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Wall Peptidoglycan in *Aerococcus viridans* Strains 201 Evans and ATCC 11563 and in *Gaffkya homari* Strain ATCC 10400*

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ABSTRACT: The wall peptidoglycans in *Gaffkya homari* ATCC 10400 and in *Aerococcus viridans* 201 Evans and ATCC 11563 are made up of peptide units L-alanyl-γ-D-glutamyl-L-lysyl-D-alanine and L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine.

The yields of the peptide fragments obtained after enzymatic degradations of the walls are consistent with a peptidoglycan structure in which all the *N*-acetylmuramic acid residues in the glycan strands are peptide-substituted and in which about half of the peptide units occur as uncross-linked monomers whereas the others form dimers by means of N^{ϵ}-(D-alanyl)-L-lysine linkages. These walls are the first known examples of lysine-containing peptidoglycans of chemotype I.

Bacterial wall peptidoglycans are networks of glycan strands interconnected through peptide chains. The glycan strands consist of alternating β-1,4-linked *N*-acetylglucosamine and *N*-acetylmuramic acid. The *N*-acetylmuramic acid residues or at least some of them are substituted by L-alanyl-γ-D-glutamyl-L-R_3-D-alanine peptide units which in turn are cross-linked by bridges extending from the C-terminal D-alanine of one peptide unit either to the ω-amino group of the L-R_3 diamino acid or to the α-carboxyl group of D-glutamic acid of another peptide unit (Ghuysen, 1968). Depending upon the composition and the location of the peptide bridges, there appear four peptidoglycan chemotypes (Ghuysen, 1968). For a long time, chemotype I peptidoglycans, in which the peptide bridging is mediated *via* direct N^{ω}-(D-alanyl)-L-R_3 bonds without any intervening additional amino acids, seemed to occur solely in wall peptidoglycans containing *meso*-diaminopimelic acid at the R_3 position (*i.e.*, in Gram-positive *Bacillaceae*, in *Escherichia coli*, and probably in all Gram-negative bacteria (van Heijenoort *et al.*, 1969). The experiments presented herein show that the same type of peptide bridging also occurs in some wall peptidoglycans containing L-lysine at the R_3 position.

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Materials and Methods

Walls. Aerococcus viridans 201 Evans, *A. viridans* ATCC 11563, and *Gaffkya homari* ATCC 10400 were grown and the corresponding walls were isolated and purified as previously described (Schleifer and Kandler, 1967). Disrupted cells were heated 20 min at 100° in order to inactivate the autolysins.

Enzymes. The following enzymes were used. *Chalaropsis* B *endo-N*-acetylmuramidase (a gift from Dr. N. A. Hash) hydrolyzes β-1,4-*N*-acetylmuramyl-*N*-acetylglucosamine in endo position in the glycan strands. *Streptomyces N*-acetylmuramyl-L-alanine amidase hydrolyzes the linkages at the junction between the peptide and the glycan moieties (after prior degradation of the glycan with an *endo-N*-acetylmuramidase). *Streptomyces* ML endopeptidase hydrolyzes N^{ϵ}-(D-alanyl)-L-lysine linkages, in endo position, in the peptide moiety. *Streptomyces* aminopeptidase liberates the L-alanine residue from the amino terminus of L-alanyl-D-isoglutaminyl peptides. There is a strict requirement for the α-carboxyl group of D-glutamic acid to be substituted by an amide group. The enzyme is known to be inactive or similar amide-free L-alanyl-γ-D-glutamyl peptides. Pig epididymis *exo-N*-acetylglucosaminidase specifically hydrolyzes β-linked *N*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharides. The use of these enzymes in determination of wall structure has been recently reviewed (Ghuysen, 1968).

Analytical Methods. Reducing groups (Park and Johnson procedure), acetamido sugars (Morgan–Elson reaction), amino sugars (Morgan–Elson reaction after chemical *N*-acetylation), glucosamine (using yeast D-glucosamine 6-phosphate *N*-acetylase), amino acids (fluorodinitrobenzene technique), D-alanine (enzymatic procedure), and N- and C-terminal groups (fluorodinitrobenzene and hydrazine techniques, respectively) were measured as previously described (Ghuysen *et al.*, 1966b, 1968). Edman degradation was carried out as described in Tipper *et al.* (1967). Partial acid hydrolyses of walls, chromatographic analyses of the hydrolysates, and identification of peptides were carried out as previously described (Schleifer and Kandler, 1967). $NaBH_4$ reduction was carried out as described in Leyh-Bouille *et al.* (1966).

