Amidation and Cross-Linking of the Enzymatically Synthesized Peptidoglycan of Bacillus stearothermophilus*

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SUMMARY

A particulate enzyme system from Bacillus stearothermophilus which catalyzes the synthesis of peptidoglycan from uridine nucleotide precursors has been investigated. The preparation catalyzed extensive cross-linking of the enzymatically synthesized peptidoglycan. In addition to the presence of amidated glucosamine, the enzymatically synthesized peptidoglycan was elucidated by a comparison with similar materials obtained after lysozyme digestion of isolated vegetative cell walls of this organism. In the present paper the products obtained on lysozyme digestion of enzymatically synthesized peptidoglycan of B. stearothermophilus will be compared to those found on similar treatment of isolated vegetative cell walls of this organism.

Efficient systems for peptidoglycan synthesis in vitro have been demonstrated in B. megaterium (12) and in B. steator-thermophilus NCTC 10339 (12). Less efficient systems have been described in B. licheniformis (3, 4) and B. stearothermophilus ATCC 15952 was grown to produce spores on a medium containing 2 g of glucose, 1 g of vitamin-free Casamino Acids (Difco), 1.0 g of KH₂PO₄, 2.09 g of KH₂PO₄, 1 g of NaCl, 1 g of KCl per liter of distilled water to which was added a spore salt solution, sterilized separately, which contained per liter of medium 200 mg of KNO₃, 40 mg of MgCl₂·6H₂O, 15 mg of CaCl₂·2H₂O, 2 mg of MnSO₄·H₂O, 2 mg of ZnSO₄, 1 mg of NH₄ molybdate, and 0.2 mg of CaCl₂. A 15-liter culture containing silicone antifoam was grown at 60°C with maximum aeration and maximum stirring in a Microferm fermenter (New Brunswick Corp.) for about 1.5 days, and then harvested by centrifugation. The resulting pellet containing mainly spores with some vegetative and sporulating forms was washed twice with 200 ml of water at 0°C, centrifuging at 2500 x g for 5 min. This spore suspension was mixed with 100 ml of water and 1 ml of CHCl₃ and allowed to autolyze at 4°C for 6 days. The resulting pellet was washed five times with water by centrifugation at 2500 x g to remove membranous material. The cleaned spores were suspended in 25 ml of H₂O and layered on top of a two-layer system formed from 15 ml each of 50% and 30% NaBr in 50 mM Na phosphate buffer, pH 6.9 in 6 x 40 ml polyallomer tubes. After centrifugation in the SB-110 rotor (IEC ultracentrifuge) at 23,000 rpm for 1 hour, membranes, whole cell ghosts, vegetative and sporulating cells were removed at the interface. The resulting spore pellet free from contamination by vegetative forms was washed twice with water and then lyophilized for storage. Heat-activated spore suspensions in H₂O (100°C for 10 min) were used as the inoculum for vegetative growth in 5 g of tryptone, 3 g of yeast extract, 2 g of glucose, 4 g of KH₂PO₄, 2 g of KH₂PO₄ per liter of distilled water with silicone antifoam agent at 60°C with maximum aeration and maximum stirring in a Microferm fermenter for about 6 days. The resulting pellet was washed five times with water by centrifugation at 2500 x g to remove membranous material. The cleaned spores were suspended in 25 ml of H₂O and layered on top of a two-layer system formed from 15 ml each of 50% and 30% NaBr in 50 mM Na phosphate buffer, pH 6.9 in 6 x 40 ml polyallomer tubes. After centrifugation in the SB-110 rotor (IEC ultracentrifuge) at 23,000 rpm for 1 hour, membranes, whole cell ghosts, vegetative and sporulating cells were removed at the interface. The resulting spore pellet free from contamination by vegetative forms was washed twice with water and then lyophilized for storage. Heat-activated spore suspensions in H₂O were used as the inoculum for vegetative growth in 5 g of tryptone, 3 g of yeast extract, 2 g of glucose, 4 g of KH₂PO₄, 2 g of KH₂PO₄ per liter of distilled water with silicone antifoam agent at 60°C with maximum possible aeration and agitation. The cells were harvested in the log phase and frozen at -15°C.

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‡ The abbreviations used are: Dap, 2,6-diaminopimelic acid; DS-P, disaccharide-peptide (GlcNAc-MurNAc-1-Ala-o-Glu-meso-Dap-(o-Ala)-(o-Ala)).
§ O. Kandler, personal communication.

