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Isolation and Chemical Structure of the Peptidoglycan of *Spirillum serpens* Cell Walls

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The peptidoglycan layer of *Spirillum serpens* cell walls was isolated from intact cells after treatment with sodium dodecylsulfate and digestion with Pronase. The isolated peptidoglycan contained glucosamine, muramic acid, alanine, glutamic acid, and *meso*-diaminopimelic acid in the approximate molar ratio of 1:1:2:1:1. Aspartic acid and glycine were the only other amino acids found in significant quantities. N-terminal amino acid analyses of the tetrapeptide amino acids in the peptidoglycan revealed that 54% of the diaminopimelic acid molecules are involved in cross-linkage between tetrapeptides. This amount of cross-linkage is greater than that found in the peptidoglycan of previously studied cell walls of gram-negative bacteria. The polysaccharide backbone was isolated, after myxobacter AL-1 enzyme digestion of the peptidoglycan, by fractionation with ECTEOLA-cellulose and Sephadex G-100. An average length of 99 hexosamines for the polysaccharide chains was found (ratio of total hexosamines to reducing end groups).

The bacterial cell wall is unique in its chemical composition and structure. The peptidoglycan polymer is the major single constituent of cell walls of gram-positive bacteria, but it accounts for only 10 to 20% of the cell wall of gram-negative bacteria. There is evidence that the peptidoglycan is involved in maintaining cell rigidity and shape (31, 32). The peptidoglycan of different bacterial cell walls is similar but not identical in composition and structure. It is composed of a carbohydrate "backbone" containing alternating units of *N*-acetylglucosamine and *N*-acetylmuramic acid linked through β-1,4-glycosidic bonds. A tetrapeptide, composed of L-alanine, D-glutamic acid (or D-isoglutamine), a dibasic amino acid (usually either L-lysine or α-ϵ-diaminopimelic acid), and D-alanine, is attached by an amide bond to the lactyl group of muramic acid. Some of the tetrapeptides are cross-linked by peptide bonds between the ϵ-amino group of the dibasic amino acid and the C-terminal D-alanine. In some bacteria the cross-linkage is direct; in others, amino acid bridges connect the tetrapeptides. These subjects have been thoroughly treated in recent reviews (15, 25, 32).

Recently, bacteriolytic enzymes have been employed to prepare intact ribbons of polysaccharide backbone free from tetrapeptides. The length of this ribbon from walls of *Staphylococcus aureus* strain Copenhagen was found to average 25 hexosamine units (30). Cell walls of *Arthrobacter crystallopoietes* grown as spheres contain ribbons averaging 34 hexosamine units in length; the length in walls of the organisms grown as rods was found to be 115 to 135 hexosamines (9). This marked variation in the size of the polysaccharide backbone in the peptidoglycan of rod- and spherical-shaped cells prompted us to investigate a spiral-shaped cell. Another impetus for this study was the extreme susceptibility of several *Spirillum* species to the bacteriolytic peptidase AL-1 produced by a myxobacterium. This enzyme did not attack a number of other gram-negative bacteria (1).

MATERIALS AND METHODS

Growth conditions. The culture of *Spirillum serpens* was obtained from R. J. Martinez. Cells were grown in a complex medium of Nutrient Broth (Difco) supplemented with 0.1% yeast extract (Difco).

Large numbers of cells were obtained by the inoculation of 100 ml of medium in a 500-ml Erlenmeyer flask with 5.0 ml of a cell suspension washed from a 3-day-old agar slant culture. The culture was incubated at 30 C on a New Brunswick rotary shaker (225 rev/min) until it reached the mid-exponential phase of growth (approximately 36 hr). A 10-ml sample was then transferred into each of three 3-liter Erlenmeyer flasks which each contained 800 ml of medium. These cultures were then incubated as above.

[1] This work was submitted in partial fulfillment of the requirements for the M.S. degree at the University of Wisconsin while the senior author was a predoctoral fellow of the U.S. Public Health Service.

When the culture reached mid-exponential growth (approximately 12 hr), the entire contents of each flask was used to inoculate the 16 liters of medium contained in each of three 20-liter carboys. These carboys were aerated vigorously at 30 C with filter-sterilized air passed through two sintered-glass spargers located near the bottom. To minimize foaming, 12 drops of sterilized Antifoam A (Dow-Corning Corp., Midland, Mich.) was added to each carboy.

Cells were harvested by use of a Sharples Super-Centrifuge during exponential growth (approximately 8 hr after inoculation). Just prior to the harvesting, sufficient crushed ice was added to each carboy so that some ice remained in the medium throughout centrifugation. Approximately 2.0 g of cells (wet weight) was obtained per liter of medium. The pinkish-red cell paste was stored at −16 C.

Purification of cell walls and peptidoglycan. Frozen cell paste (253 g wet weight) was added to 500 ml of water and was suspended by mixing with a magnetic stirrer at 5 C. The suspension was made 1.0% with respect to sodium dodecylsulfate (SDS) by the addition, drop by drop, of a 25% stock solution. The temperature of the suspension was raised to 25 C, and 1 mg of deoxyribonuclease was added to reduce the viscosity. The mixture was then stirred for 12 hr at room temperature.

