LL-Diaminopimelic Acid Containing Peptidoglycans in Walls of *Streptomyces* sp. and of *Clostridium perfringens* (Type A)*

Mélina Leyh-Bouille, Roger Bonaly,† Jean-Marie Ghuysen, Regina Tinelli,‡ and Donald Tipper§

ABSTRACT: In a major part of the wall peptidoglycans of *Streptomyces* sp. and of *Clostridium perfringens*, L-alanyl-D-isoglutaminyl-(L_1)-LL-diaminopimelyl-(L_1)-D-alanine peptides are cross-linked *via* D-alanylglycyl-(L_2)-LL-diaminopimelic acid linkages (peptidoglycan of the chemotype II group). The *Myxobacter* AL-I endopeptidase hydrolyzes both D-alanylglycine and glycyl-LL-diaminopimelic acid linkages in the walls of *C. perfringens*, liberating free glycine. In contrast, the *Myxobacter* AL-I endopeptidase hydrolyzes only D-alanylglycine linkages in walls of *Streptomyces* sp. and the liberation of the glycine residues requires subsequent treatment with an aminopeptidase. No explanation for this observation can be proposed at this time. A minor component of the AL-I endopeptidase hydrolysate of the *Streptomyces* and *C. perfringens* walls is a resistant peptide dimer. Analyses indicate that the cross-link in this dimer may be mediated through LL-diaminopimelylglycyl-LL-diaminopimelic acid linkages.

The C termini of the peptide moieties in the wall peptidoglycans are either D-alanine or LL-diaminopimelic acid but never D-alanyl-D-alanine, thus indicating the presence in these microorganisms of carboxypeptidases similar to those of *Escherichia coli*.

Bacterial wall peptidoglycans are networks of glycan chains substituted by peptide subunits that, in turn, are cross-linked by peptide bridges (for a review, see Ghuysen, 1968). The glycan chains consist, basically, of alternating β-1,4-linked *N*-acetylglucosamine and *N*-acetylmuramic acid residues. The peptide subunits have the general structure L-alanyl-γ-D-glutamyl-R_3-D-alanine[1] with a strictly alternating LDLD sequence. The glutamyl linkage is always γ whereas the other peptide linkages are always α. Depending upon the bacterial species, the R_3 residue may be L-homoserine[2] or a diamino acid such as L-diaminobutyric acid, L-ornithine, L-lysine, or *meso*-diaminopimelic acid. In this latter case, it has been proved that both the amino group linked to D-glutamic acid and the carboxyl group linked to D-alanine are located on the same asymmetric carbon, that which has the L configuration (van Heijenoort *et al.*, 1969).

From an integration of the structural studies dealing with the nature of the bridges that cross-link the peptide subunits, four main peptidoglycan chemotypes emerge, which appear to be criteria of taxonomic importance. In chemotype I, a direct bond involves the C-terminal D-alanine of one peptide subunit and the amino group located on the D carbon of the *meso*-diaminopimelic acid residue of another peptide subunit. In chemotype II, one additional amino acid or an intervening short peptide extends between the C-terminal D-alanine of one peptide subunit and the free ω-amino group of L-ornithine or L-lysine of another peptide subunit. Chemotype III has only been found, so far, among a few micrococcal L-lysine-containing peptidoglycans, and is a variation of chemotype II. The peptide bridge is made up from one or several peptides, each having the same amino acid sequence as the peptide subunits (Ghuysen, 1968; Campbell *et al.*, 1969). Chemotype IV differs from the former types in that one additional diamino acid such as D-diaminobutyric acid (Perkins, 1969), D-ornithine, or D-lysine (Perkins, 1967; Guinand *et al.*, 1969b) or a short peptide such as N^{α}-glycyl-L-lysine (Schleifer *et al.*, 1968a) extends between the α-carboxyl group of D-glutamic acid of one peptide subunit and the C-terminal D-alanine of another peptide subunit. This type of bridging, which is necessarily involved in the peptide cross-linking of those peptide subunits that have no diamino acids in their sequence, also occurs between L-diaminobutyric acid-, L-ornithine-, and L-lysine-containing peptide subunits.

Little is known on the structure of LL-diaminopimelic acid containing peptidoglycans. *Actinomyces* and *Streptomyces* (Hoare and Work, 1957; Yamaguchi 1965; Pine and Boone, 1967; Szaniszlo and Gorder, 1967; Lechevalier and Lechevalier, 1967) contain LL-diaminopimelic acid most often associated with glycine, *meso*-diaminopimelic acid being absent. *Clostridium perfringens* and several strains of *Propionibacterium* and of *Corynebacterium* (Pickering, 1966; Hoare and Work, 1957; Tinelli, 1966; Arima *et al.*, 1968; Schleifer *et al.*, 1968b) also contain LL-diaminopimelic acid. According to Pickering (1966) the *C. perfringens* peptidoglycan would belong to chemotype III group, being composed of L-alanyl-γ-(α-D-glutamylglycine)-LL-diaminopimelyl-D-alanine peptides interlinked by means of D-alanyl-L-alanine and D-alanyl-LL-diaminopimelic acid linkages. Pickering's proposal, however, was not supported by more recent studies of Schleifer *et al.* (1968b). The peptides glycyl-LL-diaminopimelyl-D-alanine and D-alanylglycine were isolated from partial acid hydrolysates of walls of *C. perfringens* as well as from walls of *P. petersonii*. These results

* From the Service de Bactériologie, Université de Liège, Liège, Belgium. *Received December 8, 1969.* This research has been supported in part by the Fonds de la Recherche Fondamentale Collective, Brussels, Belgium (Contracts 515 and 1000).

† From the Service de Chimie Biologique, Faculté de Pharmacie de Nancy. Supported by a North Atlantic Treaty Organization Fellowship.

‡ From the Institut Pasteur, Paris, France.

§ From the Pharmacology Department, University of Wisconsin, Madison, Wis.

[1] With the exception that L-alanine is sometimes replaced by L-serine or glycine.

[2] The L configuration was established by Dr. H. R. Perkins (private communication).

indicated that glycine extends from the C-terminal D-alanine to one amino group of LL-diaminopimelic acid, *i.e.*, a structure reminescent of the above described chemotype II. According to data recently reported by Arima *et al.* (1968), the actinomycetal peptidoglycans would have a similar type of structure.

