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Effect of Growth Conditions on Peptidoglycan Structure and Susceptibility to Lytic Enzymes in Cell Walls of *Micrococcus sodonensis**

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ABSTRACT: Cell walls of *Micrococcus sodonensis* were isolated and purified from cultures grown in a synthetic medium and a complex medium. Chemical analyses revealed that two preparations were free of cell membrane contaminants and were qualitatively identical. Glutamic acid, glycine, alanine, and lysine were present in a 1:1:2:1 ratio. *N*-Acetylmuramic acid and *N*-acetylglucosamine were present in equimolar amounts, although walls from cells grown in synthetic medium contained twice as many hexosamine residues as those from cells grown in the complex medium. The subunit peptide structure was determined to be N^{α}-[L-alanyl-γ-(α-glutamylglycine)]-L-lysyl-D-alanine in both cases. Sequential enzymic digestions and isolation of peptide fragments revealed that peptide cross bridging was accommodated by "head-to-tail" assembly of peptide subunits. Such assembly was facilitated by N^{ϵ}-(D-alanyl)-L-lysine and D-alanyl-L-alanine linkages. The distribution and length of cross bridges varied significantly in the two preparations. Controlled digestion studies disclosed significant differences in lysozyme susceptibility of the two preparations. Forty-nine per cent of the dry weight of walls purified from cells grown in complex medium, as opposed to 75% from cells grown in defined media were solubilized by lysozyme. Analysis revealed a greater degree of peptide substitution of the glycan and shorter cross bridges in the lysozyme-insoluble portion than the lysozyme-susceptible portion of the cell walls from both sources. Overall, the peptidoglycan structure was more complex in the walls of cells grown in complex than in synthetic medium. The chemical analyses, plus electron microscopic examination led to the conclusion that in both cases the cell wall is composed of at least two peptidoglycan matrices, one of which is insensitive to lysozyme. This resistance to lysozyme results from a combination of two factors associated with this portion of the wall, (a) an increased level of *O*-acetyl substitution and (b) an increased complexity of cross-linked structure.

In several members of Micrococcaceae, the peptide subunit N^{α}-[L-alanyl-γ-(α-glutamylglycine)]-L-lysyl-D-alanine (Ghuysen, 1968; Schleifer and Kandler, 1970) is the building block of the cell wall peptidoglycan. These subunits are interlinked by peptide bridges extending from the ϵ-amino group of lysine of the one peptide subunit to the α-carboxyl group of the C-terminal alanine of another peptide subunit. "Head-to-tail" assembly of several identical peptide subunits occurs in several micrococcal cell walls and is facilitated by two types of peptide linkages: N^{ϵ}-(D-alanyl)-L-lysine linkages which extend from the ϵ-amino group of lysine to the carboxyl groups of D-alanine, and D-alanyl-L-alanine linkages which actually assemble the peptide subunits in head-to-tail sequence (Schleifer and Kandler, 1967; Ghuysen *et al.*, 1968a,b; Campbell *et al.*, 1969).

The first observation that altered cellular nutrition produces significant changes in the cell wall was made in walls obtained from *Streptococcus faecalis* grown under conditions of threonine depletion (Toennies and Shockman, 1959). Variations in peptidoglycan structure under altered conditions of growth have been documented (Smith and Henderson, 1964; Smith *et al.*, 1965) but these changes involve substitution of one amino acid for another in the peptide subunit. This paper re-

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ports the results of studies involving chemical and enzymic degradation of the cell walls of *Micrococcus sodonensis* prepared from cells grown under two different conditions.

Materials and Methods

Cell Walls. Walls were obtained from *M. sodonensis* (ATCC 11880) grown in Trypticase soy broth, pH 7.3 (Baltimore Biological Laboratories), and a synthetic medium containing lactic acid and glutamic acid for carbon and nitrogen sources, respectively (Campbell *et al.*, 1961). Cells were grown at 30° in 14-l. batches in a Microferm fermentor, aerated at 2 l./min and using as inoculum (0.3%(v/v)) an 18-hr culture of *M. sodonensis*. Cultures were permitted to grow 36 hr at which time they were harvested by centrifugation, washed three times with distilled water, and, if not required immediately, stored at −20°.

Cells were disrupted mechanically by grinding with glass beads and walls purified by a sequence of differential centrifugation and washings in saline and distilled water. The purification procedure included a heat treatment to inactivate autolytic enzymes (Janczura *et al.*, 1961), phenol extraction (Westphal and Jann, 1964) to remove pigment, and a trypsin treatment. Cell walls so obtained were lyophilized and stored at −23° in sealed containers. Preparations derived from trypticase soy broth-grown cells were designated "TCS-grown cell walls" and those derived from cells grown in the synthetic basal medium were designated "basal-grown cell walls."

Analytical Methods. N- and C-terminal groups were measured by the fluorodinitrobenzene technique and the hydrazinolysis technique, respectively (Ghuysen *et al.*, 1966; Ghuysen 1968). Total hexosamines were determined by the modified Morgan–Elson technique (Ghuysen *et al.*, 1966), glucosamine by the glucosaminostat reagent (Lüderitz *et al.*, 1964), reducing sugars by the modified Park–Johnson ferricyanide procedure (Ghuysen *et al.*, 1966), glucose by assaying dried acid hydrolysates with the glucostat reagent (Worthington Biochemical Corp., Freehold, N. J.), and esters by colorimetric estimation of their hydroxamate salts (Hestrin, 1949). Peptide sequences were determined by a modification of the Edman degradation (Tipper *et al.*, 1967).

Chromatographic solvents were (I) chloroform–methanol–acetic acid (85:13:2, v/v) and (II) benzyl alcohol–chloroform–methanol–ammonia–water (30:30:30:2:6, v/v).

Chromatographic Separation of Amino Acid Derivatives. (1) DNP-glutamic acid,[1] DNP-glycine, DNP-alanine, and $N^{\alpha\epsilon}$-di-DNP-lysine were separated on thin-layer plates of Stahl's silica gel H (Merck) using solvent I. (2) N^{ϵ}-Mono-DNP-lysine was isolated by development of thin-layer plates in solvent II.

Gel Filtration. Separations of the products of enzymic degradation of cell walls were effected by gel filtration in 0.1 M LiCl on columns of Sephadex G-50 (medium) and Sephadex G-25 (medium) connected in series as described by Ghuysen *et al.* (1968a). Materials to be desalted were eluted from calibrated Sephadex G-25 columns, or Bio-Gel P-20 or P-2 columns with distilled water.

Conditions for Enzyme Digestion of Cell Walls. Lysozyme digestions were performed in 0.01 M potassium phosphate buffer (pH 7.0) at 37° with a lysozyme:cell walls (w/w) ratio of 1:100. The final volume of digestion mixtures was 2.0 ml. Activity was followed by reduction in turbidity and release of reducing groups in the reaction mixtures. The lysozyme-soluble portion of cell walls was defined as that portion of lysozyme digests of cell walls which was not sedimented by centrifugation at 39,000*g* for 1 hr.

