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Sphere-Rod Morphogenesis in *Arthrobacter crystallopoietes*[1]

II. Peptides of the Cell Wall Peptidoglycan

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Cell walls of *Arthrobacter crystallopoietes* grown as spheres and as rods were solubilized by treatment with the B enzyme from *Chalaropsis*, an *N*-acetylmuramidase. The neutral glycopeptides were then isolated by chromatography on ECTEOLA cellulose. The glycopeptides, consisting of disaccharide-peptide units interlinked by peptide cross-bridges, were fractionated by gel filtration on Sephadex columns into oligomers of various sizes. The size distribution ranged from monomers with no cross-bridges to polymers with a high degree of polymerization, but did not differ significantly between cell walls from cells grown as spheres or rods. Some small differences in the distribution of C- and N-terminal amino acids were found. Analyses revealed that all the peptide bridges in the glycopeptide fractions from rod cell walls were formed by one L-alanine residue. In sphere cell walls, L-alanine was also found, but, in addition, higher oligomers of the glycopeptide contained glycine in their cross-bridges. These results were confirmed by determinations of C- and N-terminal amino acids released after lysostaphin and AL-1 enzyme digestions and by Edman degradations. Models representing the structures of the sphere and rod cell walls are presented. These structures indicate that the sphere cell wall is probably a more loosely knit macromolecule than is the rod cell wall.

The peptidoglycan of the bacterial cell wall is a large macromolecule that is largely responsible for maintenance of cellular shape (18). The polymer is composed of polysaccharide chains with alternating residues of *N*-acetylmuramic acid and *N*-acetylglucosamine. The tetrapeptides are bound to the polysaccharide through the carboxyl groups of acetylmuramic acid. There are variations in these tetrapeptides in different organisms, but they generally consist of L-alanine, D-glutamic acid (or D-isoglutamine), either L-lysine or α-ϵ-diaminopimelic acid (DAP), and D-alanine. Cross-bridges linking the free ϵ-amino group of lysine or DAP of one peptide unit and the carboxyl group of the terminal D-alanine residue from another contribute an additional dimension of polymerization to the wall. In some species, cross-bridges may exist between almost all the peptides, resulting in a highly polymerized wall (5), or in others may involve as few as 60% of the peptides (13). They may be formed directly between lysine or DAP and D-alanine, as in *Micrococcus lysodeikticus* (11) or *Escherichia coli* (13), or may contain one or more specific amino acids, as in the pentaglycine bridges of *Staphylococcus aureus* strain Copenhagen (5) or in the tri-L-alanine-L-threonine bridge of *M. roseus* strain R27 (11). Integrity of both the polysaccharide chains and the peptide network is necessary for maintenance of structural rigidity. Sufficient breakage of a single linkage in either polymer results in solubilization of the peptidoglycan (12*a*).

In the preceding paper (10), we reported that sphere-rod morphogenesis in *Arthrobacter crystallopoietes* involves changes in the length of the polysaccharide of the cell wall peptidoglycan. The polysaccharides of the sphere cell walls are shorter and more heterogeneous in length than those of the rod walls. The studies to be described here concern the peptide fractions of cell wall peptidoglycans isolated from cells of *A. crystallopoietes* grown as spheres and as peptone- and succinate-induced rods.

[1] These studies were conducted in partial completion of the requirements for an M.S. degree in the Department of Bacteriology, University of Wisconsin, Madison, while the senior author was a predoctoral Graduate Fellow of the National Science Foundation.

MATERIALS AND METHODS

Growth of *A. crystallopoietes*, preparation of sphere and rod cell walls, and many of the analytical methods were identical to those described in the preceding paper (10). One preparation each of sphere and rod cell walls was incubated at 100 C for 30 min during the early stages of purification to inactivate endogenous autolytic enzymes. All subsequent determinations were performed with the heated preparation as well as with unheated cell walls.

N-terminal and free amino acids were determined by thin-layer chromatography of dinitrophenylated amino acids (6). C-terminal amino acids were determined after hydrazinolysis (6). Egg white lysozyme was used for solubilization of whole walls for end group analyses (*see below*). D-Alanine and L-alanine were assayed enzymatically (6).

Digestions with AL-1 protease were carried out as before (10). Lysozyme (Sigma Chemical Co., St. Louis, Mo.) was employed at concentrations of 40 μg/ml in 0.02 M tris(hydroxymethyl)aminomethane buffer (*p*H 8.5) containing 10^{-4} M disodium ethylenediaminetetraacetic acid. *Chalaropsis* B enzyme (a gift from J. Hash; 7) was used in reaction mixtures containing 50 mg of cell walls, 5 ml of 0.01 M sodium acetate buffer (*p*H 4.6), and 1.0 mg of enzyme. Lysostaphin (a gift from the Mead-Johnson Co., Evansville, Ind.) digests contained about 200 μmoles of substrate in 200 μliters of 0.01 M potassium phosphate buffer (*p*H 7.5) with 30 μg of enzyme. All enzymatic digestions were incubated at 37 C.

The method of Konigsberg and Hill (9) was used for Edman degradations. The products were identified by dinitrophenylation and thin-layer chromatography (6).

ECTEOLA and carboxymethyl (CM) celluloses (BioRad Laboratories, Richmond, Calif.) were used for fractionations of enzymatic digests. Gel filtration was carried out on Sephadex G-25 fine grade and G-50 medium grade (Pharmacia Inc., Rochester, Minn.). Water was used for equilibration and elution. All columns were run at room temperature.