Simultaneous with the studies of Schleifer *et al.* (1968b) and of Arima *et al.* (1968), the structures of the LL-diaminopimelic acid containing peptidoglycans in walls of *C. perfringens* type A, BP6K (tox$^+$) (synonym: *C. welchii* or *Welchia perfringens*), and of several strains of *Streptomyces* sp. were studied in this laboratory. The experiments hereby presented mainly deal with the determination of the relative position of the three substituents, D-glutamic acid, D-alanine, and glycine, on the LL-diaminopimelic acid residue[3] and with the involvement of the glycine residues in the peptide cross-linking. Preliminary reports have appeared (Leyh-Bouille and Tinelli, 1969; Ghuysen, 1969).

Materials and Methods

Assay of Penicillin Sensitivity of Streptomyces Strains. Plates of nutrient broth agar containing plugs of penicillin-containing agar (cylinder technique) were seeded with *Streptomyces* conidia and incubated for 48 hr at 25°. The minimal concentrations of penicillin G that provided a visible zone of growth inhibition were greater than 1000 μg/ml for 2 strains, and equal to 1000 μg/ml for *S. albus* G and 46 other strains, 100 μg/ml for 15 strains, and 10 μg/ml for strains R 61 and K 27.

Walls. Streptomyces. Strains *albus* G, R 61, and K 27 were grown at 28° on a gyratory shaking machine, in 1-l. flasks containing 500 ml of the following medium: Oxoid peptone, 10 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 4H_2O$, 1 g; $NaNO_3$, 1 g; KCl, 0.5 g; water, 1 l. The cells were harvested after 48 hr and disrupted with glass beads in a Bühler disintegrator (Tubingen, Germany). The walls were purified by differential centrifugation in water, heated for 7 min at 100°, treated at 37° for 2 hr with trypsin (4 mg/ml) in 0.1 M phosphate buffer pH 7.5, and finally washed several times with phosphate buffer and water. Examination under the electron microscope revealed an homogeneous preparation of long, empty mycelial fragments.

C. perfringens. The bacteria were grown anaerobically as described earlier (Tinelli, 1968). The cells were disrupted with glass beads in a Mickle disintegrator. The walls were not heat treated since they do not contain any autolysin (Tinelli, 1968) but they were purified by trypsin treatment.

Enzymes. Streptomyces F_1 *endo-N*-acetylmuramidase, *Streptomyces N*-acetylmuramyl-L-alanine amidase, *Streptomyces* aminopeptidase, *Streptomyces* MR, ML, SA, and KM endopeptidases, *Chalaropsis endo-N*-acetylmuramidase (a gift from Dr. N. A. Hash), *Myxobacter* AL-I enzyme, and egg white lysozyme (Armour) were used. The mechanism of action of these enzymes has been recently reviewed (Ghuysen, 1968). The *Streptomyces* R_1 preparation (Dierickx and Ghuysen, (1962), a mixture containing all the lytic enzymes secreted by *S. albus* G, was also used. The site of action of each enzyme can be summarized as follows (see Figure 9 for an illustration): (1) *endo-N*-acetylmuramidases (F_1 and *Chalaropsis* enzymes; egg-white lysozyme) hydrolyze the glycan strands into β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid fragments. (2) Following the action of *endo-N*-acetylmuramidases, the *Streptomyces N*-acetylmuramyl-L-alanine amidase hydrolyzes the linkages at the junction between the glycan and the peptide moieties. (3) *Myxobacter* AL-I enzyme also hydrolyzes the *N*-acetylmuramyl-L-alanine linkages at the junction between the glycan and the peptide moieties (amidase action). Prior splitting of the glycan into disaccharides is not required. (4) The N-terminal L-alanine residues exposed as a result of the action of either the *Streptomyces* amidase or the *Myxobacter* AL-I enzyme can be subsequently liberated as free residues by treatment with the *Streptomyces* aminopeptidase. (5) *Myxobacter* AL-I enzyme also hydrolyzes interpeptide linkages (endopeptidase action) within the D-alanyl-glycyl-LL-diaminopimelic acid sequence (Figure 9).

Model Peptides. The synthetic peptide *meso*-diaminopimelyl-(D)-L-alanine (Bricas *et al.*, 1962; Bricas and Nicot, 1965), and the natural peptides L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelic acid and L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine, isolated from walls of *Bacillus megatherium* and of *Escherichia coli* (van Heijenoort *et al.*, 1969) were used as models for the determination of the structure of the peptides isolated during the course of the present studies.

Analytical Methods. Reducing groups (Park and Johnson procedure); acetamido sugars (Morgan–Elson reaction); 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.*, 1966, 1968). Edman degradation was carried out as previously described (Tipper *et al.*, 1967).

Chromatographic Solvents. The following solvents were used: (I) 1-butanol–acetic acid–pyridine–water (30:6:20:24, v/v); (II) chloroform–methanol–acetic acid (88:10:2, v/v); (III) isobutyric acid–1 M NH_4OH (5:3); (IV) 1-butanol–acetic acid–water (3:1:1, v/v).

Thin-layer chromatography was performed on plates (0.3–0.4 mm) of Stahl's silica gel (Merck) (for the determination of the dinitrophenylamino acids, using solvent II), and of MN-cellulose 300 HR, Machery Nagel and Co.

Electrophoreses were carried out on Whatman No. 3MM paper using an Electroheophor apparatus, Pleuger, in water–pyridine–acetic acid (1000:2:6, v/v), pH 3.9.

Gel Filtrations. Fractionations were carried by gel filtrations on two columns, 400 ml each, of Sephadex G-50, fine (20–80 μ), and Sephadex G-25, fine (20–80 μ), connected in series and using 0.1 M LiCl solution as eluent (combined V_0; 370 ml; combined $V_0 + V_i$; 750 ml). The filtration properties of the compounds 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 always performed by filtration on Sephadex G-25 in water.

Chromatographic identification of diaminopimelic acid isomers was carried out as previously described (Bricas *et al.*, 1967). LL-Diaminopimelic acid was the only diaminopimelic acid isomer present in the walls.

[3] In order to specify the locations of the substituents of a LL-DAP residue, it is proposed to distinguish the two asymmetric carbons by the notation (L_1) and (L_2). The notation (L_1) or (L_2) written immediately before LL-diaminopimelic acid specifies its substituted amino groups. The notation (L_1) or (L_2) written immediately after LL-diaminopimelic acid specifies its carboxyl groups.