Myxobacter AL-1 protease (Ensign and Wolf, 1966) digestions were performed at 37° in 0.01 M Veronal–HCl buffer (pH 9.0), containing 1×10^{-3} M Versene with 1:1000 enzyme:cell walls (w/w). The final volume of digests was 2.0 ml. Activity was measured by reduction in turbidity and the release of free N- and C-terminal alanine groups in the digestion mixtures.

A purified preparation of Streptomyces *N*-acetylmuramyl-L-alanine amidase was obtained from J.-M. Ghuysen, Université de Liège, Belgium. Five microliters of a 1:100 dilution of this prepration was sufficient to completely hydrolyze 30 nmoles of *Staphylococcus aureus* disaccharide peptide in 4 hr at 37° (J.-M. Ghuysen, personal communication, 1970). All digestions were performed with 100 μl of diluted enzyme preparation/mg of cell wall in 0.02 M sodium acetate buffer (pH 5.5) at 37°. Hydrolysis was monitored by assaying for release of N-terminal alanine.

Digestions with Streptomyces ML-endopeptidase were performed in 0.01 M Veronal–HCl buffer (pH 9.0) with 1:50 enzyme:cell walls (w/w). Activity was followed by reduction in turbidity of digestion mixtures and release of C-terminal alanine groups.

Gas–liquid chromatography was performed with a Varian 700 gas chromatograph utilizing a thermal conductivity detector and helium as the gas carrier. Products of alkali digestion of cell walls were identified by gas–liquid chromatography on a 0.25 in. × 5 ft 15% FFAP (Byars and Jordan, 1964) on 60–80 Chromosorb W column. Column temperature was held at 135° while detector and injector temperatures were maintained, respectively, at 228 and 170°. Filament current was held at 175 mA and gas flow at 60.0 cc/min.

Electron Microscopic Analysis. Samples were examined in a Philips 200 electron microscope and thin sections prepared using a Porter-Blum automatic microtome, Model MT-2. Negative contrast staining was achieved by the application of 3% phosphotungstic acid. Procedures for fixation, embedding, and washing of samples were those described by Kellenberger *et al.* (1959).

Experimental Section

Estimation of Peptidoglycan Components. Analyses were performed upon whole lysozyme digests of cell walls. Both cell wall preparations contained glutamic acid, glycine, alanine, and lysine in a 1:1:2:1 ratio (Table I). Although muramic acid and glucosamine were present in equimolar amounts, basal-grown cell walls contained almost twice as many hexosamine residues per milligram as did TCS-grown cell walls (Table I). The absence of DNP-muramic acid and DNP-glucosamine in hydrolysates of dinitrophenylated cell walls indicated that the hexosamines of the glycan were N-acetylated. Based upon glycine content, TCS- and basal-grown cell walls contained, respectively, 523 and 531 peptide subunits per mg of cell wall.

Enzymic Degradation of Cell Walls by Myxobacter AL-1 Protease. Clarification of cell wall suspensions digested with Myxobacter AL-1 protease was compared with the rate of release of N-terminal alanine (Figure 1). Basal-grown cell wall suspensions were essentially clarified by this enzyme prior to any significant release of free N-terminal alanine groups. In contrast, clarification of TCS-grown cell wall suspensions

[1] Abbreviations used are: DNP-amino acid, dinitrophenyl derivative of the designated amino acid; FFAP, free fatty acid phase (Varian Aerograph).

TABLE I: Peptidoglycan Components of *M. sodonensis* Cell Walls.

Component	TCS-Grown Cell Walls Amt[a]	TCS-Grown Cell Walls Molar Ratio	Basal-Grown Cell Walls Amt[a]	Basal-Grown Cell Walls Molar Ratio
Glutamic acid	514	1.00	546	1.03
Glycine	523	1.02	531	1.00
Alanine	1070	2.08	1060	2.01
Lysine	514	1.00	530	1.00
Total Hexosamines	567		1070	
Glucosamine	278	1.00	540	1.00
Muramic acid	289	1.04	528	0.98

[a] Data are expressed as nanomoles per milligram of cell wall.

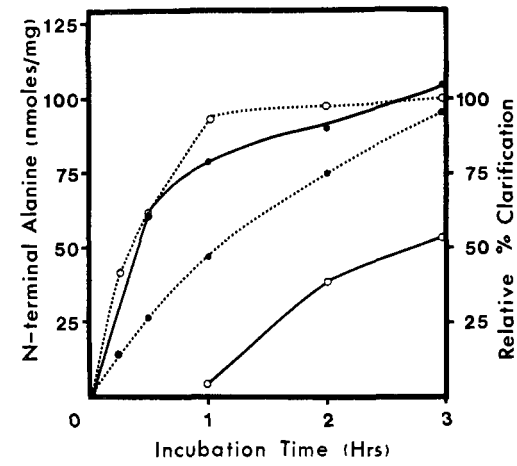

FIGURE 1: Comparison of *N*-terminal alanine release and clarification of Myxobacter AL-1 protease digests of *M. sodonensis* cell walls. Cell walls (2.5 mg/ml) were digested with Myxobacter AL-1 protease under standard specified conditions. Triplicate 100-μl aliquots were withdrawn at intervals and assayed for their content of N-terminal alanine. The turbidity of the digestion mixture was simultaneously determined at 550 nm. (●—●) N-Terminal alanine release in TCS-grown cell walls. (○—○) N-Terminal alanine release in basal-grown cell walls. (●----●) Relative per cent clarification of TCS-grown cell wall digest. (○----○) Relative per cent clarification of basal-grown cell wall digest.

and release of N-terminal alanine groups occurred almost simultaneously.

Free N- and C-terminal amino acid groups released by digestion with Myxobacter AL-1 protease were estimated. Five milligrams of each preparation in a final volume of 2.0 ml was digested for 24 hr with the protease under previously specified conditions. Triplicate 50- and 100-μl aliquots, respectively, were assayed for their content of free N- and C-terminal amino acid groups. Neither intact wall preparation contained detectable free α-amino groups (Table II). Comparison of Tables I and II shows that in intact TCS-grown walls there are no detectable free α-amino groups and 94% of N^{ϵ}-amino groups of lysine and 90% of carboxyl groups of glycine are free. After digestion with protease in excess of 96% of lysine and 99% of glycine residues had free N^{ϵ}-amino groups and carboxyl groups, respectively. By contrast, intact basal-grown cells exhibited only 73% of the lysine residues and 66% of the glycine residues with free N^{ϵ}-amino and carboxyl groups, respectively. Furthermore protease digestion failed to release the remainder of these groups, leaving approximately 25% of each substituted. Only a small percentage of the total alanine content of each cell wall preparation was released by Myxobacter AL-1 protease digestion.

