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^{α}-(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 C-terminal D-alanine residue of another peptide subunit. D-Lysine and D-ornithine bridges occur in the ratio of 2:1.

The *Streptomyces* KM endopeptidase hydrolyses the N^{α}-(D-alanyl)-D-lysine and N^{α}-(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* F_1 endo-*N*-acetylmuramidase and *Streptomyces* *N*-acetylmuramyl-L-alanine amidase are all β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid.

In many eubacteria, the peptide subunits N^{α}-(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 D-carbon of *meso*-diaminopimelyl of one peptide subunit, to the carboxyl group of the C-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).

In some micrococcal species (Schleifer and Kandler, 1967; Ghuysen *et al.*, 1968; Campbell *et al.*, 1969) the peptide subunits N^{α}-(L-alanyl-γ-α-D-glutamylglycine)-L-lysyl-D-alanine are cross-linked by means of two types

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of linkages: N^{ϵ}-(D-alanyl)-L-lysine linkages which again extend from the ϵ-amino group of L-lysine to the carboxyl group of D-alanine and D-alanyl-L-alanine linkages which assemble several identical peptide subunits in "head-to-tail" sequences.

In *Corynebacterium poinsettiae* and some other related *Corynebacteria* (Perkins, 1965, 1967), peptide subunits glycyl-γ-D-glutamylhomoseryl-D-alanine are bridged by means of a D-ornithine residue linked through its δ-amino group to the α-carboxyl group of the glutamic acid of one peptide subunit and through its α-amino group to the carboxyl-terminal D-alanine of another peptide subunit. Previous studies (Miller *et al.*, 1966, 1968) have indicated that the wall peptidoglycan in *Butyribacterium rettgeri* presents similar peculiarities. The nucleotide precursor accumulated in the presence of D-cycloserine has the sequence N^{α}-(UDP-N-acetyl-muramyl-L-seryl-γ-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 α-carboxyl group of D-glutamic acid is substituted by the ϵ-amino group of an 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° to inactivate autolytic enzymes.

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

Chromatographic Solvents. (I) Chloroform–methanol–acetic acid (88:10:2, v/v), (II) chloroform–methanol–acetic acid–water (65:25:13:8, v/v), (III) 1-butanol–pyridine–acetic acid–water (30:10:20:30, v/v), (IV) isopropyl alcohol–acetic acid–water (75:10:15, v/v), (V) α-picoline–concentrated NH$_4$OH–water (70:2:28, v/v), and (VI) 1-butanol–acetic acid–water (3:1:1, v/v).

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) N^{α}-DNP-Orn, N^{α}-DNP-Lys, N^{δ}-DNP-Orn, and N^{ϵ}-DNP-Lys (cited in order of increasing R_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 Electrorheophor 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 Ghuysen *et al.* (1968) and Campbell *et al.* (1969). The *Streptomyces* KM endo-
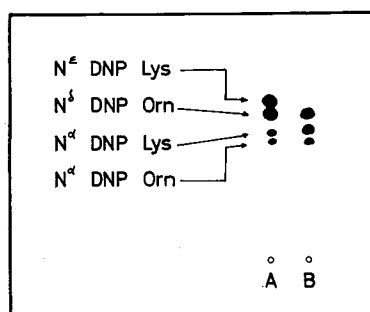

FIGURE 1: Separation of N^{α}-DNP-Orn, N^{α}-DNP-Lys, N-DNP-Orn, N^{ϵ}-DNP-Lys by silica gel thin-layer chromatography (solvent II). A = synthetic mixture; B = derivatives after dinitrophenylation of the KM endopeptidase-treated walls and hydrolysis.

peptidase used in the course of the present studies was prepared as described in Ghuysen *et al.* (1969).