The SDS suspension was centrifuged at 1,000 × *g* for 10 min and then at 13,000 × *g* for an additional 10 min. A pink layer covered a brown pellet of debris. The dark-brown supernatant fluid was discarded. The pink layer was washed from the pellet with water, suspended in 1,000 ml of water, and immediately placed into a boiling-water bath for 60 min to inactivate any autolytic enzymes which might have been present. The heated suspension was then centrifuged as above. All centrifugations were carried out at 4 C. The light-amber supernatant fluid, which contained small pieces of debris and granular material, was discarded. The pellet was suspended in a total volume of 650 ml of water, adjusted to a final concentration of 1.0% SDS, mixed for 12 hr with a magnetic stirrer at room temperature, and centrifuged as above. The pellet obtained was suspended in water and centrifuged at 12,000 × *g* for 20 min; this washing procedure was repeated 16 times. The final brownish-cream colored residue was suspended in 380 ml of 0.025 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.85). The suspension was brought to 65 C, and $CaCl_2 \cdot 2H_2O$ was added to it to give a final concentration of 10^{-4} M. A solution containing 5.0 g of Pronase (grade B, Calbiochem, Los Angeles, Calif.) was activated by incubation at 80 C for 5 min and then added to the suspension. The suspension was shaken at 200 rev/min at 65 C for 4 hr, and then quickly placed in a boiling-water bath for 20 min to destroy residual Pronase activity. The suspension was then centrifuged at 12,000 × *g* for 30 min. The pellet was washed six times by suspending it in 5.0×10^{-4} M sodium ethylenediaminetetraacetic acid (EDTA) followed by centrifugation at 13,000 × *g* for 50 min. This was followed by 10 distilled water washes. The resulting white peptidoglycan was lyophilized and stored in a desiccator.

Enzymatic digestions. Digestions with egg white lysozyme (Sigma Chemical Co., St. Louis, Mo.) were carried out at 37 C in 0.01 M Tris buffer (*p*H 8.5) containing 10^{-4} M $MgSO_4$ and 100 μg of lysozyme per ml. The AL-1 protease was purified according to the method of Ensign and Wolfe (2). Digestions with this enzyme were carried out at 37 C in an incubation mixture which contained 0.025 M Tris buffer (*p*H 9.0), 5.0×10^{-4} M EDTA, and 10 μg of enzyme per mg of peptidoglycan.

Diaminopimelic acid configuration. The isomeric form of diaminopimelic acid (DAP) in the purified peptidoglycan was determined. A 200-μg sample of acid-hydrolyzed peptidoglycan was applied to Whatman no. 1 filter paper and developed by descending chromatography using the solvent system (methanol-water-10 N HCl-pyridine, 80:11.5:2.5:10) of Rhuland et al. (23). The procedure of Ghuysen et al. (3) was also employed. This involved thin-layer chromatography of the fluorodinitrophenylated acid hydrolysate in a solvent composed of the upper phase from a mixture of equal volumes of *n*-butyl alcohol and 1% (w/v) ammonia. A standard mixture of *meso*- and L,L-DAP was co-chromatographed in each experiment.

Analytical procedures. Samples of peptidoglycan containing approximately 50 mμmoles of amino acids and amino sugars were hydrolyzed in 100 μliters of 4 N HCl at 104 to 106 C in sealed tubes for 8 hr. The samples were then diluted to 2.2 ml with 0.2 N citrate buffer (*p*H 2.2) and analyzed on a Beckman model 120-C amino acid analyzer (Beckman Instruments Inc., Fullerton, Calif.). Total amino acids were also determined after dinitrophenylation of acid-hydrolyzed peptidoglycan (5, 6).

The peptidoglycan was solubilized with lysozyme prior to end group analyses. Free amino acids and N-terminal groups were determined by thin-layer chromatography of the products of dinitrophenylation (5). C-terminal amino acids were determined after hydrazinolysis and thin-layer chromatography (5). Total phosphate was assayed by the method of Lowry et al. (12). The phenol-sulfuric acid method of Hodge and Hofreiter (8) was used to measure carbohydrate. Reducing power was determined by the method of Park and Johnson (19), and total hexosamines were measured by the modified Morgan-Elson procedure of Reissig et al. (22).

Column chromatography and gel filtration. ECTEOLA-cellulose (high capacity, Mann Research Laboratories, Inc., New York, N.Y.) was used for fractionation of the AL-1 enzyme digest of peptidoglycan (4). Columns of Sephadex G-100, G-50, and G-25 (Pharmacia Fine Chemicals, Inc., New Market, N.J.) were employed for gel filtration of enzymatically digested peptidoglycan. All columns were developed at room temperature and equilibrated with water.