The peptidoglycans of the vegetative cell walls of the Bacillus subtilis (2), Bacillus licheniformis (3, 4), Bacillus megaterium (5), Bacillus stearothermophilus (6, 7), Bacillus subtilis (8, 9), and Bacillus thuringiensis (10). In the less common type the ε-NH₂ groups of L-lysine are cross-linked through either ε-aspartagine in B. sphaericus (11) and in one strain of B. pasteurii or by β-D-aspartyl-L-alanine (or L-serine) in the other strains of B. pasteurii to the COOH-terminal ε-amino residues of adjacent tetrapeptide substituents.

In the present paper the products obtained on lysozyme digestion of enzymatically synthesized peptidoglycan of B. stearothermophilus will be compared to those found on similar treatment of isolated vegetative cell walls of this organism.

MATERIALS AND METHODS

Organism and Growth Conditions—B. stearothermophilus ATCC 15952 was grown to produce spores on a medium containing 2 g of glucose,ishige-free Casamino Acids (Difco), 1 g of NaCl, 1 g of KCl per liter of distilled water at 0°C, centrifuging at 2500 x g for 5 min. This spore suspension was mixed with 100 ml of water and 1 ml of CHCl₃ and allowed to autolyze at 4°C for 6 days. The resulting pellet was washed five times with water by centrifugation at 2500 x g to remove membranous material. The cleaned spores were suspended in 25 ml of H₂O and layered on top of a two-layer system formed from 15 ml each of 50% and 30% NaBr in 50 mM Na phosphate buffer, pH 6.9 in 6 x 40 ml polyallomer tubes. After centrifugation in the SB-110 rotor (IEC ultracentrifuge) at 23,000 rpm for 1 hour, membranes, whole cell ghosts, vegetative and sporulating cells were removed at the interface. The resulting spore pellet free from contamination by vegetative forms was washed twice with water and then lyophilized for storage. Heat-activated spore suspensions in H₂O (100°C for 10 min) were used as the inoculum for vegetative growth in 5 g of tryptone, 3 g of yeast extract, 2 g of glucose, 4 g of KH₂PO₄, 2 g of KH₂PO₄ per liter of distilled water with silicone antifoam agent at 60°C with maximum possible aeration and agitation. The cells were harvested in the log phase and frozen at -15°C.

1 L. L. Campbell, personal communication. We are grateful to Dr. Campbell for providing us with this information on an efficient sporulation medium for Bacillus stearothermophilus.
Preparation of Particulate Enzyme—The frozen cells were washed with 50 mM Tris-HCl, pH 8.5, 10 mM MgCl₂ buffer and then broken in the Mini-mill (Gifford-Wood) with glass beads in the Tris-MgCl₂ buffer as described previously (13). Particulate enzyme from 6 g of frozen cells was suspended in 1 ml of the Tris-MgCl₂ buffer and stored frozen at -80° in small aliquots (25 mg per ml of protein). Full activity was preserved for extended periods of time by this method.

Preparation of Cell Walls—Frozen vegetative cells (49 g) were broken in batches in the Mini-mill with water (13), cycling the unbroken cells twice more through the procedure to maximize the yield of dry cell walls plus period of time by this method. Particulate membranes was 1.1 g.

Lysozyme Degradation of Cell Walls—Five hundred milligrams (dry weight) of cleaned cell walls plus membranes were suspended in 50 ml of 10 mM NaPO₄ buffer, 2.5 mM EDTA, pH 6.9 and 0.5 ml of toluene and shaken gently at 37° with 50 mg of salt-free lysozyme ( Worthington Biochemicals) for 7 hours. Digestion was continued overnight and for 7 hours longer with two additions of 25 mg of fresh lysozyme. The mixture was heated at 100° for 2 min, cooled, and spun down at 20,000 × g for 20 min. The pellet was washed with 2 × 80 ml of water and then lyophilized extensively. The yield of dry cell walls plus membranes was 1.1 g.