Isomeric Configuration of D-Alanine, D-Lysine, and D-Ornithine. ENZYMATICALLY. D-Alanine concentration was estimated using D-amino acid oxidase (electrophoretically purified DAOFF, 6506, Worthington Biochemicals) by (a) quantitating the amount of pyruvate formed (Ghuysen *et al.*, 1966) or by (b) following the loss of total alanine by the fluorodinitrobenzene technique (Ghuysen *et al.*, 1966). This latter method was also used for D-ornithine determination. The specificity and completion of the reaction were confirmed by controls consisting of a synthetic mixture of DL-Ala, DL-Orn, DL-Glu, DL-Lys, and L-Ser (25 mμmoles of each) incubated for 2 hr at 37° in the presence of FAD (1 μg) and D-amino acid oxidase (170 μg) in a final volume of 50 μl of 0.1 M K$_4$P$_2$O$_7$ (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 diamino acids, *i.e.*, 2 ornithine or 1 ornithine and 1 lysine per peptide subunit, the above figures indicate the occurrence of about 320 mμmoles 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* F$_1$ endo-N-acetylmuramidase (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 mμmoles of reducing groups equivalent to β-1,4-N-acetylglucosaminyl-N-acetylmuramic acid disaccharides are liberated, *i.e.*, 80% of the total number of disaccharide peptide subunits.

Streptomyces KM endopeptidase also completely

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TABLE I: Terminal Groups of Walls of *B. rettgeri* after Enzymatic Degradations.[a]

Enzyme Used	N^α-Orn	N^δ-Orn	N^α-Lys	N^ϵ-Lys	C-Ala	C-Orn	C-Lys
F₁ endo-N-acetylmuramidase	0.18	0.92	0.26	0	0.47	0.55	0.62
KM endopeptidase	0.35	0.90	0.62	0	0.90	0.60	0.58

[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]

Enzymatic Treatment	Monomer				Dimer	Trimer	Oligomer with K_D 0.4
	Total	M₁	M₂	M₃			
F₁ + amidase	18	4	8	6	24	17	41
KM endopeptidase + F₁ + amidase	78	13	26	39	0	0	22

[a] The yields are expressed per 100 total glutamic acid residues in the original walls.

solubilizes the walls. Under the following conditions, 80 μg 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°. Table I presents the quantitation of N- and C-terminal groups after exhaustive wall degradation with F₁ 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 μg of enzyme/mg of degraded products in 300 μl of 0.03 M acetate buffer (pH 5.4), 24 hr at 37°) resulted in the liberation of 260 mμ-equiv of disaccharide units. Similar results were obtained with the F₁ endo-N-acetylmuramidase.

Streptomyces N-acetylmuramyl-L-alanine amidase acting (under standard conditions) on walls previously degraded either with F₁ 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 incontrollable variations in the amount of DNP-serine destroyed under the experimental conditions. Actual data ranged from 150 to 260 mμmoles 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 F₁ endo-N-acetylmuramidase and N-acetylmuramyl-L-alanine amidase were fractionated by gel filtration in 0.1 M LiCl on two linked Sephadex G-50 and Sephadex G-25 columns under the conditions previously described (Ghuysen 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_D values and comparison with results of similar experiments carried out in previous studies (Ghuysen 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
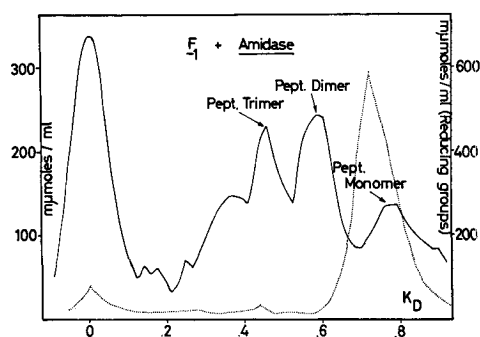