RESULTS

N- and C-terminal amino acids in whole cell walls. The tetrapeptide found in the *A. crystallopoietes* peptidoglycan is *N*-α(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine. Many of the ϵ-NH_2 groups of its lysine residues are substituted by L-alanyl residues (14). The average extent of peptide cross-linking was studied by determinations of C-terminal amino acids and free ϵ-amino groups of lysine in cell walls solubilized by lysozyme (Table 1). Alanine and lysine occur N-terminally and C-terminally in all three walls. The sphere walls contained a greater amount of N-terminal lysine and C-terminal alanine than either of the rod walls. The rod walls contained, on the other hand, twice as much N-terminal alanine and more C-terminal lysine than did the sphere walls. The total N-terminal groups exceeded the total C-terminal groups. In part, this may be due to low recovery of C-terminal lysine by the hydrazinolysis procedure employed (J.-F. Petit, *personal communication*). The excess N-terminal groups are not the result of autolytic enzyme activity, since the results were obtained with cell walls boiled during purification. Moreover, the only detectable autolytic activity in these cell walls is due to an acetylmuramidase (T. A. Krulwich and J. C. Ensign, Bacteriol. Proc., p. 130, 1967). The difference between N-terminal and C-terminal groups may also be due to breaks in acetylmuramyl-alanine bonds, since the amino groups of the alanine, but not the carboxyl groups of the acetylmuramic acid, are detected by the procedures employed. Such breaks

TABLE 1. *C- and N-terminal amino acids in lysozyme digests of whole cell walls*

Component	Preparation from				
	Spheres	Peptone rods		Succinate rods	
	Ratio to glutamate	Ratio to glutamate	Ratio to spheres	Ratio to glutamate	Ratio to spheres
Total alanine	2.29	2.52	1.16	2.46	1.12
N-terminal alanine	0.24	0.50	2.11	0.40	1.69
C-terminal alanine	0.30	0.17	0.54	0.16	0.54
Total lysine	0.81	0.92	1.13	0.80	0.98
N-terminal lysine	0.33	0.23	0.72	0.20	0.60
C-terminal lysine	0.09	0.12	1.29	0.16	1.71
Total N-terminal	0.57	0.73	1.30	0.60	1.06
Total C-terminal	0.40	0.29	0.72	0.32	0.82
Excess N-terminal[a]	0.16	0.44	2.70	0.28	1.66

[a] Total N-terminal minus total C-terminal, presumably a measure of N-terminal alanine arising from breakage of acetylmuramyl-L-alanine linkages.

have been found in other bacterial cell walls (5). However, total N-terminal alanine is somewhat greater than the apparent excess of N-terminal groups over total C-terminal groups. Some of the N-terminal alanine presumably, therefore, represents L-alanine residues present in unformed or broken cross-bridges.

Studies of Chalaropsis B enzyme digests of cell walls. Chalaropsis B enzyme is a bacteriolytic acetylmuramidase with a broader specificity than lysozyme (7, 17). This enzyme splits the polysaccharide backbone of the cell wall peptidoglycan into disaccharides which remain linked to their peptide subunits. The peptide cross-bridges link these subunits into glycopeptide oligomers of varying size. These oligomers can be fractionated by gel filtration.

Approximately 50 mg of each of the preparations of *A. crystallopoietes* cell walls was digested. The turbid cell wall suspensions began to clear instantaneously upon addition of enzyme. Release of reducing power (resulting from cleavage of glycosidic bonds) was followed during digestion (Fig. 1). Reducing power was released very rapidly from all three walls. Release was initially slower from the sphere than from the rod cell walls, and took considerably longer to reach maximal level. Samples from the three digests were dried, then reduced with 0.1 M sodium borohydride at room temperature for 3 hr. They were next neutralized with acetic acid, dried, and acid-hydrolyzed for analysis with the amino acid analyzer. Muramitol (produced from *N*-acetylmuramic acid by reduction of the disaccharides in the digests and freed during hydrolysis) is eluted in the same position as muramic acid, but gives almost no color with ninhydrin. Calculations from the maximal amount of muramic acid remaining in the reduced samples indicate that the digestions were at least 95% complete.

Each digest was applied to a column of ECTEOLA cellulose (void volume, 70 ml) and was eluted with water followed by a linear gradient of LiCl to a final concentration of 1.0 M. Fractions containing approximately 15 ml were collected and assayed for reducing power and phosphate. The results for the fractionation of the digest of peptone-induced rod cell walls are shown in Fig. 2. The other two digest fractionated similarly. A phosphate-free peak eluted with water contained the glycopeptide, which has no net charge at neutral *p*H. A second peak of reducing power with no phosphate was eluted at the beginning of the salt gradient. This peak may contain free oligosaccharides originating from those units of peptidoglycan in which the acetylmuramyl-L-alanine bond was absent in the native wall. A third peak containing a complex of phosphate polymer and glycopeptide was eluted last.