Amino Acid Analysis—Samples of approximately 50 nmole were hydrolyzed with 100 µl of degassed 4 N HCl at 105° for 17 hours in sealed tubes. The HCl was removed on a rotary evaporator and the residue was dissolved in 5.0 ml of sample diluter buffer (Beckman, Spinco Div.). Aliquots of 1.0 µl were analyzed together with 1.0 ml of a solution of 10.0 µM 2-NH₂-3-guanidinopropionic acid and norleucine as internal standards for the short and long column, respectively. A modified Beckman-Spinco Auto-Analyzer model 1203 was used with the technique of Peterson and Bernlohr (23) modified as follows. Both columns were run at 49-50°. The first buffer for the long column was adjusted to pH 3.09 with HCl and the change to pH 4.26 buffer was timed to occur after alanine but before 2,6-diaminopimelic acid (87 min under the conditions used). GlcN was quantitated from both the long and the short columns, and a mean is presented under "Results." For NH₃ quantitation, samples of 5 to 10 nmole were treated with 100 µl of 0.02 N NaOH and then lyophilized to remove free NH₃ in the sample. Hydrolysis and analysis on the column were carried out as above, except that a blank was taken through the same procedure, and the NH₃ values were adjusted accordingly.

Dinitrophenylation—The method of Jarvis and Strominger (24) was modified. Aliquots of about 50 nmole were dissolved in 25 µl of H₂O treated with 10 µl of 1% triethylamine in ethanol and 25 µl of 0.1 x 1-fluoro-2,4-dinitrobenzene in ethanol, and then incubated in the dark in closed tubes at 57° for 40 min. Solvents were removed under reduced pressure and the residues were hydrolyzed as above in the dark. The hydrolyzates were added directly to 2 ml of sample diluter buffer and analyzed on the long column as above. Mono-DNP-Dap could not be estimated accurately because of variable amounts of other material absorbing at 440 nm at a similar elution time (a broad peak where valine and leucine eluted). Quantitation of the remaining diamino-pimelic acid was used to estimate the amount derivatized, with glutamine and alanine acting as internal standards in the hy-
RESULTS

Peptidoglycan Synthesizing System from B. steatorhermolophilus—
The particulate enzyme preparation obtained from vegetative cells of B. steatorhermolophilus ATCC 15952 catalyzed synthesis of peptidoglycan in the presence of UDP-MurNAc-pentapeptide and UDP-[\(^{14}\)C]GlcNAc. The reaction was optimal at pH 8.5 in Tris-HCl buffer in the presence of 10 mM MgCl\(_2\). Both the initial rate of peptidoglycan synthesis and the over-all yield after 2 hours were optimal at 37°C, although appreciable synthesis of peptidoglycan still occurred in vitro at 55°C (Fig. 1). In B. steatorhermolophilus NCTC 10339, peptidoglycan synthesis in vitro was optimal at 55°C (12).

Lysozyme Digestion of Peptidoglycan Synthesized in Vitro—Lysozyme digestion of peptidoglycan synthesized from UDP-[\(^{14}\)C]GlcNAc or from UDP-MurNAc-L-Ala-D-Glu-(L)-meso-Dap-(L)-d-Ala-d-Ala labeled with \(^{14}\)C in either the MurNAc, Dap, or d-Ala moieties, and separation of the resulting supernatant solutions by high voltage electrophoresis on paper at pH 4.0 showed that label was found in the positions expected for both disaccharide-peptide monomers (GlcNAc-MurNAc-peptides) and cross-linked dimers (bis(GlcNAc-MurNAc-peptides)) as compared with authentic standards obtained from lysozyme digestion of Escherichia coli cell walls (comparing C6 and C3 in Reference 29). Development of the electrophoresis strips in the second dimension by paper chromatography with Solvent A yielded a pattern of products which was identical with that obtained from a B. megaterium particulate enzyme system (Fig. 1 in Reference 13). This confirmed the formation of interpeptide cross-links by transpeptidation in the in vitro system from B. steatorhermolophilus. The incorporation from UDP-[\(^{14}\)C]GlcNAc into the monomeric, dimeric, and oligomeric disaccharide-peptide products (Table I) indicated that the enzyme system achieved about 42% cross-linking (from the ratio of dimeric and oligomeric products to total monomers plus dimers plus oligomers) under the conditions employed.

Labeled compounds with less mobility toward the anode at pH 4.0 than the usual monomeric or dimeric products and present in variable amounts were also noted on electrophoretograms. Four such products (Products A, B, C, and D, see Fig. 2) were seen. It was of interest to determine the structures of these products since they had electrophoretic mobilities close to the lysozyme-digestion products of the spore cortex which contained muramic lactam (16, 17).