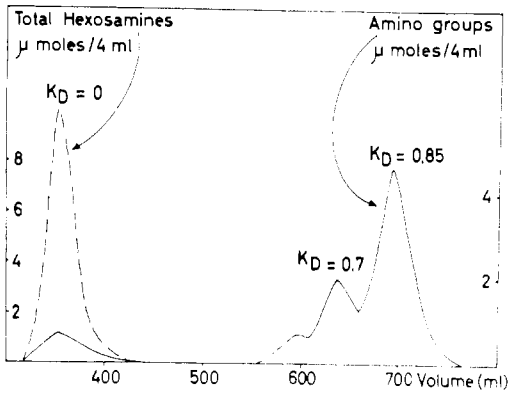

FIGURE 1: Sephadex filtration in LiCl of walls of *S. albus* G degraded with *Myxobacter* enzyme. For conditions, see text; solid line, N-terminal groups; dotted line, total hexosamine residues. Results are expressed in micromoles per fraction.

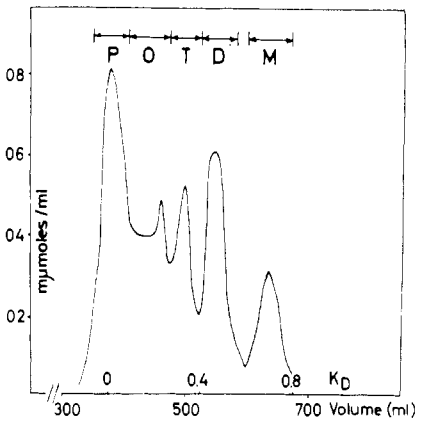

FIGURE 3: Sephadex filtration in 0.1 M LiCl of soluble products from the degradation of walls of *S. albus* G with *Chalaropsis endo-N*-acetylmuramidase. For conditions, see text; solid line, reducing groups expressed in *N*-acetylglucosamine equivalents; M, disaccharide peptide monomer; D, bisdisaccharide peptide dimer; T, trisdisaccharide peptide trimer; O, oligomer; P, polymer.

Experimental Results

I. Chemical Composition of Walls. WALLS OF *Streptomyces* sp. The walls of *S. albus* G contained, per milligram, about 550 mμequiv of a peptidoglycan composed of total hexosamines, L-alanine, D-alanine, D-glutamic acid, LL-diaminopimelic acid, and glycine, in the molar ratios 2:1:0.6:1:1:1. Estimation of N-terminal glycine groups, and of C-terminal D-alanine plus C-terminal LL-diaminopimelic acid groups indicated an average size for the peptide moiety of about 2.5 to 3 cross-linked peptide units. The walls of *S.* K 27 and *S.* R 61 had, within the range of experimental errors, chemical compositions virtually identical with that of *S. albus* G.

WALLS OF *C. perfringens*. They contained, per milligram, about 350 mμequiv of a peptidoglycan composed of total hexosamines, L-alanine, D-alanine, D-glutamic acid, LL-diaminopimelic acid, and glycine, in the molar ratios 2:1:0.6:1:1:0.65. They also contain 0.4 mol of lysine and 1.6 moles of ethanolamine per glutamic acid as well as organic phosphate, hexoses, and additional acetamido sugars (Pickering, 1966). Estimation of N-terminal groups (glycine and mono-LL-diaminopimelic acid) and C-terminal groups (glycine, LL-diaminopimelic acid, and D-alanine) indicated an average size for the peptide moiety of 2 to 2.5 cross-linked peptide units.

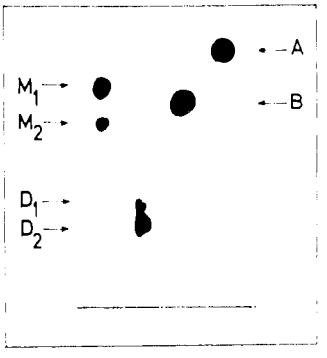

FIGURE 2: Separation of the *S. albus* G monomer fraction (K_D 0.85 in Figure 1) into compounds M_1 and M_2 and of the *S. albus* G dimer fraction (K_D 0.7 in Figure 1) into compounds D_1 and D_2, by preparative cellulose thin-layer chromatography (solvent I): A, L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine tetrapeptide; B, L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelic acid tripeptide (see Material and Methods, and van Heijenoort *et al.*, 1969).

II. Enzymatic Degradation of Walls of S. albus G. The walls prepared from strains *albus* G, K 27, and R 61 exhibited similar sensitivities toward lytic endomuramidases and endopeptidases. The ensuing paragraphs deal with the degradation and detailed structure of the wall peptidoglycan of *S. albus* G only.

DEGRADATION BY ENDOPEPTIDASE AND AMIDASE FROM *Myxobacter* AL-I ENZYME. The isolated *Streptomyces* MR, ML, SA, and KM endopeptidases (in 0.01 M Veronal buffer, pH 8.5; molar ratio of enzyme to substrate, 1 to 10) were without visible effect on the walls of *S. albus*, but *Myxobacter* AL-I enzyme (in 0.01 M Veronal buffer, pH 8.9) dissolved the walls. At completion of the reaction, about 400 mμequiv of N-terminal alanine groups, per milligram of walls, was exposed as a result of the *N*-acetylmuramyl-L-alanine amidase activity, and about 200 mμequiv of D-alanylglycine linkages, per milligram of walls, was hydrolyzed as a result of the endopeptidase activity. No free glycine residues were detected. Gel filtration of 100 mg of *Myxobacter*-degraded walls (Figure 1) on the Sephadex column systems yielded a peptide monomer fraction (K_D 0.85; yield, 50% in terms of total glutamic acid) and a peptide dimer fraction (K_D 0.7; yield, 15%). After desalting, preparative cellulose thin-layer chromatography with solvent I (Figure 2) resolved the K_D 0.85 fraction into two monomeric compounds M_1 and M_2 and resolved the K_D 0.7 fraction into two dimeric compounds D_1 and D_2.

DEGRADATION BY *endo-N*-ACETYLMURAMIDASE FROM *Chalaropsis*. The *Streptomyces* R_1 preparation (Materials and Methods) completely hydrolyzed the glycan portion of the wall peptidoglycan into *N*-acetylhexosamines residues (970 mμmoles/mg), as a result of the sequential action of *endo-N*-acetylmuramidases and *exo-N*-acetylhexosaminidases (Ghuysen *et al.*, 1968). Both egg-white lysozyme (in 0.02 M phosphate buffer pH 5.5; ratio of enzyme to substrate, 1 to 10; 24 hr at 37°) and the *Chalaropsis* enzyme (in water or in 0.01 M

TABLE I: Analyses of the Disaccharide Peptide Monomer and Oligomer Fractions Prepared from Walls of *S. albus* G.

