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Structure of the Walls of *Lactobacillus acidophilus* Strain 63 AM Gasser*

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ABSTRACT: Peptidoglycan, neutral polysaccharide, and anionic polysaccharide are the main structural components of the walls of *Lactobacillus acidophilus* strain 63 AM Gasser. The peptide moiety of the peptidoglycan is essentially composed of N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine peptides, cross-linked by N^{ϵ}-(D-alanyl-D-isoasparaginyl)-L-lysine bridges. The extent of peptide cross-linking is low. In log phase walls, the average size is of 2.3 cross-linked peptides, 10% of the peptide subunits occurring as monomers, 37% as dimers, and 30% as trimers. All of the peptide monomers and oligomers have D-alanyl-D-alanine sequences at their C termini, identified and measured with the help of a *Streptomyces* DD carboxypeptidase. The glycan moiety of the peptidoglycan is composed of β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid units. All of the muramic acid residues are peptide substituted and about 60 to 70% of them have *O*-acetyl substituents on C_6. The neutral polysaccharide is composed of equimolar amounts of glucose, galactose, and rhamnose. It is apparently linked to muramic acid residues of the peptidoglycan *via* phosphodiester groups. The extent of bridging is about 1 phosphate group for (glucose$_{70}$-rhamnose$_{70}$-galactose$_{70}$) units. The anionic polysaccharide is a mixture of (α or β)-1,6-linked polyglucose polymers with monomeric α-glycerol phosphate side chains attached to them on C_2 or C_4 position. Peptidoglycan, neutral polysaccharide, and anionic polysaccharide represent 42, 32, and 17%, respectively, of the log-phase walls. Stationary-phase walls have similar composition and structure, except that substantial amounts of neutral polysaccharide are replaced by anionic polysaccharide.

Walls of *Lactobacillus* sp. have excited considerable interest for several reasons. (i) A criterion of taxonomic importance exhibited by the lactobacilli and the streptococci of the lactic group (Kandler, 1967; Plapp *et al.*, 1967) is that the wall peptidoglycan component (Ghuysen, 1968; see also Leyh-Bouille *et al.*, 1970) always contains D-aspartic acid residues (Cummins and Harris, 1956; Swallow and Abraham, 1958; Ikawa and Snell, 1960; Ikawa, 1964) that serve to cross-link adjacent peptide-substituted glycan chains. The chemistry of these D-aspartic acid bridges, however, has only been elucidated in two cases. In *Streptococcus faecalis* strain ATCC 9790 (Ghuysen *et al.*, 1967) (reidentified as *S. faecium* variety *durans* by Kandler *et al.*, 1968) and in *Lactobacillus casei* RO 94 (Hungerer *et al.*, 1969), it has been proved that N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine subunits are cross-linked by single D-isoasparaginyl residues through N^{ϵ}-(D-alanyl-D-isoasparaginyl)-L-lysine sequences. (ii) Neutral polysaccharides composed of rhamnose, glucose, galactose, and sometimes of galactosamine (Knox and Brandsen, 1962; Knox, 1963; Hall and Knox, 1965; McCarty and Morse, 1964) and anionic ribitol or glycerol teichoic acids (Archibald *et al.*, 1961; Shaw and Baddiley, 1964; Sharpe *et al.*, 1964; Archibald *et al.*, 1968) are other major components of the lactobacillal walls, with important immunological properties. Attempts have been made to correlate the serological classification of the lactobacilli (Glastonbury and Knox, 1963; McCarty and Morse, 1964; Sharpe *et al.*, 1964) with either the presence of teichoic acids or the presence of neutral polysaccharides, the two types of polymers being seemingly exclusive. (iii) Although, at the moment, little is known about the way in which the various polymers are held together in the bacterial walls, it is noteworthy that phosphomuramic acid, now thought to be one means used by bacteria to link together the peptidoglycan and the other wall polymers (Liu and

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Gotschlich, 1967), was first identified in *L. casei* (Ågren and de Verdier, 1958; Hall and Knox, 1965; Knox and Hall, 1965).

The experiments hereby presented deal with: (i) the structure of the peptidoglycan in walls of *L. acidophilus* strain 63 AM Gasser (Pasteur Institute, Paris); (ii) the structure of the other, nonpeptidoglycan wall polymers. A preliminary report has appeared (Coyette, 1969).

Materials and Methods

Growth Conditions. L. acidophilus was grown at 37°, on a gyratory shaking machine, in 1-l. flasks containing 200 ml of the following medium: Tryptone Difco, 10 g; beef extract Difco, 10 g; yeast extract Difco, 5 g; Tween 80, 1 ml; glucose, 20 g; cysteine, 0.2 g; K_2HPO_4, 2 g; KH_2PO_4, 2 g; Na acetate·$3H_2O$, 5 g; NH_4 citrate, 2 g; saline solution, 5 ml; water to 1 l. The saline solution contained, per 100 ml: $MgSO_4 \cdot 7H_2O$, 11.5 g; $FeSO_4 \cdot 7H_2O$, 1.68 g; $MnSO_4 \cdot 2H_2O$, 2.4 g; 10 N H_2SO_4, 2 drops. The pH of the broth medium was adjusted to 7. Under the above conditions, the mean generation time during the log phase was 1 hr.

Walls. The cells were disrupted in a Bühler disintegrator (Tubingen, Germany) for 5 min at 10°, using a ratio of 15 g of wet cells to 160 ml of glass beads to 80 ml of water. The walls were purified by differential centrifugations, at 4°, successively in water, in 1 M NaCl solution, and, again, in water. The walls were not heat treated nor trypsin treated, unless specified. After purification, they were lyophilized.

