The Peptidoglycan in Walls of Butyribacterium rettgeri

Micheline Guinand, Jean-Marie Ghuysen, Karl H. Schleifer, and Otto Kandler

ABSTRACT: The wall peptidoglycan in Butyribacterium rettgeri is composed of N^a-(L-seryl-γ-D-glutamyl)-L-ornithyl-D-alanine subunits. The peptide subunits are cross-linked by means of a D-lysine or a D-ornithine residue, extending from the α-carboxyl group of the glutamic acid of one peptide subunit, to which D-lysine is linked through its ε-amino group or D-ornithine through its δ-amino group, to the carboxyl group of the ε-carboxy-terminal D-alanine residue of another peptide subunit. D-Lysine and D-ornithine bridges occur in the ratio of 2:1.

In many euubacteria, the peptide subunits N^a-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine (Muñoz et al., 1966) and L-alanyl-γ-D-glutamyl-(L)-meso-diaminopimelyl-(L)-D-alanine (van Heijenoort et al., 1969) are the building blocks of the peptide moiety of many wall peptidoglycans. These subunits are interlinked by peptide bridges which extend from the ε-amino group of L-lysine or from the amino group on the δ-carbon of meso-diaminopimelyl of one peptide subunit, to the carboxyl group of the ε-carboxy-terminal D-alanine of another peptide subunit. According to the species, the bridges consist of direct peptide linkages between peptide subunits or they are built up of additional intervening amino acids such as glycine, L-amino acids, or D-isoasparagine (Ghuysen, 1968).

The Streptomyces KM endopeptidase hydrolyses the N^a-(D-alanyl)-D-lysine and N^a-(D-alanyl)-D-ornithine linkages and causes wall solubilization. The walls, as they are prepared, have a low degree of cross-linking. About 59% of the peptide subunits occur as monomers (18%), dimers (24%), and trimers (17%). Higher oligomers account for the remaining 41%. The disaccharide units obtained after sequential treatment with Streptomyces Fi, endo-N-acetylmuramidase and Streptomyces N-acetylmuramyl-L-alanine amidase are all β-1,4-N-acetylglicosaminyl-N-acetylMuramic acid.

* From the Service de Bactériologie, Université de Liège, Belgium. Received August 1, 1968. This research has been supported by U. S. Department of Agriculture Grant UR-E4-10-2 under Public Law 480, and by the Fonds de la Recherche Fondamentale Collective, Brussels, Belgium (Contract No. 515).
† On leave for absence from the Service de Chimie Biologique, Faculté des Sciences de Lyon, Villeurbanne, France.
‡ Permanent address: Institut für Angewandte Botanik, Technische Hochschule, München, Germany.
of linkages: \( N'-(\text{d}-\text{alanyl})-\text{L-lysine} \) linkages which again extend from the \( \epsilon \)-amino group of \( \text{L-lysine} \) to the carboxyl group of \( \text{d-alanine} \) and \( \text{d-alanyl-L-alanine} \) linkages which assemble several identical peptide subunits in "head-to-tail" sequences.

In *Corynebacterium poinsetiae* and some other related *Corynebacteria* (Perkins, 1965, 1967), peptide subunits glycyl-\( \gamma \)-D-glutamyldonorsetine-D-alanine are bridged by means of a \( \delta \)-ornithine residue linked through its \( \delta \)-amino group to the \( \alpha \)-carboxyl group of the glutamic acid of one peptide subunit and through its \( \alpha \)-amino group to the carboxyl-terminal \( \delta \)-alanine of another peptide subunit. Previous studies (Miller et al., 1966, 1968) have indicated that the wall peptidoglycan in *Butyrivibacterium rettgeri* presents similar peculiarities. The nucleotide precursor accumulated in the presence of \( \text{D-}\text{cycloserine} \) has the sequence \( \text{N}''-(\text{UDP}-\text{N-acetyl-}
\text{muramyl-L-seryl-}\gamma\text{-D-glutamyl-L-ornithine}\). Moreover partial acid hydrolysis of the walls yielded significant concentration of peptides, the established structures of which were consistent with the hypothesis that the \( \alpha \)-carboxyl group of \( \text{D-glutamic acid} \) is substituted by the \( \epsilon \)-amino group of an \( \text{L- or D-lysine} \) residue. This paper reports the results of studies involving chemical and enzymatic degradations of the peptidoglycan of *B. rettgeri* by which means a direct establishment of its structure has been obtained.