FIGURE 2: Sephadex filtration in LiCl of *B. rettgeri* walls degraded with *Streptomyces* F₁ endo-N-acetylmuramidase and N-acetylmuramyl-L-alanine amidase. Solid line: terminal amino group. Dotted line: reducing group.
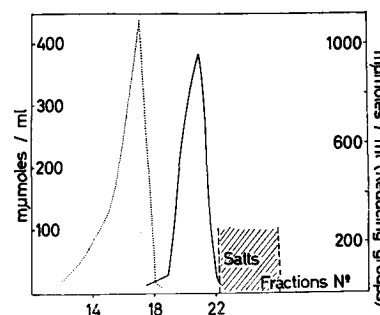

FIGURE 3: Separation of peptide monomer (solid line) and disaccharide units (dotted line) by filtration on Sephadex G-25 in water.
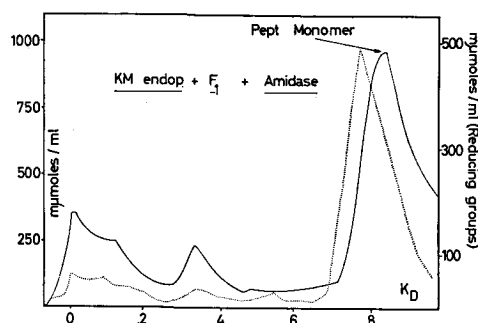
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FIGURE 4: Elution profile, from Sephadex G-50–G-25 in 0.1 M LiCl, of *B. rettgeri* walls degraded with *Streptomyces* KM endopeptidase, *Streptomyces* F_1 endo-N-acetylmuramidase and *Streptomyces* N-acetylmuramyl-L-alanine amidase. Solid line: terminal amino groups. Dotted line: reducing groups.

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* F_1 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: M_1 (the compound with smaller R_F), M_3 (the compound with higher R_F), and M_2 (a mixture of two compounds M_2A and M_2B which could not be separated from each other under the present conditions). Table II also presents the yields with which M_1, M_2, and M_3 were obtained after enzymatic degradation of cell walls. Table III shows the amino acid and terminal group compositions of the two peptide monomers M_1 and M_3 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 M_1, M_2, and M_3 monomers by the action of KM endopeptidase (Table IV). Conditions for these degradations were as
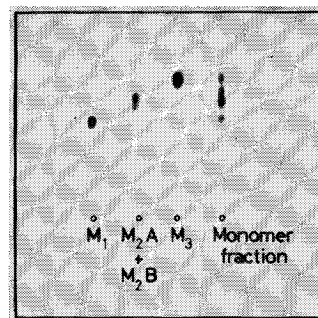

FIGURE 5: Separation of the monomer fraction into compounds M_1, M_2-A + M_2-B, and M_3, by preparative cellulose thin-layer chromatography using solvent III.

follows. Peptide subunit (1 μequiv) was incubated at 37°, 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°, 18 hr) were separated by equilibrium chromatography at 30° 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, DNPOH was removed from di-DNP diamino acids by sublimation. Molar optical rotation, $[M]_D$, of di-DNP-Lys 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), $[M]_D$ 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 D 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 M_1 and M_3 was also determined quantitating the loss of D-ornithine (using the fluorodinitrobenzene technique) after reaction with D-amino acid oxidase. The results are presented in Table III.
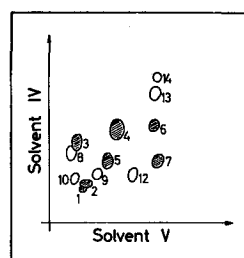
FIGURE 6: Chromatogram of a partial acid hydrolysate of dinitrophenylated cell walls of *B. rettgeri*. 1 = ornithine; 2 = lysine; 3 = glutamic acid; 4 = alanine; 5 = serine; 6 = muramic acid; 7 = glucosamine; 8 = L-Ser-D-Glu; 9 = N^α-D-Ala-D-Lys; 10 = N^ϵ-D-Glu-D-Lys; 12 = N^α-[L-Ser-γ-(N^ϵ-α-D-Glu-D-Lys)]-N^δ-DNP-L-Orn-D-Ala; 13 = N^α-(L-Ser-γ-D-Glu)-N^δ-DNP-L-Orn-D-Ala; 14 = N^α-(Mur-L-Ser-γ-D-Glu)-N^δ-DNP-L-Orn-D-Ala.