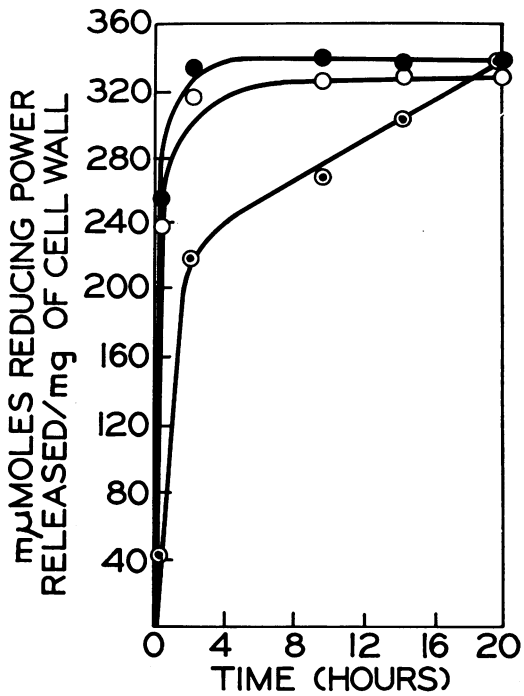

FIG. 1. *Release of reducing power during digestion of Arthrobacter crystallopoietes cell walls by Chalaropsis B enzyme. A 50-mg amount of cell walls was incubated at 37 C with 1.0 mg of enzyme in 5.0 ml of 0.01 M sodium acetate buffer at pH 4.6. Samples were removed at various times and assayed for reducing power, with N-acetylglucosamine as a standard. Symbols:* ⊙ = *sphere cell walls;* ● = *peptone-induced rod cell walls; and* ○ = *succinate-induced rod cell walls.*

The glycopeptide fractions were lyophilized, redissolved in water (2 ml), and applied to columns of Sephadex G-50 and G-25 connected in series so that the effluent from the G-50 column entered directly into the G-25 column. Fractions of 3 ml were collected and assayed for reducing power and total amino groups. Since virtually every acetylmuramic acid residue in the peptidoglycan had its reducing group freed by treatment with the *Chalaropsis* enzyme, the reducing power of a given glycopeptide fraction is proportional to its content of disaccharide-peptide subunits. The patterns of separation obtained for the three cell walls are shown in Fig. 3. In each fractionation, there was a peak of amino groups at the salt peak.

The elution volume of the peak just preceding the salt peak was identical to that of the monomer (*N*-acetylglucosamine and *N*-acetylmuramic acid disaccharide substituted by a single peptide unit) of the glycopeptide produced from *S. aureus* cell

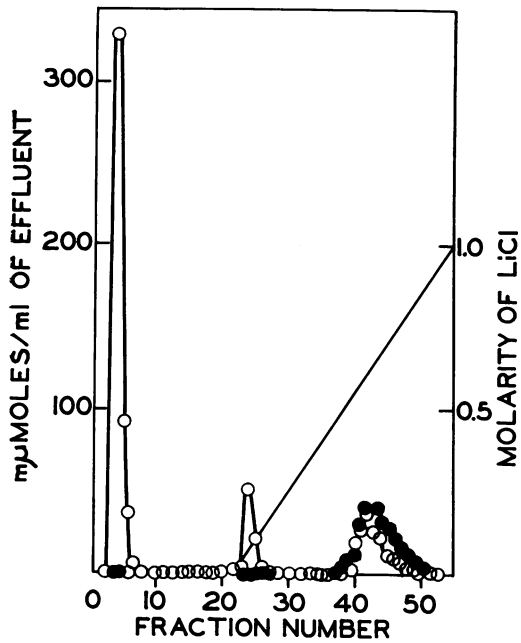

FIG. 2. *Fractionation of a Chalaropsis B enzyme digest of cell walls from peptone-induced rods of Arthrobacter crystallopoietes on a column of ECTEOLA cellulose. The products from digestion of 50 mg of cell walls were applied to the column and eluted with water, followed by a LiCl gradient. Fractions of 15 ml were collected and assayed for reducing power and total phosphate. Symbols:* ○ = *reducing power, and* ● = *phosphate.*

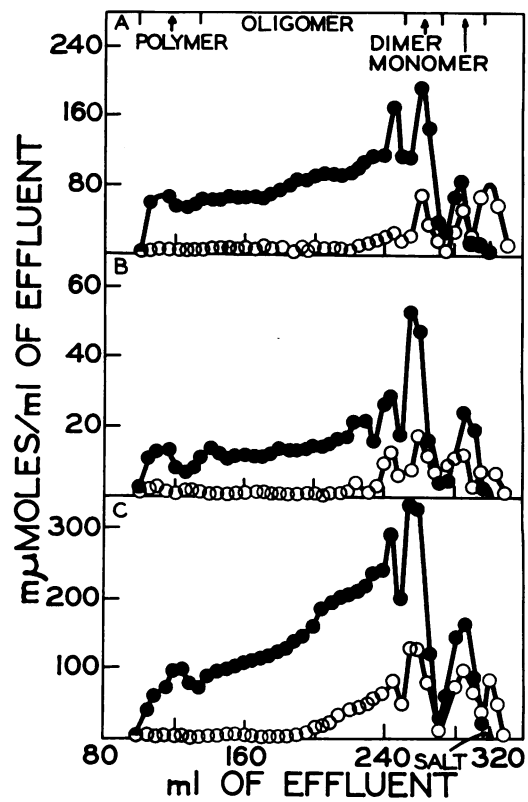

FIG. 3. *Fractionation of the glycopeptide obtained from Chalaropsis B enzyme digestion of Arthrobacter crystallopoietes cell wall preparations on Sephadex columns. Sphere and succinate- and peptone-induced rod cell walls were digested. Fractions of each, eluted with water from an ECTEOLA cellulose column (Fig. 2), were pooled, concentrated, and applied in turn to a 1.6 by 118 cm Sephadex G-50 column. The effluent from this column fed directly into a 2.6 by 100 cm column of Sephadex G-25. Fractions of 3 ml were collected and assayed for reducing power and amino groups. Differences in the ordinate scales reflect the different amounts of each glycopeptide applied to the columns. (A) Spheres, (B) peptone rods, and (C) succinate rods. Symbols:* ○ = NH_2 *groups, and* ● = *reducing power.*