Materials and Methods

Cell walls of *B. rettgeri* ATCC 10825 were prepared as previously described (Miller et al., 1966) from a cell suspension previously heated 30 min at 100° in inactivating autolytic enzymes.

Analytical Methods. They have been previously described (Ghuyse et al., 1966) and are summarized elsewhere in this journal (Campbell et al., 1969; van Heijenoort et al., 1969).


Chromatographic Separation of Amino Acid Derivatives and Peptides. (1) DNP-Ser, DNP-Glu, di-DNP-Orn, DNP-Ala, and di-DNP-Lys were separated on thin-layer plates of Stahl's silica gel (Merck) using solvent I; (2) \( \text{N}''\text{-DNP-Orn, N}''\text{-DNP-Lys, N}''\text{-DNP-}
\text{Orn, and N}''\text{-DNP-Lys (cited in order of increasing R} \) \text{f}) \) were separated (Figure 1) on silica gel using solvent II; (3) separation of peptide subunits (vide infra) was carried out on plates of cellulose MN 300 HR (Macherey, Nagel and Co., Düren) using solvent III.

Paper electrophoresis was carried out on Whatman No. 3MM paper in a Electrophoraphor apparatus Pleuger, Antwerp, Belgium, at pH 4 in pyridine–acetic acid–water (2.5:9:1000, v/v), at 20 V/cm for 2 hr.

Enzymes and conditions for isolation of products by gel filtration are described in Ghuyse et al. (1968) and Campbell et al. (1969). The Streptomyces KM endopeptidase used in the course of the present studies was prepared as described in Ghuyse et al. (1969).

Isomeric Configuration of \( \text{D-Alanine} \), \( \text{D-Lysine} \), and \( \text{D-Ornithine} \). Enzymatically, \( \text{D-Alanine} \) concentration was estimated using \( \text{D-amino acid oxidase} \) (electrophoretically purified DAOFF, 6506, Worthington Biochemicals) by (a) quantitating the amount of pyruvate formed (Ghuyse et al., 1966) or by (b) following the loss of total alanine by the fluorodinitrobenzene technique (Ghuyse et al., 1966). This latter method was also used for \( \text{D-ornithine} \) determination. The specificity and completion of the reaction were confirmed by controls consisting of a synthetic mixture of \( \text{DL-Ala}, \text{DL-Orn}, \text{DL-Glu}, \text{DL-Lys}, \) and L-Ser (25 mmol of each) incubated for 2 hr at 37° in the presence of FAD (1 μg) and \( \text{D-amino acid oxidase} \) (170 μg) in a final volume of 50 μl of 0.1 M \( \text{K}_2\text{HPO}_4 \) (pH 8.3). OPTICALLY. The configuration of the lysine and the ornithine was determined by measuring the optical rotation of the di-DNP derivatives, using a Perkin-Elmer Model M polarimeter with tubes of 1 dm and about 1-ml content.

Experimental Section

Estimation of the Number of Disaccharide Peptide Units. Analyses showed the presence in millimicromoles per milligram of walls of: glucosamine, 360; total hexosamines, 730; L-serine, 320; D-glutamic acid, 350; D-alanine, 290; LD-ornithine, 370; and D-lysine, 210.

If one assumes that, on the average, there are two disaccharide units, i.e., 2 ornithine or 1 ornithine and 1 lysine per peptide subunit, the above figures indicate the occurrence of about 320 mmol of disaccharide peptide units/mg of walls, with the following ratio: disaccharide, 1.12; L-Ser, 1; D-Glu, 1.10; D-Ala, 0.94; LD-Orn, 1.16; and D-Lys, 0.66.

Enzymatic Degradation of Walls of *B. rettgeri*. Streptomyces \( \text{F} \), endo-N-acetylaminosacharidase (50 μg of enzyme/ mg of walls, final volume 1 ml, 24 hr at 37°, in water) completely solubilizes the walls. At completion of the reaction 260 mmol of reducing groups equivalent to \( \beta,1,4\)-N-acetylglucosaminyl-N-acetylmuramic acid disaccharides are liberated, i.e., 80% of the total number of disaccharide peptide subunits.