Electrophoreses were carried out on Whatman No. 3MM paper using an Electrorheophor apparatus, Pleuger, in water–pyridine–acetic acid (1000:3:9, v/v, pH 3.7).

Chromatographic Solvents. The following solvents were used: (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) 1-butanol–acetic acid–water (3:1:1, v/v); (V) 2-propanol–acetic acid–water (75:10:15, v/v); (VI) α-picoline–ammonia (*d* 0.91)–water (70:2:28, v/v).

Chromatography. Separations of dinitrophenyl- (DNP)-glutamic acid, DNP-alanine, and di-DNP-lysine were performed on plates of Stahl's silica gel G (Merck) using solvent I and separation of N^{ϵ}-DNP-lysine on silica gel plates using solvent II. Peptide units were chromatographed on plates of cellulose MN 300 HR (Machery, Nagel and Co., Düren, West Germany) using solvent III. Disaccharides were chromatographed on paper Whatman No. 1 using solvent IV. Products of partial acid hydrolysis of walls were separated by bidimensional chromatography on Schleicher and Schüll 2043 b paper using first solvent IV and then solvent V.

Gel filtrations were carried out on two columns, 400 ml each, of Sephadex G-50 fine and Sephadex G-25 fine connected in series. The filtration properties were expressed in terms of distribution coefficients $K_D = (V_e - V_0)/V_i$ with V_e = elution volume, $V_0 = V_e$ of totally excluded material, and $V_i = V_e$ of NaCl $- V_0$. Desalting was performed by filtration on Sephadex G-25 in water.

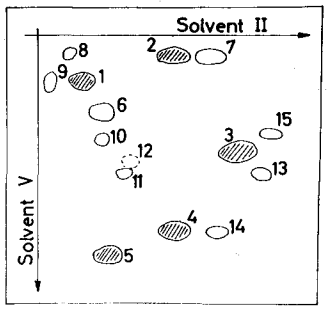

FIGURE 1: Chromatogram of a partial acid hydrolysate of walls of *G. homari* and *A. viridans* 201 and 11563: 1 = lysine; 2 = glutamic acid; 3 = alanine; 4 = muramic acid; 5 = glucosamine; 6 = L-lysyl-D-alanine; 7 = L-alanyl-D-glutamic acid; 8 = γ-D-glutamyl-L-lysine; 9 = a mixture of peptides containing glutamic acid, lysine, and alanine; 10 = N^{ϵ}-(D-alanyl)-L-lysine; 11 = N^{ϵ}-(*D*-alanyl)-L-lysyl-D-alanine; 12 = L-lysyl-D-alanyl-D-alanine; 13 = D-alanyl-D-alanine (spots 12 and 13 were detected only in hydrolysates from walls of *G. homari*); 14 = muramyl-L-alanine; 15 = muramyl-L-alanyl-D-glutamic acid (spots 14 and 15 were detected only after 10-min hydrolyses). Configuration of lysine and glutamic acid has been determined by optical rotatory dispersion of the dinitrophenyl derivatives (to be published elsewhere).