walls by digestion with *Chalaropsis* B enzyme (D. J. Tipper, Federation Proc. **18**:344, 1966), and has the position expected for material of this molecular weight. It is therefore called monomer, and subsequent analyses substantiated its chain length (Tables 2 and 3). Similarly, the major peak preceding the monomer peak is called dimer (two disaccharides with their peptides linked through a cross-bridge). These peaks were preceded in turn by trimer and progressively larger sized fractions ranging up to highly cross-linked glycopeptide which is eluted at the void volume.

Monomer, dimer, oligomer, and polymer fractions were pooled separately, as shown in Fig. 3, and were lyophilized. Samples of each were analyzed for amino acids, amino sugars, and D- and L-alanine (Table 2). All fractions of all three wall preparations contained approximately 2 moles of L-alanine per mole of glutamic acid. Since the tetrapeptide contains one L-alanine, the second L-alanine must be contained in the interpeptide cross-bridges. This L-alanine is the only amino acid in addition to the tetrapeptide amino acids in the rod cell wall glycopeptide, and therefore the bridges in these cell walls consist solely of an average of one L-alanine residue.

The ratio of D-alanine to glutamic acid is approximately 1 in the high molecular weight fractions, but is considerably less in the monomer and dimer fractions. This suggests that C-terminal D-alanine may have been removed from uncross-linked tetrapeptides by the action of a D-alanine carboxypeptidase (1, 2, 8; K. Izaki, Federation Proc. **26**:388, 1967) during peptidoglycan synthesis.

Sphere cell walls contain small amounts of

TABLE 2. *Composition of glycopeptide fractions from Chalaropsis B enzyme digests of Arthrobacter crystallopoietes cell walls*[a]

Cell wall	Fraction	Total Alanine	D-Alanine	L-Alanine	Ratio of D- to L-alanine	Lysine	Glycine[b]	Muramic	Glucosamine
Sphere	Monomer	2.12	0.50	1.61	0.31	0.90	0.25	0.85	1.03
	Dimer	2.37	0.68	1.69	0.40	0.92	0.15	0.90	1.27
	Oligomer	3.18	0.91	2.24	0.40	1.01	0.35	0.99	1.06
	Polymer	2.60	0.90	1.70	0.53	1.04	1.68	0.78	0.97
Peptone rod	Monomer	2.56	0.46	2.10	0.22	0.89	0.05	0.94	0.83
	Dimer	2.70	0.84	1.86	0.45	0.81	0	0.90	0.89
	Oligomer	2.84	0.84	2.00	0.42	0.89	0	0.88	1.08
	Polymer	2.78	0.81	1.97	0.41	0.84	0	0.97	0.77
Succinate rod	Monomer	2.69	0.73	1.97	0.37	0.90	0	0.81	0.99
	Dimer	2.86	0.85	2.02	0.42	1.19	0	0.87	0.90
	Oligomer	3.29	—[c]	—[c]	—[c]	0.83	0	0.89	0.88
	Polymer	2.82	0.85	1.97	0.43	0.89	0	0.80	0.82

[a] All values are relative to total glutamic acid taken as 1.0, except for the ratio of D-alanine to L-alanine.

[b] These data were obtained from an analysis of one batch of cell walls. Another preparation of sphere glycopeptide was found to contain approximately one-third as much glycine, but the relative pattern of distribution was the same in the fractions. The average glycine content in several preparations of sphere cell walls was 0.14 mole per mole of glutamic acid as was reported in the previous paper (10).

[c] No data obtained.

glycine in the monomer and dimer fractions, more in the oligomers, and still greater amounts in the polymers. One preparation, the composition of which is shown in Table 2, contained about 1.7 moles of glycine per mole of glutamic acid in the polymer fraction. Another batch of sphere cell walls had the same pattern of distribution of glycine among the fractions of the glycopeptide produced from it by digestion with the *Chalaropsis* enzyme, but had a total of only about 0.5 mole of glycine per mole of glutamic acid in the polymer. The amount of glycine thus seems to vary, but is always associated with the more highly cross-linked fractions of the sphere glycopeptide. Glycine is presumably a bridge amino acid. Data in support of this conclusion are presented below.

The results of N- and C-terminal amino acid determinations of the gel filtration fractions are presented in Table 3. The recovery of end groups was low, especially that of C-terminal amino acids. The amounts of N-terminal amino acids generally confirm the degree of polymerization of the fractions. C-terminal and N-terminal alanine and lysine were found in all fractions. As in the analyses of whole cell walls (Table 1), larger proportions of N-terminal lysine groups were found in the sphere than in the rod cell walls.