Streptomyces \( \text{KM} \) endopeptidase also completely...
TABLE I: Terminal Groups of Walls of *B. rettgeri* after Enzymatic Degradations.\(^a\)

<table>
<thead>
<tr>
<th>Enzyme Used</th>
<th>(N)(^a)-Orn</th>
<th>(N)(^b)-Orn</th>
<th>(N)(^a)-Lys</th>
<th>(N)(^a)-Lys</th>
<th>C-Ala</th>
<th>C-Orn</th>
<th>C-Lys</th>
</tr>
</thead>
<tbody>
<tr>
<td>(F_1) endo-(N)-acetylMuramidase</td>
<td>0.18</td>
<td>0.92</td>
<td>0.26</td>
<td>0</td>
<td>0.47</td>
<td>0.55</td>
<td>0.62</td>
</tr>
<tr>
<td>KM endopeptidase</td>
<td>0.35</td>
<td>0.90</td>
<td>0.62</td>
<td>0</td>
<td>0.90</td>
<td>0.60</td>
<td>0.58</td>
</tr>
</tbody>
</table>

\(^a\) Results expressed in moles per mole of peptide subunit. On the average, peptide subunits are composed of L-Ser, 1; D-Glu, 1.10; D-Ala, 0.94; Ld-Orn, 1.16; and D-Lys, 0.66. All the lysine residues are thus C terminal.

TABLE II: Yields of Peptide Fragments Produced by Enzymatic Degradation of Walls of *B. rettgeri*.\(^a\)

<table>
<thead>
<tr>
<th>Enzymatic Treatment</th>
<th>Monomer</th>
<th>Oligomer with (K_0)</th>
<th>0.4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>(M_1)</td>
<td>(M_2)</td>
</tr>
<tr>
<td>(F_1) + amidase</td>
<td>18</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>KM endopeptidase + (F_1) + amidase</td>
<td>78</td>
<td>13</td>
<td>26</td>
</tr>
</tbody>
</table>

\(^a\) The yields are expressed per 100 total glutamic acid residues in the original walls.

Solubilizes the walls. Under the following conditions, 80 \(\mu\)g of enzyme/mg of walls, final volume 1 ml in water, complete clarification of the wall suspension and maximum liberation of \(N\)- and \(C\)-terminal groups occurred after 12-hr incubation at 37\(^\circ\). Table I presents the quantitation of \(N\)- and \(C\)-terminal groups after exhaustive wall degradation with \(F_1\) enzyme, \(i.e.,\) those groups present in the native walls and with KM endopeptidase.

Egg-white lysozyme only partially solubilizes the walls. However, lysozyme treatment of a KM endopeptidase digest of the walls (100 \(\mu\)g of enzyme/mg of degraded products in 300 \(\mu\)l of 0.03 \(M\) acetate buffer (pH 5.4), 24 hr at 37\(^\circ\)) resulted in the liberation of 260 \(\mu\) equiv of disaccharide units. Similar results were obtained with the \(F_1\) endo-\(N\)-acetylmuramidase.

*Streptomyces* \(N\)-acetylMuramyl-L-alanine amidase acting (under standard conditions) on walls previously degraded either with \(F_1\) enzyme alone or with KM endopeptidase and lysozyme used in sequence liberated terminal amino groups of serine. However, the quantitation of these amino groups was not reliable because of uncontrollable variations in the amount of DNP-serine destroyed under the experimental conditions. Actual data ranged from 150 to 260 \(\mu\) moles per mg of walls.