Amidation of Peptidoglycan by Particulate Enzyme—Preliminary experiments suggested that the presence of these labeled products of lysozyme digestion might be dependent on ATP and NH\(_4\). After the nucleotide substrates required for peptidoglycan synthesis had been freed from NH\(_4\)\(^+\) by DEAE-cellulose chromatography, a clearer pattern for the requirement of ATP and sources of NH\(_4\) emerged. Peptidoglycan was synthesized from UDP-[\(^{14}\)C]GlcNAc or UDP-[\(^{14}\)C]MurNAc-Ala-Glu-Dap-Ala-Ala,

| Product | Amount formed (\(\mu\)mol) |
|---------|----------------------------|
| R-meso-Dap-d-Ala-d-Ala | (monomer) 45.3 |
| R-meso-Dap-d-Ala | (monomer) 22.2 |
| eso-Dap | (monomer) 45.1 |
| eso-Dap-(n-Ala)-(n-Ala) | (dimer) 66.5 |
| R-meso-Dap-d-Ala | (trimer) 16.0 |
| R-meso-Dap-d-Ala | (trimer) 16.0 |

TABLE I

Peptidoglycan Synthesized from UDP-[\(^{14}\)C]GlcNAc by particulate enzyme from vegetative cells of B. steatorhermolophilus and then digested with lysozyme as outlined under “Materials and Methods.” The entire lysozyme digest was separated by electrophoresis at pH 4.0 and 34 volt per cm for 4 hours, followed by chromatography in Solvent A for 40 hours on Whatman No. 3MM paper. The pattern of products was identical to that in Reference 13 but included a higher oligomer, probably trimer (tris (disaccharide-peptide)). R- = GlcNAc-MurNAc-L-Ala-d-Glu-.

*Parentheses indicate that dimer and oligomer were probably mixtures of materials with varying amounts of COOH-terminal d-Alanine.*

Fig. 1. Temperature dependence of peptidoglycan synthesis from UDP-[\(^{14}\)C]GlcNAc by B. steatorhermolophilus particulate enzyme. The assays contained 4 \(\mu\)moles of Tris HCl adjusted to pH 8.5 at the temperature employed, 0.2 \(\mu\)mole of MgCl\(_2\), 5 \(\mu\)mole of UDP-MurNAc-L-Ala-d-Glu-meso-Dap-d-Ala-d-Ala, 5.3 \(\mu\)mole of UDP-[\(^{14}\)C]GlcNAc (2.1 \(\mu\)Ci per \(\mu\)mole, 2.3 \(\times\) 10^4 cpm) and 80 \(\mu\)l of particulate enzyme protein, in a total volume of 20 \(\mu\)l. After incubation for the time indicated, the reactions were stopped by the addition of 10 \(\mu\)l of Solvent A at 0°C and the entire reaction mixtures were spotted for chromatography in Solvent A overnight. Peptidoglycan remained at the origin. Solid lines refer to peptidoglycan synthesis dependent on both UDP-GlcNAc and UDP-MurNAc-pentapeptide, while dashed lines refer to the synthesis of some polymeric material remaining at the origin from UDP-[\(^{14}\)C]-GlcNAc, not dependent on UDP-MurNAc-pentapeptide. The solid lines have been corrected for the blank represented by the dotted lines.
FIG. 2 (left). Electrophoretic separation of lysozyme-digested peptidoglycan formed from UDP-[\(^{14}\)C]GlcNAc by particulate enzyme from B. stearothermophilus, dependent on added ATP and NH\(_4\)Cl or l-glutamine. Purification of the nucleotide substrates from NH\(_4\) contamination and all other experimental techniques are covered under "Materials and Methods." ATP, NH\(_4\)Cl, and glutamine were added where applicable at final concentrations of 8.5, 17, and 14 mM, respectively. Electrophoresis was carried out as in Table I and the dried paper was autoradiographed for 4 days.

