Characterization of the *Micrococcus lysodeikticus* Type of Peptidoglycan in Walls of Other *Micrococcaceae**

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ABSTRACT: Peptidoglycans in walls of *Micrococcus lysodeikticus*, *Micrococcus flavus*, *Micrococcus citreus*, and *Sarcina lutea* are composed of N^{α}-[L-alanyl-γ-(α-D-glutamylglycine)]-L-lysyl-D-alanine subunits, cross-linked through D-alanyl-L-alanine and N^{ϵ}-(D-alanyl)-L-lysine linkages, and do not contain any additional intervening amino acids. The D-alanyl-L-alanine peptide cross-linkages largely predominate. Although the number of peptide subunits is generally, but not always, greater than the number of disaccharide units in the glycan moiety, not all of the *N*-acetylmuramic acid residues are peptide substituted. Such a structure is of taxonomic importance since it differentiates the four aforementioned species from other members of the family *Micrococcaceae*. In *Micrococcus lysodeikticus*, *Micrococcus flavus*, and *Sarcina lutea*, segments of the glycan, consisting of 8–18 disaccharide units, are linked through 1 muramic acid phosphate to a segment, consisting of about 10 glucose residues, of a second wall polymer.

In all bacterial walls so far examined, the glycan portion of the rigid peptidoglycan layer consists of alternating β-1,4-linked *N*-acetylglucosamine and *N*-acetylmuramic acid residues. This consistency of structure, however, is not reflected in the peptide portion. In many *Micrococcaceae* (Muñoz *et al.*, 1966; Ghuysen *et al.*, 1967), the peptide subunits, which substitute through their N termini the D-lactic acid groups of the *N*-acetylmuramic acid residues of the glycan strands, have the sequence N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine. These peptide subunits are cross-linked by means of additional amino acids which are visualized as peptide bridges. In all cases, the peptide bridges have identical locations extending from the ε-amino group of L-lysine of one peptide subunit to the carboxyl group of the C-terminal D-alanine of another peptide subunit. The wall peptidoglycan in *Micrococcus lysodeikticus* has been shown to present marked differences (Ghuysen *et al.*, 1968). (1) The peptide subunits have the sequence N^{α}-[L-alanyl-γ-(α-D-glutamylglycine)]-L-lysyl-D-alanine. (2) Peptide cross-linking is not mediated by additional amino acid residues, but results from direct bonding between peptide subunits. (3) Two types of peptide cross-linkages are involved: N^{ϵ}-(D-alanyl)-L-lysine linkages extending from the ε-amino group of lysine of one peptide subunit to the C-terminal D-alanine of another, and D-alanyl-L-alanine linkages, or the so-called "head-to-tail" linkages, extending between the N-terminal alanine of one peptide subunit to the C-terminal alanine of another. The involvement of these two peptide cross-linkages is visualized as follows, if one considers two *N*-acetylmuramic acid residues occurring in adjacent glycan strands. (a) One of the *N*-acetylmuramic acid residues is substituted by a peptide subunit with the aforementioned structure. (b) The second *N*-acetylmuramic acid residue is substituted by a head-to-tail sequence of the same peptide subunits assembled by means of D-alanyl-L-alanine linkages. Variations in the lengths of these latter sequences occur throughout the peptidoglycan net, with, however, an average polymer size of six peptide subunits occuring in major part of it. (c) The ε-amino group of L-lysine of the substituent peptide subunit and the C-terminal D-alanine of the substituent head-to-tail sequence are joined together through a N^{ϵ}-(D-alanyl)-L-lysine linkage. (4) Because of this particular structure of the peptide moiety, long segments of the glycan strands are not substituted with peptide. Actually, at least 50% of the *N*-acetylmuramic acid residues occur with free carboxyl groups. A representation of the structure of the *M. lysodeikticus* peptidoglycan is shown in Figure 13 of Ghuysen *et al.* (1968). The purpose of the present studies is to demonstrate that this type of structure is not restricted to *M. lysodeikticus* but is found in some other members of the same family and may, therefore, be of taxonomic importance. In addition, the role of organic phosphate in these walls, which lack teichoic acids, has been studied.

Materials and Methods

Cell Walls. The cell walls of *M. lysodeikticus*, strain NCTC 2665, of *Sarcina lutea*, strain R 262 (Pasteur Institute, Paris), of *Micrococcus citreus*, strain R 266 (Pasteur Institute), and of *Micrococcus flavus*, strain 53160 (Pasteur Institute), were prepared as in previous studies (Petit *et al.*, 1966; Leyh-Bouille *et al.*, 1966;

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TABLE I: Chemical Composition and Terminal Groups of Walls of *M. lysodeikticus*, *S. lutea*, *M. flavus*, and *M. citreus*, before and after Enzymatic Degradations.