Estimation of the number of *N*-acetylmuramyl-L-alanine linkages in intact walls was achieved by subtracting the number of N-terminal alanine groups released by the hydrolysis of D-alanyl-L-alanine linkages from the total number of free N-terminal alanine groups released by Myxobacter AL-1 protease digestion of cell walls (Table III). N-Terminal groups released by cleavage of D-alanyl-L-alanine bonds was defined as the difference in free C-terminal alanine content before and after Myxobacter AL-1 protease digestion. On the average, two in every five *N*-acetylmuramic acid residues are peptide substituted in TCS-grown cell walls whereas approximately one in ten *N*-acetylmuramic acid residues is so substituted in basal-grown cell walls.

Myxobacter AL-1 protease digests of cell walls were fractionated by gel filtration as previously described (Figure 2). The free amino groups of TCS-grown cell wall digests treated in this manner yielded four main peaks having K_{av} values of

TABLE II: Free N- and C-Terminal Amino Acids before and after Digestion with Myxobacter AL-1 Protease.[a]

Walls	*N*-Ala	*N*-Lys	*C*-Ala	*C*-Gly
TCS grown				
Before digestion	*b*	490	88	487
After digestion	134	501	106	518
Basal grown				
Before digestion	*b*	387	30	352
After digestion	86	415	46	397

[a] Data are expressed as nanomoles per milligram of cell wall. [b] Below levels of detection.

TABLE III: *N*-Acetylmuramic Acid Peptide Substitution in *M. sodonensis* Cell Walls.[a]

	TCS-Grown Cell Walls	Basal-Grown Cell Walls
Total N-terminal alanine released by Myxobacter AL-1 protease (A)	134	86
N-Terminal alanine released by cleavage of D-alanyl-L-alanine linkages (B)	18	16
Total *N*-acetylmuramyl-N^{α}-L-alanine linkages (A–B)	116	70
Total muramic acid	289	528
Per cent muramic acid substituted	40	13

[a] Data are expressed as nanomoles per milligram of cell wall.

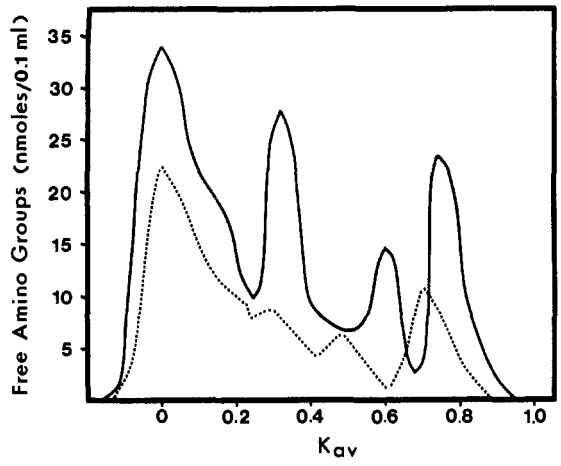

FIGURE 2: Fractionation of Myxobacter AL-1 protease digests of *M. sodonensis* cell walls. Each cell wall preparation (50 mg) was digested with Myxobacter AL-1 protease under standard specified conditions. Digests were concentrated to a volume of 5.0 ml and applied to the linked Sephadex G-50–G-25 system and eluted with 0.1 M LiCl. Fractions (5 ml) were collected and 100 μl of each assayed for its content of free amino groups relative to an L-alanine standard. (——) TCS-grown cell walls. (------) Basal-grown cell walls.

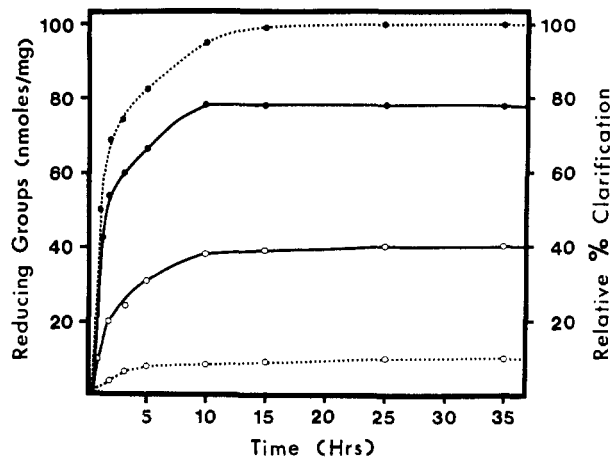

FIGURE 3: Kinetics of lysozyme digestion of *M. sodonensis* cell walls. Each cell wall preparation (5 mg) was digested at 37° with lysozyme under standard specified conditions. The final volume of each digest was 2.0 ml. At intervals, 50-μl aliquots were withdrawn, centrifuged to sediment insoluble material, and 10- or 20-μl portions of the supernatant fractions assayed for their content of reducing groups relative to a glucosamine standard. The pellet was resuspended in 400 μl of distilled water and the turbidity at 550 nm determined. (O——O) Reducing groups liberated in TCS-grown cell walls. (●——●) Reducing groups liberated in basal-grown cell walls. (O----O) Relative per cent clarification of TCS-grown cell wall digests. (●----●) Relative per cent clarification of basal-grown cell wall digests.

0.75, 0.6, 0.32, and 0 (excluded). The first two corresponded to the peptide monomer and dimer fractions observed in a similar fractionation of *Sarcina lutea* cell walls (Campbell *et al.*, 1969). Digests of basal-grown cell walls did not yield such clearly resolved monomer or dimer fractions.

The fraction from TCS-grown cell walls with a K_{av} of 0.75 was provisionally designated "peptide monomer," desalted on a 2.5 × 30 cm column of Bio-Gel P-2, eluted with distilled water, and concentrated to a volume of 3.0 ml. A 0.5-ml aliquot was subjected to sequential Edman degradation. Total and C- and N-terminal amino acids were determined on samples containing 25–50 nmoles of each amino acid from (a) intact monomer, (b) monomer subjected to one Edman cycle (Edman I), and (c) monomer subjected to two Edman cycles (Edman II). The results are summarized in Table IV. The values were corrected for nonspecific amino acid destruction by the technique, using alanine as the reference.

TABLE IV: Analysis of the *M. sodonensis* Cell Wall Peptide Monomer Subjected to Sequential Edman Degradation.

Material Assayed	Total Amino Acids[a]	Molar Ratio	N-Terminal Groups[a]	C-Terminal Groups[a]
A. Peptide monomer				
Glutamic acid	1840	1.03	*b*	*b*
Glycine	1780	1.00	*b*	1790
Alanine	3640	2.04	1790	1850
Lysine	1780	1.00	1770	*b*
B. Edman I				
Glutamic acid	1160	1.00	1130	*b*
Glycine	1200	1.04	*b*	1190
Alanine	1160	1.01	*b*	1160
Lysine	1160	1.00	1150	*b*
C. Edman II				
Glycine	790		790	

[a] Data are expressed as nanomoles per milliliter of peptide monomer solution. [b] Below levels of detection.