Analytical Techniques. Reducing groups (Park–Johnson procedure), acetamido sugars (Morgan–Elson reaction), hexosamines (Morgan–Elson reaction after chemical acetylation), D-glucosamine (Morgan–Elson reaction after enzymatic *N*-acetylation), amino acids (fluorodinitrobenzene technique), D-alanine (enzymatic procedure), and N- and C-terminal groups (fluorodinitrobenzene and hydrazinolysis techniques, respectively) were measured as previously described (Ghuysen *et al.*, 1966, 1968). Amide ammonia was measured and Edman degradation was carried out as described by Tipper *et al.* (1967). Free D-glucose was measured using D-glucose oxidase *O*-anisidine reagent (Glucostat reagent; Worthington Biochemical Corp.). Rhamnose was measured according to the technique of Dische and Shettles (1948) as modified by Heymann *et al.* (1967) (molar extinction coefficient $\epsilon^{396-427}$ 14,500). Measurement of total hexoses and methylpentoses was done using the anthrone–sulfuric acid reagent of Shields and Burnett (1960). Molar extinction coefficients, at 620 mμ, vary according to the sugars: 6750 for glucose; 4500 for rhamnose; 3600 for galactose. Measurement of *O*-acetyl groups was performed with alkaline hydroxylamine according to Hestrin (1949). Measurement of glycerol was performed using *Aerobacter aerogenes* glycerol dehydrogenase (Worthington Biochemical Corp.) in the presence of NAD as previously described (Ghuysen, 1964). Measurement of phosphomonoester groups was performed with the help of the *Escherichia coli* alkaline phosphatase type III (10 mg/ml) of Sigma Chemical Co. Measurement of inorganic and organic phosphate was performed according to Lowry *et al.* (1954). Reduction with $NaBH_4$ and periodate oxidation were performed as described by Leyh-Bouille *et al.* (1966).

Electrophoreses. They were carried out on Whatman No. 3MM paper using an Electrorheophor apparatus, Pleuger, at pH 5 (acetic acid–pyridine–water, 2:4:1000), at pH 3.9 (6:2:1000), at pH 3.6 (9:2:1000), and at pH 2 (0.2 N formic acid).

Chromatography. The following solvents were used: (I) chloroform–methanol–acetic acid (88:10:2); (II) benzyl alcohol–chloroform–methanol–water–15 N NH_4OH (30:30:30:6:2); (III) isobutyric acid–0.5 N NH_4OH (5:3); (IV) 1-butanol–acetic acid–water (3:1:1); (V) 1-butanol–pyridine–water (6:4:3). Chromatography was performed on Whatman No. I papers and on thin-layer plates (0.25 mm) of Stahl's silica gel (Merck). Solvent I was used for the separation of dinitrophenyl derivatives of amino acids (DNP amino acids) and solvent II for the separation of mono-DNP derivatives of diamino acids (Ghuysen *et al.*, 1966).

Detection Reagents. Oligosaccharides from peptidoglycan were detected by fluorescence (Sharon, 1964). Amino sugars, amino acids, and free amino groups were detected with ninhydrin (0.5% in isopropyl alcohol–water, 9:1). Sugars, amino sugars, and glycerol were detected with alkaline silver nitrate (Trevelyan *et al.*, 1950). Polyols were detected using the periodate–Schiff reagents of Spinco Division Beckman Instruments Inc., as previously described (Ghuysen, 1961). Inorganic phosphate was detected with the molybdate–perchloric acid reagent of Bandurski and Axelrod (1951). Organic phosphate was detected using the same procedure except that the paper, after spraying, was maintained 2 min at 85° and then exposed to ultraviolet light for 15 min.

Enzymes. Besides the D-glucose oxidase, the *A. aerobacter* glycerol dehydrogenase and the *E. coli* alkaline phosphatase type III (see Analytical Techniques), the following enzymes were also used: the *Streptomyces* F1 *endo-N*-acetylmuramidase, *N*-acetylmuramyl-L-alanine amidase, aminopeptidase, SA endopeptidase, and KM endopeptidase (recently characterized as a DD carboxypeptidase: Guinand *et al.*, 1969; Ghuysen and Leyh-Bouille, 1969). All these enzymes were previously described (Ghuysen, 1968; Ghuysen *et al.*, 1968). Finally, an *exo-β-N*-acetylglucosaminidase was prepared from pig epididymis according to Sanderson *et al.* (1962).

Gel Filtrations. Sephadex G-75 fine, Sephadex G-50 fine, and Sephadex G-25 fine (particle size: 20–80 μ) were used. The gel filtration properties of the compounds are expressed in terms of distribution coefficient $K_D = (V_e - V_0)/V_i$ with V_e = elution volume, $V_0 = V_e$ of totally excluded material, and $V_i = V_e$ of NaCl $- V_0$.

Experimental Section

I. The L. acidophilus Peptidoglycan. WALL COMPOSITION. Chemical composition of walls from log-phase cells and from stationary-phase cells are reported in Table I. As will be shown, glycerol occurs in amounts almost equivalent to those of organic phosphate. Rhamnose, glucose, and galactose were the only neutral sugars detected by paper chromatography, in solvent V, of wall acid hydrolysates (2 N HCl, 100°, 2 hr). Galactose was estimated on the basis of the color development in the anthrone–H_2SO_4 reaction using, as standards, solutions containing various amounts of galactose in presence of fixed amounts of glucose and rhamnose equivalent to those contained in the wall samples. Taking into account the presence of *O*-acetyl and amide

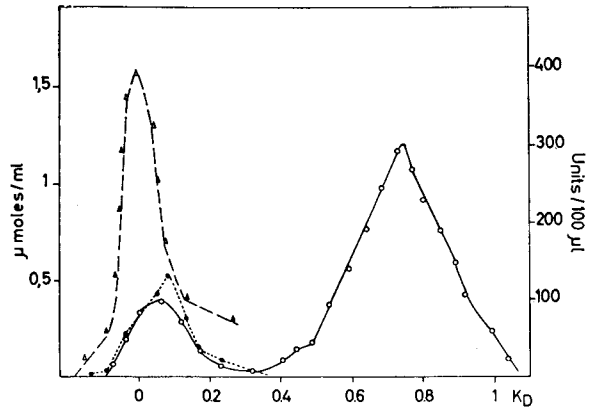

FIGURE 1: Sephadex G-75 filtration in 0.05 M citrate buffer pH 5 of an autolysate of *L. acidophilus* log walls: solid line, total hexosamine residues; broken line, lytic activity; dotted line, organic phosphate. Fractions (100 μl) were incubated at 37° with 500 μg of heat-treated log wall in a final volume of 0.05 M citrate buffer pH 5. One lysis unit equals a decrease of 0.001 OD/hr.

groups (*vide infra*), the aforelisted components amounted to about 90% of the dry weight of the walls.