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TABLE III: Characterization of the Peptides Isolated from Walls of B. rettgeri (Chemical Data).[a]

Peptides	Amino Acid Composition					Treatment with Peptidase	Terminal Groups[c] before and after Treatment with KM Endopeptidase					
	Ser	Glu	Orn	Lys	Ala		N^{α}-Orn	N^{δ}-Orn	N^{α}-Lys	N^{ϵ}-Lys	C-Ala	C-Orn
Monomer M₁	1	1.15	1.85 {D 1, L 0.85}	0	0	0	0.75	0.93	0	0	0	2.70
Monomer M₃	1	1.17	1.11 {D 0, L 1.11}	1.18	1.17	0	0	1.0	0.84	0	1.17	0
Dimer (experimental)	1.12	0.90	1.10	0.55	0.80	0	0.16	1.13	0.27	0	0.35	0.50
						+	0.34	1.2	0.51	0	0.74	0.50
Dimer[b] (proposed model)	1	1	1.33	0.67	1–0.5	0	0.17	1.0	0.33	0	0.5–0	0.33–0.83
Trimer (experimental)	1.08	1.08	1.12	0.57	0.90	0	0.11	1.0	0.17	0	0.26	0.43
						+	0.29	1.03	0.43	0	0.85	0.44
Trimer[b] (proposed model)	1	1	1.33	0.67	1–0.67	0	0.11	1.0	0.12	0	0.33–0	0.33–0.55

[a] Results are expressed as moles per mole of peptide subunit (determined as the average value relative to Ser, Glu, and the data are not reported in this table. As shown in Table I, lysine is C terminal and the data are not reported in this table. [b] See Discussion and Figure 8. [c] For terminal amino groups of serine, see text.

TABLE IV: Yields of Peptide Monomers Produced by KM Endopeptidase Degradation of Dimer and Trimer Fractions.

	Actual Yields[a]			Theoretical Yields[b]		
Fraction	M₁	M₂	M₃	M₁	M₂	M₃
Dimer	10	44	46	8	42	50
Trimer	12	42	46	6	38	56

[a] The yields are expressed per 100 total glutamic acid residues for each fraction. [b] Theoretical yields are calculated assuming that each of the two peptides present both in 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 D-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°, 1 hr) of B. rettgeri walls yields the dipeptide L-Orn-D-Ala. This dipeptide has been isolated by bidimensional paper chromatography under the conditions described in Figure 6 (spot 9). A new analysis carried out on the extracted no. 9 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°, 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 N^{ϵ}-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 N^{α}-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 dipeptides 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.

Cycle	N-Glu	N^{α}-Orn	N^{δ}-Orn	N^{α}-Lys	N^{ϵ}-Lys
1	1	0	0.18	0	0
2	0	0	0.30	0	0.30
3	0	0	0.10	0	0.10

12–14 led to their tentative identifications (see legend of Figure 6).