TABLE II

Utilization of UDP-[\(^{14}\)C]GlcNAc and UDP-[\(^{14}\)C]MurNAc-Ala-Glu-Dap-Ala-Ala for formation of Products A, B, and C

The compounds shown in Fig. 2 were cut out and counted.

| Labeled substrate | Additions | Amount of product formed (pmoles) |
|-------------------|-----------|----------------------------------|
|                   |           | A  | B  | C  |
| UDP-[\(^{14}\)C]GlcNAc | None      | 7  | 6  | 13|
|                    | ATP       | 21 | 8  | 13|
|                    | NH\(_4\)Cl| 10 | 8  | 30|
|                    | Glutamine | 9  | 7  | 15|
|                    | ATP + NH\(_4\)Cl | 83| 30| 26|
|                    | ATP + Glutamine | 90| 33| 15|
| UDP-[\(^{14}\)C]MurNAc-Ala-Glu-Dap-Ala-Ala | None | 6  | 4  | 6|
|                    | ATP       | 19 | 10 | 6|
|                    | NH\(_4\)Cl| 16 | 11 | 21|
|                    | Glutamine | 14 | 10 | 6|
|                    | ATP + NH\(_4\)Cl | 62| 35| 23|
|                    | ATP + Glutamine | 61| 44| 12|

digested with lysozyme and separated by electrophoresis at pH 4.0 (Fig. 2). The areas corresponding to Products A, B, and C were cut out and counted. (The amount of Product D was small and was ignored in this experiment.) The results (Table II) showed that formation of Products A and B was dependent on ATP and either NH\(_4\)Cl or l-glutamine, with glutamine being slightly more efficient than NH\(_4\)Cl. The formation of Product C appeared to be dependent on NH\(_4\)Cl alone rather than on ATP or glutamine. Penicillin G was utilized to investigate the formation of these products further (Table III and Fig. 3). As expected, penicillin G inhibited formation of the normal cross-linked dimer (Product G) (22, 26, 27). The formation of Product A was stimulated by penicillin G in the presence of ATP and NH\(_4\)Cl as would be expected for a monomeric disaccharide-pentapeptide with COOH-terminal d-Ala-d-Ala, while the formation of Product B in the presence of ATP and NH\(_4\)Cl was inhibited. The formation of Product C in the presence of ATP and NH\(_4\)Cl appeared also to be inhibited but the changes were

TABLE III

Effect of penicillin G on formation of Products A, B, and C by B. stearothermophilus particulate enzyme from UDP-[\(^{14}\)C]GlcNAc in presence of ATP and NH\(_4\)Cl

The compounds shown in Fig. 3 were cut out and counted.

| Antibiotic | Other additions | Amount of product formed (pmoles) |
|------------|-----------------|----------------------------------|
|            |                | A  | B  | C  |
| None       | None           | 7  | 5  | 12|
| None       | ATP + NH\(_4\)Cl | 65| 24| 24|
| Penicillin G | None          | 10 | 6  | 17|
| Penicillin G | ATP + NH\(_4\)Cl | 102| 12| 13|
small and may not be significant. The results for Product B could be explained by inhibition of d-alanine carboxypeptidase by penicillin G and for Product C by inhibition of transpeptidase (26, 27), assuming the structures elucidated below.

Products A, B, C, and D were investigated further by a larger scale preparation from UDP-[14C]GlcNAc, followed by their isolation from the pH 4.0 electrophoretogram. Electrophoretic and chromatographic data for these products are shown in Table IV. Reduction of these products with NaBH4 and then electrophoresis at pH 4.0 gave only bands at the original positions for A, B, C, and D. The absence of products with much reduced anionic character at pH 4.0 showed the absence of muramic acid lactam in these products (16, 17). Treatment of A, B, C, and D with Streptomyces amidase (a gift of Dr. J.-M. Ghysen) followed by separation at electrophoresis at pH 4.0 and chromatography in Solvent C (28) showed that only the disaccharide, GlcNAc-MurNAc, was released. No traces of the tetrasaccharide (GlcNAc-MurNAc-GlcNAc-MurNAc) the isomeric disaccharide (MurNAc-GlcNAc), muramic acid lactam-containing oligosaccharides, or disaccharides lacking N-acetyl groups were detected.

The combined data suggested that Products A, B, C, and D were normal disaccharide-peptide containing the original peptide chain (since these products were also obtained when UDP-MurNAc-Ala-Glu-Dap-[14C]Ala-[14C]Ala was the labeled substrate). The most probable explanation for their reduced anionic character at pH 4.0 was the masking of a COOH group by an amidation reaction, since amidated peptide units are known in other strains of Bacillaceae (2-4, 8, 9).