Walls	D-Glucose[b]	P[b]	Peptide[a,b] Subunits	Disac Units[b,h,i]	Amino Acid Composition[c] Ala	Glu	Gly	Lys	Asp	Terminal Groups after Enzymatic Degradations[c] Enzyme Used	Reducing Groups[e]	*N*$^{\epsilon}$-Lys	*N*-Ala	*C*-Ala	*C*-Gly	*C*-Glu + *C*-Asp
										Glycosidase[d]	0.41	0.60	0.00	0.20	0.97	
M. lysodeikticus	260	26	480	480	1.95	0.95	1.0	1.02	0.00	ML endop[e]		0.82	0.00	0.35	1.00	
										Myxo[f]		0.60	0.80	0.63	0.97	
										Glycosidase	0.50	0.99	0.02	0.00	1.20	0.7
S. lutea	230	19	400	240	2.04	1.37	1.0	0.98	0.27	ML endop		1.31	0.02	0.00	1.10	0.7
										Myxo		0.93	0.73	0.54	1.00	0.7
										Glycosidase	0.45	0.90	0.03	0.00	1.03	
M. flavus	275	36	610	280	1.93	1.10	1.0	0.92	0.00	ML endop		1.12	0.04	0.20	1.12	
										Myxo		0.90	0.80	0.80	0.94	
										Glycosidase[g]	0.26	0.87	0.07	0.00	0.98	
M. citreus	46	70	455	340	1.97	0.95	1.0	0.92	0.00	ML endop		0.99	0.10	0.09	0.95	
										Myxo		0.68	0.98	0.72	0.93	

[a] Estimated on the basis of the glycine content of the walls. [b] Estimated on the basis of the glucosamine content of the wall. [c] Expressed relative to a glucose standard. [d] Glycosidase = F_1 endo-*N*-acetylmuramidase. [e] ML endop = *Streptomyces* ML endopeptidase. [f] Myxo = *Myxobacter* ALI protease. [g] *M. citreus* walls were only partially solubilized by this enzyme (36%). [h] In millimicromoles per milligram of walls. [i] In moles per mole of peptide subunit. [j] Disac, disaccharide.

Ghuysen *et al.*, 1968). Their chemical composition is given in Table I.

Analytical Methods. Identification and quantitation of free amino acids and N-terminal groups (fluorodinitrobenzene technique), of D- and L-alanine (enzymatic procedures), of C-terminal groups (hydrazine technique), of reducing groups (Park–Johnson method), of acetamido sugars (Morgan–Elson reaction), of amino sugars (Morgan–Elson reaction after chemical reacetylation) and of glucosamine (using yeast D-glucosamine 6-phosphate *N*-acetylase) have been previously described (Ghuysen *et al.*, 1966, 1968). Measurement of free D-glucose was done using the D-glucose oxidase–*o*-anisidine reaction (glucostat reagent, Worthington Biochemical Corp.). Measurement of inorganic and organic phosphate was performed according to Lowry *et al.* (1954). Reduction with $NaBH_4$ was performed as described by Leyh-Bouille *et al.* (1966) and Edman degradations as described by Tipper *et al.* (1967a).

Paper electrophoresis was carried out on Whatman No. 3MM paper (40 × 40 cm) in an Electrorheophor Pleuger apparatus, at pH 5 in pyridine–acetic acid–water (4:2:1000, v/v), and at pH 2 in 0.1 *M* formic acid.

Chromatography. A. The solvents used were: (I) 1-butanol–pyridine–water (6:4:3, v/v), (II) 1-butanol–acetic acid–water (3:1:1, v/v), (III) pyridine–water (4:1, v/v), (IV) isobutyric acid–ethanolamine–water (3:325:1675, v/v).

B. Locating reagents. Oligosaccharides from the peptidoglycan were detected by fluorescence after alkaline treatment of the paper (Sharon, 1964). Amino sugars, amino acids, and free amino groups were detected with ninhydrin. Sugars and amino sugars were detected with alkaline silver nitrate. Phosphorylated compounds were detected by the procedure of Bandurski and Axelrod (1951) using the spray of Hanes and Isherwood (1949).

Gel Filtration. Sephadex G-50 fine (particle size 20–80 μ) and Sephadex G-25 fine (particle size 20–80 μ) were used. The gel filtration properties of the compounds are exposed in terms of distribution coefficient, K_D: $(V_e - V_0)/V_i$, where V_e is the elution volume of the compound, V_i is the volume of the solvent imbibed by the gel particles, and V_0 is the void volume of the column.

Enzymes. The following enzyme preparations were those used in the study of the *M. lysodeikticus* walls (Ghuysen *et al.*, 1968): egg-white lysozyme, *Streptomyces* F_1 endo-*N*-acetylmuramidase, *Streptomyces* ML endopeptidase, and *Myxobacter* AL I protease (a generous gift from Dr. J. C. Ensign). *N*-Acetylmuramyl-L-alanine amidase was prepared as described in Ghuysen *et al.* (1969). Yeast-D-glucosamine 6-phosphate *N*-acetylase (Brown, 1962) was a gift from Dr. R. Tinelli. Pig epididymis exo-β-*N*-acetylglucosaminidase was prepared according to Sanderson *et al.* (1962). D-Glucose oxidase (Worthington Biochemical Corp.) and alkaline phosphatase from *Escherichia coli*, type III (10 mg/ml) (Sigma Chemical Co.), were obtained commercially.