FRACTIONATION OF LOG WALL AUTOLYSATE. Walls from both log-phase and stationary-phase cells readily autolyze and the rate of clarification is maximal in a 0.05 M citrate buffer pH 5 (Coyette and Ghuysen, 1970). A log-phase wall autolysate (200 mg, in 0.05 M citrate buffer, pH 5) was filtered, in the same buffer, on a 210-ml Sephadex G-75 column. Autolysin and organic phosphate-, sugar-, and glycerol-containing materials were excluded from the gel (Figure 1). The flow sheet (Figure 2) outlines further processing of the fractions. Fraction K_D, 0.5–0.9 (Figure 1), which contained at least 85% of the wall peptidoglycan in the form of disaccharide peptide fragments, was again filtered, in 0.1 M LiCl, on two columns of Sephadex G-50 and Sephadex G-25 connected in series (400 ml each; combined V_0: 370 ml; combined $V_0 + V_i$: 750 ml) (Figure 3). This filtration yielded three main fractions of which the K_D values were those expected for a disaccharide monomer (K_D 0.6; yield = 10% in terms of total glutamic acid residues of the original walls), for a bisdisaccharide peptide dimer (K_D 0.4; yield 37%), and for a trisdisaccharide peptide trimer (K_D 0.3; yield 30%). The fractions were separately desalted by filtration in water on Sephadex G-25 and were found homogeneous by paper electrophoresis: in 0.2 M formic acid, the three compounds were slightly cationic and they exhibited similar but distinct migrations. The three fractions, however, could be separated by thin-layer chromatography into subfractions which differed only in the degree of *O*-acetylation of the disaccharide units (*vide infra*). Fractionation of stationary-phase walls was carried out under the same conditions as above, and yielded a disaccharide peptide monomer fraction (13%), a bisdisaccharide peptide dimer fraction (24%), and a trisdisaccharide peptide trimer fraction (22%).

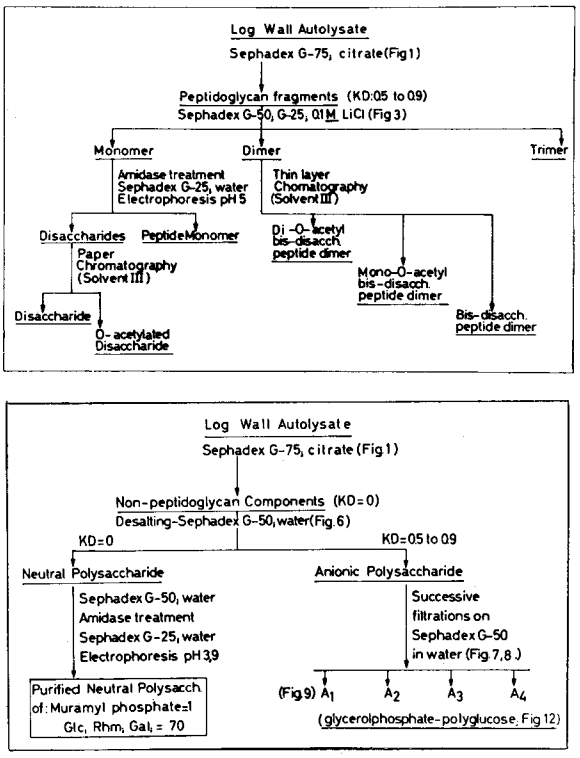

FIGURE 2: Fractionation of *L. acidophilus* log wall autolysate. Flow sheet.

TABLE I: Chemical Composition of *L. acidophilus* Walls (Data Expressed in mμmoles/mg).

	Log-Phase Walls	Stationary-Phase Walls
Total hexosamines	680	980
Lysine	270	470
Alanine	750	1270
Glutamic acid	350	550
Aspartic acid	340	500
Organic phosphate	150	225
Glucose	1320	1800
Rhamnose	570	310
Galactose	570	310

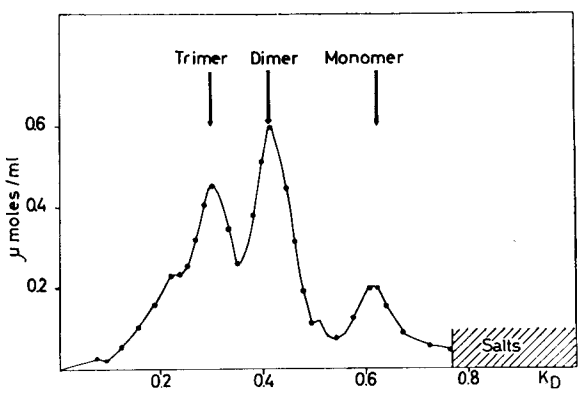

FIGURE 3: Filtration on two connected columns of Sephadex G-50 and Sephadex G-25, in 0.1 M LiCl, of the peptidoglycan fragments (see Figure 1; fraction K_D 0.5 to 0.9) of an autolysate of *L. acidophilus* log walls. Solid line: reducing groups.

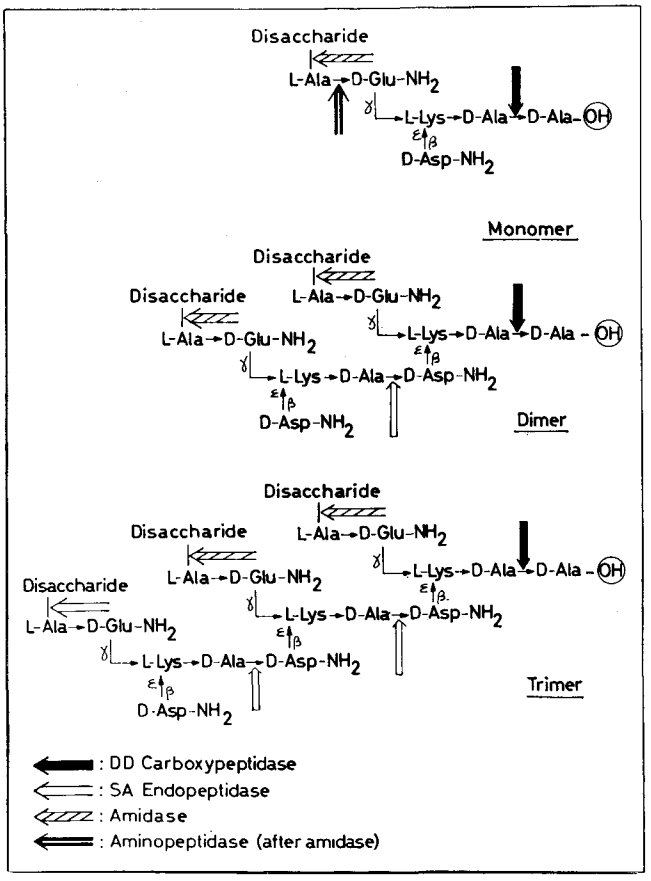

FIGURE 4: Structure of disaccharide peptide monomer, dimer, and trimer from log walls of *L. acidophilus*.