RESULTS

The procedure adopted for the isolation of *S. serpens* peptidoglycan (Fig. 1) made it relatively easy to separate the peptidoglycan from cyto-

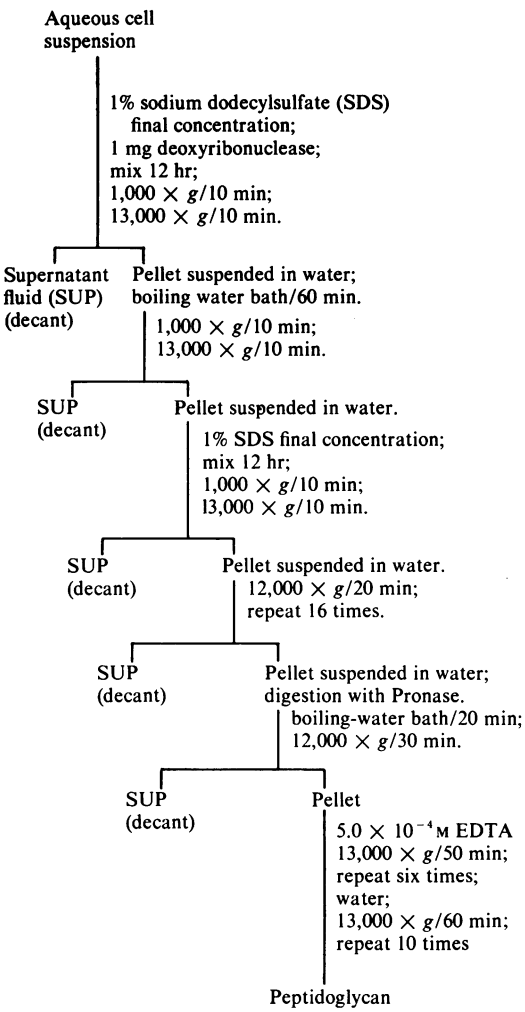

FIG. 1. *Flow diagram of the procedure for purifying Spirillum serpens peptidoglycan.*

plasmic materials and from other cell wall components. The SDS treatment removed the outer lipoprotein layer (16) and disrupted the cytoplasmic membrane, thereby freeing intracellular materials. Amino acid analyses of the washed residue showed a heterogeneous mixture of amino acids, which indicated the presence of protein (Table 1). Two compounds unique to cell walls, DAP and muramic acid, as well as glucosamine, were found in the approximate molar ratios of 1:1:1 as was expected for peptidoglycan. Pronase digestion of the SDS residue removed essentially all of the protein. The components commonly associated with bacterial cell walls were found in the purified peptidoglycan in the expected molar ratios of glutamic acid-alanine-DAP-glucosamine-muramic acid, 1:2:1:1:0.7 (Table 1). The only significant variation was the low muramic acid, which probably reflects instability of this compound during acid hydrolysis (5). Analyses revealed all of the DAP to be of the *meso-* configuration.

The final yield of purified peptidoglycan was 2.4% of the dry weight of the intact vegetative cells. Electron micrographs of uranyl acetate-stained peptidoglycan revealed a thin, delicate, almost transparent material (Fig. 2). Observation with the phase-contrast microscope revealed that the peptidoglycan retained the characteristic cell shape. Granules were observed inside the cell wall sacculi; these stained with Sudan Black B. Martinez (17) reported poly-β-hydroxybutyrate (PHB) granules in *S. serpens*. The sedimentable residue after AL-1 digestion was assayed for PHB by the method of Law and Slepecky (11). Approximately 13% of the dry weight of purified peptidoglycan was found to be PHB. The purified peptidoglycan contained 0.096 μmole of phosphate and 0.071 μmole of carbohydrate per mg. This corresponds to the ratios of phosphate-carbohydrate-DAP of 0.2:0.17:1.0. These components may have come from contamination with

TABLE 1. *Amino acid and amino sugar analyses of Spirillum serpens*

Component	Peptidoglycan (PG)		Sodium dodecylsulfate-treated whole cells	
	Amt/mg of PG	Ratio to diamino-pimelic acid	Amt/mg of cells	Ratio to diamino-pimelic acid
	μmoles		*μmoles*	
Diaminopimelic acid	0.47	1.0	0.074	1.0
Alanine	0.91	1.9	0.53	7.1
Glutamic acid	0.48	1.0	0.43	5.8
Muramic acid	0.32	0.67	0.061	0.82
Glucosamine	0.47	1.0	0.075	1.0
Ammonia	0.14	0.30	0.23	3.1
Aspartic acid	0.10	0.22	0.060	0.80
Lysine	0.039	0.083	0.18	2.4
Histidine	0.013	0.028	0.070	0.94
Arginine	0.047	0.097	0.17	2.3
Threonine	0.0012	0.0025	0.16	2.1
Serine	0.037	0.078	0.18	2.5
Proline	—	—	0.16	2.1
Glycine	0.047	0.10	0.31	4.2
Valine	0.038	0.081	0.14	1.9
Methionine	—	—	0.046	0.62
Isoleucine	0.034	0.007	0.093	1.3
Leucine	0.035	0.075	0.27	3.6
Tyrosine	0.033	0.069	0.082	1.1