The intact monomer contained only glutamic acid, glycine, alanine, and lysine in a 1:1:2:1 ratio, with one-half the total alanine N terminal and the other half C terminal. All the N^{ϵ}-amino groups of lysine and the carboxyl groups of glycine were free. The Edman I fraction had lost one-half of the total alanine and all of the N-terminal alanine, leaving a 1:1:1:1 ratio and the glutamic acid as the N terminal. The second cycle (Edman II) destroyed the N-terminal glutamic acid and left free glycine as evidenced by the equivalence of the total and N-terminal values and chromatographic analysis. The only peptide structure consistent with these data is N^{α}-[L-alanyl-γ-(-glutamylglycine)]-L-lysyl-D-alanine, the type C peptide subunit described by Ghuysen (1968).

Lysozyme Activity on Cell Walls. Clarification of cell wall digests and lysozyme-mediated release of reducing groups as a function of time (Figure 3) disclosed that the response of the two wall preparations to lysozyme was markedly different. Basal-grown cell walls exhibited rapid clarification accompanied by a concommitant release of reducing groups. Lysozyme digestion of TCS-grown walls produced only 10% clarification and released less than half as many reducing groups as were observed in basal-grown walls. Neither wall preparation was ever completely solubilized by lysozyme digestion. That digestion was complete and ceased because of unavailability of substrate and not because of product inhibition was established by the fact that addition of fresh lysozyme to digestion mixtures or resuspension of lysozyme-insoluble material in fresh reaction mixtures did not increase the liberation of reducing groups.

Basal-grown cell walls digested with lysozyme until release of reducing power stopped (*i.e.*, the lysozyme-insoluble por-

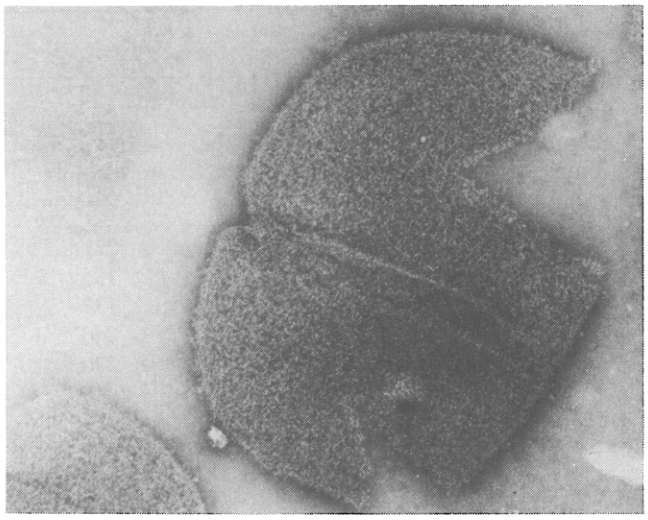

PLATE I: Electron photomicrograph of the lysozyme-insoluble portion of basal-grown cell walls of *M. sodonensis*. Basal-grown cell walls were digested with lysozyme under standard specified conditions until addition of fresh lysozyme produced no increase in the release of reducing groups. The preparation was negative contrast stained with 3% phosphotungstic acid. Magnification, 36,600×.

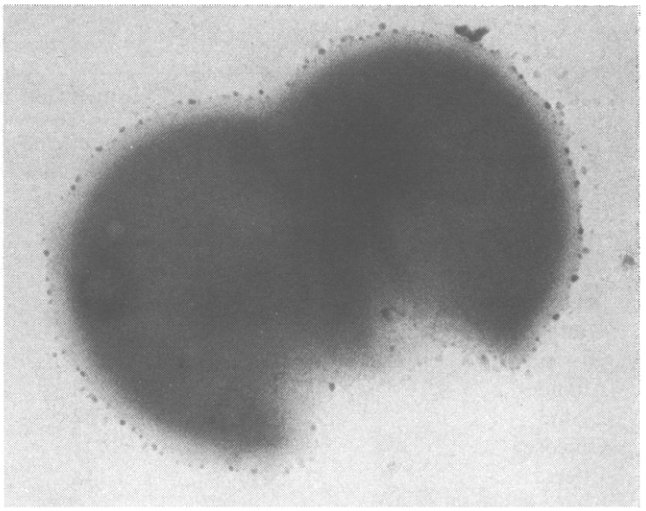

PLATE II: Electron photomicrograph of undigested basal-grown cell walls of *M. sodonensis*. Preparations were negative contrast stained with 3% phosphotungstic acid. Magnification 56,950×.

tion) were examined in the electron microscope (Plate I). Although the matrix of the wall was perceptibly more diffuse than that seen in whole, undigested cell walls (Plate II), structural integrity was maintained even though 75% of the wall had been solubilized. Thin sections of the lysozyme-insoluble portion (Plate III) revealed that wall thickness was 920 Å, only slightly less than the 980 Å thickness of intact cell walls.

The distribution of components in the lysozyme-soluble and -insoluble portions of walls was determined. Five milligrams of each wall preparation was digested 24 hr with lysozyme under specified conditions. The following samples (in triplicate) were removed for assay: 200 μl for esters, and 50 μl each for hexosamines, for glucose, for glucosamine, and for amino acids. Parallel digests were centrifuged at 39,000*g* for 1 hr. The supernatant (lysozyme-soluble fraction) decanted into fresh containers, and the same quantities of this material assayed as described previously. The lysozyme-insoluble precipitate was resuspended to a final volume of 1.0 ml with distilled water, and the previously designated quantities assayed for total esters, hexosamines, glucose, glucosamine, and amino acids.

These data (Table V) show that gross cell wall composition varies under altered growth conditions. Intact TCS-grown cell walls contained more esters, fewer hexosamine residues, and less glucose than basal-grown walls. There was excellent agreement between the total amount of each component and the sum of components in the respective lysozyme-soluble and -insoluble portions of each cell wall preparation. On the basis of hexosamine content, 49 and 75% of TCS- and basal-grown cell walls, respectively, were lysozyme soluble. In TCS-grown walls, the most insensitive to lysozyme, the ester:muramic acid ratio of the insoluble portion was double that in the analogous portion of basal-grown walls. Esterification in the form of O-acetylation, therefore is a key, but not the sole, factor in the diminished response of these cell walls to lysozyme (*vide infra*).

Distribution of the peptidoglycan components in lysozyme-soluble and -insoluble portions likewise varies under differing growth conditions. Although a 1:1 ratio of muramic acid to glucosamine and 1:1:2:1 ratio of glutamic acid, glycine, alanine, and lysine were preserved in all cases, the lysozyme-insoluble portion of TCS-grown walls contains 87% of the total amino acids, slightly more than twice the amino acid content of the analogous portion of basal-grown walls.