TABLE II: Analyses of the Disaccharide Peptide Monomer, Bisdisaccharide Peptide Dimer, and Trisdisaccharide Peptide Trimer Isolated from Walls of *L. acidophilus*.

	Monomer	Dimer	Trimer
Disaccharide[a]	1000	1000	1000
Amino acid composition			
Glutamic acid	1160	950	1120
Lysine	1040	975	970
Aspartic acid	1020	1000	1060
Total alanine	3320	2540	2370
D-Alanine	2030		
Amide ammonia	2000	2000	2260
Terminal groups			
N-Aspartic acid[b]	510	250	135
C-Alanine[c]	940	535	330
C-D-Alanyl-D-alanine[d]	1000	450	270
N-Aspartic acid[b] after SA endopeptidase treatment			470
C-Alanine[c] after SA endopeptidase treatment			1180
N-Alanine[b] exposed by amidase	820	840	850
L-Alanine liberated by aminopeptidase after amidase treatment	1020		
N-Glutamic acid[b] exposed by aminopeptidase after amidase treatment	735		

[a] Estimated by total hexosamine and reducing group determinations and on the basis of the color development in the Morgan–Elson reaction (30 min of heating in 1% borate). [b] Uncorrected data (fluorodinitrobenzene technique). [c] Corrected data using glycylglycylalanine as model (hydrazinolysis). [d] Estimated on the basis of the D-alanine liberated by the *Streptomyces* DD carboxypeptidase. Note that the D-alanine liberated from the monomer is exactly half of the total D-alanine.

STRUCTURE OF THE PEPTIDE MOIETY OF THE *L. acidophilus* WALL DISACCHARIDE PEPTIDE COMPONENTS. The structures proposed for the disaccharide peptide monomer, bisdisaccharide peptide dimer, and trisdisaccharide peptide trimer (Figure 4) rested upon the following evidence. (i) Composition in disaccharide units, amino acids residues, and amide ammonia (Table II), determination of C- and N-terminal groups (Table II), and specific estimation of the D-alanyl-D-alanine sequences at the C termini of the peptides (using the *Streptomyces* DD carboxypeptidase; Tables II and III). (ii) Hydrolysis of the *N*-acetylmuramyl-L-alanine linkages by the *Streptomyces* amidase followed by the hydrolysis of the L-alanyl-D-isoglutamine linkages by the *Streptomyces* aminopeptidase (Tables II and III). (iii) Edman degradation of the disaccharide peptide monomer resulted in the disappearance of N-terminal aspartic acid without the concomitant exposure of any other terminal amino groups (as expected for a β-aspartyl linkage). (iv) The disaccharide peptide monomer was treated by NaOH under conditions known to catalyze the β elimination of the lactyl peptide together with the deamidation of the isoasparaginyl residue and the interconversion of the peptide into a mixture of N^{ϵ}-(α- and β- aspartyl)lysyl peptides, (Ghuysen *et al.*, 1967; Tipper, 1968; Hungerer *et al.*, 1969). Under these conditions, the disaccharide peptide monomer gave rise to a mixture of two peptide compounds giving at pH 5 and at pH 2 the electrophoretic mobilities expected for the peptide N^{α}-(lactyl-L-alanyl-D-isoglutaminyl)-N^{ϵ}-(β-aspartyl)-L-lysyl-D-alanyl-D-alanine and for the peptide N^{α}-(lactyl-L-alanyl-D-isoglutaminyl)-N^{ϵ}-(α-aspartyl)-L-lysyl-D-alanyl-D-alanine, respectively, and in which the β isomer greatly predominated (establishment of the β-isoasparaginyl-L-lysine sequence) (Figure 5). (v) Hydrolysis of the D-alanyl-D-isoasparagine linkages in the dimer and the trimer compounds by the means of the *Streptomyces* SA endopeptidase (Tables II and III). Kinetics of the degradation of the trimer was followed by end groups and electrophoretic analyses. Using this latter technique, the trimer appeared to be directly degraded into monomers. The transitory formation of dimer was not observed during the course of the degradation. As shown in Table III, the trimer is a much better substrate than the dimer. That the specific activity of the SA endopeptidase depends upon the size of the substrates (Table III) is strengthened by the peptidase degradation of *L. acidophilus* heat-treated log walls. Kinetics of wall solubilization suggested

TABLE III: Specific Activities of *Streptomyces* *N*-Acetylmuramyl-L-alanine Amidase, SA Endopeptidase, and DD Carboxypeptidase on Various Wall Peptides. Results Expressed in mμequiv of Hydrolyzed Linkages per hr per mg of Protein.

	Amidase[a]	SA endopeptidase[b]	DD carboxypeptidase[c]
L. acidophilus			
Disaccharide peptide monomer	6,500	0	
Peptide monomer	0	0	450
Bisdisaccharide peptide dimer	11,000	≪50	95
Peptide dimer	0		220
Trisdisaccharide peptide trimer	10,000	50	60
S. aureus			
Disaccharide pentapeptide pentaglycine[d]	30,000	0	5,000
Disaccharide tetrapeptide pentaglycylpentapeptide disaccharide[e]	15,000	2,800	1,250