	M	D	T	O	P
Amino acid composition					
L-Ala	93	97	99	97	103
D-Ala	71	56	46	60	61
Glu	100	100	100	100	100
LL-Diaminopimelic acid	106	108	110	115	115
Gly	96	113	95	94	96
β-1,4-GlNAc-MurNAc[a]	96	91	80	78	82
N-Terminal groups					
Gly[b]	90	43	35	24	30
Ala	2	4	4	5	8
Peptide chain length[c]	1	2.1	2.6	3.3	2.6
Recovery in % total Glu	5.6	15.7	13	23.7	42

[a] GlNAc = *N*-acetylglucosamine; MurNAc = *N*-acetylmuramic acid. Results are expressed in terms of glutamic acid residue. [b] Corrected for normal recovery of 65%. [c] 100/total N terminals. Average peptide chain length of the peptide moiety = 100/Σ% contribution to total N terminals = 2.5.

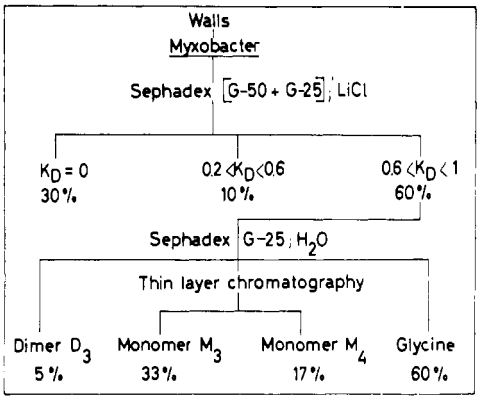

FIGURE 4: *Myxobacter* degradation of walls of *C. perfringens*. Flow sheet of the Sephadex filtrations and of the ensuing preparative cellulose thin-layer chromatography. Yields are expressed in per cent of the total glutamic acid residues, except that of free glycine which is expressed in % of the total glycine residues. Note that the fraction K_D 0 contains all the acetamido sugars and 90% of total lysine and ethanolamine residues.

acetate buffer pH 4.5; ratio of enzyme to substrate, 1 to 100; 24 hr at 37°) also dissolved the walls and liberated, per milligram of walls, reducing and acetamido sugars which were maximally equivalent to about 480 mμmoles of β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharides. Gel filtration of 200 mg of *Chalaropsis*-degraded walls in 0.1 M LiCl, on the Sephadex G-50–Sephadex G-25 column systems (Figure 3) followed by desalting of the pooled fractions on Sephadex G-25 in water yielded a disaccharide peptide monomer M (K_D 0.70; yield expressed in total glutamic acid, 5.6%), a bisdisaccharide peptide dimer D (K_D 0.45; yield, 15.7%), a trisdisaccharide peptide trimer T (K_D 0.33; yield, 13%), and a mixture of oligomers O (K_D 0.20; yield, 23.7%) and excluded polymers P (yield, 42%). The purity of the monomeric, dimeric, and trimeric compounds (K_D 0.7, K_D 0.45, and K_D 0.33, respectively) was confirmed by thin-layer chromatography in solvent III (R_F values, 0.44 for M, 0.34 for D, and 0.24 for T) and their structures were confirmed by analyses (Table I). These also indicated that both the oligomer and polymer fractions had an average chain length of about 3 cross-linked peptide units. These fractions are probably composed of nonpeptidoglycan polymers in covalent association with small peptidoglycan polymers.

DEGRADATION BY *endo-N*-ACETYLMURAMIDASE FROM *Chalaropsis* AND *N*-ACETYLMURAMYL-L-ALANINE AMIDASE FROM *Streptomyces*. *Chalaropsis*-degraded walls (100 mg) were treated with the *Streptomyces N*-acetylmuramyl-L-alanine amidase (in 0.03 M acetate buffer, pH 5.5), resulting in the exposure of about 300 mμmoles of N-terminal L-alanine per mg of walls. Gel filtration of the degraded products on the Sephadex column system yielded a fraction containing 27 μmoles of free disaccharide units (K_D 0.75). After filtration on Sephadex G-25, in water, the disaccharide was further purified by preparative paper chromatography in solvent IV. It was characterized as *N*-acetylglucosaminyl-*N*-acetylmuramic acid (Azuma *et al.*, 1970).

III. Enzymatic Degradation of Walls of C. perfringens. DEGRADATION BY ENDOPEPTIDASE AND AMIDASE FROM *Myxobacter* AL-I ENZYME. Solubilization of the walls by *Myxobacter* enzyme followed the appearance, per milligram, of 180 mμequiv of N-terminal L-alanine (as a result of the *N*-acetylmuramyl-L-alanine amidase activity), the liberation of free glycine residues, and the exposure of C-terminal D-alanine and mono-N-terminal LL-diaminopimelic acid (as a result of the endopeptidase activity). Data showed that the endopeptidase activity induced the hydrolysis, per milligram of walls, of about 130 mμequiv of D-alanylglycyl-LL-diaminopimelic acid sequences at both the D-alanylglycine and the glycyl-LL-diaminopimelic acid sites. Approximately 80% of the glycine, or 0.5 mole per mole of total glutamic acid, was in the endo position in the native walls, and out of this, 75% or 0.39 mol was liberated as free amino acid. Gel filtration of 100 mg of *Myxobacter*-treated walls on the Sephadex column system (Figure 4) followed by desalting yielded free glycine, a peptide monomer fraction (yield, 50% in total glutamic acid), and a peptide dimer fraction D_3 (yield, 5% in total glutamic acid). Cellulose thin-layer chromatography with solvent I of the peptide monomer fraction (Figure 5) yielded two monomers M_3 and M_4.