Experimental Section

Cell Wall Composition and Degradation with Myxo-

bacter AL I and ML-Endopeptidases. The demonstration of the structure in *M. lysodeikticus* rested upon the use of two lytic enzymes (Ghuysen *et al.*, 1968): (1) the *Myxobacter* AL I enzyme (Ensign and Wolf, 1966) whose activity on this type of walls is restricted to the hydrolysis of the D-alanyl-L-alanine linkages in the peptide moiety and to the hydrolysis of *N*-acetylmuramyl-L-alanine linkages at the junction between the glycan strands and the peptide substituents and (2) the *Streptomyces* ML endopeptidase whose activity is restricted to the hydrolysis of N^{ϵ}-(D-alanyl)-L-lysine linkages. Table I presents the composition of whole walls of *M. lysodeikticus*, *Sarcina lutea*, *M. flavus*, and *M. citreus* as well as the results of determination of reducing groups and of N- and C-terminal amino acids after degradation with an endo-*N*-acetylmuramidase (*Streptomyces* F_1 enzyme or egg-white lysozyme), and with either *Streptomyces* ML endopeptidase or *Myxobacter* AL I protease. The data reported in Table I were those obtained at completion of the enzymatic degradations, as evidenced by kinetic experiments. Conditions for the degradations were as follows: F_1 enzyme, 50 μg/mg of walls and 0.01 M citrate buffer (pH 5.0); lysozyme, 50 μg/mg of walls and 0.01 M phosphate buffer (pH 6.5); ML endopeptidase, 6 μg/mg of walls and 0.01 M Veronal buffer (pH 9); and *Myxobacter* AL I protease, 70 units/mg of walls and 0.01 M Veronal buffer (pH 9). It should be understood that the N- and C-terminal groups detected in the endo-*N*-acetylmuramidase degraded products are those present in the native walls. From Table I, one observes that (1) alanine, glutamic acid, glycine, and lysine occur in walls of *M. lysodeikticus*, *M. citreus*, and *M. flavus* in the 2:1:1:1 ratio. The same ratio is true for the peptidoglycan of *S. lutea* although aspartic acid and an excess of glutamic acid are detected with walls. As shown by hydrazinolysis, the aspartic acid and the excess glutamic acid both occur with the two C-terminal groups free and further studies have shown them to be constituents of nonpeptidoglycan polymers. (2) In native walls, N- and C-alanine termini occur as traces or are below the limit of detection whereas all the glycine residues have their carboxyl groups unsubstituted. (3) Solubilization of the walls by *Myxobacter* AL I protease evidently involves cleavage of alanylalanine linkages. (4) Hydrolysis of *N*-acetylmuramyl-L-alanine linkages through the action of *Myxobacter* AL I enzyme is difficult to detect (this hydrolysis should be reflected by the difference between the N- and C-terminal alanine groups liberated by the enzymatic treatment). (5) The lytic action of the ML endopeptidase is accompanied by a release of a small but detectable amount of C-terminal alanine. The parallel increase in ε-amino group of lysine that one would expect is below the sensitivity of the detection procedure because of the presence of a large amount of ε-amino groups of lysine in the native walls. The foregoing properties are, of course, compatible with the hypothesis that the peptidoglycans of the four *Micrococcaceae* have the same type of structure, that is to say that N^{α}-[L-Ala-γ(α-D-Glu-Gly)]-L-Lys-D-Ala subunits are cross-linked mainly through D-Ala-L-Ala linkages, and for a minor part through N^{ϵ}-(D-Ala)-L-Lys linkages and that the extent of peptide substitution of the glycan strands is very low. (6) Amino sugar determination (after HCl hydrolysis of the walls) shows that in all cell walls, but those of *M. lysodeikticus*, there are much fewer disaccharide units in the glycan moiety than there are peptide subunits in the peptide moiety of the respective peptidoglycans. (7) In all the cell walls tested, with the exception of *M. citreus*, the ratios P/glucose are approximately equal, one phosphate occuring for 8 to 12 glucose residues.

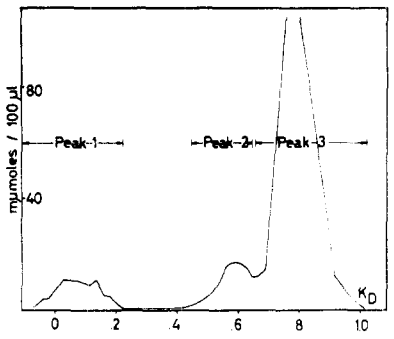

FIGURE 1: Sephadex filtration in LiCl of *S. lutea* cell walls degraded with *Myxobacter* AL 1 protease. For conditions, see text. Solid line: terminal amino groups.

Characterization of the Peptide Subunits and of the N^{ϵ}-(D-Alanyl)-L-lysine Linkages. Because of the two enzymatic activities of the *Myxobacter* AL I protease (see above), the one incubation of the walls with this enzyme results both in the degradation of the peptide moiety and in the liberation of the peptide fragments from the glycan strands. In the case of *M. lysodeikticus* (Ghuysen *et al.*, 1968), it has been proved that the two main peptide fragments liberated are (1) the peptide subunits (monomer) with the aforementioned structure and (2) a peptide dimer in which two peptide subunits are linked by N^{ϵ}-(D-alanyl)-L-lysine linkages (the so-called peptide dimer Ala-Lys). Walls of *S. lutea*, *M. flavus*, and *M. citreus* were degraded with *Myxobacter* AL I enzyme and the degraded products were fractionated by filtration in 0.1 M LiCl on two columns of Sephadex G-50 and G-25 connected in series. The conditions for the enzymatic degradations and for the gel filtration were identical with those previously described (Ghuysen *et al.*, 1968). The amino groups fractionated mainly into three fractions: a peptide monomer (K_D = 0.75), a peptide dimer (K_D = 0.60), and a high molecular weight fraction (excluded). This excluded material contained all the hexosamine residues, the organic phosphate, and the glucose residues present in the walls. Figure 1 is an illustration of such a fractionation. Table II presents the composition and the determination of N- and C-terminal groups of the peptide monomers and peptide dimers, as well as terminal groups determination of the peptide dimers after subsequent treatment with the ML endopeptidase. This latter enzyme is active on N^{ϵ}-(D-alanyl)-L-lysine linkages (see above) and the degradation of the peptide dimers into monomers was confirmed by thin-layer chromatography on silica gel in solvent IV as previously described (Ghuysen *et al.*, 1968). Moreover, a ratio of L-lysine to D-alanine to L-alanine of 1:1:1 was found in the peptide subunits of *S. lutea* and