[a] Hydrolysis of *N*-acetylmuramyl-L-alanine linkages in endo position. Experimental conditions: substrate (containing 30 mμequiv of substrate linkages) was incubated with the enzyme in 30 μl (final volume) of 0.033 M acetate buffer pH 5.4. [b] Hydrolysis of D-alanyl-D-isoasparagine linkages (*L. acidophilus*) or D-alanylglycine (*S. aureus*) linkages in endo position. Experimental conditions: substrate (containing 20 mμequiv of substrate linkages) was incubated with the enzyme in 40 μl (final volume) of 0.01 M Veronal buffer pH 9. [c] Hydrolysis of D-alanyl-D-alanine linkages at the C termini of the peptide moieties. Experimental conditions: substrate (containing 15 mμequiv of substrate linkages) was incubated with the enzyme in 40 μl (final volume) of 0.02 M Veronal buffer pH 8.9. [d] N^{α}-(Disaccharide-L-Ala-D-isoGlN); N^{ϵ}-(penta-Gly)-L-Lys-D-Ala-D-Ala. [e] *I.e.*, one N^{α}-(disacch-L-Ala-D-isoGlN)-L-Lys-D-Ala-D-Ala monomer and one N^{α}-(disacch-L-Ala-D-isoGlN)-Lys-D-Ala monomer, cross-linked by a pentaglycine bridge between the ε amino group of lysine of the first monomer and the C-Ala of the second.

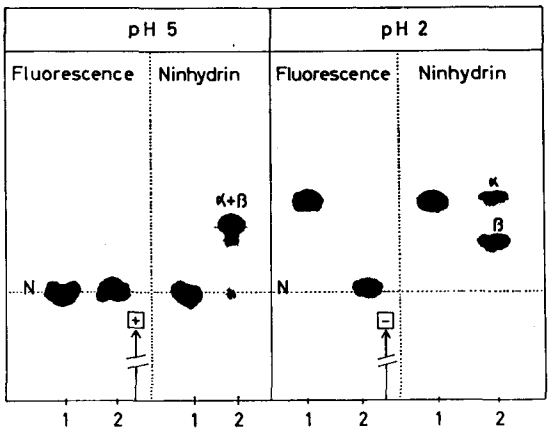

FIGURE 5: Electrophoresis of *L. acidophilus* disaccharide peptide monomer before and after treatment with 0.05 N NaOH (using 10 equiv of NaOH per peptide; 1 hr; 37°; Ghuysen *et al.*, 1967), at pH 5 (20 V/cm; 90 min) and at pH 2 (0.1 M formic acid; 20 V/cm; 90 min) (N = position of neutrality); 1 = untreated disaccharide peptide: detected by fluorescence (Sharon, 1964) and with ninhydrin; 2 = NaOH-treated disaccharide peptide. Under NaOH treatment, the disaccharide peptide is split into a lactylless disaccharide (neutral at both pH 5 and pH 2; detected by fluorescence) and a mixture of N^{α}-(lactyl-L-alanyl-D-isoglutaminyl)-N^{ϵ}-(α- and β-aspartyl)-L-lysyl peptides (detected by ninhydrin). At pH 5, the two lactyl peptides are anionic but equally charged and thus they are not separable. Electrophoresis at this pH, however, shows that the NaOH degradation has been complete since under these conditions the untreated disaccharide peptide is neutral. At pH 2 the α peptide (present in a minor amount) is more cationic than the β peptide (present in a major amount).

that about 900 mμequiv of D-alanyl-D-isoasparagine linkages were hydrolyzed per hr per mg of enzyme, a value which is about 20 times higher than that of the specific activity observed with the peptide trimer.

STRUCTURE OF THE DISACCHARIDE UNITS OF THE *L. acidophilus* WALL PEPTIDOGLYCAN. Free disaccharide was isolated and purified from the amidase-treated disaccharide peptide monomer (Table II) by gel filtration on Sephadex G-25 in water, followed by paper electrophoresis at pH 5. After acid hydrolysis, glucosamine and muramic acid were the only two hexosamines present. Quantitation of D-glucosamine by the D-glucosamine 6-phosphate *N*-acetylase revealed half of the hexosamine residues to be glucosamine. $NaBH_4$ reduction destroyed half of the total hexosamine, all the muramic acid, and none of the glucosamine. Pig epididymis *exo*-β-*N*-acetylglucosaminidase, an enzyme specific for β-glycosidic linkages, converted the disaccharide into free *N*-acetylhexosamine residues (on the basis of the color development in the Morgan–Elson reaction after 7 min of heating in 1% borate). Determination of the molar extinction coefficient in the Morgan–Elson reaction (30 min of heating in 1% borate) indicated a 1:4 and not a 1:6 glycosidic linkage. Hestrin's test, however, revealed the presence of 0.7 *O*-acetyl groups per muramic acid residue and paper chromatography, using solvent IV, of the *exo*-β-*N*-acetylglucosaminidase-treated disaccharide, yielded *N*-acetylglucosamine (R_F 0.37), *N*-acetylmuramic acid (R_F 0.63), and *N*,*O*-diacetylmuramic acid (R_F 0.82). Finally, preparative paper chromatography of the disaccharide preparation, using solvent III, allowed the isolation of β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharide (R_F 0.40) and of β-1,4-*N*-acetylglucosaminyl-*N*,*O*-diacetylmuramic acid disaccharide (R_F 0.55) in the molar ratio of 1 to 2, respectively. These two disaccharides were found identical with those previously obtained from the walls of *S. aureus* (Tipper *et al.*, 1965). The bisdisaccharide peptide dimer fraction (K_D 0.4 in Figure 3) was separated by silica gel thin-layer chromatography using solvent III, into three compounds of R_F 0.47, 0.35, and 0.27, occurring in the ratios of 2 to 2 to 1, respectively. Each dimer was further purified by filtration in water on Sephadex G-25. Determination of *O*-acetyl groups revealed the compound R_F 0.47 to be a peptide dimer substituted by two *O*-acetyl disaccharides

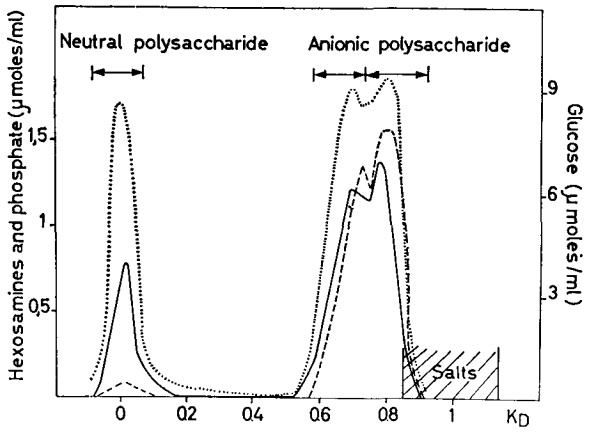

FIGURE 6: Sephadex G-50 filtration in water of the nonpeptidoglycan polymers isolated from a *L. acidophilus* log wall autolysate (excluded fraction K_D 0 in Figure 1): solid line, total hexosamine residues; broken line, organic phosphate; dotted line, glucose (after HCl hydrolysis).