Experimental Results

Chemical Composition of Walls and Sensitivity to Lytic Enzymes. The walls of the three selected bacteria contained, per milligram, about 650 to 700 nequiv of a peptidoglycan solely composed of glucosamine, muramic acid, L- and D-alanine, L-lysine, and D-glutamic acid. These components occurred in equimolar amounts with the exception of D-alanine (0.3–0.7 residue per L-lysine residue). Such a composition is reminiscent of that of the peptidoglycan of *E. coli* except that in the latter *meso*-diaminopimelic acid replaces L-lysine. The nonpeptidoglycan components of the walls were not thoroughly studied. Paper chromatography of acid hydrolysates of each of the three selected walls, however, revealed the presence of polysaccharides composed, at least in part, of glucose, rhamnose, and galactosamine. In addition, walls of *G. homari* also contained galactose. The selected walls of *A. viridans 201*, *A. viridans* 11563, and *G. homari* contained 0.7, 0.44, and 0.48% by weight of organic phosphate, respectively. No teichoic acids were detected. Egg-white lysozyme exerted a weak lytic effect on walls of *G. homari* and *A. viridans* sp. No complete solubilization could be obtained. On the contrary, *Chalaropsis* B *endo-N*-acetylmuramidase readily solubilized them (2.5 μg of enzyme per mg of walls, in 100 μl of 0.02 M acetate buffer, pH 4.5, at 37°). At completion of the reaction (2 hr under the above conditions), the color development obtained with the Morgan–Elson reaction (30 min of heating in borate) was consistent with the liberation of about 400 nmoles of peptide-substituted disaccharide units (yield of hydrolysis, 60%). Subsequent treatment with *Streptomyces N*-acetylmuramyl-L-alanine amidase (in 0.025 M acetate buffer, pH 5.4) liberated one terminal amino group of L-alanine per L-lysine residue, thus demonstrating that L-alanine was solely engaged in the binding of the peptide moiety to the glycan strands and that all the

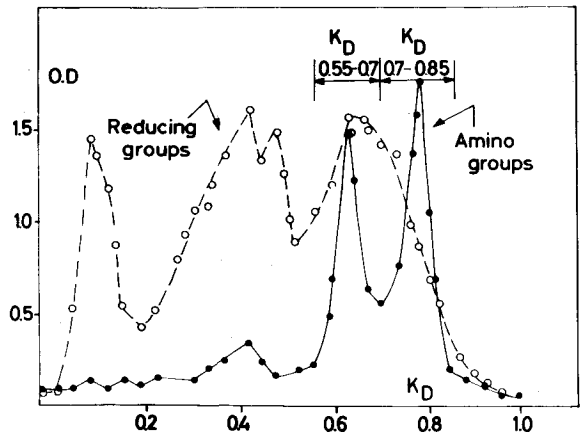

FIGURE 2: Elution profile, from Sephadex G-50–G-25 in 0.05 M LiCl, of walls of *A. viridans* 201 degraded with *Chalaropsis* B *endo-N*-acetylmuramidase and *Streptomyces N*-acetylmuramyl-L-alanine amidase: solid line, terminal amino groups; dotted line, reducing groups. Results are expressed in optical density. Aliquots of 100 μl and 200 μl (from 7-ml fractions) were used for amino groups and reducing groups estimations, respectively. Degraded walls of *A. viridans* 11563 and of *G. homari* gave almost identical elution profiles.

N-acetylmuramic acid residues were substituted by peptide units. Terminal amino group estimations indicated that about 60–80% of the lysine residues had their ε-amino groups not engaged in cross-linking. Treatment with the *Streptomyces* ML endopeptidase (in 0.01 M Veronal buffer, pH 9) either on intact walls (which were solubilized during the process) or on *Chalaropsis*- and amidase-degraded products resulted in the hydrolysis of N^{ϵ}-(D-alanyl)-L-lysine linkages. At completion of the reaction, one terminal ε-amino group of lysine occurred per peptide unit.

Partial Acid Hydrolysis of Walls. Partial acid hydrolyses (1 hr, 100°, 4 N HCl, 1 ml for 5 mg of walls) of the walls from the three selected bacteria gave rise (Figure 1) to dipeptide N^{ϵ}-(D-alanyl)-L-lysine and tripeptide N^{ϵ}-(D-alanyl)-L-lysyl-D-alanine as well as to dipeptide γ-D-glutamyl-L-lysine and tripeptide γ-D-glutamyl-L-lysyl-D-alanine, thus providing evidence for the occurrence of N^{ϵ}-(D-alanyl)-L-lysine interpeptide linkages and for the occurrence of γ-glutamyl linkages.