**Isolation of Peptides and Disaccharide Fragments from Walls of *B. rettgeri*. Procedure I.** The degraded products from 100 mg of walls treated sequentially with \(F_1\) endo-\(N\)-acetylmuramidase and \(N\)-acytylmuramyl-L-alanine amidase were fractionated by gel filtration in 0.1 \(M\) LiCl on two linked Sephadex G-30 and Sephadex G-25 columns under the conditions previously described (Ghuyesen et al., 1968; Campbell et al., 1969). The elution profile of the amino and reducing groups is shown in Figure 2. Calculation of the \(K_0\) values and comparison with results of similar experiments carried out in previous studies (Ghuyesen et al., 1968; Campbell et al., 1969) permitted the identification of a peptide monomer (mixed with free disaccharide units), a peptide dimer, and a peptide trimer. The peptide dimer and

![Figure 2](image_url)

**Figure 2:** Sephadex filtration in LiCl of *B. rettgeri* walls degraded with *Streptomyces* \(F_1\) endo-\(N\)-acetylmuramidase and \(N\)-acetylmuramyl-L-alanine amidase. Solid line: terminal amino group. Dotted line: reducing group.

![Figure 3](image_url)

**Figure 3:** Separation of peptide monomer (solid line) and disaccharide units (dotted line) by filtration on Sephadex G-25 in water.
the peptide trimer were purified by rechromatography on the linked Sephadex columns in 0.1 M LiCl and were desalted on Sephadex G-25 in water. This latter filtration also effected the separation of the peptide monomer and of the free disaccharide units (Figure 3).

PROCEDURE II. Walls (100 mg) were sequentially treated with KM endopeptidase, Streptomyces F1, endo-N-acetylmuramidase, and N-acetylmuramyl-L-alanine amidase. The elution profile of the degraded products filtered in 0.1 M LiCl on the two linked Sephadex G-50 and Sephadex G-25 columns is shown in Figure 4. Peptide monomer and disaccharide units were separated and purified by filtration on Sephadex G-25 in water. Peptide monomer, dimer, and trimer migrate as single cationic entities by paper electrophoresis at pH 4 (see Materials and Methods) but were not separable under those conditions. Table II presents the yields with which the four peptide fractions were obtained.

Characterization of the Peptide Monomer, Dimer, and Trimer. Cellulose thin-layer chromatography of the two peptide monomer fractions in solvent III (Figure 5) revealed the presence of four distinct compounds and yielded three fractions: M₃ (the compound with smaller Rₓ), M₄ (the compound with higher Rₓ), and M₅ (a mixture of two compounds M₆A and M₆B which could not be separated from each other under the present conditions). Table II also presents the yields with which M₁, M₂, and M₃ were obtained after enzymatic degradation of cell walls. Table III shows the amino acid and terminal group compositions of the two peptide monomers M₁ and M₂ and of the peptide dimer and trimer, as well as the terminal group compositions after a subsequent treatment of peptide dimer and peptide trimer with KM endopeptidase. Although not shown in Table III, terminal amino groups of serine were present in all the cases in amounts ranging from 0.6 to 0.9 per peptide subunit. As pointed out above, quantitation of these amino groups was not satisfactory and the data were not used as criteria for structural determination. Cellulose thin-layer chromatography using solvent III showed the peptide dimer and the peptide trimer to be quantitatively degraded into a mixture of M₄, M₅, and M₆ monomers by the action of KM endopeptidase (Table IV). Conditions for these degradations were as follows. Peptide subunit (1 μequiv) was incubated at 37°C, for 15 hr, with 150 μg of enzyme in a final volume of 500 μl of 0.01 M Veronal buffer (pH 9). Edman degradation of the peptide trimer yielded the data reported in Table V (see Discussion).

Configuration of Lysine and Ornithine. The lysine and ornithine residues present in acid hydrolysates of walls (6 n HCl, 100°C, 18 hr) were separated by equilibrium chromatography at 30°C on an Amberlite CG120 H⁺ column (0.9/45 cm) using 0.38 n (in Na⁺) sodium acetate buffer (pH 4.25). Desalting of lysine and ornithine was performed by adsorption on Dowex 50-X2 H⁺ and subsequent elution with 1 n aqueous ammonia. After freeze drying and dinitrophenylation, DNPH was removed from di-DNP diaminoc acids by sublimation. Molar optical rotation, [M]₀, of di-DNP-L-lysine and of di-DNP-orn in acetic acid was measured and found to equal +130 ± 13° and −197 ± 46°, respectively. According to Rao and Sober (1954), [M]₀ for di-DNP-d-lysine is +127° and for di-DNP-L-ornithine is −339°. All of the lysine residues of the wall peptidoglycan are thus L isomers. The major part of the ornithine residues occur as the L form but some D isomers are also present. The configuration of the ornithine residues present in acid hydrolysates of the peptide monomers M₁ and M₂ was also determined quantitatively by the loss of D-ornithine (using the fluorodinitrobenzene technique) after reaction with d-amino acid oxidase. The results are presented in Table III.
### TABLE IV: Yields of Peptide Monomers Produced by KM Endopeptidase Degradation of Dimer and Trimer Fractions.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimer</td>
<td>10</td>
<td>44</td>
<td>46</td>
<td>8</td>
<td>42</td>
<td>50</td>
</tr>
<tr>
<td>Trimer</td>
<td>12</td>
<td>42</td>
<td>46</td>
<td>6</td>
<td>38</td>
<td>56</td>
</tr>
</tbody>
</table>