Products of lysozyme digestion were fractionated by gel filtration. On the basis of K_{av} values, there was little correlation between digestion products of the two wall preparations (Figure 4). Only one peak of the lysozyme-soluble portion of TCS-grown cell walls contained peptidoglycan components. This material, which was excluded from the system, contained 13% of the total cell wall amino acids and 49% of the total cell wall hexosamine residues. Glucose, which represented 38% of the total cell wall glucose, was present as low molecular weight polysaccharide fragments free of peptidoglycan components with a K_{av} of 0.9. In contrast, lysozyme-soluble components of basal-grown cell walls gave three main peaks which accounted for 75 and 56%, respectively, of the total wall hexosamine and amino acid residues. In this case, glucose was excluded from the system with peptidoglycan components and accounted for 79% of the total cell wall glucose.

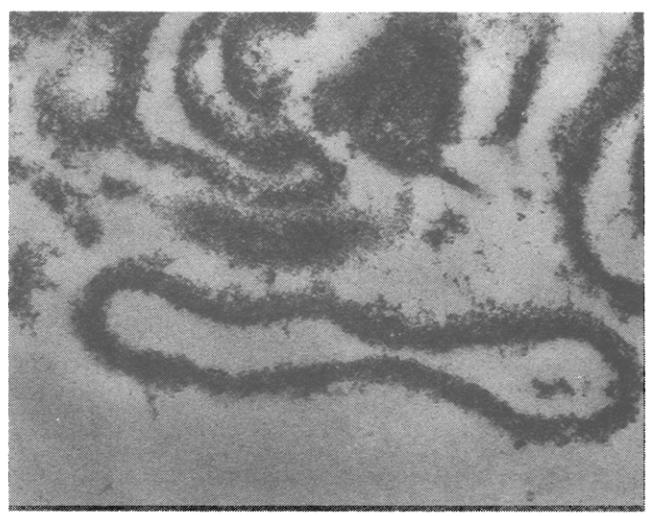

PLATE III: Electron photomicrograph of the lysozyme-insoluble portion of basal-grown *M. sodonensis* cell walls in thin section. Magnification 64,800×.

TABLE V: Distribution of *M. sodonensis* Cell Wall Components in Lysozyme Digests.[a]

Component	TCS-Grown Cell Walls			Basal-Grown Cell Walls		
	Whole	Lysozyme-Soluble Portion	Lysozyme-Insoluble-Portion	Whole	Lysozyme-Soluble Portion	Lysozyme-Insoluble Portion
Total hexosamines	567	273	295	1070	796	271
Muramic acid	289	137	151	528	397	136
Glucosamine	278	136	144	540	400	137
Glutamic acid	514	63	458	546	300	240
Glycine	523	64	451	531	305	230
Alanine	1070	123	913	1060	590	450
Lysine	514	63	452	530	300	230
Glucose	350	134	214	580	460	125
Esters	470	116	354	338	175	160
Esters:muramic acid	1.62	0.85	2.34	0.64	0.46	1.19
Alanine:muramic acid	3.70	0.90	6.05	2.01	1.49	3.29

[a] Data are expressed as nanomoles per milligram of cell wall.

This high molecular weight glucose-containing material is the subject of a separate study.

O-Acetylation of Cell Walls and Lysozyme Sensitivity. Cell wall O-acetylation was established by hydrolyzing cell wall preparations in alkali (Brumfitt, 1959), and analyzing soluble portions of such mixtures for acetate. Following incubation of 50 mg of cell walls in 0.01 N NaOH for 48 hr at 23°, insoluble material was removed by centrifugation at 30,000*g* for 20 min. The supernatant fraction, which contained no detectable amino acids, neutral sugars, or hexosamines, was passed through Dowex 50W-X4 $(H)^+$ to remove Na^+. Effluent portions were extracted into diethyl ether and analyzed by gas–liquid chromatography. Two components, acetic acid and acetic anhydride, were found which coupled with the fact that the preparations were Hestrin positive confirmed the presence of *O*-acetyl substituents in *M. sodonensis* cell walls.

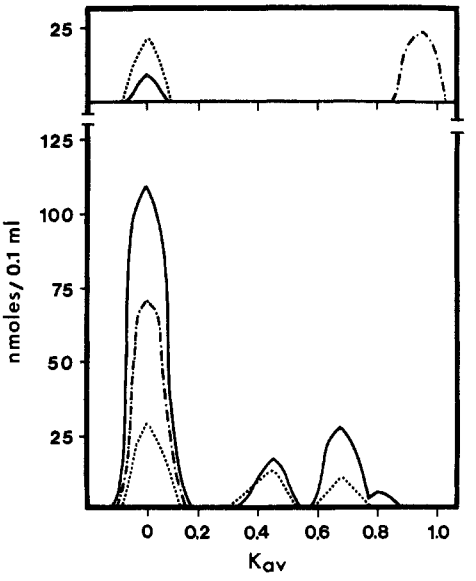

FIGURE 4: Fractionation of the lysozyme-soluble protion of *M. sodonensis* cell walls. The lysozyme-soluble portion obtained from 100 mg of cell walls was concentrated to a volume of 5.0 ml and applied to the linked Sephadex G-50–G-25 system and eluted with 0.1 M LiCl. Fractions of 5 ml were collected. The content of glucose, hexosamines, and free amino groups in 0.1-ml portions of each fraction was determined as described in Materials and Methods. Top profile: lysozyme-soluble portion of TCS-grown cell walls. Bottom profile: lysozyme-soluble portion of basal-grown cell walls. (——-) Glucose, (--------) free amino groups, and (——) hexosamines.

Only 57 and 30% of the total esters of intact TCS- and basal-grown cell walls, respectively, could be removed by 72-hr incubation of walls in 0.01 N NaOH at 23°. However, the lysozyme-insoluble portions of both wall preparations were completely deacetylated in 72 hr under these conditions.

Whole deacetylated cell walls and preparations of the deacetylated lysozyme-insoluble portions of cell walls were digested with lysozyme under standard specified conditions. Comparison of the lysozyme-mediated release of reducing groups in intact and deacetylated cell walls (Figure 5) revealed that the latter were significantly more sensitive to lysozyme. On the basis of hexosamine content, deacetylated TCS-grown walls were 76% solubilized by lysozyme, compared to 92% in deacetylated basal-grown walls—increases of 28 and 17%, respectively. Deacetylation of the lysozyme-insoluble portions of walls also increased lysozyme susceptibility. Lysozyme solubilized 73 and 97% of the deacetylated TCS- and basal-grown preparations, respectively (Figure 6) but, significantly, complete deacetylation did not render the lysozyme-insoluble portions of cell walls wholly sensitive to this enzyme.

The ester:hexosamine ratio of the lysozyme-insoluble portion of TCS-grown walls was 0.85 while that of the analogous portion of basal grown was 1.72. Since these ratios are greater than 0.5, acetylation likely occurs on both *N*-acetylhexosamine residues.

Involvement of Peptide Substitution with Lysozyme Sensitivity. Two types of digestions were performed on deacetylated cell walls or portions thereof: a sequential digestion with Myxobacter AL-1 protease and lysozyme, and digestion with lysozyme alone. Lysozyme activity was estimated as nanomoles of reducing groups liberated per milligram of cell wall (Table VI). In every case, pretreatment of wall material by Myxobacter AL-1 protease permitted enhanced lysozyme activity.