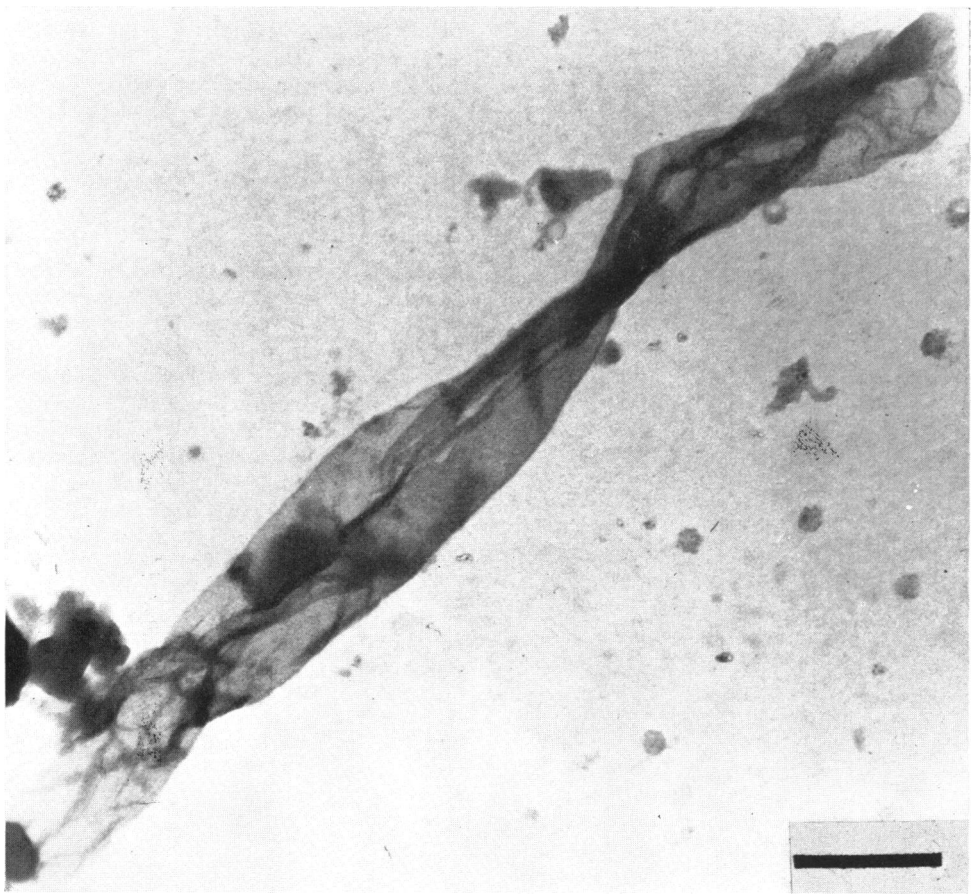

FIG. 2. *Peptidoglycan of Spirillum serpens, stained with 3% uranyl acetate (pH 4.3). The sample was stained on the Formvar-coated grid for 20 sec. Marker represents 0.5 μ.*

TABLE 2. *Free carboxyl and amino groups of amino acids in Spirillum serpens peptidoglycan*

Component	Amt/mg of peptidoglycan	Ratio to diaminopimelic acid
	μmoles	
Alanine		
Total	0.91	1.9
Free amino groups	0.02	0.040
Free carboxyl groups	0.22	0.48
Diaminopimelic acid		
Total	0.47	1.0
Free ε-amino groups	0.21	0.45
Free carboxyl groups	0.04	0.087
Total free amino groups	0.23	0.49
Total free carboxyl groups	0.27	0.56

residual membrane or lipopolysaccharide, both of which are reported to contain carbohydrate and phosphate (7, 26). Similar contamination was found in *Escherichia coli* peptidoglycan (31).

The peptidoglycan may have been altered during Pronase digestion. There was no decrease in turbidity and no release of N-terminal amino groups, free amino acids, or mono-dinitrophenylatable DAP during a lengthy period of digestion of purified peptidoglycan by Pronase. Thus, the final product appeared to be insensitive to the enzyme. This does not prove that Pronase did not attack some peptide bonds in the glycopeptide during the purification treatment. This appears unlikely, however, since data to be presented later (Table 2) show the peptidoglycan to contain few N- or C-terminal groups. Proteo-

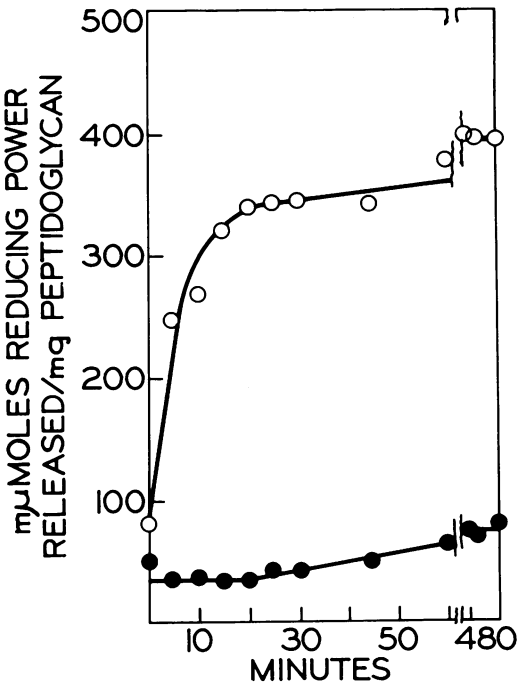

FIG. 3. *Release of reducing power during digestion by lysozyme of Spirillum serpens peptidoglycan. The digestion mixture containing 0.244 mg of peptidoglycan, 100 μliters of 10^{-3} M $MgSO_4$, 100 μliters of Tris buffer (0.1 M, pH 8.5), 100 μg of lysozyme and 800 μliters of water was incubated at 37 C. Samples of 50 μliters were removed at various times, and the reducing power was measured. A control mixture without lysozyme was assayed in parallel. Symbols: ●, without lysozyme; ○, with lysozyme.*

lytic enzymes such as trypsin, pepsin, and Pronase have been used by others for obtaining the peptidoglycan of gram-negative bacteria (13, 16, 31).