- The yields are expressed per 100 total glutamic acid residues for each fraction. Theoretical yields are calculated assuming that each of the two peptides present in both the dimer and in the trimer fractions (see Figure 8 for the trimer) is itself a mixture of two compounds, in equimolar amounts, terminated at the C termini either by a β-alanine or by a L-ornithine residue.

### Partial Acid Hydrolysis of Dinitrophenylated Cell Walls

It has been reported by Miller et al. (1966) that partial acid hydrolysis (4 N HCl, 100°C, 1 hr) of B. reiteri walls yields the dipetide L-Orn-L-Ala. This dipetide has been isolated by bidimensional paper chromatography under the conditions described in Figure 6 (spot 9). A new analysis carried out on the extracted material showed also the presence of a small amount of a lysine-containing peptide. In order to ascertain the identity of this latter compound, dinitrophenylated walls (using the technique of Takebe, 1965) were submitted to partial acid hydrolysis (4 N HCl, 100°C, 1 hr). Bidimensional paper chromatography (Figure 6) revealed, at position 9, a spot of low intensity. Complete acid hydrolysis of the extracted material yielded alanine, lysine, and traces of ornithine; on the other hand, dinitrophenylation followed by acid hydrolysis yielded Nβ-DNP-lysine and DNP-alanine (in smaller amounts, which is probably due to the formation of diketopiperazine), thus demonstrating the presence in the walls of the sequence Nβ-Ala-Lys.

Bidimensional paper chromatography of the partial acid hydrolysate of the dinitrophenylated walls also yielded several dinitrophenyl derivatives. Identification of free amino acids and DNP-amino acids after complete acid hydrolysis and identification of dipetides arising from partial acid hydrolysis of compounds

### TABLE V: Release of N-Terminal Amino Groups by Repeated Cycles of Edman Degradation of the Peptide Trimer.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>N-Glu</th>
<th>Orn</th>
<th>N&lt;sup&gt;α&lt;/sup&gt;-Orn</th>
<th>Lys</th>
<th>N&lt;sup&gt;α&lt;/sup&gt;-Lys</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0.18</td>
<td>0</td>
<td>0.18</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0.30</td>
<td>0</td>
<td>0.30</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0.10</td>
<td>0</td>
<td>0.10</td>
</tr>
</tbody>
</table>
12-14 led to their tentative identifications (see legend of Figure 6).

The Disaccharide Units. Authentic β-1,4-N-acetylglucosaminy-N-acetylmuramic acid disaccharides isolated from Micrococcus lysodeikticus (Leyh-Bouille et al., 1966) and the B. rettgeri disaccharide were found indistinguishable under the following conditions: (1) paper chromatography in solvent VI, (2) estimation of glucosamine and muramic acid, (3) quantitative hydrolysis into N-acetylglucosamine and N-acetylmuramic acid with the help of the pig epididymis exo-β-N-acetylg glucosaminidase, and (4) estimation of the molar extinction coefficient with the Morgan-Elson reaction (acetamido groups) and with the Park-Johnson reaction (reducing groups).