Degree and Nature of Glycan Peptide Substitution. I. *Strep-*

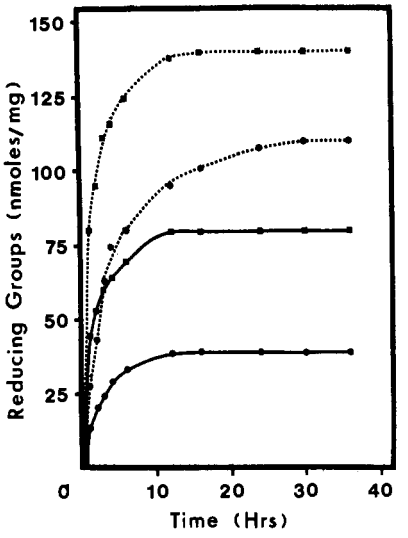

FIGURE 5: Comparison of the response of native and deacetylated *M. sodonensis* cell walls to lysozyme digestion. Each wall preparation (5 mg) digested with lysozyme under standard specified conditions. The final volume of each digest was 2.0 ml. At various times, 50-μl aliquots were removed, centrifuged to sediment insoluble material, and 20 μl of the supernatant fractions assayed for their content of reducing groups. (●——●) Reducing groups liberated in untreated TCS-grown cell walls. (●----●) Reducing groups liberated in deacetylated TCS-grown cell walls. (■——■) Reducing groups liberated in native basal-grown cell walls. (■----■) Reducing groups liberated in deacetylated basal-grown cell walls.

tomyces N-ACETYLMURAMYL-L-ALANINE AMIDASE was employed to digest the lysozyme-soluble and -insoluble portions from 5 mg of each cell wall preparation. Following 24-hr digestion at 37°, aliquots of each mixture containing 50–100 nmoles of alanine were assayed for N-terminal alanine. Lysozyme-insoluble portions of both cell wall preparations contained significantly more *N*-acetylmuramic acid-linked alanine than did the lysozyme-soluble portions. Glycans of these portions are, therefore, more highly peptide substituted than their corresponding lysozyme-soluble portions. Approximately three in five of the *N*-acetylmuramic acid residues are peptide substituted in the lysozyme-insoluble portion of TCS-grown cell walls compared to two in five in the analogous fraction of basal-grown walls. A greater difference in the degree of peptide substitution was observed in the lysozyme-soluble portions of the two preparations: in TCS-grown walls, one in five *N*-acetylmuramic acid residues is peptide substituted compared to one in twenty in analogous material from basal-grown walls. Significantly, both portions of the TCS-grown wall, which is the least sensitive to lysozyme, are more highly peptide substituted than the corresponding basal-grown wall portions. Amidase digestion was complete in that the total release of N-terminal alanine groups (117 and 70 nmoles per mg in TCS- and basal-grown cell walls, respectively) was identical with values obtained by Myxobacter AL-1 protease digestion of whole cell walls. Relatively low amounts of total cell wall alanine were released by amidase digestion (Table VII), and residual L-alanine, which must be involved in peptide cross bridging, was highest in the lysozyme-soluble portion of basal-grown cell walls, an indication that long-peptide cross bridges occur in this portion of the walls.

II. MYXOBACTER AL-1 PROTEASE ACTIVITY ON PRESOLUBILIZED CELL WALLS. To determine if the L-alanine not present in *N*-acetylmuramyl-L-alanine linkages occurred in D-alanyl-L-alanine linkages, the aforementioned amidase digests were adjusted to pH 9.0 with NaOH, and 10 μg of Myxobacter AL-1 protease and sufficient 0.01 M Veronal–HCl buffer (pH 9.0) added to yield a final volume of 3.0 ml. Reaction proceeded at 37° for 24 hr at which time triplicate 100-μl aliquots were assayed for N-terminal alanine content. Triplicate 200-μl aliquots were assayed for C-terminal alanine content before and after Myxobacter AL-1 protease digestion. Table VIII shows that this protease released in excess of 94% of the total L-alanine content as N terminal in these presolubilized preparations. The release of equimolar amounts of N- and C-terminal alanine unequivocally establishes the presence of D-alanyl–L-alanyl bonds.

TABLE VI: Response of Deacetylated *M. sodonensis* Cell Wall Material to Sequential Myxobacter AL-1 Protease and Lysozyme Digestion.[a]

Deacetylated Material	Reducing Groups Liberated by Digestion 1[b]	Digestion 2[b]	Δ
A. TCS-grown cell walls	110	142	32
B. Basal-grown cell walls	134	170	36
C. Lysozyme-insoluble portion of TCS-grown cell walls	68	140	72
D. Lysozyme-insoluble portion of basal-grown cell walls	130	152	22

[a] Data are expressed as nmoles per mg of cell wall material. [b] Digestion 1: digestion of the cell wall material with lysozyme alone; digestion 2: digestion of the cell wall material with Myxobacter AL-1 Protease followed by digestion by lysozyme.

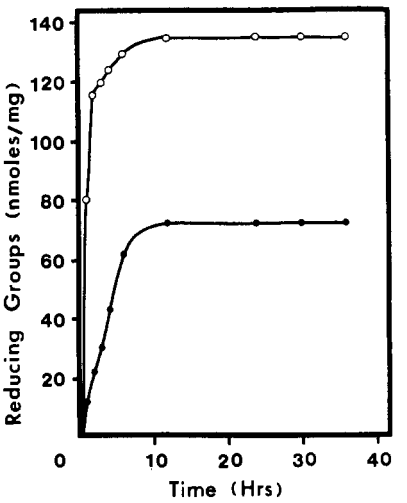

FIGURE 6: Response of the deacetylated lysozyme-insoluble portions of *M. sodonensis* cell walls to lysozyme digestion. Each deacetylated lysozyme-insoluble portion of cell walls (5 mg) was digested with lysozyme under standard specified conditions. The final volume of each digest was 2.0 ml. At intervals, 50-μl aliquots were withdrawn, centrifuged to sediment insoluble material, and 20 μl of the supernatant fractions was assayed for their content of reducing groups. (●——●) Reducing groups liberated in the lysozyme-insoluble portion of TCS-grown cell walls. (○——○) Reducing groups liberated in the lysozyme-insoluble portion of basal-grown cell walls.