To rule out the possibility that Products A, B, C, and D might be artifacts of the preparation procedure, a particulate enzyme preparation from E. coli prepared by grinding the cells with alumina (26, 27, 29) was used to synthesize peptidoglycan from UDP-[14C]GlcNAc in the presence of ATP and NH4Cl. The resulting peptidoglycan was degraded with lysozyme directly or with trypsin followed by lysozyme and the resulting supernatants were separated by electrophoresis at pH 4.0 (Fig. 4). No bands were found with mobilities similar to Products A, B, C, and D from B. stearothermophilus, except for a weak band at Rf 0.82 which was present only in the trypsin-pretreated lysozyme digests. This weak band could represent the disaccharide tetrapeptide, GlcNAc-MurNAc-Ala-o-Glu-(L)-meso-Dap-(L)-Lys, which has been reported as a trypsin degradation product of the lipoprotein-peptidoglycan conjugate from E. coli (30, 31).


gia Digestion Products from Vegetative Cell Walls of B. stearothermophilus—Crude cell walls were prepared from B. stearothermophilus vegetative cells by a similar method to that used for preparing the particulate enzyme. After washing well with water, the cell wall and membrane mixture was degraded with lysozyme. The digestion products were separated by preparative paper electrophoresis at pH 4.0. Bands which were ninhydrin positive and which showed amino sugar fluorescence (21) were found with mobilities corresponding to the enzymatically synthesized Products B, C, and D as well as the nonamidated disaccharide-peptide Products E, F, and G. These latter correspond to products seen in lysozyme digests of E. coli cell walls. As will be shown below, E is the cyclic dimer corresponding to E. coli compound C4; F is the monomer, C6; and G is the normal dimer C3 (25). Only a very weak band was seen corresponding to enzymatically synthesized Product A. These bands were eluted from the paper and further separated by paper chromatography in Solvent A followed by two separations in Solvent B. The final separation utilized Whatman No. 3MM paper pre-washed by continuous elution with 1% acetic acid and then dried to minimize contamination with extraneous amino acids from the paper. Product B gave a single major band in Solvent A, together with a small band at Rf 1.46 which corresponded to Product A (Table IV). Chromatography of Product B in Solvent B gave a poor separation into two compounds, B1 and B2. Products C, E, and G gave only single bands in Solvents A and B. Product D was separated in Solvent A into a major band (D1) and a minor band (D2) with Rf 0.87 which was not further investigated. Product F separated further in both Solvents A and B into two broad, overlapping bands, F1 and F2. The

![Fig. 4. Electrophoretic separation of lysozyme-digested peptidoglycan formed from UDP-[14C]GlcNAc by particulate enzyme from E. coli. Particulate enzyme was prepared by alumina grinding of E. coli (27, 28, 30). The peptidoglycan-synthesizing system, in the presence of ATP and NH4Cl where applicable, and the lysozyme digestion technique were identical with those used for B. stearothermophilus (Materials and Methods). Where indicated the boiled peptidoglycan-synthesizing mixtures were treated with 1 mg per ml of trypsin for 2 hours at 23°, heated to 100° for 5 min and then treated with lysozyme as before. The electrophoretogram prepared as in Fig. 2 was autoradiographed for 4 days.](http://www.jbc.org/)
The electrophoresis and chromatography systems are described under “Materials and Methods” as well as preparation of the lysozyme digest. C6 and C3 are from E. coli.

| Product | Moles at pH 1.0 | R3 in Solvent A | R3i in Solvent B |
|---------|----------------|----------------|-----------------|
| B1      | 0.53           | 1.14           | 0.70            |
| B2      | 0.53           | 1.14           | 0.82            |
| C       | 0.67           | 0.72           | 0.45            |
| D       | 0.93           | 0.86           | 0.59            |
| E       | 1.12-1.20      | 0.60           | 0.50            |
| F1      | 1.21-1.28      | 0.87           | 0.80            |
| F2      | 1.21-1.28      | 1.00           | 0.95            |
| G       | 1.29-1.36      | 0.54           | 0.52            |

**TABLE VI**

**Analysis of purified products of lysozyme-digested cell walls of B. stearothermophilus ATCC 19588**

All procedures are described under “Materials and Methods.” The total yield of products from 0.5 g of the cell walls preparation was corrected for losses due to staining of guide strips only; other losses were not taken into account. Mole ratios were based on diaminopimelic acid (1.0) except for the analysis of B2 which was based on glutamic acid.