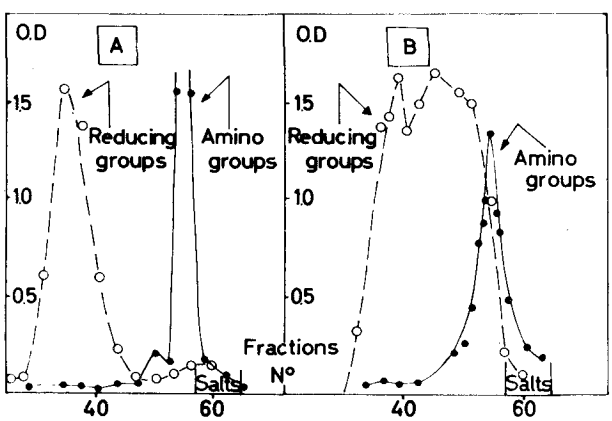

FIGURE 3: Elution profile, from Sephadex G-25 in water, of disaccharide units (dotted line) and peptide compounds (solid line) from degraded walls of *A. viridans* 201: A, fractions K_D 0.7–0.85 of Figure 2; B, fractions K_D 0.55–0.7 of Figure 2. Results are expressed as in Figure 2. Identical results were obtained with degraded walls of *A. viridans* 11563 and of *G. homari*.

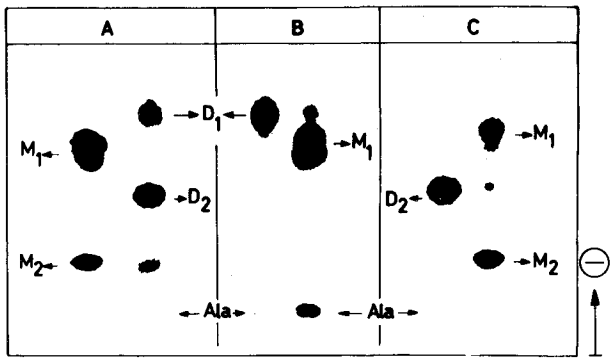

FIGURE 4: Paper electrophoresis at pH 3.75 of: (A) peptides M_1 and M_2 (from fractions K_D 0.7–0.85; Figure 2) and peptides D_1 and D_2 (from fractions K_D 0.55–0.7; Figure 2); (B) isolated peptides D_1 before and after treatment with ML endopeptidase; (C) isolated peptides D_2 before and after treatment with ML endopeptidase; Ala = position of alanine; detection, ninhydrin.

Small amounts of D-alanyl-D-alanine and of L-lysyl-D-alanyl-D-alanine were also detected in hydrolysates of walls of *G. homari*, thus showing that in this latter case some of the peptide units had retained the C-terminal D-alanyl-D-alanine sequence that is believed to occur in all peptidoglycan nucleotide precursors.

Enzymatic Degradation of Walls. ISOLATION OF PEPTIDES M_1 AND M_2, OF PEPTIDES D_1 AND D_2, AND OF DISACCHARIDE UNITS. Walls (200 mg) were treated sequentially with *Chalaropsis* B *endo-N*-acetylmuramidase and with *Streptomyces N*-acetylmuramyl-L-alanine amidase and the degraded products were fractionated by gel filtration in 0.05 M LiCl on two columns of Sephadex G-50 and Sephadex G-25 connected in series (see Materials and Methods). The elution profile of the products from walls of *A. viridans* 201 is shown in Figure 2. Similar profiles were obtained with the degraded walls of *G. homari* and *A. viridans* 11563. Fractions K_D 0.55–0.7 and K_D 0.7–0.85, altogether, contained in all cases 70–80% of the wall peptide units, and 50–60% of the wall glycan moieties in the form of free disaccharide units. Glycan fragments larger than disaccharides resulting from incomplete hydrolysis of the glycan moiety (yield of hydrolysis was 60% of the theoretical, see above) as well as the non-peptidoglycan polysaccharide components of the walls were eluted at K_D values 0–0.5. These fractions were not further studied. Fractions K_D 0.7–0.85 from the three selected bacteria

TABLE I: Actual Yields in Peptides M_1, M_2, D_1, and D_2 after Degradation of the Walls with *Chalaropsis* B *endo-N*-acetylmuramidase and *Streptomyces N*-Acetylmuramyl-L-alanine Amidase. Results Are Expressed in Per Cent of Total Peptide Units.

	Fractions K_D 0.7–0.85[a]		Fractions K_D 0.55–0.70[a]	
	M_1	M_2	D_1	D_2
A. viridans 201	8.5	25.5	20	30
A. viridans 11563	8	24	7	23
G. homari	34	0	26	14

[a] See Figures 2 and 3.