TABLE II: Characterization of the Peptides Isolated from the Walls of *S. lutea*, *M. flavus*, and *M. citreus*.

Walls	Peptides	Yields[a] and Endopeptidase[b] Used (%)	Amine Acid Composition				Terminal Groups before and after Subsequent Treatment with Endopeptidase				
			Ala	Glu	Gly	Lys	Endopeptidase[b] Used	N^{ϵ}-Lys	N-Ala	C-Ala	C-Gly
S. lutea	Monomer	Myxo (60)	2.04	1.08	1.0	1.10	None	1.30	1.10	1.20	1.05
		ML (7)	1.40	0.97	1.0	0.95	None	0.95	0.84	0.70	0.93
	Dimer	Myxo (23)	1.89	0.99	1.0	1.01	None	0.58	0.81	0.47	1.04
							ML	1.0	0.82	1.01	1.05
		ML (31)	1.85	0.91	1.0	0.90	None	0.98	0.54	0.49	0.88
							Myxo	0.92	0.99	0.88	0.94
M. flavus	Monomer	Myxo (88)	2.0	1.08	1.0	1.0	None	1.10	0.93	1.07	1.17
	Dimer	Myxo (6)	2.0	1.14	1.0	0.86	None	0.52	0.90	0.52	1.10
							ML	0.98	0.84	0.90	0.87
M. citreus	Monomer	Myxo (61)	1.95	0.97	1.0	1.0	None	1.08	0.95	0.90	0.96
	Dimer	Myxo (8)	2.10	0.96	1.0	0.99	None	0.52	0.94	0.60	0.97
							ML	1.03	0.90	0.88	0.91

[a] Actual recoveries expressed in per cent of total amino acids. [b] Myxo = *Myxobacter* AL 1 protease; ML = *Streptomyces* ML endopeptidase. Data relevant to cell walls of *M. lysodeikticus;* see Tables II and III in Ghuysen *et al.* (1968).

the sequence Ala → Glu → Gly was established by Edman degradation. The yields obtained in this sequential degradation were similar to those previously reported for the peptide monomer of *M. lysodeikticus* (Tipper *et al.*, 1967a; Ghuysen *et al.*, 1968). From the foregoing it is thus evident that N^{α}-[L-Ala-γ-(α-D-Glu-Gly)]-L-Lys-D-Ala is the peptide subunit in the four micrococcal walls and that N^{ϵ}-(D-Ala)-L-Lys linkages are involved in peptide cross-linking.

Characterization of D-*Alanyl*-L-*alanine Linkages.* As it is pointed out above, the existence of such peptide cross-linkages is obvious from the N- and C-terminal groups which are liberated from the walls by *Myxobacter* AL I enzyme (Table I). Nevertheless, the peptide dimer in which two peptide subunits are linked through a D-Ala-L-Ala linkage, the so-called peptide dimer Ala-Ala, has been isolated from walls of *S. lutea*. The walls were degraded with the ML endopeptidase and the liberation of the degraded peptides was accomplished by subsequent splitting of the glycan strands with lysozyme and a final treatment with *N*-acetylmuramyl-L-alanine amidase (to be operational, this latter enzyme requires the glycan first to be split). The ensuing fractionation of the degraded products by filtration in 0.1 M LiCl on the linked Sephadex G-50 and G-25 columns (Figure 2) yielded the peptide subunit (monomer), the peptide dimer (mixed with oligosaccharide debris from which they were not further separated), and a gel-excluded material containing the organic phosphate and the glucose polymer with some peptidoglycan debris attached to them. Peptide monomer and peptide dimer Ala-Ala were characterized by the usual techniques (Table II) and the degradation of the dimer into monomers using the *Myxobacter* AL I enzyme was confirmed by thin layer chromatography in solvent IV. Table II, finally, presents the actual yields with which peptide monomers and the two isomeric peptide dimers were produced from the micrococcal cell walls.