DEGRADATION BY *endo-N*-ACETYLMURAMIDASE AND *N*-ACETYLMURAMYL-L-ALANINE AMIDASE FROM *Streptomyces*. *Streptomyces* F_1 *endo-N*-acetylmuramidase (in 0.01 M phosphate buffer pH 7.7; ratio of enzyme to substrate, 1 to 100; 16 hr at 37°) dissolved the walls and, eventually, exposed per milligram of walls, reducing groups equivalent to about 110 mμmoles of disaccharide units. Subsequent action of the *Streptomyces N*-acetylmuramyl-L-alanine amidase exposed about 140 mμequiv of N-terminal alanine per mg of walls. Gel filtration of 100 mg of degraded walls on

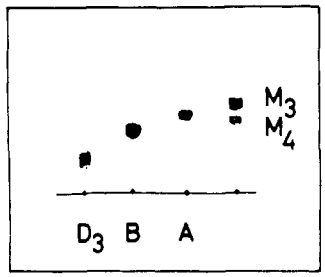

FIGURE 5: Separation of the *C. perfringens* monomer fraction (see Figure 4) into compounds M_3 and M_4 by preparative cellulose thin-layer chromatography (solvent I). Under the same conditions, dimer D_3 (see Figure 4) was not resolved into individual compounds. A and B: model peptides, see Figure 2. The chromatographic mobilities of the peptide monomers are in the following order: M_3 monomer from *C. perfringens;* L-Ala-γ-D-Glu-(L)-*meso*-diaminopimelyl-(L)-D-Ala; M_4 monomer from *C. perfringens* or M_1 from *S. albus* G; L-Ala-γ-D-Glu-(L)-*meso*-diaminopimelic acid; M_2 monomer from *S. albus* G.

the Sephadex column system did not give rise to any well-characterized compound. Although the excluded fraction had been preferentially enriched in ethanolamine, lysine, hexoses, and organic phosphate, these nonpeptidoglycan components were detected in all samples from K_D 0 to K_D 0.9 together with peptidoglycan fragments. Three main fractions (K_D 0; K_D 0.2–0.6; K_D 0.6–1) were separately refiltered on Sephadex G-25 in water and yielded a series of complex fractions A_1, A_2, A_3, B_1, B_2, C_1, and C_2 and a small amount of peptide monomers (yield, 8% in total glutamic acid) (Figure 6). Cellulose thin-layer chromatography of this latter fraction with solvent I gave rise to two peptide monomers which were found indistinguishable from the peptide monomers M_3 and M_4 (Figure 5) isolated from walls of *C. perfringens* degraded by *Myxobacter* enzyme. Evidently, the yield of the above degradation was quite unsatisfactory. The presence of many phosphate groups which probably reflects a considerable cross-linking between the peptidoglycan and the other wall polymers may provide an explanation for the limited number of linkages that were susceptible to enzymatic hydrolysis and for the poor resolution of the fractionation.

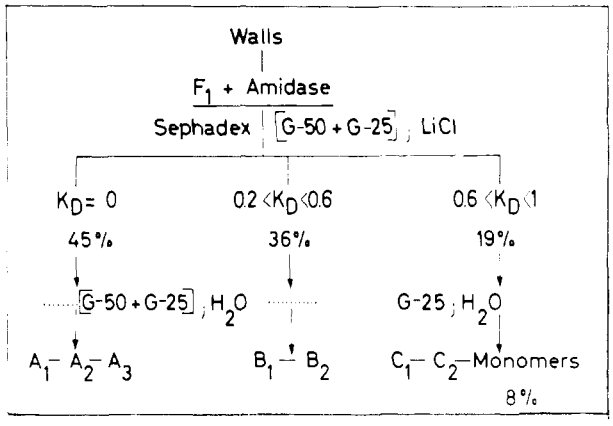

FIGURE 6: F_1 muramidase and amidase degradation of walls of *C. perfringens*. Sephadex fractions and yields (in per cent of the total glutamic acid residues).

TABLE II: Action of *Myxobacter* Enzyme on Soluble Peptide Fractions from *C. perfringens* (see Figure 6).[a]

Fractions	Enzyme Treatment[b]	Terminal Amino Groups: Mono-Diamino pimelic acid	Terminal Amino Groups: Gly	Free Gly	C-Terminal Ala
A_1	None	0.60	0	0	
	+ Myxo	0.94	0.05	0.35	
	+ Myxo + Amino	1.02	0.05	0.38	
B_2	None	0.15	0	0	0
	+ Myxo	0.18	0.15	0	0.24
	+ Myxo + Amino	0.31	0.05	0.16	0.25
C_2	None	0.30	0	0	0
	+ Myxo	0.30	0.14	0	0.35
	+ Myxo + Amino	0.64	0.04	0.32	0.35

[a] Data are expressed in mμmoles or mμequiv per μl. Values for C- and N-terminal groups were not corrected. [b] Myxo = *Myxobacter* ALI enzyme; Amino = *Streptomyces* aminopeptidase.

DEGRADATION OF SOLUBLE GLYCOPEPTIDE FRAGMENTS BY ENDOPEPTIDASE FROM *Myxobacter* AL-I ENZYME. The fractions A_1, A_2, A_3, B_1, B_2, C_1, and C_2 (Figure 6) obtained after gel filtration of the endomuramidase- and amidase-degraded walls were separately treated by *Myxobacter* enzyme. Free glycine was liberated from fraction A_1 which was excluded from the gels as was observed with the intact walls. In marked contrast to this, however, the *Myxobacter* enzyme, when acting upon all the other fractions, catalyzed the hydrolysis of D-alanylglycine linkages (*i.e.*, linkage a in Figure 9) but not the hydrolysis of glycyl-LL-diaminopimelic acid linkages (*i.e.*, linkage b in Figure 9). Free glycine residues were only liberated in subsequent hydrolysis with the *Streptomyces* aminopeptidase. The enzymatic behavior of all *C. perfringens* fractions but A_1 was thus reminiscent of that of the intact walls of *S. albus* G. Some typical results are shown in Table II.