The Disaccharide Units. Authentic β-1,4-*N*-acetylglucosaminyl-*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*-acetylglucosaminidase, 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 N^{α}-(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 expected sequence for the peptide subunits located at endo positions in the wall peptidoglycan is N^{α}-(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 M₃, were isolated and identified. M₁ is a tetrapeptide (Table III), *i.e.*, the tripeptide of the nucleotide precursor containing an additional D-ornithine residue (Figure 7C). The two ornithine residues are C terminal. The L-ornithine residue has the δ-amino group free while the D-ornithine residue has the α-amino group free. The structure of this tripeptide monomer is thus N^{α}-[L-Ser-γ-(N^{δ}-α-D-Glu-D-Orn)]-L-Orn. M₃ is a pentapeptide (Table III), *i.e.*, the tripeptide of the nucleotide precursor containing an additional D-lysine residue with its α-amino group free and an additional D-alanine residue which is C terminal (Figure 7D). The structure of this pentapeptide monomer is thus N^{α}-[L-Ser-γ-(N^{ϵ}-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 M₂ fraction, was isolated from the enzymatic lysates. Chromatographic properties are compatible with the presence of two peptide monomers: tetrapeptide M₂-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 M₃ with a D-ornithine residue instead of a D-lysine. It should be understood that peptide monomers M₂-A and -B were not characterized because
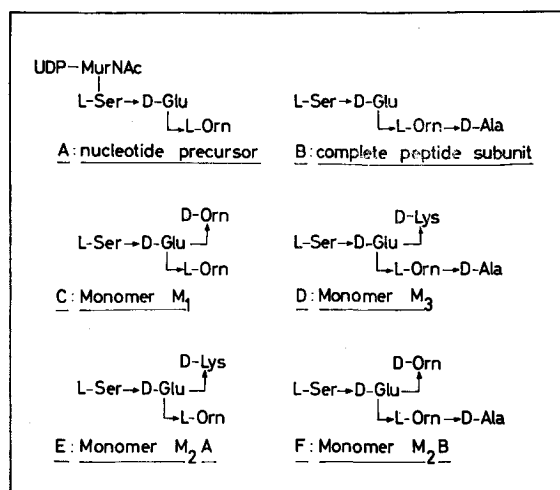

FIGURE 7: Structure of a precursor and of peptide fragments from walls of *B. rettgeri*.

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%; M₃, 6%; and M₂-A + M₂-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 α-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 α-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 N^{α}-(D-Ala)-D-Lys from the wall partial acid hydrolysate (Figure 6). In other words, D-lysine is the bridge between peptide subunits for a major part of the peptidoglycan (2:3) and D-ornithine for a minor part (1:3). These "bridge" amino acids extend from the α-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 N^{α}-(D-Ala)-D-Lys and N^{α}-(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 M₂-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-
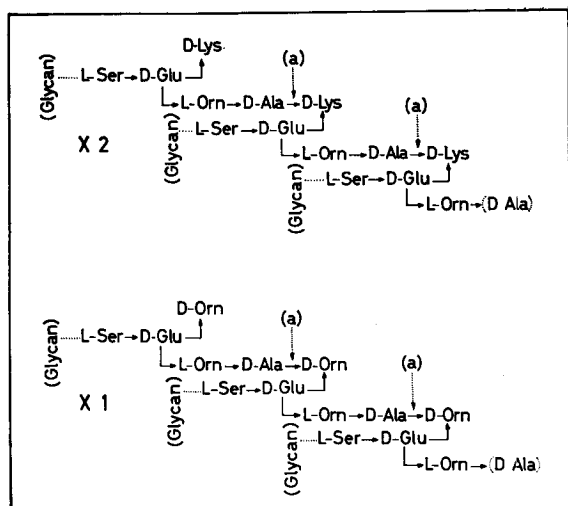
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FIGURE 8: Structural model for the peptide trimer fraction. For explanation, see text. (a) = site of action of the KM endopeptidase.

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 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 ϵ- 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^ϵ-D-lysine and 0.22 N^δ-D-ornithine to be exposed by the subsequent

degradation cycle. Table V shows that N^ϵ-lysine was exposed with an actual yield of 70% of the theoretical one. N^δ-Ornithine was also exposed. The actual yield, however, is difficult to estimate since after the first degradation cycle about 18% of the original N^δ-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), glycyl-threo-3-hydroxy-glutamyl-L-lysyl-D-alanine subunits are cross-linked by means of N^α-(glycyl)lysine dipeptides extending from threo-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^α-(L-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 Micrococcus lysodeikticus; Katz et al., 1967). Similar substitutions by a D-lysine, a D-ornithine, or an N^α-(glycyl)lysine 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 ϵ-amino group of L-lysine in the case of M. lacticum.

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