Degradation of cell walls and glycopeptide polymers by lysostaphin. Lysostaphin is a mixture of two lytic enzymes produced by a strain of *Staphylococcus* which actively lyses other members of this genus (12). The preparation contains an endo-*N*-acetylglucosaminidase and a peptidase that releases N-terminal alanine and glycine from

TABLE 3. *C- and N-terminal amino acids of glycopeptide fractions from Chalaropsis B enzyme digests of Arthrobacter crystallopoietes cell walls*[a]

Cell wall preparation	Fraction	N-terminal groups		C-terminal groups	
		Alanine	Lysine	Alanine	Lysine
Sphere	Monomer	0.31	0.67	0.25	0.37
	Dimer[b]	0.15	0.15	0.06	0.12
	Oligomer	0.04	0.03	0.03	0.07
	Polymer	0.04	0.04	—[c]	—[c]
Peptone rod	Monomer	0.36	0.34	0.23	0.21
	Dimer[b]	0.22	0.03	0.10	0.14
	Oligomer	0.13	0.08	0.11	0.16
	Polymer	0.07	0.08	0.04	0.14
Succinate rod	Monomer	0.39	0.29	0.24	0.23
	Dimer[b]	0.21	0.12	0.23	0.16
	Oligomer	0.15	0.01	0.06	0.04
	Polymer	0.08	0.04	0.03	0.04

[a] Data are expressed as moles per mole of total glutamic acid.

[b] Low values for the N-terminals of the dimer fraction may be due to some contamination by higher oligomers. See Fig. 3.

[c] No data obtained.

cell walls of *S. aureus* strain Copenhagen (3). The peptidase hydrolyzes polyglycine cross-bridges and, at a slower velocity, *N*-acetylmuramyl-L-alanine linkages (3). Experiments were conducted to determine whether *A. crystallopoietes* cell walls are susceptible to lysostaphin. If so, it was anticipated that the enzyme would be useful in studying the peptide cross-bridge structure.

Lysostaphin causes a 90% reduction in optical density of suspensions of sphere and rod walls within 2 hr. N-terminal alanine was released during the digestion (0.3 to 0.6 mole of N-terminal alanine per mole of glutamic acid). Two preparations of sphere glycopeptide polymer fractions containing 0.5 and 1.68 moles of glycine per mole of glutamic acid were incubated with lysostaphin for 2 hr. Approximately 0.2 mole of both N-terminal alanine and N-terminal glycine per mole of glutamic acid were released from each preparation. Thus, lysostaphin evidently caused breakage of peptide cross-bridges in the soluble polymers.

Samples (containing 0.1 and 0.5 μmole of total glutamic acid) from two preparations of soluble sphere glycopeptide polymer were digested with lysostaphin for 9 hr. N-terminal amino acids were measured before and after lysostaphin treatment. The products were then subjected to two rounds of Edman degradation. Alanyl-glycyl-glycine was the control for the first degradation, and glycyl-glycyl-alanine, for the second. All of the N-terminal alanine disappeared at the first stage of degradation; at that time, only a small amount of N^{ϵ}-terminal lysine appeared, and N-terminal glycine increased slightly (Table 4). N-terminal glycine disappeared at the second round of Edman degradation and was replaced by N^{ϵ}-terminal lysine.

Studies of AL-1 enzyme digests of cell walls. The AL-1 enzyme is a peptidase which hydrolyzes peptide cross-bridges as well as the *N*-acetylmuramyl-L-alanine bond (16). Both sphere and rod cell walls were digested with the enzyme. N-terminal alanine (0.7 to 0.9 mole per mole of glutamic) was released from each of the cell wall preparations. This amount of N-terminal alanine indicates that the digestions were only partial. AL-1 also released equal amounts of free alanine and C-terminal lysine (0.09 to 0.25 mole per mole of glutamic acid) from all the walls. Digests of sphere walls contained, in addition, 0.05 to 0.07 mole of free glycine per mole of glutamic. Free ϵ-NH_2 groups of lysine increased by an equal amount over those present in the lysozyme-digested cell walls.

A 50-mg amount of sphere and rod cell walls was digested with AL-1 and fractionated on ECTEOLA cellulose columns, as described in the preceding paper (10). Fractions from each wall preparation containing free amino acids, peptide, and short chain length oligosaccharide were eluted with water. These were further fractionated on columns of CM cellulose. Fractions containing 15 ml were collected and assayed for total amino groups and reducing power. The results of the CM cellulose fractionation of the ECTEOLA cellulose fraction from succinate rods is shown in Fig. 4. Virtually the same pattern was obtained with the other wall preparations. Oligosaccharides and free amino acids were eluted by water, and basic peptides were subsequently eluted in a sharp peak with LiCl.

The configuration of the free alanine eluted from the CM cellulose columns was determined. Free alanine from both rod cell wall digests was entirely D-alanine. The free alanine from the sphere walls contained mainly D-alanine but also a small amount of L-alanine (5 to 15% of the total).

TABLE 4. *Edman degradation of the lysostaphin product of sphere glycopeptide polymers*[a]

Substance	Treatment			
	None	Lysostaphin digestion	First Edman cycle	Second Edman cycle
N-terminal alanine	0.04	0.43	0	0
N-terminal glycine	0	0.67	0.88	0.12
N^{ϵ}-terminal lysine	0.04	0.12	0.25	0.86
N-terminal glutamic acid	0	0	0.06	0
Sum	0.08	1.22	1.19	0.98

[a] Data are expressed as moles per mole of total glutamic acid. Note that N^{ϵ}-terminal lysine is converted into a 4-phenyl thiocarbamyl derivative on reaction with phenyl isothiocyanate and that this product is reconverted to free N^{ϵ}-amino groups during cyclization to only a small extent. Consequently, N^{ϵ}-terminal lysine present after a degradation cycle must have largely been released during that cycle. Thus the N^{ϵ}-terminal lysine, present after the second degradation cycle (column 5), was derived from the N-terminal glycine in column 4. The experiment shown here was repeated with a second preparation of sphere glycopeptide and similar data were obtained.