IV. Structure of the Peptide Monomers M_1 and M_2 Isolated after Myxobacter Degradation of Walls of S. albus G and of the Peptide Monomers M_3 and M_4 Isolated after Myxobacter Degradation of Walls of C. perfringens. Analyses of the peptide monomers and the results of degradation of these compounds by the *Streptomyces* aminopeptidase are given in Table III. Liberation of L-alanine by the aminopeptidase always resulted in the exposure of an equivalent amount of N-terminal isoglutamine while liberation of glycine (from monomers M_1 and M_2) always resulted in the exposure of an equivalent amount of mono-N-terminal LL-diaminopimelic acid. The aminopeptidase-catalyzed liberation of the terminal L-alanine residue established that the amide group was located on the α-carboxyl of glutamic acid, *i.e.*, the presence of an isoglutamine residue. Indeed, it is known that this enzyme is ca-

TABLE III: Analyses of the Peptide Monomers Prepared from Walls of *S. albus* G and of *C. perfringens*.[a]

	S. albus G		*C. perfringens*	
	M_1	M_2	M_3	M_4
Amino acid composition				
L-Ala	1.1	1	1.06	0.96
D-Ala	1.1	0.1	1.03	0
Glu	1	1	1	1
LL-Diaminopimelic acid	1.3	1.3	1.1	1
Gly	1	1.1	0	0
Amide	1	1	0.93	1
N-Terminal groups[b]				
L-Ala	0.63	0.71	0.97	0.84
Mono-*N*-diaminopimelic acid	0	0	0.97	0.86
Gly	0.57	0.65	0	0
C-Terminal groups[b]				
D-Ala	1.1	0	0.82	0
Diaminopimelic acid	0	0.74	0	0.99
Free amino acid Released by Aminopeptidase				
L-Ala	1.08	1.07	0.96	0.85
Gly	0.80	0.87	0	0

[a] Results are expressed in terms of glutamic acid residue. [b] Uncorrected values.

pable of hydrolyzing an L-alanyl-D-isoglutaminyl linkage (Muñoz *et al.*, 1966) whereas it has no action on peptides such as L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine (van Heijenoort *et al.*, 1969) or N^{α}-[L-alanyl-γ-(α-D-glutamylglycine)]-L-lysyl-D-alanine (Ghuysen *et al.*, 1968). At pH 3.8, the peptide monomers (as well as the peptide dimers) were neutral (Figure 7). This property was in agreement with the presence of an isoglutamine residue, since under the same conditions, the model peptides L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelic acid and L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine were anionic (Figure 7). Evidently, monomer M_4 (from *C. perfringens*) was the tripeptide L-alanyl-D-isoglutaminyl-LL-diaminopimelic acid (Figure 8) and monomer M_2 (from *S. albus* G) was the tetrapeptide (L-alanyl-D-isoglutaminyl)-(L_1)-(glycyl)-(L_2)-LL-diaminopimelic acid (Figure 8). Moreover, monomer M_3 (from *C. perfringens*) was a tetrapeptide and monomer M_1 (from *S. albus* G) a pentapeptide differing from tripeptide M_4 and tetrapeptide M_2, respectively, by the presence of an additional C-terminal D-alanine residue. The location of the D-alanine residue was proved by Edman degradations (Table IV). Degradation of tetrapeptide M_3 (after one cycle) and degradation of pentapeptide M_1 (after two cycles) did not give rise to free alanine whereas, under the same conditions and after one cycle of degradation, L-alanine was liberated from the model peptide *meso*-diaminopimelyl-(D)-L-alanine. Consequently, the C-terminal D-alanine residue in the tetrapeptide M_3 of *C. perfringens* was not in position α to the free amino group of LL-diaminopimelic acid (Figure 8). Similarly, in the pentapeptide M_1 of *S. albus* G, the C-terminal D-alanine and the N-terminal glycine were not located on the same asymmetric carbon of LL-diaminopimelic acid (Figure 8). From the above analytic data, together with the aforestudied mechanism of action of the *Myxobacter* enzyme, it is evident that in a major part of the *S. albus* G and *C. perfringens* wall peptidoglycans, the peptide cross-linking is mediated by single glycine residues as shown in Figure 9.

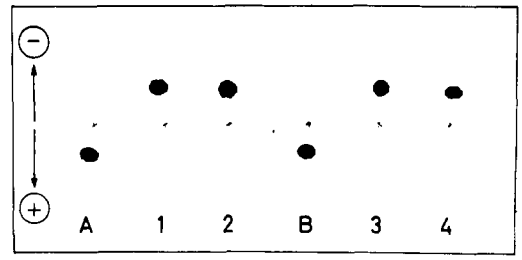

FIGURE 7: Paper electrophoresis at pH 3.9 of peptide monomers M_1 + M_2 of *S. albus* G (1), of the peptide monomers M_3 + M_4 of *C. perfringens* (3), of peptide dimers D_1 + D_2 of *S. albus* G (2), of peptide dimer D_3 of *C. perfringens* (4), and of model peptide A and B (see Figure 2).

V. Structure of the Peptide Dimers Isolated after Myxobacter Degradation of Walls of S. albus G and of C. perfringens. Table V presents the composition of the peptide dimers D_1 and D_2 isolated from *S. albus* G and of the peptide dimer D_3 isolated from *C. perfringens*, determinations of N- and C-terminal groups, and results of the degradation by the *Streptomyces* aminopeptidase. Again, the liberation of free L-alanine and of free glycine was followed by the exposure of N-terminal isoglutamine and of mono-N-terminal LL-diaminopimelic acid, respectively. The dimeric structure of compounds D_1 and D_2 of *S. albus* G rested upon their chromatographic migrations (Figure 2) and the demonstration that, per glutamic acid, 0.5 glycine was in the endo position and 0.5 glycine occurred as N-terminal groups. Furthermore, the liberation of this N-terminal glycine exposed, per glutamic acid, 0.5 mono-N-terminal LL-diaminopimelic acid groups. Similarly, the

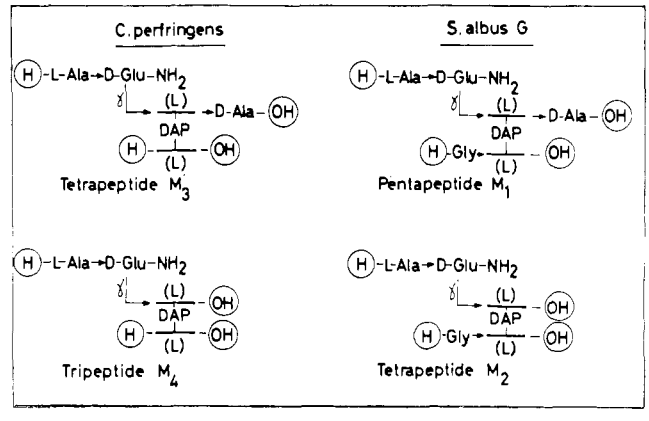

FIGURE 8: Structure of the peptide monomers from walls of *C. perfringens* and *S. albus* G.