Peptide Substitution of the Glycan Strands. Cell walls of *S. lutea* were degraded with lysozyme. Filtration of the degraded products in 0.1 M LiCl on linked Sephadex G-50–Sephadex G-25 columns yielded four fractions (Figure 3): 1 (K_D = 0), 2 (K_D = 0.2), 3 (K_D = 0.5), and 4 (K_D = 0.7). They were desalted on Sephadex G-25. The excluded material, K_D = 0, was treated with *N*-acetylmuramyl-L-alanine amidase and refiltered on the same Sephadex columns system (Figure 4). Fractions A (K_D = 0) and B (K_D = 0.7) were desalted as above. Characterization of fractions 4 and B as β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharides rested upon the following determinations. (1) Acid hydrolysis followed by quantitation of total hexosamines and of glucosamine revealed half the hexosamine residues to be glucosamine; (2) treatment with pig epididymis exo-β-*N*-acetylglucosaminidase, an enzyme specific for β-glycosidic linkages, liberated *N*-acetylglucosamine and *N*-acetylmuramic acid in equimolar amounts and established the β anomery of the link; (3) $NaBH_4$ reduction destroyed all of the muramic acid, half the total hexosamines, and none of the glucosamine; and (4) determination of the molar extinction coefficient in the Morgan–Elson reaction (30-min heating in borate) showed the glycosidic link to be 1:4, not 1:6. Analyses of fractions 2 and 3 revealed oligosaccharide com-

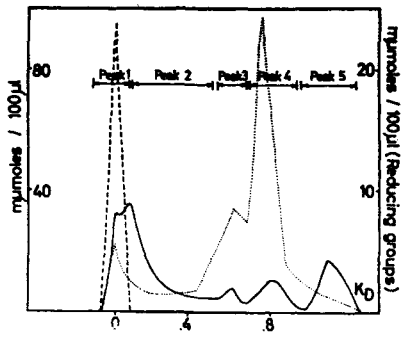

FIGURE 2: Sephadex filtration in LiCl of *S. lutea* cell walls degraded sequentially with ML endopeptidase, lysozyme, and *N*-acetyl-muramyl-L-alanine amidase. For conditions, see text. Broken line: glucose; solid line: terminal amino groups; dotted line: reducing groups (right ordinate).

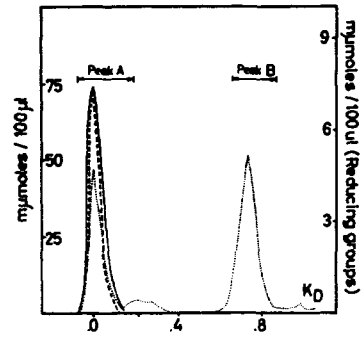

FIGURE 4: Sephadex filtration in LiCl of peak I (Figure 3) of a lysozyme digest of *S. lutea* cell walls, treated subsequently with *N*-acetylmuramylalanine amidase. For conditions, see text. Broken line: terminal amino groups; solid line: glucose; dotted line: reducing groups (right ordinate).

pounds with equivalent amounts of *N*-acetylglucosamine and *N*-acetylmuramic acid residues. Reduction with $NaBH_4$, R_F values in solvent II, together with the K_D values on the Sephadex columns (Figure 3), showed that they were, respectively, tetrasaccharide and oligosaccharide, with, in each case, *N*-acetylmuramic acid at the reducing end. Walls of *M. flavus* and *M. citreus* were similarly treated by lysozyme and fractionated on the linked Sephadex columns. Figure 5 is the elution profile of the products of lysozyme digestion of *M. flavus* cell walls, and is also typical of the results obtained with *M. citreus*. The material trailing off after the single excluded peak was, in both cases, pooled, desalted, concentrated, and examined for the presence of free unsubstituted di- or tetrasaccharides by descending paper chromatography in solvent II. No such substances could be detected as a result of lysozyme action on the walls of these two organisms. Thus depending upon the species, up to 52% of the total *N*-acetylmuramic acids of the glycan strands are not peptide substituted. These values are minimal since not all the *N*-acetylmuramic acid residues present in the gel-excluded fractions are necessarily linked to the intact peptide moiety. They may behave as high molecular compounds because of their attachment to wall polymers other than the peptidoglycan.

Muramic Acid Phosphate in Cell Walls of M. lysodeikticus, S. lutea, and M. flavus. The walls of *M. lysodeikticus* are known to contain a polysaccharide which is solely composed of D-glucose and 2-acetamido-2-deoxymannuronic acid, most likely in equimolar amounts (Perkins, 1963). Although Perkins (1963) and Prasad and Litwack (1965) reported that *M. lysodeikticus* walls were free of phosphate, the studies of Liu and Gotschlich (1967) and Montague and Moulds (1967) confirmed previous observations made by Salton (1953) according to which organic phosphate occurs in amounts ranging from 0.09 to 0.13% of the dry weight of the walls. Phosphate in walls of *M. lysodeikticus* and in those of *S. lutea* and *M. flavus* was detected in the course of the present studies in amounts ranging from 25 to 35 mμmoles per mg of walls (Table I). Organic phosphate and, glucose were always quantitatively recovered in the gel-excluded material from the degraded cell walls. Their concentration is often used to obtain an accurate estimate of non-peptidoglycan wall components. Kinetics of the release of phosphomonoester groups upon HCl hydrolysis of these soluble wall compounds were followed. The conditions finally selected were 6 N HCl, 100°, 90 min because they effected a quantitative conversion into phosphomonoester groups with a minimum release of P_i (about 10%). Acid hydrolysates of the gel-excluded fractions from walls of *M. lysodeikticus*, *M. flavus*, and *S. lutea* were adsorbed on 20-ml columns of AG 50W-

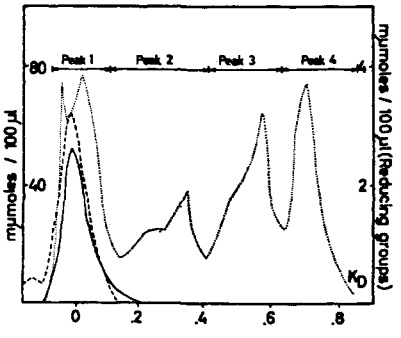

FIGURE 3: Sephadex filtration in LiCl of *S. lutea* cell walls degraded with lysozyme. For conditions, see text. Solid line: terminal amino groups; broken line: glucose; dotted line: reducing groups (right ordinate).