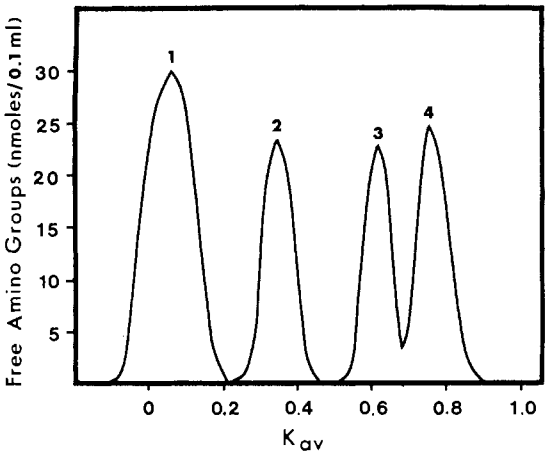

FIGURE 7: Fractionation of peptide fragments of sequentially digested TCS-grown *M. sodonensis* cell walls. TCS-grown cell walls (100 mg) were sequentially digested with Streptomyces ML-endopeptidase for 60 hr, lysozyme for 36 hr, and Streptomyces *N*-acetylmuramic acid-L-alanine amidase for 24 hr. Standard specified conditions for digestion were employed at each successive stage. The digest was concentrated to a volume of 5.0 ml and applied to the Sephadex G-50–m-25 system eluted with 0.1 M LiCl. Fractions (5 ml) were collected and 0.1-ml portions of each assayed for free amino groups relative to an L-alanine standard.

III. *Streptomyces* ML-ENDOPEPTIDASE digests of basal-grown cell walls achieved 100% relative clarification in 45–50 hr at 37° compared to 82% in TCS-grown walls and digestion was complete only after 60 hr. *N*-(D-Alanyl)-L-lysine linkages were measured by assaying free C-terminal alanine groups released by the ML-endopeptidase. TCS- and basal-grown cell walls contained respectively, 41 and 34 *N*-(D-alanyl)-L-lysine linkages per mg of cell wall.

IV. SEQUENTIAL ENZYMIC DEGRADATION OF CELL WALLS. Peptide cross-bridge fragments were obtained by sequential digestion of 100 mg of each wall preparation with Streptomyces ML-endopeptidase for 60 hr, lysozyme for 36 hr, and Streptomyces *N*-acetylmuramyl-L-alanine amidase for 24 hr under previously specified conditions. Digests were concentrated to 5.0 ml and fractionated by gel filtration as previously described. Amino groups of TCS-grown cell walls (Figure 7) yielded four peaks with K_{av} values of 0.045 (peak 1), 0.34 (peak 2), 0.605 (peak 3), and 0.75 (peak 4). The elution profile of basal-grown cell walls was similar (Figure 8), yielding three peaks with K_{av} values of 0 (excluded, peak 1), 0.34 (peak 2), and 0.75 (peak 3). Fractions containing each of the peaks were pooled, desalted on 2.5 × 45 cm Bio-Gel P-2 columns, eluted with distilled water, evaporated to dryness, and resuspended in 10.0 ml of 0.01 M Veronal–HCl buffer (pH 9.0) containing 0.001 M Versene. Appropriate aliquots containing between 20 and 50 nmoles of alanine were assayed for total- and N-terminal alanine content. Mean chain length of each peptide fragment was determined by dividing the L-alanine content of each fraction by the total number of free N-terminal alanine groups. The length and distribution of peptide fragments differs significantly in the two preparations of cell walls (Table IX). Of the total TCS-grown wall peptide subunits, 39% were released as octamers, 25% as tetramers, 15% as dimers, and 20% as monomers. These represent cross bridges of seven-, three-, and one-peptide subunits length, respectively. Monomeric units represent material which was covalently bound to the glycan of the intact cell wall. The corresponding figures for basal-grown walls are oligomers (18 subunits length) 72%, tetramers, 16%, and monomers 12%.

All the fragments larger than one subunit were then digested with Myxobacter AL-1 protease at 37° for 24 hr as previously

TABLE VII: Peptide Substitution in the Glycans of Lysozyme-Soluble and -Insoluble Portions of *M. sodonensis* Cell Walls.[a]

	Lysozyme-Soluble Portion		Lysozyme-Insoluble Portion	
	TCS Grown	Basal Grown	TCS Grown	Basal Grown
Amidase-released N-terminal alanine groups (A)	27	18	88	52
Total muramic acid (B)	137	397	151	136
% muramic acid peptide substituted (A/B × 100)	20.0	4.6	58.4	38.0
Total L-alanine (C)	62	295	457	224
% muramic acid linked L-alanine (A/C × 100)	43.6	6.1	19.3	23.1

[a] Data are expressed as nanomoles per milligram of original cell wall.

TABLE VIII: Enzymic Release of N- and C-Terminal Alanine in Predigested *M. sodonensis* Cell Walls.[a]

	Lysozyme-Soluble Portion		Lysozyme-Insoluble Portion	
	TCS Grown	Basal Grown	TCS Grown	Basal Grown
Total N-terminal alanine released by digestion 1 (A)	58	290	450	220
% of total L-alanine	94	98	98	98
Total N-terminal alanine released by digestion 2 (B)	27	18	88	52
N-Terminal alanine released by hydrolysis of D-alanyl-L-alanine linkages (A − B)	31	272	362	168
C-Terminal alanine released by digestion 1 (C)[b]	34	285	381	183
Ratio of C- to N-terminal alanine in D-alanyl-L-alanine linkages [C/(A − B)]	1.13	1.05	1.05	1.09

[a] Data are expressed as nanomoles per milligram of original cell wall. [b] Release of C-terminal alanine groups above control level of free C-terminal alanine. Digestion 1: sequential digestion of the cell wall fractions with Streptomyces *N*-acetylmuramic acid-L-alanine amidase followed by digestion with Myxobacter AL-1 protease; digestion 2: digestion with *Streptomyces N*-acetylmuramic acid-L-alanine amidase only.

TABLE IX: Analysis of Peptide Fragments of Sequentially Digested *M. sodonensis* Cell Walls.[a]

Material	Total L-Ala in Each Peak	% of Total Cell Wall Ala in Each Peak	N-Terminal L-Ala in Each Peak	Fragment Length
TCS-grown cell wall				
Peak 1	533	39.5	68	8
Peak 2	335	24.8	83	4
Peak 3	201	14.9	97	2
Peak 4	270	20.0	265	1
Basal-grown cell wall				
Peak 1	954	72.0	53	18
Peak 2	212	16.0	53	4
Peak 3	159	12.0	156	1

[a] Data are expressed as nanomoles per milliliter of each peptide preparation.