The susceptibility of fresh exponential cells, SDS-treated cells, and purified peptidoglycan to lysis by AL-1 enzyme was determined. Intact and SDS-treated cells were highly susceptible to the enzyme; suspensions of both were almost completely cleared within 15 min. Peptidoglycan required approximately 30 min for maximal clearing. The turbidity of the whole cell suspension decreased to 5%, SDS-treated cells to 5%, and peptidoglycan to 42% of that at the beginning. In each case, residual turbidity was accounted for by PHB granules observed by phase microscopy.

Solubilization of the peptidoglycan by lysozyme was determined by following the release of reducing power with time. Reducing power was liberated rapidly and linearly for 5 min (Fig. 3). This was accompanied by a decrease in turbidity. A slow release of reducing power followed, and reached a maximal value of 400 mμmoles released per mg of peptidoglycan. After centrifugation at 15,000 × *g* for 15 min, 93% of the total reducing power was recovered in the supernatant fluid.

The extent of peptide cross-linking in the peptidoglycan was determined by N- and C-terminal amino acid and free ε-amino DAP analyses (Table 2). The small amount of free N-terminal alanine probably reflected breaks in the muramyl-alanine bond. C-terminal DAP probably resulted from enzymatic cleavage of the DAP-alanine bond or from incompletely synthesized tetrapeptides. The amounts of free C-terminal alanine and ε-amino DAP were approximately equal. This was a good indication that cross-linkage of tetrapeptides occurs between these amino acids. Calculations based upon the free C-terminal alanine and ε-amino DAP indicated that 54% of these amino acids are

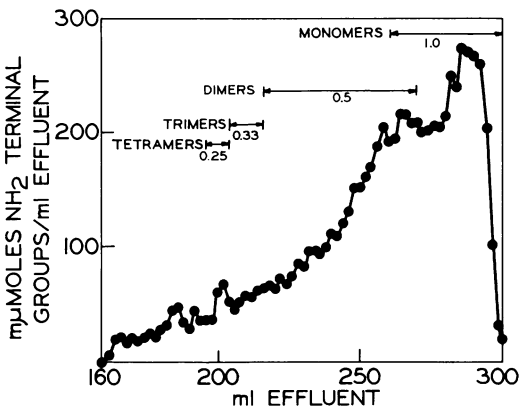

FIG. 4. *Sephadex G-50:G-25 filtration of a lysozyme digest of Spirillum serpens peptidoglycan. A 58-mg sample of purified peptidoglycan was digested with 0.5 mg of lysozyme for 10 hr at 37 C. Following centrifugation to remove residual particulate matter (mostly PHB), the supernatant fluid was applied to a 1.7 × 112 cm Sephadex G-50 column. The effluent from this column fed directly into a 1.5 × 80.5 cm Sephadex G-25 column. The columns were eluted with water, 2.0-ml fractions were collected, and NH_2-terminal groups were determined by dinitrophenylation. The amount of total DAP and free ε-amino-DAP in the fractions was determined by thin-layer chromatography. The ratio of total DAP to free ε-amino-DAP determined whether the fractions contained monomers, dimers, trimers, or tetramers. A ratio of 1.0 could only result from an uncross-linked monomer unit. Similarly, ratios of 0.5, 0.33, and 0.25 would correspond to dimers, trimers, and tetramers, respectively.*

involved in cross-linking. In contrast, only 30% of the DAP is involved in cross-linkage in *E. coli* peptidoglycan (28).

An alternate procedure for studying the tetrapeptide cross-linkages was employed. Purified peptidoglycan was digested with lysozyme, then centrifuged at 15,000 × *g* for 15 min to sediment PHB granules and any undigested residue. The supernatant fluid was filtered through a Sephadex G-50 column which fed directly into a G-25 column. The columns were eluted with water. Fractions of 2 ml were collected, and each was assayed for content of total free amino groups. The ratio of total DAP to free ε-amino DAP was determined and used as the indicator of polymer size. The resulting pattern of separation into disaccharide-tetrapeptides of differing sizes, dependent upon the extent of cross-linking between them, is shown in Fig. 4. Those fractions found to contain a ratio of 1.0 were comprised of monomers (a disaccharide-peptide unit). Those fractions containing the ratios of 0.5, 0.33, and 0.25 were comprised, respectively, of dimers (two disaccharide-peptide units linked by a peptide bond between the ε-amino DAP of one and the terminal alanine of the other), trimers, and tetramers. Relatively small amounts of tetramers and trimers were found in comparison to the large amounts of dimers and monomers. These data correlated with the results of end group analyses which indicated approximately 50% cross-linkage.

The polysaccharide component (backbone) of the peptidoglycan, consisting of polymeric ribbons of repeating *N*-acetyl muramic acid and *N*-acetyl glucosamine, was isolated. The procedures employed were patterned after those used to prepare polysaccharide backbones of *A. crystallopoietes* (9) and *S. aureus* Copenhagen (30) cell walls. The procedure is based upon fractionation of peptidoglycan after hydrolysis with the AL-1 peptidase. This enzyme splits muramyl-alanine bonds in all susceptible cell walls, and in some cases attacks the cross-bridges (10, 30). The mode of lysis of *S. serpens* peptidoglycan by AL-1 was determined by following the release of N- and C-terminal amino acids during digestion. N-terminal alanine was released rapidly accompanied by clearing of the suspension. Approximately 80% of the total muramyl-alanine bonds were cleaved within a 7-hr incubation period. There was absolutely no release of N- or C-terminal DAP or C-terminal alanine. Thus, the enzyme acted only upon the muramyl-alanine bonds of *S. serpens* peptidoglycan.