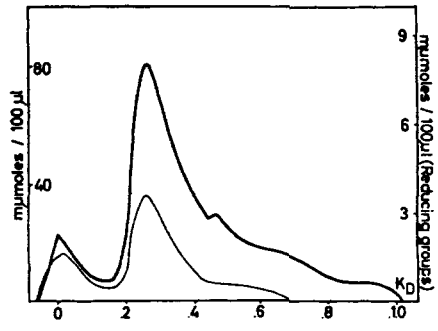

FIGURE 5: Sephadex filtration in LiCl of *M. flavus;* cell walls degraded with lysozyme. For conditions, see text. Thin line: terminal amino groups; thick line: reducing groups (right ordinate).

TABLE III: Chemical Composition of Glucose–Aminohexuronic acid–Glycopeptide Complexes.

	Polysaccharide Moiety		Peptidoglycan Moiety	
	Glucose	Phosphate[a]	Glucosamine	Peptide Subunit
Walls	10	1.0	17.3[d]	17.3
Perkin's preparation	10	?	2.6	1.6
ML preparation[b]	10	1.0	2.5[d]	0.8
Lys preparation[c]	10	1.0	3.3[d]	1.1

[a] As muramic acid phosphate exclusively. [b] Obtained after degradation of the walls with *Streptomyces* ML endopeptidase, lysozyme, and *N*-acetylmuramyl-L-alanine amidase. [c] Obtained after degradation of the walls with lysozyme. [d] Quantitated by the enzymatic assay (Ghuysen *et al.*, 1966). Results are expressed in millimicromoles or millimicroequivalents, relative to glucose.

X8 H^+ Bio-Rad resin which were further washed with water. Glucose, muramic acid 6-phosphate, previously identified in walls of *M. lysodeikticus* and of other gram-positive bacteria by Liu and Gotschlich (1967), and a phosphorylated amino sugar neutral at pH 5 were sequentially eluted with little mutual contamination. Muramic acid phosphate was characterized by quantitation of the phosphomonoester groups and of the hexosamine residues (found in the ratio 1:1) and by paper chromatography in solvents I and II before and after alkaline phosphatase treatment. The phosphorylated amino sugar compound neutral at pH 5 is readily distinguishable from muramic acid phosphate which is anionic under these conditions. The amino sugar moiety liberated by the alkaline phosphatase was found to be cationic at pH 5 but presented paper chromatographic properties different from these of glucosamine. This phosphorylated amino sugar, also detected in wall acid hydrolysates of *M. lysodeikticus* by Montague and Moulds (1967), is actually a degradation product as evidenced by the fact that known muramic acid 6-phosphate, when treated with 6 N HCl for 90 min at 100°, was partially transformed into a phosphorylated amino sugar neutral at pH 5. Muramic acid 6-phosphate from wall fractions of *M. lysodeikticus*, *M. flavus*, and *S. lutea* was isolated with actual yields of 70–80% with respect to the total organic phosphate residues. Evidently, all of the organic phosphate present in these walls is engaged solely in this type of grouping.

Significance of Muramic Acid Phosphate in Walls of M. lysodeikticus. Table III presents the chemical composition of three preparations of the glucose–aminohexuronic acid polymer of *M. lysodeikticus*. The three preparations present striking similarities, albeit they were obtained through entirely different procedures. One preparation was isolated by Perkins (1963) from trichloroacetic acid extracts of the walls by paper electrophoresis using a continuous flow apparatus. The presence of organically bound phosphorus was not, however, recognized by this author. A second preparation was obtained from walls which had been thoroughly degraded by ML endopeptidase, lysozyme, and *N*-acetylmuramyl-L-alanine amidase used in sequence. The material excluded from the linked Sephadex G-50 and Sephadex G-25 columns, in 0.1 M LiCl, was desalted on Sephadex G-50. In terms of glucose and phosphate, the polysaccharide polymer was eventually obtained with a yield of 96%. A third preparation was isolated from wall which had been treated with lysozyme only. A series of gel filtrations (1) on Sephadex G-50 in 0.1 M LiCl; (2) on linked Sephadex G-50–Sephadex G-25 columns first in 0.1 M LiCl and then in water (two runs) yielded fractions progressively enriched in polysaccharide polymer (final yields, 56%). Paper chromatography in a variety of solvents of the two polysaccharide fractions, prepared by the enzymatic procedures, did not provide any separation of the constituents. They behaved as a single anionic compound by paper electrophoresis at pH 5. Exhaustive treatment with ML endopeptidase or with *Myxobacter* AL I enzyme did not cause liberation of peptides.