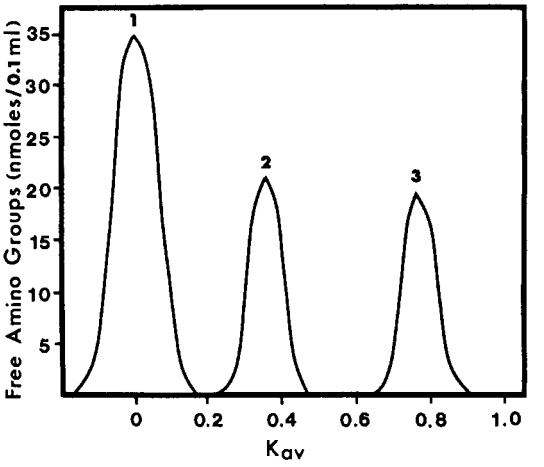

FIGURE 8: Fractionation of peptide fragments of sequentially digested basal-grown *M. sodonensis* cell walls. Basal-grown cell walls (100 mg) were sequentially digested with Streptomyces ML-endopeptidase for 60 hr, lysozyme for 36 hr, and Streptomyces *N*-acetylmuramic acid-L-alanine amidase for 24 hr. Standard specified conditions for digestion were employed at each successive stage. The digestion was concentrated to a volume of 5.0 ml and applied to the Sephadex G-50–G-25 system and eluted with 0.1 M LiCl. Fractions (5 ml) were collected and 0.1-ml portions of each assayed for free amino groups relative to an L-alanine standard.

specified, concentrated to 5.0 ml, and fractionated by gel filtration. Peaks 2 (tetramer) and 3 (dimer) of TCS-grown cell walls and peak 2 (tetramer) of basal-grown cell walls produced single peaks with K_{av} of 0.75 (peptide monomer). However, peak 1 (octamer) of the TCS-grown cell walls and peak 1 (oligomer) of the basal-grown cell walls were incompletely hydrolyzed by the protease yielding some monomer and high molecular weight residue.

Discussion

Micrococcus sodonensis cell wall peptidoglycan composition was found to resemble that of other Micrococaceae (Table I). The number of peptide subunits is intermediate between *Micrococcus lysodeikticus* (480 peptide subunits/mg) and *Micrococcus flavus* cell walls (610 peptide subunits/mg), and the structure is identical with the type C subunit, found also in *S. lutea, M. flavus, M. lysodeikticus, and Micrococcus citreus* cell walls (Campbell *et al.*, 1969).

Nickerson and Day (1971) reported a difference in muramic acid contents in strains of *Bacillus cereus* differing by a point mutation in penicillinase production. This is not, however, completely analagous to the observation in *M. sodonensis* that the disaccharide:peptide subunit ratio is nearly twice as high in basal—as in TCS-grown walls (540 and 278 disaccharide units per mg of wall, respectively), which represents a phenotypic alteration in peptidoglycan rather than the selection of a different genotype. That the approximate 1:1 ratio of disaccharide to peptide subunits in the basal-grown cell walls reflects a more highly ordered structure is refuted by the following pieces of evidence. (1) Studies with Myxobacter AL-1 protease (Table III) revealed that in TCS-grown cell walls 40% of the available *N*-acetylmuramic acid residues are peptide substituted compared to only 13% in basal-grown cell walls. (2) Digestion of the lysozyme-soluble and -insoluble portions of cell walls with Streptomyces *N*-acetylmuramyl-L-alanine amidase (Table VII) disclosed that the degree of peptide substitution was greater in both portions of TCS-grown walls than in the analogous fractions of basal-grown walls and that only a small proportion of the total wall L-alanine was involved in *N*-acetylmuramyl-*N*-α-L-alanine linkages. Assuming the head-to-tail assembly of peptide subunits (Ghuysen, 1968; Schleifer and Kandler, 1970), the TCS- and basal-grown cell walls are calculated to possess cross bridges 2 to 3 subunits long and 18 to 19 subunits long, respectively, in the lysozyme-soluble portions, and 3 and 4 subunits long, respectively, in the lysozyme-insoluble portions. (3) Sequential digestion of cell walls with Streptomyces ML-endopeptidase, lysozyme, and Myxobacter AL-1 protease, showed that shorter peptide cross bridges occurred with greater frequency in TCS-grown cell walls (Table IX). The distribution of the peptidoglycan amino acids in the lysozyme-soluble and -insoluble portions of cell walls (Table IV) suggests that shorter cross bridges predominate in the latter portion of cell walls. (4) Digestion of walls with lysozyme, Streptomyces ML-endopeptidase, or Myxobacter AL-1 protease was consistently more rapid and more complete in basal-grown than in TCS-grown cell walls which supports the theory that the latter are more highly organized.

Three conclusions may be drawn from the foregoing facts: first, that some areas of the peptidoglycan of intact cell walls are more highly organized than others; second, that the peptidoglycan of TCS-grown cell walls is more highly ordered than that of basal-grown cell walls; and third, that subtle changes in growth conditions have a profound effect on peptidoglycan structure. The long segments of unsubstituted glycan strands and large peptide cross bridges observed in the lysozyme-soluble portion of basal-grown cell walls supports the mechanism postulated for the head-to-tail assembly of peptide units in Micrococcaceae (Schleifer and Kandler, 1967; Ghuysen, 1968).

Other differences between the peptidoglycan of TCS- and basal-grown cell walls are suggested by the observations that release of *N*-amino groups of lysine and carboxyl groups of glycine by Myxobacter AL-1 protease was significantly greater in the basal- than in the TCS-grown cell walls. It is, therefore,

possible that an *N*-(α-glycyl)-L-lysine linkage occurs in *M. sodonensis* cell walls. The inability to detect free glycine carboxyl groups suggests they may be amidated as are the α-carboxyl groups of diaminopimelic acid in Corynebacteria, and of glutamic acid in *S. aureus*, Copenhagen, *Micrococcus roseus* R27, *Streptococcus pyogenes*, and *Corynebacterium diphtheriae* (Ghuysen *et al.*, 1968a; Muñoz *et al.*, 1966; Tipper *et al.*, 1967).

The structure of the subunit peptide of *M. sodonensis* cell walls and the head-to-tail assembly of peptide subunit to facilitate cross bridging established in this investigation have been recently confirmed in another laboratory (Schleifer and Kandler, 1970). However, their technique destroys the samples thus precluding any further tests to confirm the distribution and size of peptide crosss-bridging structures.

Lysozyme susceptibility of the two types of walls differed both with respect to per cent solubilized and to size distribution of the fragments produced. This difference clearly involves at least three factors: complexity of the peptidoglycan matrix, O-acetylation of the cell walls, and configuration of the peptide cross bridges. In the deacetylation experiments since the soluble portion contained no detectable amino acids, it was assumed that base-catalyzed elimination from *N*-acetylmuramic acid had not occurred (Tipper, 1968).

The role of O-acetylation in lysozyme resistance is well established in this and other systems. The observation that the lysozyme susceptibility of deacetylated walls was further increased by disruption of the peptide–bridge complex by Myxobacter AL-1 protease shows that the configuration imposed by the peptide–bridge complex (*i.e.*, accessibility of the glycosidic bond) is also of importance. The role of stereochemical factors in lysozyme activity was also noted by Raferty and Dahlquist (1969). The observation that lysozyme digestion did not significantly change wall shape or thickness, in spite of the fact that up to 75% of the wall had been solubilized precludes both the hypotheses that the lysozyme-sensitive and -resistant polymers are covalently integrated or that they exist as superimposed layers in the intact wall. The obviously more diffuse matrix in lysozyme-digested walls suggests that in the native cell wall the two exist as interwoven peptidoglycan nets, one of which is resistant to lysozyme because of extensive peptide cross bridging and increased levels of O-acetylation.

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