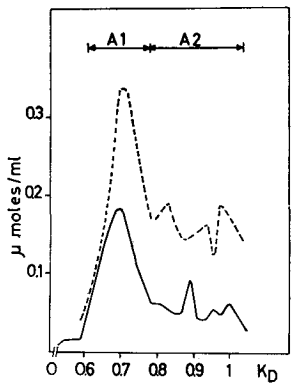

FIGURE 7: Fractionation of the anionic polysaccharide isolated from a *L. acidophilus* log wall autolysate. Sephadex G-50 filtration in water of fraction K_D 0.6 to 0.7 of Figure 6: solid line reducing groups; broken line, organic phosphate.

(actual data: 1.01 *O*-acetyl group per disaccharide unit), the compound R_F 0.35 to be a peptide dimer substituted by one *O*-acetyl disaccharide and one disaccharide (actual data: 0.6 *O*-acetyl group per disaccharide unit), and the material with R_F 0.27 to be mainly composed of a peptide dimer substituted by two disaccharide units (actual data: 0.27 *O*-acetyl group per disaccharide unit). The trisdisaccharide peptide trimer preparation (K_D 0.3 in Figure 3) was also submitted to thin-layer chromatography using solvent III and was shown to be composed of four distinct compounds. Evidently, these results are compatible with the presence of four glycopeptide trimers that differ from each other by the number of disaccharide and *O*-acetylated disaccharide substituents (three disaccharides; two disaccharides and one *O*-acetyl disaccharide; one disaccharide and two *O*-acetyl disaccharides; three *O*-acetyl disaccharides; Tipper and Strominger, 1968).

II. The Nonpeptidoglycan Polymers in Walls of L. acidophilus. ISOLATION OF A NEUTRAL POLYSACCHARIDE COMPLEX AND OF AN ANIONIC POLYSACCHARIDE COMPLEX. The Sephadex G-75 excluded material (600 mg) isolated from a log-phase wall autolysate (fraction K_D 0 in Figure 1) was desalted by filtration on Sephadex G-25 in water. Further filtration on a 450-ml Sephadex G-50 column, in water, yielded two main fractions (Figure 6). One of them was excluded from the gel (K_D 0) and was found neutral by paper electrophoresis at pH 3.9. The second fraction presented an average K_D value of 0.6 to 0.85 and was found anionic at pH 3.9. Both fractions contained glucose, hexosamines, and amino acids residues. The neutral fraction contained virtually all the rhamnose and the galactose present in the original fraction and only traces of organic phosphate. The anionic fraction contained no rhamnose or galactose but virtually all the glycerol and the organic phosphate present in the original fraction. Neither fractions exhibited lytic activity on heat-treated *L. acidophilus* walls. Indeed, autolysin undergoes denaturation during the lyophilization of wall autolysates (but not of intact walls).

NEUTRAL POLYSACCHARIDE. About 65% of the glucose content of the neutral polysaccharide fraction remained excluded from the gel when the crude fraction was refiltered in water in the 450-ml Sephadex G-50 column. At this stage, the high molecular weight polymer was found to be composed of organic phosphate 1; total hexosamines (glucosamine and muramic acid) 3; peptidoglycan peptide subunits 1.7; glucose 64; galactose 66; rhamnose 72. It did not contain any detectable phosphomonoester or reducing groups. Part of the peptidoglycan components remaining attached to the bulk of the high molecular weight polysaccharide were eliminated by treatment with *Streptomyces N*-acetylmuramyl-L-alanine amidase, followed by filtration on Sephadex G-25 in water and finally by preparative paper electrophoresis at pH 3.9. Analyses of the purified polysaccharide showed that the amidase had removed about half of the total hexosamine residues (as free disaccharide residues) and about 10% of the peptide units. Total hexosamines still occurred in the ratio of 2 residues per organic phosphate residue or per about (glucose$_{70}$-rhamnose$_{70}$-galactose$_{70}$) units. Hydrolysis of the polysaccharide with 6 N HCl (2 hr; 100°) quantitatively transformed the organic phosphate into phosphomonoester residues which, under paper electrophoresis at pH 5, migrated as muramyl 6-phosphate. The phosphomonoester compound was isolated by electrophoresis under these conditions. It was actually characterized as muramyl phosphate (using the techniques described in Campbell *et al.*, 1969) with the help of the *E. coli* alkaline phosphatase which yielded equivalent amounts of inorganic phosphate and muramic acid (yield of the enzymatic hydrolysis: 80% in terms of total organic phosphate groups).

FRACTIONATION OF THE ANIONIC POLYSACCHARIDE COMPLEX. The anionic polysaccharide complex isolated from the log wall autolysate (Figure 6) was divided into fraction K_D 0.6 to 0.7 and fraction K_D 0.7 to 0.85. Both fractions were separately submitted to further filtrations, in water, on the 450-ml Sephadex G-50 column and yielded fractions A1 and A2 (Figure 7) and fractions A3 and A4 (Figure 8), respectively. The molar ratios of glucose to organic phosphate were found to be equal to 17 to 1 for fraction A1, 13 to 1 for fraction A2, 9 to 1 for fraction A3, and 4 to 1 for fraction A4. These different ratios were reflected by the increasing anionic properties of the corresponding fractions (Figure 9). Evidently, the anionic polysaccharide complex present in *L. acidophilus* log-phase wall is a polydisperse system of which

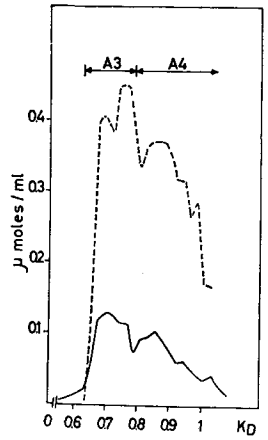

FIGURE 8: Fractionation of the anionic polysaccharide isolated from a *L. acidophilus* log wall autolysate. Sephadex G-50 filtration in water of fraction K_D 0.7 to 0.85 of Figure 6: solid line, reducing groups; broken line, organic phosphate.