Amino acid analyses and C- and N-terminal determination showed the presence, per phosphate residue, of a peptide subunit of established structure. *N*-Acetylmuramyl-L-alanine amidase, however, failed to remove it. Besides the few amino acid residues, glucose, glucosamine, a smaller amount of muramic acid, and muramic acid phosphate were detected by paper chromatography of acid hydrolysates (6 N HCl, 90 min, 100°) in solvents I–III. Chemical acetylation of the acid hydrolysates, followed by Morgan–Elson reaction (7-min heating in borate), gave a color development indicative of 9.5 amino sugars/phosphate (expressed in glucosamine equivalents). This figure, however, very imperfectly reflects the aminohexuronic acid content of the fractions since this compound is rapidly broken under acid treatment and since its molar coefficient extinction in the above reaction is not known (Perkins, 1963). Exo-β-*N*-acetylglucosaminidase liberated about 1.40 residues of *N*-acetylglucosamine/phosphate, *i.e.*, about 40% of the total glucosamine residues from intact polysaccharide preparation. Borohydride reduction did not affect the glucose or the glucosamine content of the complexes, but destroyed 3.6 hexosamine equiv. Not all the muramic acid was destroyed and therefore the above figure suggests that some aminohexuronic acid residues are at the reducing ends of the polysaccharide complex. From the foregoing, the enzymatically pre-

pared polysaccharide complexes appear to be composed of a tetra- or hexasaccharide, *i.e.*, a glycan fragment substituted by a peptide monomer, linked to the glucose–aminohexuronic acid polymer through a phosphodiester bond emanating from C_6 of one of the *N*-acetylmuramic acid residues of the glycan fragments. On the average, such a linking group occurs for every 10 glucose residues of the polysaccharide bulk. Nothing is known, however, about the polysaccharide part of the phosphodiester bond. This part is preferentially cleaved by acid hydrolysis since muramic acid phosphate is quantitatively produced. This hydrolysis proceeds at a rather low rate; less than 10% of the phosphomonoester groups are produced under treatment with 1 N HCl at 37°, for 24 hr. On the other hand, no phosphomonoester groups were produced by alkaline treatment (1 N NaOH, 4 hr, 60°) which suggests the absence of a vicinal hydroxyl group.

Discussion

Two types of peptidoglycan emerge from an integration of the structural studies made on walls of *Micrococcaceae* and *Lactobacillaceae*. In a first type, the peptide subunits N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine (Muñoz *et al.*, 1966) are interlinked *via* peptide bridges extending from the ϵ-amino group of L-lysine to C-terminal D-alanine. All of the *N*-acetylmuramic acid residues in the glycan strands are substituted by peptide subunits, both occuring in equimolar amounts. At present, one distinguishes four subgroups in this type of peptidoglycan. Subgroup A: the bridges are composed of L-amino acid residues: *Micrococcus roseus* (Petit *et al.*, 1966), *Arthrobacter crystallopoietes* (Krulwich *et al.*, 1967), and *Streptococcus pyogenes* (Muñoz *et al.*, 1966); subgroup B: the bridges are composed of glycine residues: *Staphylococcus aureus* (Mandelstam and Strominger, 1961; Petit *et al.*, 1966); subgroup C: the bridges are composed of glycine and L-amino acids: *Staphylococcus epidermidis* (Tipper, 1968), *Gaffkya tetragena* (M. Lache, unpublished data); and subgroup D: the bridges are composed of one D-isoasparaginyl residue: *Streptococcus faecalis* (Ghuysen *et al.*, 1967), *Lactobacillus casei* (Hungerer, 1968), and *Lactobacillus acidophilus* (J. Coyette, unpublished data). In a second type of peptidoglycan, peptide subunits N^{α}[L-alanyl-γ-(α-D-glutamylglycine)]-L-lysyl-D-alanine are cross-linked *via* D-alanyl-L-alanine and N^{ϵ}-(D-alanyl)-L-lysine linkages. Examples are the peptidoglycans in *M. lysodeikticus*, *M. flavus*, *M. citreus*, and *S. lutea*. Variations occur, according to the species, with respect to the number of D-Ala-L-Ala and N^{ϵ}-(D-Ala)-L-Lys linkages although the former ones always largely predominate. Peptide substitution of the glycan strands is infrequent; hence, long unsubstituted segments of the glycan strands do not directly contribute to the rigidity and insolubility of the peptidoglycan network. In *M. lysodeikticus*, the 1:1 ratio between β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharide units and the peptide subunits, which, of course, reflects the mechanisms involved in the peptidoglycan biosynthesis, is preserved. In *M. flavus*, *M. citreus*, and *S. lutea*, there is a pronounced deficit in disaccharide units. One may hypothesize that after the peptides have been transferred from disaccharide units to form head-to-tail peptide sequences according to the mechanism previously proposed (Schleifer and Kandler, 1967; Ghuysen *et al.*, 1968), the initiation in the glycan strands of new acceptor sites through the action of an endo-*N*-acetylmuramidase autolysin results in the loss of some glycan segments which are free of peptide substituents.