TABLE IV: Edman Degradation of *S. albus* G and *C. perfringens* Peptide Monomers and of Peptide Model.[a]

	Cycles of Edman Degradation	Free Ala	Terminal Amino Groups: Di-diamino-pimelic Acid	Ala	Glu	Gly	Mono-diamino-pimelic Acid
Synthetic dipeptide	0	0	1	0	0	0	0
meso-Diaminopimelyl-D-L-Ala	1	0.9	0	0	0	0	0
C. perfringens M_3 peptide	0	0	0	1	0	0	0.97
	1	0	0	0.03	0.63	0	0.18
S. albus G M_1 peptide	0	0	0	1	0	0.7	0
	1	0	0	0	1	0	0.79
	2	0	0	0	0	0	0

[a] Data expressed per N-terminal group of L-alanine of the original peptide.

dimeric structure of the D_3 preparation of *C. perfringens* was proved by the demonstration that all the glycine residues were in the endo position and that they occurred in the ratio of 0.5 per glutamic acid. Unexpectedly, however, D-alanine apparently was not involved in the peptide cross-linking. Indeed, the dimer D_2 of *S. albus* G had only traces of D-alanine and the D_3 fraction of *C. perfringens* had only C-terminal D-alanine. These data are consistent with the structures seen in Figure 10. None of the isolated dimers was found sensitive to further treatment with the *Myxobacter* enzyme.

Discussion

In agreement with studies made by Schleifer *et al.* (1968b) and by Arima *et al.* (1968), the present studies led us to propose that a major part of the peptide moiety of the wall peptidoglycans of *Streptomyces* sp. and of *C. perfringens* consists of L-alanyl-D-isoglutaminyl-(L_1)-LL-diaminopimelyl-(L_1)-D-alanine tetrapeptides cross-linked by means of single glycine residues extending from the amino group located on the L_2 carbon of LL-diaminopimelic acid of one peptide subunit to the C-terminal D-alanine of another subunit (Figure 9). Thus, according to the proposed classification of Ghuysen (1968), these LL-diaminopimelic acid containing peptidoglycans, like other L-ornithine- and L-lysine-containing peptidoglycans, belong to the chemotype II group. The demonstration that both the amino group linked to D-isoglutamine and the carboxyl group linked to D-alanine are located on the same asymmetric carbon gives further support to the prevailing hypothesis that the peptide subunits in all the wall peptidoglycans have the al-

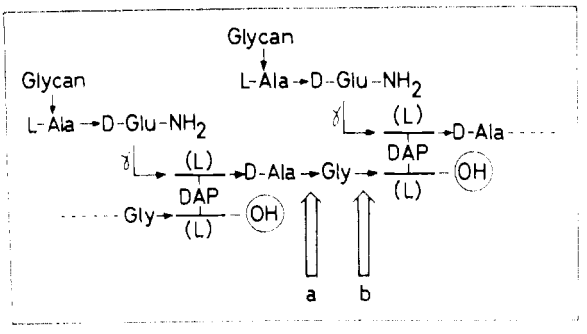

FIGURE 9: Main type of peptide cross-linking in the wall peptidoglycans of *C. perfringens* and *S. albus* G (peptidoglycan of the chemotype II group; Ghuysen, 1968). When acting on walls of *C. perfringens*, *Myxobacter* enzyme hydrolyzes a and b linkages. When acting on walls of *S. albus* G, *Myxobacter* enzyme hydrolyzes a linkages but not b linkages.

TABLE V: Analyses of the Peptide Dimers Prepared from Walls of *S. albus* G and of *C. perfringens*.[a]

	S. albus G: D_1	*S. albus* G: D_2	*C. perfringens*: D_3
Amino acid composition			
Total Ala			1.60
L-Ala	1	1	
D-Ala	0.33	0.1	
Glu	1	1	1
LL-Diaminopimelic acid	1.17	1.26	1.08
Gly	0.90	1.08	0.50
Amide NH_3	1	1	1
N-Terminal groups[b]			
L-Ala	0.63	0.65	0.73
Mono-*N*-diaminopimelic acid	0	0	0.42
Gly	0.25	0.28	0
C-Terminal groups[b]			
D-Ala			0.46[c]
Free amino acid released by aminopeptidase			
L-Ala	1	1.2	0.9[c]
Gly	0.47	0.6	0.1

[a] Results are expressed in terms of glutamic acid residue. [b] Uncorrected values. [c] The sum of these two figures (1.36 for 1.60 total alanine) show that there is no alanine in the endo position.

ternating LDLD sequence, all the peptide linkages being α, except for the γ-glutamyl linkage (see introduction). Other features of the *Streptomyces* and *C. perfringens* peptidoglycans are that: (i) the carboxyl group of LL-diaminopimelic acid not engaged in linkages is not amidated; (ii) the extents of cross-linking are similar with an average size of about 2 to 3 cross-linked peptide subunits; and (iii) the C-termini of the peptide monomers and oligomers are either D-alanine or LL-diaminopimelic acid.

The disruption of the peptide cross-linking bridges with the liberation of the glycine residues in walls of *Streptomyces* sp. was achieved through the sequential action of the endopeptidase from *Myxobacter* enzyme which hydrolyzed D-alanylglycine linkages (that are in endo position) (link a in Figure 9), and of the *Streptomyces* aminopeptidase which hydrolyzed the N-terminal glycyl-LL-diaminopimelic acid linkages. When acting on native walls of *C. perfringens*, the endopeptidase from *Myxobacter* enzyme was capable of hydrolyzing both D-alanylglycine and glycyl-LL-diaminopimelic acid linkages within a majority of the peptide cross-linking bridges (links a and b in Figure 9) so that the glycine residues were freed directly. However, prior treatment of the *C. perfringens* walls by endomuramidase and amidase from *Streptomyces* made most of the glycyl-LL-diaminopimelic acid linkages resistant to *Myxobacter* endopeptidase so that, as observed with walls of *S. albus* G, the liberation of the glycine residues again required that *Myxobacter* action be followed by aminopeptidase treatment. The hydrolysis of the glycyl-LL-diaminopimelic acid linkages within the D-alanylglycyl-LL-diaminopimelic acid sequences apparently depends upon unidentified structural features in the substrate. No explanation for this observation can be proposed at present.