Discussion

In a previous work by Miller et al. (1966), a nucleotide precursor of cell wall peptidoglycan had been isolated from B. rettgeri after inhibition by D-cycloserine. Determination of the configuration of the amino acids, end-groups analyses, and identification of hydrazinolysis and partial acid hydrolysis products established the peptide sequence N⁴-(L-Ser-γ-D-Glu)-L-Orn (Figure 7A). As is expected from the known mechanism of action of D-cycloserine (Roze and Strominger, 1966), the peptide moiety of the nucleotide precursor is a tripeptide, i.e., a complete precursor lacking the usual dipeptide D-Ala-D- Ala. The experiment sequence for the peptide subunits isolated at endo positions in the wall peptidoglycan is N⁴-(L-Ser-γ-D-Glu)-L-Orn-D-Ala (Figure 7B). This peptide monomer was not isolated from the wall enzymatic lysates, but was found as the mono-DNP derivative in the acid partial hydrolysate of dinitrophenylated walls (Figure 6). From the enzymatic lysates, two peptide monomers, namely M₁ and M₂, were isolated and identified. M₁ is a tetrapeptide (Table III), i.e., the tripeptide of the nucleotide precursor containing an additional γ-ornithine residue (Figure 7C). The two γ-ornithine residues are C-terminal. The L-ornithine residue has the ε-amino group free while the D-ornithine residue has the ε-amino group free. The structure of this tripeptide monomer is thus N⁴-[L-Ser-γ-(N⁴-α-D-Glu-D- Orn)]-L-Orn. M₂ is a pentapeptide (Table III), i.e., the tripeptide of the nucleotide precursor containing an additional γ-lysine residue with its ε-amino group free and an additional D-alanine residue which is C-terminal (Figure 7D). The structure of this pentapeptide monomer is thus N⁴-[L-Ser-γ-(N⁴-α-D-Glu-D-Lys)]-L-Orn-D-Ala. The DNP derivative of this peptide was also isolated from the partial acid hydrolysate of dinitrophenylated walls (Figure 6). Another peptide monomer fraction, the M₃ fraction, was isolated from the enzymatic lysates. Chromatographic properties are compatible with the presence of two peptide monomers: tetrapeptide M₁-A (Figure 7E), that is to say tetrapeptide M₁ with a D-lysine instead of a D-ornithine, and pentapeptide M₂-B (Figure 7F), that is to say pentapeptide M₂ with a D-ornithine residue instead of a D-lysine. It should be understood that peptide monomers M₁-A and -B were not characterized because the two compounds could not be separated from each other in a satisfactory way. In the native walls, about 18% of the peptide subunits are not cross-linked and the four monomeric species occur in approximately equivalent proportions: M₁, 4%; M₂, 6%; and M₃-A + M₃-B, 8% (Table II).

Degradation of the walls with KM endopeptidase liberates C-terminal D-alanine. Parallel to this, α-terminal amino groups of lysine and ε-terminal amino groups of ornithine are liberated in the proportion of 2:1 (Table I). It is thus evident that the D-lysine residues and the D-ornithine residues which substitute the ε-carboxyl group of the glutamic acid residues are, in the native walls, linked to C-terminal D-alanine residues. Another evidence for the existence of this kind of cross-linkage has been the isolation of N⁴-(D-Ala)-D-Lys from the wall partial acid hydrolysate (Figure 6). In other words, this amino acid is a bridge between peptide subunits for a major part of the peptidoglycan (2:3) and D-ornithine for a minor part (1:3). These "bridges" amino acids extend from the ε-carboxyl group of glutamic acid of one peptide subunit, to which they are linked through their ε- and δ-amino group, respectively, to the C-terminal D-alanine of another peptide subunit. The linkages specifically hydrolyzed by the KM endopeptidase are thus N⁴-(D-Ala)-D-Lys and N⁴-(D-Ala)-D-Orn. After degradation of the walls with KM endopeptidase, about 78% of the peptide subunits were found as monomers. The increase in monomer M₁, from 4 to 13%, after treatment with KM endopeptidase (Table II), is low. Evidently, in the native walls these subunits are at the C-terminal of peptide oligomers, since they lack the terminal D-alanine residue. The peptide monomers liberated by the endopeptidase are for the most part M₃ monomers and, in all probability, the homolog M₃-B. In the native walls, these monomers occur in the endo position as well as at the N termini.