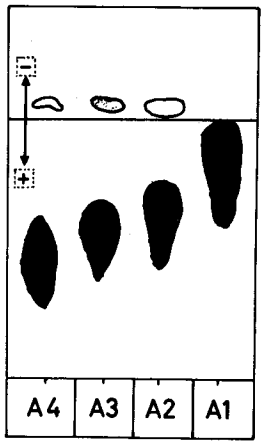

FIGURE 9: Paper electrophoresis at pH 3.6 (20 V/cm; 90 min) of the anionic polysaccharide fractions isolated from a *L. acidophilus* log wall autolysate. Fractions A1 and A2 were obtained as shown in Figure 7. Fractions A3 and A4 were obtained as shown in Figure 8. Detection = fluorescence (Sharon, 1964).

the components vary from each other by their ratios of glucose to phosphate. Because of its high content in organic phosphate, the most anionic A4 fraction was selected for the ensuing structural studies. Fraction A4, which originally contained 0.1 peptide subunit and 0.25 total hexosamine residues per organic phosphate, was treated with *Streptomyces* *N*-acetylmuramyl-L-alanine amidase. Disaccharide units, representing 40% of the total hexosamine residues, and peptide components, representing about 30% of the total glutamic acid residues, were liberated. The anionic polysaccharide complex was separated from the degraded products by filtration in water on Sephadex G-25 (K_D 0), followed by preparative paper electrophoresis at pH 3.9. The molar ratio of glucose to phosphate of the purified A4 complex was 3.5 to 1, indicating a slight enrichment in phosphate (ratio in the original A4 complex, 4 to 1).

ALKALINE DEGRADATION OF THE ANIONIC A4 POLYSACCHARIDE. Treatment of the purified A4 polysaccharide (2.5 μmoles of organic phosphate) with 2.5 ml of a 1 N NaOH solution at 60° for 6 hr quantitatively converted the organic phosphate into phosphomonoester groups (Figure 10). No free glucose was produced. NaOH was removed by adsorption on a 5-ml column of Dowex 50W-X12, H^+, 200–400 mesh, and the salt free solution containing glucose (after acid hydrolysis) and phosphomonoester groups was lyophilized. Analysis of part of this material by paper chromatography (Whatman No. 1) using solvent IV revealed the presence of a glucose polymer (R_F 0; not sensitive to silver nitrate reagent; detected with periodate–Schiff reagent) and the presence of glycerol phosphate (R_F 0.15; weakly sensitive to silver nitrate; not sensitive to periodate–Schiff reagent; detected with the Bandurski and Axelrod reagent). Another part of the NaOH-degraded A4 polysaccharide was treated with the *E. coli* alkaline phosphatase. Kinetics of the degradation showed the liberation of equivalent amounts of inorganic phosphate and of glycerol (measured by means of the *A. aerogenes* glycerol dehydrogenase) (for experimental conditions see Ghuysen, 1964). At completion of the enzymatic reaction, paper chromatography of the degraded products in solvent IV showed the disappearance of glycerol phosphate and its replacement by free glycerol (R_F 0.6).

PERIODATE OXIDATION OF THE ANIONIC A4 POLYSACCHARIDE. Periodate oxidation (Figure 11) gave rise to the rapid (5 min) consumption of 1 mole of IO_4^- per organic phosphate, simultaneously with the release of 1 mole of formaldehyde. Under prolonged conditions, no more formaldehyde was produced but, eventually, 7.5 moles of IO_4^- per glycerol phosphate residue, or 2.2 moles per glucose residue, was consumed. Analyses of the oxidized polysaccharide (after destruction of IO_4^- excess with ethylene glycol) showed that no glucose and no glycerol had been left intact.

STRUCTURE OF THE ANIONIC A4 POLYSACCHARIDE. The above data (alkaline and periodate degradation) were compatible with a structure (Figure 12) in which α-glycerol phosphate monomers substitute through phosphodiester linkages a linear polymer of (β or α ?) 1,6 linked pyranoside glucose residues. On the average one glycerol phosphate side chain occurs for every three to four glucose residues on C_2 or C_4 position.

Discussion

The walls of *L. acidophilus* are composed of at least three structural components: the peptidoglycan, a neutral polysaccharide, and an anionic polysaccharide.

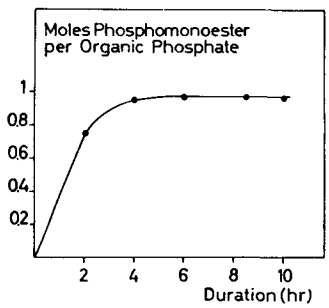

FIGURE 10: Alkaline degradation of the anionic polysaccharide fraction A4 (ratio glucose:phosphate = 3.5 to 1). Kinetics of liberation of phosphomonoester (measured with the help of the *E. coli* alkaline phosphatase).

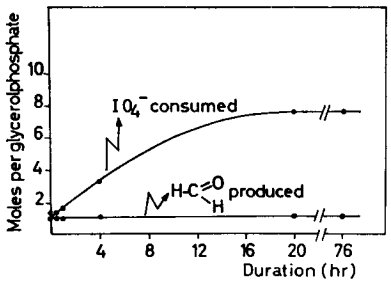

FIGURE 11: Periodate oxidation of the anionic polysaccharide fraction A4 (ratio glucose:glycerol phosphate, 3.5 to 1).

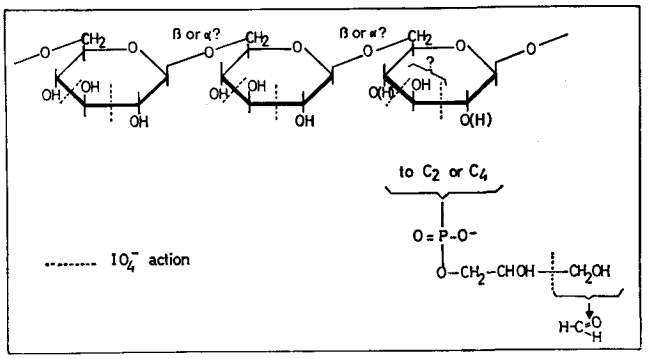

FIGURE 12: Structure of the anionic polysaccharide fraction A4. On the average, one glycerol phosphate substituent occurs for every 3.5 glucose residues.