TABLE II: Structures of Peptides M_1, M_2, D_1, and D_2. Amino Acid Composition, Terminal Groups before and after Treatment with ML Endopeptidase, and Effect of Aminopeptidase.

Peptide	Origin		Amino Acid Composition			Terminal Groups					
			Glu	Ala	Lys	N^{ϵ}-Lys	N^{ϵ}-Lys after ML Endopeptidase	C-Ala	C-Ala after ML Endopeptidase	*N*-Ala	Free L-Ala after Aminopeptidase
M_1	*G. homari*	Experimental	1.04	1.94	1.00	1.06	1.06	0.80	0.80	1.01	1.00
		Theoretical[a]	1	2	1	1	1	1	1	1	1
	A. viridans										
	11563	Experimental	1.00	1.16	1.00	0.96	0.96	0.21	0.21	0.98	0.95
	201	Experimental	1.11	1.18	1.00	1.01	1.01			0.67	0.70
		Theoretical[b]	1	1.2	1	1	1	0.2	0.2	1	1
M_2	*A. viridans*										
	11563	Experimental	1.16	1.22	1.00	0.90	0.90	0.20	0.20	0.78	0.10
		Theoretical[c]	1	1.2	1	1	1	0.2	0.2	1	0
D_1	*G. homari*	Experimental	1.01	2.07	1.00	0.44	0.96	0.46	1.09	1.00	0.91
		Theoretical[d]	1	2	1	0.5	1	0.5	1	1	1
	A. viridans										
	11563	Experimental	1.11	1.79	1.00	0.53	0.97	0.30	0.76	0.91	0.77
	201	Experimental	0.85	1.56	1.00	0.51	1.28	0.32	0.73	0.89	0.92
		Theoretical[e]	1	1.6	1	0.5	1	0.25	0.75	1	1
D_2	*G. homari*	Experimental	1.00	2.34	0.80	0.55	1.13	0.74	1.27	1.00	0.60
		Theoretical[f]	1	2.25	1	0.5	1	0.5	1	1	0.5
	A. viridans										
	11563	Experimental	0.79	1.34	1.00	0.38	0.76	0.20	0.32	0.80	0.25
	201	Experimental	1.02	1.50	1.00	0.52	1.08	0.52	0.51	0.99	0.45
		Theoretical[g]	1	1.5–2	1	0.5	1	0–0.5	0.5–1	1	0.5

[a] 100% of amidated tetrapeptide. [b] Amidated tripeptide plus amidated tetrapeptide (8 to 2). [c] Nonamidated tripeptide and nonamidated tetrapeptide (8 to 2) [d] Bisamidated octapeptide. [e] Bisamidated heptapeptide and bisamidated octapeptide (3 to 1). [f] Monoamidated nonapeptide and monoamidated octapeptide (1 to 1). [g] Monoamidated heptapeptide and monoamidated octapeptide.

were separately filtered in water on Sephadex G-25. The peptides were completely separated from the disaccharides (Figure 3A) and they were further submitted to preparative paper electrophoresis at pH 3.75 (10 V/cm, 150 min; see Materials and Methods). *A. viridans* 11563 and *A. viridans* 201 gave rise to two peptides, M_1 and M_2 (Figure 4A) and *G. homari* to peptide M_1 only. Similarly, Sephadex filtration of fractions K_D 0.55–0.7 (Figure 3B) followed by paper electrophoresis of the enriched peptide materials gave rise, in all cases, to two peptides, D_1 and D_2 (Figure 4A) (and sometimes to a small amount of a third peptide presenting the same mobility as the above peptide M_2 of fractions K_D 0.7–0.85). Finally, the peptide-free disaccharides isolated by Sephadex filtration in water of fractions K_D 0.7–0.85 (Figure 3A) were also purified by preparative paper electrophoresis at pH 3.75. Under these conditions, the disaccharide units were separated from small amounts of free *N*-acetylmuramic acid (which is more anionic) and of free *N*-acetylglucosamine (which is neutral). These free *N*-acetylhexosamine residues were produced during the treatment with the *N*-acetylmuramyl-L-alanine amidase which exhibited a weak *exo*-β-*N*-acetylhexosaminidase activity (Ghuysen *et al.*, 1966a). With respect to their cationic properties, the four isolated peptides were in the following order: $D_1 > M_1 > D_2 > M_2$ (Figure 4A). Table I gives the actual yields of peptide M_1, peptide M_2, peptide D_1, and peptide D_2. Of the total wall peptides, 84, 62, and 74% have been accounted for by the isolated M_1, M_2, D_1, and D_2 peptides. These figures are the actual yields of recovery at the end of a procedure which was applied to relatively small amounts of walls (200 mg, *i.e.*, about 140 μequiv of peptide compounds) and which involved two sequential enzymatic degradations as well as several purifications by Sephadex filtration and paper electrophoresis. Under these conditions, peptides M_1, M_2, D_1, and D_2, altogether, must represent most, if not all, of the peptide moieties of the wall peptidoglycans of the three selected bacteria. Materials containing free amino group and presenting K_D values 0–0.5 (Figure 2) were eluted from the Sephadex column in such minute amounts that they could not be subjected to any chemical characterization.