A 50-mg portion of purified peptidoglycan was digested for 10 hr with the AL-1 enzyme. The digest was centrifuged to remove residual PHB granules, and the supernatant fluid was applied to an ECTEOLA-cellulose column. Digestion products were eluted first with water then by a linear LiCl gradient. Fractions of 5.0 ml were collected and assayed for total hexosamines, reducing power, free amino groups, and total

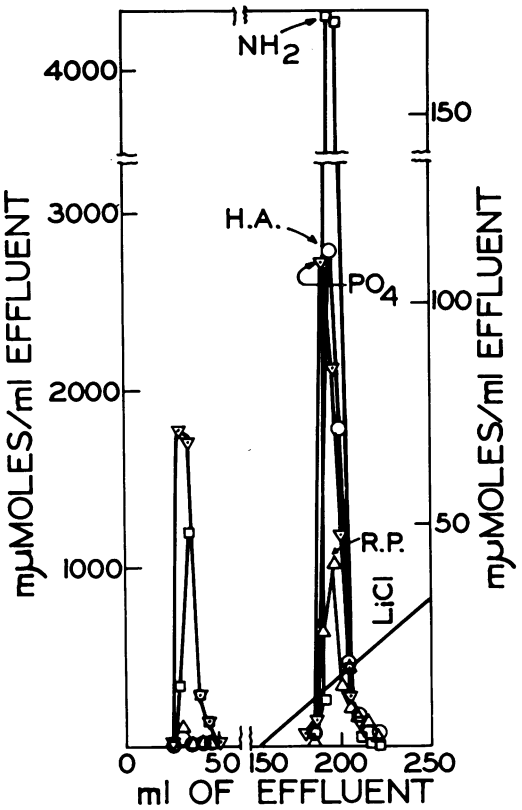

FIG. 5. *ECTEOLA-cellulose fractionation of an AL-1 enzyme digest of Spirillum serpens peptidoglycan. A 5.0-ml digestion mixture, containing 50 mg of peptidoglycan, 2.50 ml of 0.05 M Tris buffer (pH 9.0) with 10^{-3} M EDTA, 2.25 ml of water, and 0.25 ml of AL-1 enzyme solution (10 mg/ml) was incubated at 37 C with frequent mixing. An additional 100 μliters of AL-1 enzyme solution was added after 5 hr and incubated another 5 hr. At this time, the digestion was complete. Following centrifugation to remove residual particulate matter (mostly PHB), the supernatant fluid was applied to a 1.5 × 20.5 cm ECTEOLA-cellulose column. The digestion mixture was eluted with water followed by a 0 to 1.0 M linear gradient of LiCl, and 5.0-ml fractions were collected. Symbols: Plotted on left-hand ordinate (0 to 4,000 mμmoles/ml effluent): ○, total hexosamines (H. A.); □, total free amino groups (NH_2). Plotted on right-hand ordinate (0 to 150 mμmoles/ml of effluent): ▽, total phosphate (PO_4); △, reducing power (R. P.).*

phosphate (Fig. 5). The first peak, which was eluted with water, contained nonpeptidoglycan peptides and amino acids. This was determined by thin-layer chromotography and analyses using the amino acid analyzer. Virtually no hexosamines or reducing power was eluted with water. The peak of material eluted at approximately 0.3 M LiCl contained hexosamines and amino groups. Analysis of a hydrolyzed sample from this peak revealed only hexosamines and peptidoglycan amino acids in the expected molar ratios. Approximately 150 mμmoles of phosphate were found in the first peak, and 300 mμmoles in the second. The total phosphate amounted to 0.09 mole per mole of DAP. The purified peptidoglycan before AL-1 digestion and gel filtration contained 0.2 mole of phosphate per mole of DAP. Thus, more than half of the phosphate was not recovered; presumably, it was sedimented during centrifugation of the digest. The nature of the phosphate in the two peaks is not known.

The fractions comprising the second peak (between 190 and 215 ml effluent) were pooled and lyophilized. The residue was dissolved in 5.0 ml water and applied to a Sephadex G-100 column having external (V_o) and internal (V_i) solvent volumes of 36 and 108 ml, respectively. The column was eluted with water, and 3.0-ml fractions were collected. Each fraction was assayed for content of total hexosamine, free amino groups, total phosphate, and reducing power. A peak emerging immediately after the V_o contained mostly hexosamine essentially free of peptides (Fig. 6). This, presumably, was the polysaccharide backbone of the glycopeptide. The peak of amino terminal groups eluted just after the V_i probably contains small molecular weight peptides or amino acids. A small amount of phosphate appeared well after the V_i. This phosphate is either small in size or interacts in some way with the dextran, thus retarding its filtration. A peak containing low molecular weight reducing power elutes at the V_i. This probably contains mono- or disaccharide units, possibly the products of autolytic enzyme activity or newly synthesized units not incorporated into polymer.