The peptidoglycan belongs to the chemotype II group (Ghuysen, 1968). Indeed N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine peptide units are cross-linked by "specialized" bridges which extend between the C-terminal D-alanine residues to the ϵ-amino groups of lysine. Single D-isoasparagine residues serve as peptide cross-linking bridges, thus strengthening the prevailing hypothesis (Kandler, 1967; Ghuysen, 1968) according to which N^{ϵ}-(D-alanyl-D-isoasparaginyl)-L-lysine bridges are valid as a taxonomic criterion for the lactobacilli and streptococci of the lactic group. The peptide moiety of the peptidoglycan has an average size of about 2.3 cross-linked peptide units. The yield of fragments isolated from log-phase wall autolysates are consistent with a structure in which 10% of the peptide units occur as monomers, 37% as dimers, and 30% as trimers. In stationary-phase walls, monomers, dimers, and trimers represent 13, 24, and 22%, respectively, of the total peptide units. D-Alanyl-D-alanine sequences were identified at the C-termini of all the peptide monomers and oligomers by means of a *Streptomyces* DD carboxypeptidase which releases the terminal D-alanine residue (Ghuysen and Leyh-Bouille, 1969). It should be emphasized that the amounts of peptide monomers and oligomers estimated after wall fractionation do really reflect the low average size of the peptide moiety *in vivo*. Indeed, the demonstration of D-alanyl-D-alanine sequences at the C-termini of the peptides provide firm evidence that there was not enzymatic cleavage during wall preparation, purification, and fractionation. The *E. coli* wall peptidoglycan is another example of a peptide moiety with, probably, a very low extent of peptide cross-linking (1.5 cross-linked peptides on the average). In contrast to the walls of *L. acidophilus*, however, the peptides in walls of *E. coli* have no D-alanyl-D-alanine sequences at their C termini. It has been proposed (Izaki and Strominger, 1968) that the small size of the peptide moiety in *E. coli* might be related to the presence in this organism of a DD carboxypeptidase. Indeed, by hydrolyzing the terminal D-alanyl-D-alanine sequences of the peptide units at some stage during the peptidoglycan biosynthesis, this enzyme might reduce (and control) the number of peptides capable of undergoing the final transpeptidation reaction which leads to the formation of peptide cross-linking. Quite obviously, such a mechanism cannot be involved in the limitation of the size of the peptide moiety in walls of *L. acidophilus*.

The glycan portion of the *L. acidophilus* peptidoglycan consists of linear chains of repeating β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharide units, *i.e.*, a structure which appears to be ubiquitous in the bacterial world. All of the *N*-acetylmuramic acid residues are peptide substituted, about 70% of them have an *O*-acetyl group on C_6, and about 3% of them occur as *N*-acetylmuramyl 6-phosphate residues. The presence in wall autolysates of three bisdisaccharide peptide dimers and, probably, of four trisdisaccharide peptide trimers that differ from each other by the number of *O*-acetyl substituents indicates that peptide bridging occurs randomly between disaccharide peptides, or between *O*-acetyldisaccharide peptides, or between disaccharide peptides and *O*-acetyldisaccharide peptides, a structure which is reminiscent of that of *S. aureus* (Ghuysen *et al.*, 1966; Tipper and Strominger, 1968). It is not known, however, whether or not the *S. aureus* and *L. acidophilus* walls contain two types of glycan strands, one being solely composed of *N*-acetylglucosaminyl-*N*-acetylmuramic acid units and the other being solely composed of *N*-acetylglucosaminyl-*N*,*O*-diacetylmuramic acid units.

The neutral polysaccharide is composed of equivalent amounts of glucose, rhamnose, and galactose. It is covalently linked to some muramic acid residues of the peptidoglycan by means of phosphodiester bridges. On the average, there seems to be one phosphate bridge for each ($glucose_{70}$-$rhamnose_{70}$-$galactose_{70}$) hypothetical unit. Another polysaccharide moiety of the walls is a mixture of (β or α) 1,6-linked polyglucose polymers with side chains of α-glycerol phosphate residues attached through phosphodiester linkages on the C_2 or C_4 position. Enriched fractions were isolated of which the molar ratios glucose to glycerol phosphate varied from 17 to 1, to 3.5 to 1. According to the definition proposed by Archibald *et al.* (1968), these anionic polysaccharides are not teichoic acids since the phosphodiester linkages do not form an integral part of the polymer backbones. Similar structures with glycerol phosphate side chains were found in the specific polysaccharide of type 18 (Estrada-Parra *et al.*, 1962) and type 11 a (Kennedy, 1964) *Pneumococcus*, and probably in the phosphomucopolysaccharide of walls of *Bacillus megaterium* KM (Ghuysen, 1964). Chemical analyses revealed that neutral polysaccharide and anionic polysaccharides represent, expressed in dry weight, 32 and 17%, respectively, of the log-phase walls and 17 and 30%, respectively, of the stationary-phase walls. Thus, in the course of the bacterial growth, substantial amounts of neutral polysaccharides are replaced by anionic polysaccharides. It is known (Ellwood

and Tempest, 1969) that the occurrence and the nature of wall anionic polymers (teichoic acids and teichuronic acids) in gram positive bacteria are markedly influenced by the growth environment. It appears that a similar phenomenon occurs in *L. acidophilus* with respect to the occurrence of the neutral and anionic wall polysaccharides. This observation may provide an explanation of earlier findings (Ikawa and Snell, 1960) according to which the walls of a *L. acidophilus* strain contained glucose, phosphate, and glycerol (*i.e.*, the anionic polysaccharide) but no galactose and only traces of rhamnose (*i.e.*, no neutral polysaccharide). The above observation may also be related to the fact that, whereas the group specificity of many lactobacilli can be associated with intracellular or wall teichoic acids, or with wall neutral polysaccharides (Sharpe *et al.*, 1964; McCarty and Morse, 1964), no satisfactory grouping is available for the *L. acidophilus* sp. (Indeed, no antiserum able to react with the majority of the strains has even been obtained.)

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