The walls of *M. flavus* and *M. citreus* are only partially digested by lysozyme, but all of the material solubilized by this enzyme appears to be substituted with peptide. This suggests that essentially all of the glycan which lacks peptide substitution (as well as some of the substituted glycan) is protected from lysozyme action and remains insoluble, either because of a liaison with non-peptidoglycan polymers, or *O*-acetyl substitution, or both. In any case continuous segments large enough to remain insoluble after lysozyme treatment obviously exist.

Another character of the walls of *M. lysodeikticus*, *M. flavus*, and *S. lutea* is that they all contain organic phosphate and glucose in a ratio close to 1:10. The glycan moiety of the wall peptidoglycan is linked to the glucose-containing polysaccharide through a phosphodiester bond emanating from C_6 of one of the *N*-acetylmuramic acid residue. A complex [(glucose–aminohexuronic acid)$_{10}$-(P)$_1$-(oligosaccharide-peptide subunit)$_1$] has been obtained from walls of *M. lysodeikticus* degraded by endo-*N*-acetylmuramidase without damaging the peptide moiety. One can thus postulate that in the native walls, phosphate bridging and peptide cross-linking do not occur in close vicinity on the glycan strands. On the other hand, one phosphate group occurs every 8–18 disaccharide units according to the species. By analogy with glycan chain length determinations carried out on *S. aureus* (Tipper *et al.*, 1967b), the figures of 8–18 disaccharide units may well represent the average chain length of the glycan strands in *M. lysodeikticus*, *M. flavus*, and *S. lutea*. Since phosphate bridging and peptide cross-linking seem not to occur on the same portion of the glycan strands, not many phosphate bridges can substitute a single strand. Hence, it may be that, on the average, each glycan strand of 8–18 disaccharides in length is joined through one phosphodiester bridge to a portion of the other wall polysaccharide constituent containing 10 glucose residues. It is not known if aminouronic acid occurs in the walls of *M. flavus* and *S. lutea* as it does in those of *M. lysodeikticus*. It should be understood that the above hypothesis does not imply that the wall polysaccharides of these three micrococci are identical.

References

Bandurski, R. S., and Axelrod, B. (1951), *J. Biol. Chem. 193*, 405.

Brown, D. H. (1962), *in* Methoden der Enzymatischen Analyse, Bergmeyer, H. V., Ed., Weinheim, Verlag Chemie, p 151.

Ensign, J. C., and Wolfe, R. S. (1966), *J. Bacteriol. 91*, 524.

Ghuysen, J.-M., Bricas, E., Lache, M., and Leyh-Bouille, M. (1968), *Biochemistry 7*, 1450.
Ghuysen, J.-M., Bricas, E., Leyh-Bouille, M., Lache, M., and Shockman, G. D. (1967), *Biochemistry 6*, 2607.
Ghuysen, J.-M., Dierickx, L., Coyette, J., Leyh-Bouille, M., Guiand, M., and Campbell, J. N. (1969), *Biochemistry 8*, 213 (this issue; paper IV).
Ghuysen, J. M., Tipper, D. J., and Strominger, J. L. (1966), *Methods Enzymol. 8*, 685.
Hanes, C. S., and Isherwood, F. A. (1949), *Nature 164*, 1107.
Hungerer, D. (1968), *Fed. Proc. 447*, 294.
Krulwich, T. A., Ensign, J. C., Tipper, D. J., and Strominger, J. L. (1967), *J. Bacteriol. 94*, 741.
Leyh-Bouille, M., Ghuysen, J.-M., Tipper, D. J., and Strominger, J. L. (1966), *Biochemistry 5*, 3079.
Liu, T. Y., and Gotschlich, E. C. (1967), *J. Biol. Chem. 3*, 471.
Lowry, O. H., Roberts, N. R., Leiner, K. Y., Wu, M. L., and Farr, A. L. (1954), *J. Biol. Chem. 207*, 1.
Mandelstam, M., and Strominger, J. L. (1961), *Biochem. Biophys. Res. Commun. 5*, 466.
Montague, M. D., and Moulds, J. D. (1967), *Biochim. Biophys. Acta 135*, 565.
Muñoz, E., Ghuysen, J.-M., Leyh-Bouille, M., Petit, J. F., Heymann, H., Bricas, E., and Leirancier, P. (1966), *Biochemistry 5*, 3748.
Perkins, H. (1963), *Biochem. J. 86*, 475.
Petit, J. F., Muñoz, E., and Ghuysen, J.-M. (1966), *Biochemistry 5*, 2764.
Prasad, A., and Litwack, G. (1965), *Biochemistry 4*, 496.
Salton, M. R. J. (1953), *Biochim. Biophys. Acta 10*, 512.
Sanderson, A. R., Strominger, J. L., and Nathenson, S. G. (1962), *J. Biol. Chem. 237*, 3603.
Schleifer, K. H., and Kandler, O. (1967), *Biochem. Biophys. Res. Commun. 28*, 965.
Sharon, N. (1964), *Proc. 3rd Symp. Fleming's Lysozyme, Milan*, p 44/R.T.
Tipper, D. J. (1968), *Fed. Proc. 446*, 294.
Tipper, D. J., Katz, W., Strominger, J. L., and Ghuysen, J.-M. (1967a), *Biochemistry 6*, 921.
Tipper, D. J., Strominger, J. L., and Ensign, J. C. (1967b), *Biochemistry 6*, 906.

The Peptidoglycan in Walls of *Butyribacterium rettgeri**

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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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