Degradation of the walls with F₁ endo-N-acetylmuramidase yielded a peptide dimer and a peptide trimer (Table III). Both fractions are heterogeneous with respect to the nature of the C-terminal amino acid (a D-
alanine or a l-ornithine residue). The analytical data (amino acids and terminal groups composition) for a dimer fraction and for a trimer fraction in which bridging through D-lysine and through D-ornithine occurs in the ratio of 2:1, respectively, were calculated (see Table III, proposed models). In these calculations it was assumed that all the glutamic residues had their α-carboxyl groups substituted by one of the aforementioned D-diamino acids. On the other hand, the data relative to total d-alanine, C-alanine, and C-ornithine were calculated for the two extreme cases, that is to say assuming that the C-terminal of the peptide oligomers is either D-alanine or L-ornithine. Figure 8 represents the structure of such a peptide trimer fraction. As shown in Table III, the actual and the theoretical data relative to the peptide dimer and the peptide trimer fractions agree within the limit of experimental errors. This, together with the enzymatic degradation of the isolated peptide dimer and peptide trimer into monomers (Table IV), provides clear evidence that the proposed structural models are valid.

The foregoing structural demonstrations rest upon the assumption that the glutamic acid residue in the wall peptidoglycan is linked through its γ-carboxyl group to the L-ornithine residue as it is in the nucleotide precursor. Edman degradation of the peptide trimer fraction offered a way to confirm this thesis, at least with respect to that peptide trimer in which a D-lysine residue is used for peptide bridging. Indeed, in a preparation containing two peptide trimers bridged by D-lysine and one peptide trimer bridged by D-ornithine (Figure 8), four D-lysine residues and two D-ornithine residues per nine glutamic acid residues are in endo positions Consequently, their e- and δ-amino groups, respectively, should be exposed after the second cycle of an Edman degradation. Thus, for each terminal amino group of glutamic acid which appeared after the first cycle of the degradation, one could expect 0.44 N*-D-lysine and 0.22 N*-d-ornithine to be exposed by the subsequent degradation cycle. Table V shows that N*-lysine was exposed with an actual yield of 70% of the theoretical one. N*-Ornithine was also exposed. The actual yield, however, is difficult to estimate since after the first degradation cycle about 18% of the original N*-ornithine group had persisted. The wall of B. rettgeri, a species of the Propionibacteriaceae family, and that of Corynebacterium poinsettiae and other related plant pathogenic Corynebacteria (Perkins, 1965, 1967), are thus similar in that the interpeptide bridges of the peptidoglycan consist of a single d-diamino acid residue extending from the α-carboxyl group of the D-glutamic acid residue to the carboxyl group of the D-alanine residue. It had also been proposed that, in Microbacterium lacticum (Schleifer et al., 1967, 1968), glycolyl-three-3-hydroxyglutamyl-l-lysyl-d-alanine subunits are cross-linked by means of N*-glycyl)lysine dipeptides extending from three-3-hydroxyglutamic acid to d-alanine. It is not necessary to envisage any unusual reactions for the biosynthesis of this type of peptide bridging. It is known that pentapeptide subunits N*-D-Ala-γ-D-Glu-L-Lys-D-Ala-d-Ala, while in the form of lipid intermediates, undergo various modifications such as amidation of the α-carboxyl group of glutamic acid (in Staphylococcus aureus; Siewert and Strominger, 1968) or the substitution of the same carboxyl group of a glycine residue (in Microccocus lysodeikticus; Katz et al., 1967). Similar substitutions by a l-lysine, a d-ornithine, or an N*-glycyllysine grouping can thus be readily hypothesized. The final closure of the bridge between peptide subunits would then result from a transpeptidation (Wise and Park, 1965; Tipper and Strominger, 1965) involving the D-Ala-d-Ala dipeptide of a hexa- or octapeptide subunit, and an acceptor consisting of a second peptide subunit with the concomitant loss of the terminal d-alanine. This acceptor would be the α-amino group of d-ornithine in the case of C. poinsettiae, of D-lysine or d-ornithine in the case of B. rettgeri, or the e-amino group of l-lysine in the case of M. lacticum.

References

GUINAND, GHUYSEN, SCHLEIFER, AND KANDLER
Perkins, H. R. (1967), Biochem. J. 102, 29 C.