The fractions comprising the first peak were pooled arbitrarily into four samples. Sample 1 contained the effluent between 30 and 45 ml, sample 2 between 46 and 69 ml, sample 3 between 70 and 84 ml, and sample 4 between 85 and 99 ml. The average chain length of the polysaccharide in each sample was calculated from determinations of total hexosamines and of reducing end groups. The average chain length (hexosamines per reducing end group) of the polysaccharide

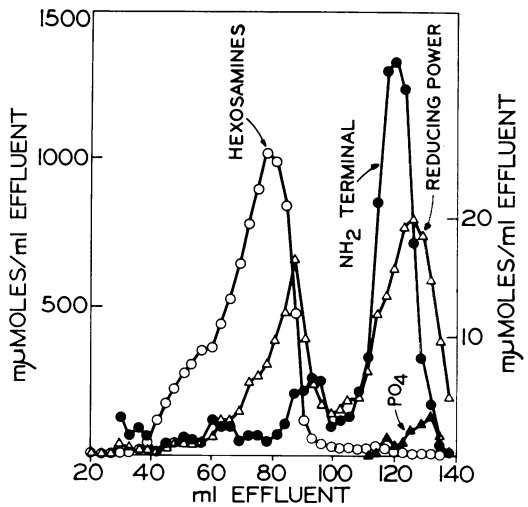

FIG. 6. *Sephadex G-100 gel filtration of pooled polysaccharide-peptide mixture from the ECTEOLA-cellulose fractionation of Spirillum serpens peptidoglycan. The pooled fractions from the ECTEOLA-cellulose fractionation were lyophilized, resuspended in 5.0 ml of water, applied to the 1.7 × 48.0 cm G-100 column, and eluted with water. Fractions of 3.0 ml were collected. Plotted on left-hand ordinate (0 to 1,500 mμmoles/ml of effluent): ○, total hexosamines; ●, total free amino groups; ▲, total phosphate. Plotted on right-hand ordinate (0.20 mμmoles/ml of effluent): △, reducing power.*

was 91 for sample 1, 117 for sample 2, 91 for sample 3, and 14 for sample 4. Considering only samples 1–3 (where the chain lengths are relatively close), an average chain length of 99 hexosamines was calculated. To arrive at this number, the relative amounts of material in the three fractions was taken into consideration. Sample 4 was excluded from the calculation because of the relatively small amount of material in this fraction (the tailing of the curve) and the wide deviation from the lengths of the other three samples.

Another procedure for determining the average lengths of cell wall carbohydrate backbone entails periodate oxidation followed by measurement of formaldehyde produced (29). Attempts to employ this procedure with the *S. serpens* backbones failed owing to excessively high values obtained in unreduced control samples. The high blank values suggested the presence of polyalcohols which readily yield formaldehyde upon periodate oxidation. The polyol could be glycerol from contaminating membrane fragments. A sample of the backbone material was extracted with chloroform–*n*-amyl alcohol (9:1, v/v) at 25 C for 5 hr. Reduced and unreduced samples were oxidized with periodate. The

amount of formaldehyde formed was the same in the untreated and solvent-extracted samples. Thus, glycerol of membrane origin was excluded as a source of the high formaldehyde blank. The reasons for the unusually high blank values could not be explained.

DISCUSSION

The procedures employed in isolating and purifying the peptidoglycan layer of the cell wall of *S. serpens* were somewhat novel in that whole cells were employed as the starting material. The cell wall isolation procedures utilized by others have involved fractionation of disrupted cells. The validity of employing intact *S. serpens* cells is confirmed by the analyses of the final product. Peptidoglycan amino acids, in the expected ratios, were practically the only amino acids present.

Martin (15) found peptidoglycan in the cell wall of a *Spirillum* sp. to be more easily purified than that in the cell walls of other gram-negative bacteria. He reported that aqueous SDS removed a protein layer from the cell wall; this did not occur with two other gram-negative walls. We found considerable protein remaining with the residue of SDS-treated cells. This protein was quantitatively removed by Pronase treatment. It appears that the cell walls of the *Spirillum* sp. and *S. serpens* differ in the manner by which the protein layer is associated with the peptidoglycan. However, the possibility that the protein remaining after SDS treatment of *S. serpens* cells is of intracellular origin was not ruled out.

The results of this investigation are summarized in a hypothetical model for the peptidoglycan polymer of *S. serpens* cell walls (Fig. 7). For convenience, the model represents a two-dimensional structure. The actual organization of the peptidoglycan macromolecular complex in the cell wall possibly involves peptide bonding between the tetrapeptides forming a three-dimensional structure. Electron micrographs of thin sections of *S. serpens* cell walls showed the peptidoglycan layer, located just exterior to the plasma membrane, to be approximately 30 A wide (18). In a recent review, Martin (15) postulated that mureins (peptidoglycan) of gram-negative bacteria are not extensively cross-linked in the third dimension and may exist as monolayer sheets. This postulate was based upon the thickness of sections of *E. coli* and *Proteus mirabilis* mureins of 17 and 25 A, respectively. Based upon the analyses of the purified material, and by analogy with the structure of other cell wall peptidoglycans, the macromolecular structure would consist of a matrix of carbohydrate ribbons composed of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid molecules. Tetrapeptides composed of L-alanyl-D-glutamyl-*meso*-DAP-D-alanine are attached to the lactyl group of muramic acid. The tetrapeptides are cross-linked by peptide bonds between the ϵ-NH_2 group of DAP of one chain and the C-terminal D-alanine group of another. C- and N-terminal amino acid analyses showed that approximately half of the tetrapeptides are cross-linked in this fashion.