Structure of Peptides M_1, M_2, D_1, and D_2. The quantitative compositions, N^{ϵ}-lysine and *C*-D-alanine terminal group estimations before and after treatment with ML endopeptidase, N-terminal L-alanine determinations, and quantitative estimations of free L-alanine liberated after treatment with aminopeptidase are given in Table II in which the experimental data are compared with the theoretical data expected for peptides with structures as represented in Table III. The action of the ML endopeptidase on peptides M_1, M_2, D_1, and D_2 was also followed by paper electrophoresis at

TABLE III

Peptide	Structure	Nomenclature
M_1	L-Ala→D-Glu-NH_2 ↳ L-Lys→D-Ala	Amidated tetrapeptide
	without C-terminal D-Ala	Amidated tripeptide
M_2	L-Ala→D-Glu-OH ↳ L-Lys→D-Ala	Nonamidated tetrapeptide
	without C-terminal D-Ala	Nonamidated tripeptide
D_1	L-Ala→D-Glu-NH_2 L-Ala→D-Glu-NH_2 ↳ L-Lys→D-Ala ↳ L-Lys→D-Ala	Bisamidated octapeptide
	without C-terminal D-Ala	Bisamidated heptapeptide
D_2[a]	L-Lys→D-Glu-OH L-Ala→D-Glu-NH_2 ↳ L-Lys→D-Ala→D-Ala ↳ L-Lys→D-Ala	Monoamidated nonapeptide
	with one C-terminal D-Ala	Monoamidated octapeptide
	without C-terminal D-Ala	Monoamidated heptapeptide

[a] In peptide D_2 the amide group has been arbitrarily located on the monomer moiety of which the D-alanine residue is engaged in peptide cross-linking.

pH 3.75. Peptides M_1 and M_2 were found not to be substrates of the endopeptidase and hence were uncross-linked peptide monomers. Under identical conditions, peptides D_1 gave rise to monomer M_1 (Figure 4B) and peptides D_2 gave rise to a mixture of monomer M_1 and monomer M_2 (Figure 4C).

Peptides M_1. From the above data (Table II) and experimental results (Figure 4A,B,C), peptide M_1 from *G. homari* was, obviously, the amidated tetrapeptide L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine. The occurrence of glutamic acid in the form of an isoglutamine residue was proved by aminopeptidase treatment which liberated, per lysine, one L-alanine residue and exposed one amino group of isoglutamine (see Materials and Methods). In its turn, N-terminal isoglutamine disappeared after one cycle of Edman degradation and was not replaced by any other terminal amino group (evidence for a γ linkage; not shown in Table II). Finally, peptide M_1 and authentic L-alanyl-D-isoglutaminyl-L-lysine-D-alanine from *Staphylococcus aureus* (Munoz *et al.*, 1966) were found indistinguishable by paper electrophoresis at pH 3.75 and by chromatography on thin-layer cellulose in solvent III.

Peptide monomers M_1 from *A. viridans* 201 and 11563 were indistinguishable from each other but they differed from peptide M_1 of *G. homari* in that they only contained about 0.2 D-alanine residue per lysine. Hence, these M_1 monomers appeared to be mixtures of amidated tripeptide L-alanyl-D-isoglutamyl-L-lysine and of amidated tetrapeptide L-alanyl-D-isoglutamyl-L-lysyl-D-alanine, occurring in a molar ratio of 8 to 2.