DISCUSSION

The average extent of peptide cross-linking in peptidoglycans of *A. crystallopoietes* rod and

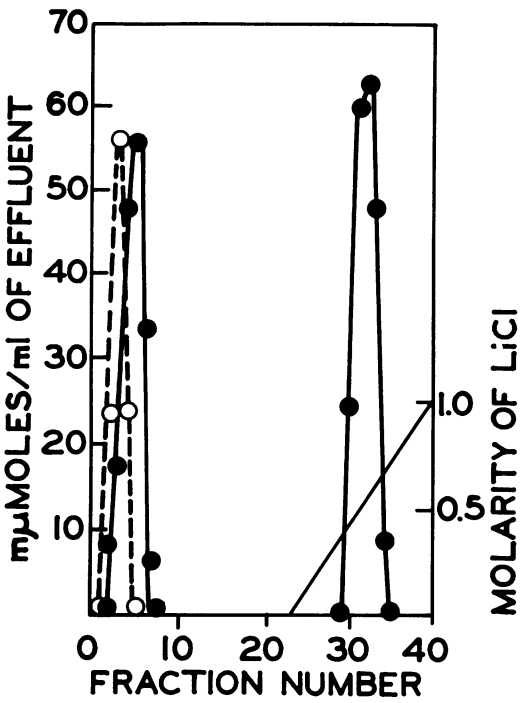

FIG. 4. *Fractionation of the neutral and basic materials isolated from an AL-1 enzyme digest of succinate-induced rods of Arthrobacter crystallopoietes on CM cellulose. The digest from 50 mg of cell walls was placed upon a 2.2 by 37 cm column of ECTEOLA cellulose. Fractions containing free amino acids, peptides, and undegraded peptidoglycan were eluted, concentrated, and applied to the CM cellulose column. The components were eluted from the column by water, followed by a LiCl gradient. Fractions of 15 ml were collected and assayed for total amino groups and reducing power. Symbols:* ○ = *reducing power, and* ● = *groups.*

sphere cell walls was determined by analyses of free peptide end groups. The sources of such free groups could be newly synthesized peptides that had not yet been cross-linked, peptides that had been altered (e.g., by a D-alanine carboxypeptidase) so that they could no longer be cross-linked, or a variety of breaks caused by activities of autolytic enzymes. The extent of interpeptide cross-linking as judged by measurements of terminal groups does not differ greatly in the sphere and rod cell walls. As calculated from C-terminal groups, approximately 60 to 70% of the peptides are linked in cross-bridges, the rod walls having a slightly greater percentage of cross-bridges than the sphere walls. These values are lower than those for the more extensively polymerized walls of *S. aureus* (5), and are comparable to the average cross-linking in the peptidoglycan in *E. coli* (13). Rod cell walls had a somewhat greater excess of N-terminal groups over C-terminal groups (probably a measure of N-terminal alanine), suggesting that they may contain more breaks between acetylmuramic acid and L-alanine than sphere cell walls.

The patterns of distribution of cross-bridges in the glycopeptides produced by hydrolysis of rod and sphere walls with the *Chalaropsis* B enzyme are quite similar (Fig. 3). The *A. crystallopoietes* walls contain large amounts of peptide-linked monomer and dimer as well as a considerable amount of highly polymerized glycopeptide. This pattern differs from that found in either the essentially completely cross-linked walls of *S. aureus* which contain very little low molecular weight material or the walls of *E. coli* (18) and *Corynebacterium diphtheriae* (Kato and Strominger, *unpublished data*), which contain exclusively monomer and dimer fractions.

The presence of a relatively large amount of glycine in the highly polymerized fraction of glycopeptide derived from sphere cell walls suggests that there are at least some glycine-containing cross-bridges in this material, in contrast to the presence of L-alanine alone in the cross-bridges of other fractions of the sphere glycopeptide and in all fractions derived from the rod cell walls. The peptidase in lysotaphin, which has a known specificity for polyglycine cross-bridges (3), catalyzed hydrolysis of the high molecular weight sphere glycopeptide polymer. The products of lysostaphin digestion of this material were subjected to two cycles of Edman degradation. The results of these experiments (Table 4) indicate that the glycine in the sphere glycopeptide polymer is indeed in the cross-bridges, since elimination of N-terminal glycine in the second degradation cycle resulted in exposure of virtually all of the ε-amino groups of the lysine of the tetrapeptides.