The study of the peptide dimers isolated after degradation of the walls by endopeptidase and amidase from *Myxobacter* enzyme provided evidence for the existence in a minor part of the peptide moieties of a new type of peptide cross-linking that does not involve a D-alanine residue (Figure 10). Such dimers might have arisen from a bridge closure reaction between L-alanyl-D-isoglutaminyl-(L_1)-LL-diaminopimelyl-(L_1)-glycyl-D-alanine and L-alanyl-D-isoglutaminyl-(L_1)-LL-diaminopimelyl-(L_1)-D-alanyl-D-alanine peptides by means of a transpeptidase-catalyzed transfer of the carboxyl group of the glycine residue of the former to the L_2-amino group of the LL-diaminopimelic acid of the latter. This would require the involvement of two types of muramylpentapeptide nucleotides in the synthesis of wall peptidoglycan, *i.e.*, UDP-*N*-acetylmuramyl-L-alanine-γ-D-glutamyl-(L_1)-LL-diaminopimelyl-(L_1)-D-alanyl-D-alanine and UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L_1)-LL-diaminopimelyl-(L_1)-glycyl-D-alanine.

As noted above, the C termini in the peptide moieties of the wall peptidoglycans of *Streptomyces* sp. and of *C. perfringens* are either LL-diaminopimelic acid or D-alanine but never D-alanyl-D-alanine, thus indicating the existence in these microorganisms of carboxypeptidases similar to those of *E. coli* recently studied by Izaki and Strominger (1968), and by Bogdanovsky *et al.* (1969). A DD carboxypeptidase which, in contrast to the *E. coli* enzyme, is not inhibited by penicillin G has been isolated from the penicillin-resistant *S. albus* G strain (Ghuysen and Leyh-Bouille, 1970; Guinand *et al.*, 1969a). Interest in the mechanism of action and physiological role of this *Streptomyces* DD carboxypeptidase has added impetus to the structural studies that have been presented in this paper.

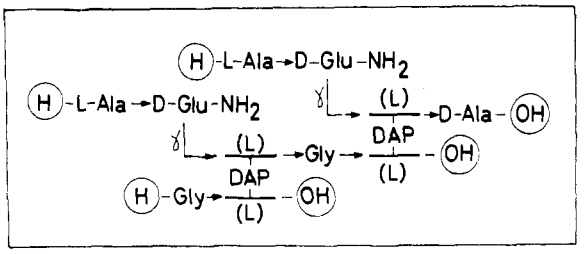

FIGURE 10: Proposed structure for a minor type of cross-linking in the wall peptidoglycans of *S. albus* G and *C. perfringens*. Dimer D_1 from *S. albus* G has the above structure. Dimer D_2 from *S. albus* G lacks the C-terminal D-alanine. Dimer D_3 from *C. perfringens* lacks the N-terminal glycine.

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Wall Autolysin of *Lactobacillus acidophilus* Strain 63 AM Gasser*

Jacques Coyette and Jean-Marie Ghuysen

ABSTRACT: The autolysin of *Lactobacillus acidophilus* strain 63 AM Gasser has the specificity of an endo-*N*-acetylmuramidase. It hydrolyzes both *N*-acetylmuramic acid and *N*, *O*-diacetylmuramic acid linkages. It does not exhibit any amidase or endopeptidase action. It is present in both log-phase and stationary-phase cells.

In stationary-phase cells its action upon the wall peptidoglycan is inhibited.

Experiments carried out with several Gram-positive bacteria (Cole, 1965) and with *Escherichia coli* (Schwarz *et al.*, 1969) strongly suggest a zonal growth of the wall peptidoglycan at least during cell division. Biochemical study of the biosynthesis of the peptidoglycan also strongly suggests that the insertion of newly synthesized β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramyl peptide fragments requires the creation of nonreducing *N*-acetylglucosamine receptor sites in the glycan strands (for a review, see Ghuysen, 1968). Wall lytic endo-*N*-acetylmuramidases could thus play the role of providing those receptor sites a nd it may be that the safe enlargement of the peptidoglycan sacculus is due, in fact, to a strict coordination between the biosynthetic and the hydrolytic processes. So far is known, *Streptococcus faecalis* appears to be one of the simplest model with regard to the study of wall growth at the cellular level. Indeed, the active wall-bound autolytic system consists of a single enzyme that has the specificity of an endo-*N*-acetylmuramidase (Shockman *et al.*, 1967b). Moreover, it has been shown that the cell equator is the site where the active autolysin is located and the region where new wall material is inserted (Shockman *et al.*, 1967a; Shockman and Martin, 1968; Shockman and Cheney, 1969; Pooley and Shockman, 1969). In contrast to *S. faecalis* which exhibits a single plane of division, other spherical microorganisms that are characteristically arranged in clusters such as *Staphylococcus aureus* (Tipper, 1969), and rod-shaped bacteria such as *E. coli* (Weidel and Pelzer, 1964) and *Bacillus subtilis* (Young, 1966a,b) have complex autolytic systems. These systems contain enzymes such as amidases, endopeptidases, and endo-*N*-acetylglucosaminidases that do not appear to be consistent with a role in wall biosynthesis but that might be involved in other phenomena such as competence, excretion, and permeation of large molecules.

The experiments hereby presented show that *L. acidophilus* 63 AM Gasser is another simple model that may be useful for the study of wall expansion and cell division in a rod-shaped microorganism.

Materials and Methods

Growth conditions, analytic techniques (measurement of reducing groups, acetamido sugars, and N-terminal groups), and *walls preparation and structure* have been described (Coyette and Ghuysen, 1970).

Enzymes. Streptomyces F_1 endo-*N*-acetylmuramidase was used (Ghuysen, 1968).

Experimental Section

Autolysis and Bacterial Growth. Strains of *Lactobacilli* are known to autolyze (Knox and Brandsen, 1962). Preliminary experiments carried out with *L. acidophilus* 63 AM Gasser showed that the rate of autolysis of log-phase cells suspension was maximal in a 0.05 M citrate buffer, pH 5. The specific autolytic activity during growth of *L. acidophilus* was determined as follows: cells were harvested at various times and washed by centrifugations with cold distilled water. The pellets were resuspended in 0.05 M citrate buffer, pH 5, and the turbidity of each cell suspension was adjusted to an optical

* From the Service de Bactériologie, Liège, Belgium. *Received April 2, 1970.* This research has been supported in part by the Fonds de la Recherche Fondamentale Collective, Brussels, Belgium (Contracts 515 and 1000). This paper is from a dissertation submitted by J. C. in partial fulfilment of the requirements for a Ph.D. degree, University of Liège, Belgium (1969).