Peptides M_2. Peptide M_2 from *A. viridans* 11563 differed from peptide monomer M_1 of the same organism in that its terminal L-alanine residue could not be removed by the *Streptomyces* aminopeptidase. This enzyme resistance together with the weak cationic property of the peptide (Figure 4A) were compatible with the presence of a free α-carboxyl group of glutamic acid. After one cycle of Edman degradation, N-terminal L-alanine disappeared and was replaced by N-terminal glutamic acid. After a second cycle, N-terminal glutamic acid disappeared and was not replaced by any other N-terminal group (not shown in Table II). Hence, this M_2 peptide was characterized as a mixture of nonamidated tripeptide L-alanyl-γ-D-glutamyl-L-lysine and of nonamidated tetrapeptide L-alanyl-γ-D-glutamyl-L-lysyl-D-alanine, occurring in the molar ratio of 8 to 2.

Peptides D_1. Peptide D_1 from *G. homari* contained equivalent amounts of L-alanine (at the amino terminus), of D-alanine (half of it occurring at the carboxyl terminus), of D-glutamic acid (in endo position), and of L-lysine (with half of the ϵ-amino group unexposed) (Table II). Aminopeptidase removed one L-alanine per lysine residue. ML endopeptidase hydrolyzed one N^{ϵ}-(D-alanyl)-L-lysine linkage per two lysine residues, giving rise to monomer M_1 (Figure 4B). Peptide D_1 from *G. homari* was thus a dimer composed of two tetrapeptides M_1, L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine, interlinked through N^{ϵ}-(D-alanyl)-L-lysine linkage (*i.e.*, a bisamidated octapeptide).

Peptides D_1 from *A. viridans* 201 and 11563 were identical with peptide D_1 from *G. homari* except that some of the dimers present in the preparations had not retained the C-terminal D-alanine residue. They thus appeared as mixtures of bisamidated heptapeptide and bisamidated octapeptide occurring in the molar ratio of 3 to 1.

PEPTIDES D_2. Peptides D_2 from *G. homari* and from *A. viridans* 201 and 11563 gave rise, under treatment with ML endopeptidase, to both monomer M_1 (with an isoglutamine residue) and to monomer M_2 (with a γ-linked, nonamidated D-glutamic acid residue) (Figure 4C). They thus differed from dimers D_1 in that only one of the two peptide units had an amide group. The D_2 peptide preparations were mixtures of octapeptide and heptapeptide in the cases of *A. viridans* 201 and 11563, and of octapeptide and nonapeptide (*i.e.*, a peptide dimer ending in a D-alanyl-D-alanyl sequence) in the case of *G. homari*. The presence of such a nonapeptide was in accord with the presence of D-alanyl-D-alanine and L-lysyl-D-alanyl-D-alanine in partial acid hydrolysates of the corresponding walls.

Disaccharide Units. Authentic β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharides from *Micrococcus lysodeikticus* (Leyh-Bouille *et al.*, 1966) and the disaccharides isolated in the course of the present studies and purified by paper electrophoresis were found undistinguishable under the following conditions: (1) paper chromatography in solvent IV, (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, (4) selective reduction of the reducing *N*-acetylmuramic acid with $NaBH_4$, (5) estimation of the molar extinction coefficient with the Morgan–Elson reaction (acetamido groups) and with the Park–Johnson reaction (reducing groups).