From the patterns of Edman degradation, the existence of more than one kind of peptide cross-bridge in the glycopeptide polymer can be deduced. The analyses reported have been repeated several times but are difficult to carry out, especially when small differences are involved. The following discussion is, therefore, presented only as a working model and with the cautionary note that further studies involving other methods may necessitate reinterpretation. The disappearance of N-terminal alanine in the first degradation cycle is accompanied by an increase of N-terminal glycine and N^{ϵ}-terminal lysine and by the appearance of a small amount of N-terminal glutamic acid, which together quantitatively account for the disappearance of N-terminal alanine. The small amount of N-terminal glutamic acid produced (equivalent to 14% of the initial N-term-

inal alanine) must have been derived from L-alanyl-glutamic acid, produced by splitting of some acetylmuramyl-L-alanine linkages by lysostaphin (3). Further degradation of this end of the peptide during the second cycle of Edman degradation is prevented by the γ-linkage of glutamic acid to lysine in the tetrapeptide (14). The observed limited acetylmuramyl-L-alanine amidase activity of lysostaphin on this substrate is consistent with the observation that the amidase activity is inhibited when the acetylmuramyl residue has a free reducing group (16).

The increase of N^{ϵ}-terminal lysine in the first degradation cycle (equivalent to 28% of the initial N-terminal alanine) indicates the proportion of L-alanyl and N^{ϵ}-lysine linkages, derived from open bridges, which was present in the material. Finally, the increase of N-terminal glycine (50% of the initial N-terminal alanine) indicates the proportion of the N-terminal alanine which was attached to glycine. This amount is of the same order of magnitude as the N-terminal glycine remaining after two cycles of Edman degradation, suggesting that these bridges may have been L-alanyl-glycyl-glycine bridges. The N-terminal glycine initially present does not decrease during the first degradation cycle, thus indicating that this N-terminal glycine residue must itself be attached to a second glycine residue. Thus, there must also have been present in the initial material some glycyl-glycine linked directly to lysine.

Therefore, the data suggest the possibility that the lysostaphin degradation product contains four types of opened interpeptide bridges (Fig. 5). The appearance of these products could be accounted for by the hydrolysis by lysostaphin at the two positions indicated in the figure. Substantiation of this hypothesis could be achieved by separation of the peptides in this mixture and their analysis. In any case, however, *more than one* cross-bridge structure must be present in the sphere glycopeptide fraction. The existence of more than one bridge structure in a peptidoglycan has been suggested for other bacterial cell walls (5, 11), but has never been demonstrated conclusively.

It has not been demonstrated that all the cells in a population of spheres have glycine-containing peptides in their peptidoglycans. Conceivably only some proportion of cells might contain these peptides, whereas the remainder have none. Such a qualitative difference in cell walls within an ostensibly homogeneous population appears unlikely. The incorporation of glycine is not a highly controlled process, however, since different preparations of cell walls contained somewhat different amounts of glycine in the polymer frac-

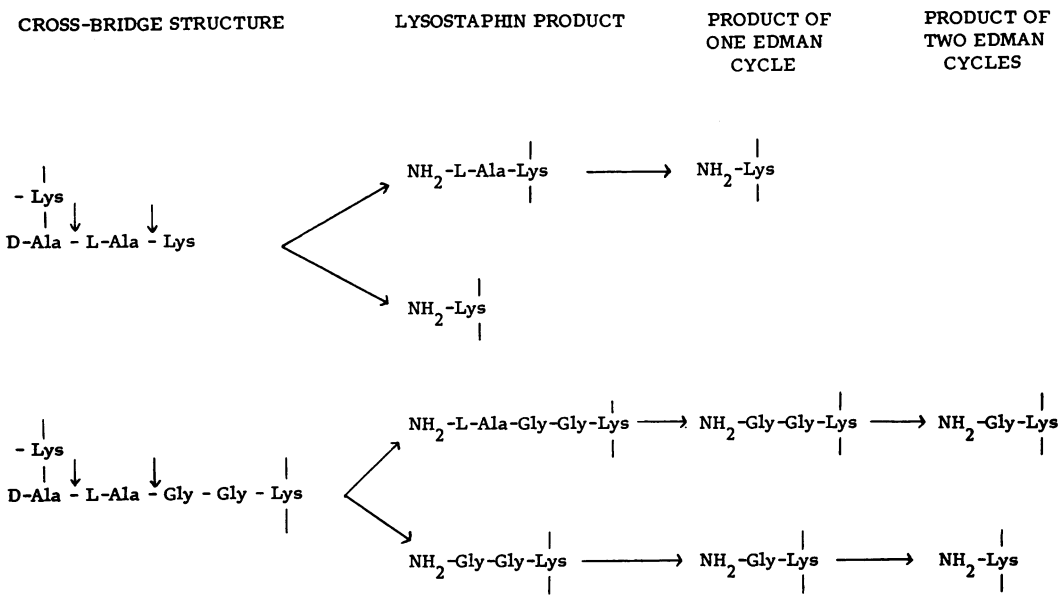

FIG. 5. *Proposed mechanism of hydrolysis of interpeptide bridges by lysostaphin and Edman degradation of the resulting peptides. It is proposed that the two types of bridges may be split by lysostaphin at either of the alternate linkages indicated. The course of two cycles of Edman degradation of the products of lysis is shown. The analytical data indicate that this product contained a mixture of glycopeptides, 27% of which had cross-linkages consisting of a single L-alanine residue, and 73% of which had cross-linkages consisting of L-alanyl-glycyl-glycine residues.*

tions. The mechanism whereby glycine is incorporated primarily into the polymer of sphere cell walls, but not into rod cell walls, requires further study. The possibility that the observed results were due to contamination with a coccus containing a polyglycine bridge was eliminated by parallel plate counts of sphere batch cultures on GS medium (10) and on GS medium plus 0.2% 2-hydroxypyridine. Pigment-producing colonies characteristic of *A. crystallopoietes* on the 2-hydroxypyridine medium (4) equalled total colonies on GS medium. No colonies lacking the characteristic green crystalline pigment were observed out of more than 500 examined.