| Product | GlcN | MurN | Ala | Glu | Dap | NH3 | % derivatized by dinitrophenylation | Total yield |
|---------|------|------|-----|-----|-----|-----|-----------------------------------|------------|
| B1      | 0.79 | 1.01 | 1.23| 1.09| 1.00| 2.44| 97                                  | 1.4        |
| B2      | 0.67 | 0.95 | 1.27| 1.00| 0.44| 2.70| 72                                  | 0.5        |
| C       | 0.90 | 0.87 | 1.81| 0.91| 1.00| 1.08| 50                                  | 0.7        |
| D1      | 0.75 | 0.97 | 1.52| 0.92| 1.00| 0.55| 67                                  | 4.4        |
| E       | 0.78 | 0.95 | 1.59| 0.90| 1.00| 0   | 67                                  | 12.0       |
| F1      | 0.85 | 1.10 | 1.15| 1.05| 1.00| 0   | 100                                 | 5.1        |
| F2      | 0.85 | 1.01 | 1.46| 1.08| 1.00| 0   | 100                                 | 5.3        |
| G       | 0.72 | 0.95 | 1.60| 1.09| 1.00| 0   | 56                                  | 28.6       |

the structures shown in Fig. 5, in which the configurations of the amino acids have been inferred by analogy to established cell wall peptides.

Products F1 and F2 were the nonamidated disaccharide tripeptide and tetrapeptide monomers, and G was the nonamidated bis(disaccharide-peptide) dimer (corresponding to product C3 from E. coli, see Reference 25). Product E gave essentially the same analytical results as G. Its mobility on electrophoresis and chromatography suggested that it was a tetrasaccharide with internal cross-linking between the diaminopimelic acid of one tripeptide or tetrapeptide unit and another tetrapeptide unit, i.e. a cyclic dimer equivalent to C4 from E. coli (25). Compounds of this type are apparently formed through transesterification of disaccharide-peptide dimers by lysozyme (21, 25). B1 appeared to be the monoamidated-tripeptide. The NH3 value (Table VI) for B1 suggested a diamide, but this is ruled out by its electrophoretic mobility at pH 4.0. This high NH3 value may have been due to some contamination with B2 which yielded excessive amounts of NH3 even after prior treatment with dilute alkali and lyophilization, or by the difficulty of doing NH3 analyses on a compound isolated in relatively small amounts. The NH3 contents of C and D1 together with their electrophoretic mobilities at pH 4.0 suggested that they were diaminated and monomomidated bis(disaccharide-peptide) dimers, respectively. The assignment of D1 as the “internal” amide is based on the greater mobility of the minor component D2 separated on chromatographs in Solvent A, by analogy with the mobilities of the equivalent peptides obtained after autolysis of B. subtilis cell walls on thin layer chromatography in isobutyric acid-triethylamine-H2O (100:7:43 by volume) (9). It is of interest that a
The occurrence of amides in bacterial walls was first demonstrated in *Staphylococcus aureus* where the amide is on the α-carboxyl of p-glutamic acid (34). Subsequently in *Corynebacterium diphtheriae* amides were found on both the glutamic acid and Dap residues (35). In *B. subtilis* only the Dap residue is amidated (16). It is noteworthy that no amides were found in the spore cortex of *Bacillus subtilis* or *Bacillus stearothermophilus* (16). Therefore, the amidating enzyme might be an enzyme which would be expected to disappear during sporulation. The occurrence of amidated products in the peptidoglycan synthesized enzymatically by preparations from vegetative cells of *Bacillus stearothermophilus* makes it difficult to search for products containing muramic acid lactam which might be synthesized by enzyme preparations from sporulating cells because the amidated products and the products containing muramic lactam have similar electrophoretic mobilities.

The extensive cross-linking of the peptidoglycan synthesized by intact cells of *Bacillus stearothermophilus* is also found in the peptidoglycan synthesized by enzyme preparations from this organism. The transpeptidase in this preparation will also catalyze the incorporation of meso- or d-diaminopimelic acid into the peptidoglycan and the penicillin-sensitivity of this reaction has already been described (36). The only other cell-free systems in which cross-linking has been demonstrated are those from *Escherichia coli* (22, 26, 29), *Dacillus nigrutum* (13, 30), *Staphylococcus aureus* (37), *Sporosarcina ureae* (38), and *Micrococcus lysodeikticus* (30).

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