyl group of the lactyl moiety of this amino sugar is potentially free and ionizable.

The low degree of peptide substitution on the amino sugar polymer in this organism is unusual. *Micrococcus lysodeikticus* was found to have roughly 40% substitution (8, 11), and the peptidoglycan of *B. subtilis* spores has been shown to be substituted on about 35% of the muramic acid residues (19). In the majority of cases, virtually complete substitution of the muramic acid is found (14).

The peptidoglycan structure which is proposed for *B. psychrophilus* is unlike any of the other *Bacillus* species which have been studied. However, Schleifer and Kandler (17), by using partially hydrolyzed cell wall, proposed a peptidoglycan for the halophilic *Planococcus* which is identical to the one suggested for *B. psychrophilus*. In most respects, however, the cell wall of *B. sphericus* is more similar to the one reported here. Both organisms contain lysine rather than diaminopimelic acid, neither have a substitution on the α-carboxyl of the D-glutamic acid residue, both have large amounts of protein and low amounts of peptidoglycan in the wall, and neither organism has teichoic acid in the wall (6). The major differences in the walls of *B. sphericus* and *B. psychrophilus* appear to be the cross-linking amino acid (asparagine and glutamic acid, respectively) and the virtually complete substitution of muramic acid by peptides in *B. sphericus* (6).

Aside from the unusual cell wall of this psychrophile, it is apparent from the peptidoglycan fragments which were liberated during autolysis that only one of the peptide linkages in the structure proposed in Fig. 3 is immune from hydrolysis by the autolysins indigenous to the walls of *B. psychrophilus*. No activity has been detected at site 6 (Fig. 3). Studies are currently in progress to resolve the several autolysins and more completely characterize the enzyme activities indicated by the fragments isolated in this study.

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