Models for the cell wall structures and sites of enzymatic cleavage by AL-1 enzyme from *Myxobacterium* and the B enzyme from *Chalaropsis* are presented in Fig. 6. Sphere cell walls are more susceptible to digestion by the AL-1 peptidase (4) and less susceptible to digestion by the *Chalaropsis* B acetylmuramidase than are rod walls. These data support the hypothesis that the polysaccharide chains of the peptidoglycan, which are three to four times longer in rod than in sphere cell walls (10), play a relatively greater role in maintaining the rigidity of the rod than the sphere walls. The presence of some cross-bridges with L-alanyl-glycyl-glycine residues in sphere cell walls could account for differences in the products of AL-1 enzyme digestion of sphere and rod walls, as well as the greater susceptibility of sphere walls to this enzyme. The ϵ-amino lysine groups freed during digestion of sphere, but not rod, walls by the AL-1 peptidase could be derived from the glycine-containing bridges upon cleavage of the glycyl-lysine bond. The L-alanine in the L-alanyl-glycyl-glycine bridge peptides might also be susceptible to removal by AL-1 enzyme. When L-alanine is bound directly to lysine, as in rod and some of the sphere cross-bridges, it is not released by AL-1.

The release of free D-alanine from *A. crystallopoietes* cell walls by AL-1 differs from results obtained with cell walls of *S. aureus* strain Copenhagen digested with this enzyme (16). No good explanation for the release of free D-alanine from the tetrapeptide in the present experiments is available. Perhaps, the presence of L-alanine in place of pentaglycine in the peptide bridge increases the susceptibility of the L-lysyl-D-alanine linkage to cleavage.

The possibility that the shorter polysaccharides of the sphere peptidoglycans represent the con-

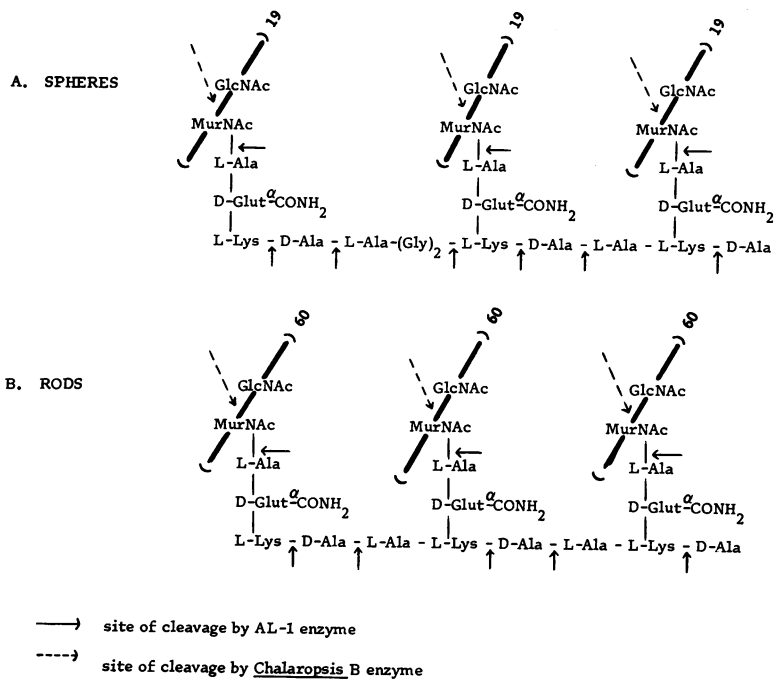

FIG. 6. *Representations of possible structures of sphere and rod cell walls of Arthrobacter crystallopoietes. MurNAC = N-acetylmuramic acid, and GlcNAC = N-acetylglucosamine. The polysaccharide strands in the sphere cell walls have an average length of 20 disaccharide units, whereas those in the rod cell walls have an average length of 60 disaccharide units. These values are taken from the calculations presented in the previous paper (10). Points of enzymatic cleavage are indicated.*

struction of a more flexible molecule has been already discussed (10). The presence of L-alanyl-glycyl-glycine bridges in the polymer fractions of sphere glycopeptide might also be related to a need for more flexible polymers for spheres than for rods. The increase in the average length of cross-bridges that must be afforded by the presence of these glycine-containing peptides in the sphere walls might result in a less rigid polymer. It is a commonly observed phenomenon that weakening of the cell wall of a rod-shaped bacterium (e.g., by penicillin or lysozyme) results in the formation of a spherical cell (spheroplast). It is reasonable that maintenance of rod shape by an organism, in a medium whose osmotic pressure is considerably lower than that inside the cell, requires a more rigid structure than maintenance of spherical shape.

The question remains as to how the specific changes associated with sphere to rod morphogenesis are brought about. Insofar as changes in cell walls are concerned, the sphere to rod transition entails alteration of the polymerization enzymes that produce peptidoglycan or of an autolysin which hydrolyzes the glycan (10) and the loss or repression of the glycine-incorporating system. Since morphogenesis can be controlled nutritionally, some features of metabolic control of cell wall synthesis and regulation of cellular shape may eventually be elucidated in the studies of *A. crystallopoietes*.

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