Discussion

The wall peptidoglycans from *G. homari* and from *A. viridans* 201 and 11563 are the first known examples of peptidoglycans of chemotype I in which the cross-linking between peptide units is mediated *via* direct N^{ϵ}-(D-alanyl)-L-lysine linkages (see Introduction). Virtually all the *N*-acetylmuramic acids of the glycans are substituted by peptide units L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine or L-alanyl-γ-D-glutamyl-L-lysyl-D-alanine. Depending upon the bacterial species, about 30–50% of these units are joined and form dimers. Oligomers higher than dimers might exist in trace amounts (fraction K_D 0.4 in Figure 2) but such oligomers could not actually be isolated. A low extent of peptide cross-linking was also a structural feature of the *meso*-diaminopimelic acid containing peptidoglycans of chemotype I in *E. coli* and in *Bacillaceae* (van Heijenoort *et al.*, 1969). No peptide dimers containing two nonamidated peptide units were isolated. It may be that dimerization requires at least one of the two peptides involved to have an isoglutamine residue. Dimers in which two N^{α}-(L-alanyl-γ-D-glutamylglycine)-L-lysyl-D-alanine units are interlinked through N^{ϵ}-(D-alanyl)-L-lysine linkages were previously isolated from walls of *Micrococcus lysodeikticus* and of related *Micrococcaceae* (Ghuysen *et al.*, 1968; Campbell *et al.*, 1969). However, in those microorganisms, only a very small percentage of the peptide units (4% in *M. lysodeikticus*) occurs in the form of such dimers. Most of the peptide units which substitute the *N*-acetylmuramic acid residues are cross-linked through bridges which consist of one or several peptides each having the same composition than the peptide units. Two types of interpeptide linkages—*i.e.*, N^{ϵ}-(D-alanyl)-L-lysine and D-alanyl-L-alanine—are involved in the formation of these oligomers. Such a peptide structure characterizes peptidoglycans of chemotype III (Ghuysen, 1968). It does not occur in walls of *G. homari* and of *A. viridans* 201 and 11563. The peptides in walls of *A. viridans* sp. and most of them in walls of *G. homari* have not retained at their C-termini the D-alanyl-D-alanine sequence that has always been found in the wall peptide precursors. Hence D-alanyl-D-alanine carboxypeptidases must occur in these bacteria. In view of the proposed identity (Leyh-Bouille *et al.*, 1970) between the D-alanyl-D-alanine carboxypeptidases and the transpeptidases involved in the bridge closure reaction between peptide units (*i.e.*, the reaction which ensures the polymerization of the nascent wall peptidoglycan), the isolation and the study of the DD-carboxypeptidases form *G. homari* and *A. viridans* would be of interest. Indeed these DD-carboxypeptidases must act on acyl-L-R_3-D-alanyl-D-alanine peptides containing a free ω amino group on the side chain of the L-R_3 residue (here an N^{ϵ}-L-lysine), a substrate structural feature which is known to inhibit the activities of the DD-carboxypeptidases from *Streptomyces albus* G (Leyh-Bouille *et al.*, 1970), *Streptomyces* R 61 (Leyh-Bouille *et al.*, 1971), and *E. coli* B (Izaki and Strominger, 1968).

References

Campbell, J. N., Leyh-Bouille, M., and Ghuysen, J. M. (1969), *Biochemistry 8*, 193.

Ghuysen, J. M. (1968), *Bacteriol. Rev. 32*, 425.

Ghuysen, J. M., Bricas, E., Lache, M., and Leyh-Bouille, M. (1966a), *Biochemistry 5*, 1455.

Ghuysen, J. M., Bricas, E., Lache, M., and Leyh-Bouille, M. (1968), *Biochemistry 7*, 1450.

Ghuysen, J. M., Tipper, D. J., and Strominger, J. L. (1966b), *Methods Enzymol. 8*, 685.

Izaki, K., and Strominger, J. L. (1968), *J. Biol. Chem. 243*, 3193.

Leyh-Bouille, M., Coyette, J., Ghuysen, J. M., Idczak, J., Nieto, M., and Perkins, H. R. (1971), *Biochemistry 10*, (in press).

Leyh-Bouille, M., Ghuysen, J. M., Bonaly, R., Nieto, M., Perkins, H. R., Schleifer, K. H., and Kandler, O. (1970), *Biochemistry 9*, 2961.

Leyh-Bouille, M., Ghuysen, J. M., Tipper, D. J., and Strominger, J. L. (1966), *Biochemistry 5*, 3079.

Munoz, E., Ghuysen, J. M., Leyh-Bouille, M., Petit, J. F., Heymann, H., Bricas, E., and Lefrancier, P. (1966), *Biochemistry 5*, 3748.

Schleifer, K. H., and Kandler, O. (1967), *Arch. Mikrobiol. 57*, 335.

Tipper, D. J., Katz, W., Strominger, J. L., and Ghuysen, J. M. (1967), *Biochemistry 6*, 921.

van Heijenoort, J., Elbaz, L., Dezélée, Ph., Petit, J. F., Bricas, E., and Ghuysen, J. M. (1969), *Biochemistry 8*, 207.