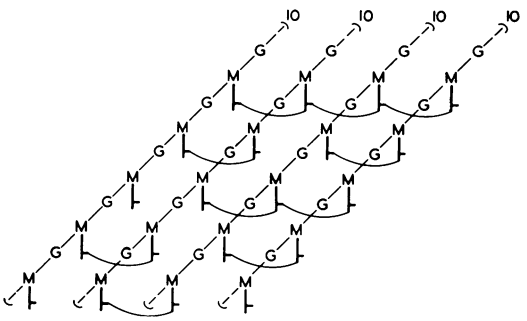

FIG. 7. *Hypothetical structural model of the peptidoglycan from Spirillum serpens. M and G represent, respectively, N-acetylmuramic acid and N-acetylglucosamine. Heavy lines represent the tetrapeptide linked to M. The straight light lines represent chemical bonds between M and G, and the curved light lines the tetrapeptide cross-bridge peptide bonds. The numbers outside the parentheses indicate the average number of such units per polysaccharide chain.*

Similar direct cross-linking between DAP and D-alanine was found previously in the peptidoglycans of *E. coli* (21), *P. mirabilis* (H. H. Martin, Abstr. Intern. Congr. Biochem. **6**:70, 1964), and *Aerobacter cloacae* (27), the three gram-negative bacteria for which such determinations have been made. The peptidoglycan of *S. serpens* seems to be more tightly knit than that of *E. coli* or *P. mirabilis*, the only other gram-negative cell walls where this has been determined. In *E. coli*, 30% of the DAP is involved in cross-linkages (28), in *P. mirabilis* the value is 40% (14). Our data showed 54% cross-linkage in *S. serpens* walls.

Aspartic acid and glycine were found in the purified peptidoglycan in amounts which might be significant. These amino acids have been found in cross-bridges between the tetrapeptides of several different peptidoglycans. These amino acids are presumably not involved in cross-bridging in *S. serpens* peptidoglycan. Neither amino acid was detected by thin-layer chromatography of acid hydrolysates of the material in the dimer peak (Fig. 4). Moreover, the tetra-

peptides of all bacterial cell walls containing DAP are directly cross-bridged. None of the gram-negative peptidoglycans studied thus far possesses amino acid-containing cross-bridges. The relationship of these two amino acids to the peptidoglycan is not known. Aspartic acid has been shown to link protein to polysaccharide in some glycoproteins (24), and it might perform a similar function in *S. serpens*.

Pelzer, Maass, and Weidel (20) digested peptidoglycan of *E. coli* with lysozyme and separated the products by paper chromatography. Monomers (disaccharide-tetrapeptide) and dimers (disaccharide - tetrapeptide - tetrapeptide - disaccharide) were found in a 1.22:1 ratio. Takebe (28) obtained virtually the same results by chemical end group analyses of a lysozyme digest. Mardarowicz (13) reported a similar pattern after fractionation of lysozyme-digested *Brucella abortus* peptidoglycan. Similar experiments with *P. mirabilis* revealed trimers in addition to dimers and monomers (15). We employed different techniques to determine the size of the peptide cross-linked products of lysozyme digestions of *S. serpens* peptidoglycan. Gel filtration revealed mostly monomers and dimers but also some trimers and tetramers. The presence of the larger cross-linked fragments supports the conclusion based upon C- and N-terminal amino acid analyses that the peptidoglycan of *S. serpens* is more extensively cross-linked than is that of *E. coli* or *P. mirabilis*.

The extent of inter-peptide cross-linkage probably determines to a considerable degree the strength or rigidity of the peptidoglycan macromolecule. One would also expect the relative length of the carbohydrate backbone polymer to have an effect upon rigidity. Conceivably, the length of the backbone is related to cell shape, perhaps being longer in rod-shaped than spherical-shaped cell walls. The determination of chain length has been made for only a few bacterial cell walls. Cell walls of *A. crystallopoietes* grown as spheres contain backbone ribbons composed of an average of 34 hexosamine units. Walls from the same organism grown as rods contain 115 to 135 hexosamines per chain (9). The peptidoglycan polysaccharide from *S. aureus* Copenhagen consists of chains containing 19 to 25 hexosamines (30). Analyses of the peptidoglycan polysaccharide of *Lactobacillus casei* (D. J. Tipper, *personal communication*) and the Porton strain of *Bacillus subtilis* (A. D. Warth, *personal communication*) revealed an average of 25 hexosamines per chain for each. From this small sampling, it appears that no direct relationship exists between cell shape and carbohydrate polymer length. The role which long versus short backbone polymers might play in rigidity and strength of peptidoglycan is not evident from these studies.

The results of this investigation do not provide an explanation for the extreme susceptibility of *S. serpens* to the myxobacter AL-1 protease. In contrast, intact cells of *E. coli* are completely resistant to lysis by the enzyme (1). There are apparently no profound differences in the peptidoglycan of the two organisms. The difference in susceptibility must be related to other wall polymers which may affect penetration of the enzyme.

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