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Peptidoglycan of Free-Living Anaerobic Spirochetes

R. JOSEPH,[1] S. C. HOLT, AND E. CANALE-PAROLA
Department of Microbiology, University of Massachusetts, Amherst, Massachusetts 01002

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Electron microscope examination of negatively stained or thin-sectioned cells of *Spirochaeta stenostrepta* treated with penicillin or lysozyme showed that the peptidoglycan was present as a thin, electron-dense layer adjacent and external to the cytoplasmic membrane. The peptidoglycan was isolated from cells of *S. stenostrepta* and *Spirochaeta litoralis* by a procedure including treatments with sodium lauryl sulfate and Pronase. Hydrolysates of the isolated *S. stenostrepta* and *S. litoralis* peptidoglycans contained glucosamine, muramic acid, glutamic acid, L-ornithine, and alanine in molar ratios of 0.90:0.85:1.00:1.00:1.40 and of 0.63:0.63:0.99:1.00:1.41, respectively. Determination of N-terminal residues suggested that nearly 50% of the ornithine in *S. stenostrepta* and *S. litoralis* peptidoglycans was involved in peptide cross-linkage. The peptidoglycan layer of *S. stenostrepta* was sensitive to lysozyme and myxobacter AL-1 protease.

All known spirochetes possess several morphological features in common: (i) a helically shaped or wavy protoplasmic cylinder which consists of the nuclear and cytoplasmic regions surrounded by a multilayered cell wall-cytoplasmic membrane complex; (ii) from two to several hundred axial fibrils wound together with the protoplasmic cylinder; (iii) an outer sheath which surrounds both the axial fibrils and the protoplasmic cylinder. Except for the presence of axial fibrils located between the outer sheath and the cell wall-cytoplasmic membrane complex, the cell envelopes of spirochetes appear to be morphologically similar to those of gram-negative bacteria (5, 10, 13, 17). Ginger (9), who isolated muramic acid and glucosamine from cell residues of *Borrelia duttoni* and of leptospires, predicted the presence of a peptidoglycan layer in spirochetes. Subsequently, Yanagawa and Faine (25) and Tinelli and Pillot (22) detected muramic acid and glucosamine in cell residues of *Leptospira interrogans* serotype *icterohaemorrhagiae* and of the Reiter treponeme. In addition, Tinelli and Pillot (22) reported the presence of ornithine, lysine, alanine, glutamic acid, and small amounts of diaminopimelic acid and other amino acids in cell wall preparations of the Reiter treponeme. Pillot and Ryter (19) postulated that the peptidoglycan layer of spirochetes was intimately associated with the cytoplasmic membrane. Although the above-mentioned investigators have demonstrated the presence of peptidoglycan in spirochetes, the peptidoglycan layer of spirochetes has neither been identified morphologically nor isolated in preparations sufficiently pure for quantitative chemical analysis.

The twofold purpose of this study was to cytologically examine the cell envelopes of two species of *Spirochaeta* and to isolate and chemically characterize the peptidoglycan from these organisms.

MATERIALS AND METHODS

Organisms and culture media. *S. stenostrepta* strain Z-1 (ATCC 25083) and *S. litoralis* strain R-1 (ATCC 2700) were cultured as previously described (13).

Isolation of peptidoglycan. The peptidoglycan of *S. stenostrepta* was isolated by a modification of the method of Kolenbrander and Ensign (14). Cells in the late logarithmic phase of growth were suspended in 300 ml of distilled water to a final concentration of 20 mg per ml (dry wt). As the suspension was continuously stirred, 25% (wt/vol) sodium lauryl sulfate (SLS) was added dropwise to a final concentration of 1% (wt/vol), and the preparation was incubated at 23 C for 4 h with mixing. Ribonuclease (1 μg/ml, final concentration) and deoxyribonuclease (10 μg/ml, final concentration) were added early in the incubation period to decrease the viscosity. The suspension was

[1] Present address: Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia, Pa., 19140

centrifuged at 5,000 × *g* for 10 min and the resulting supernatant liquid was centrifuged at 35,000 × *g* for 1 h. After centrifugation the pellet consisted of a dark lower portion and a white top layer. The white layer was removed and suspended in 100 ml of distilled water. The suspension was heated in a boiling water bath for 30 min, cooled, and centrifuged at 5,000 × *g* for 15 min. The supernatant liquid was centrifuged at 108,000 × *g* for 1 h. The pellets were suspended in 100 ml of distilled water and the SLS treatment was repeated. The preparation was then centrifuged at 108,000 × *g* for 1 h. The resulting pellets were combined, suspended in 60 ml of distilled water, and centrifuged at 5,000 × *g* for 15 min. The supernatant liquid was centrifuged at 108,000 × *g* for 1 h. The pellets were suspended in 100 ml of 0.025 M tris(hydroxymethyl)aminomethane-hydrochloride (Tris) buffer, pH 7.9, containing 10^{-4} M $CaCl_2$, and heated to 65 C. A 350-mg sample of Pronase (Calbiochem), dissolved in 5 ml of Tris buffer and heat-activated at 80 C for 5 min, was added to the suspension. After incubation at 65 C for 4 h, the preparation was boiled for 20 min to destroy residual protease activity and centrifuged at 5,000 × *g* for 15 min to remove denatured proteins. The supernatant liquid was centrifuged at 108,000 × *g* for 1 h to sediment the peptidoglycan, which was then washed once with 10^{-4} M sodium ethylenediaminetetraacetic acid and three times with distilled water. The peptidoglycan was either suspended in distilled water or lyophilized prior to storage at 5 C.

Peptidoglycan from *S. litoralis* was isolated by procedures identical to those used for *S. stenostrepta*, except that 0.01 M piperazine-*N*,*N'*-bis(2-ethanesulfonic acid) buffer, pH 6.8, containing 0.3 M NaCl and 0.002 M Na_2S, was used as the first suspending medium instead of distilled water. Distilled water or the appropriate buffer solution was used in all the steps after boiling, as described above.

The purity of the peptidoglycan preparations was verified by electron microscopy and by amino acid analysis.

Lysozyme digestion. Samples (whole cells or purified peptidoglycan; final concentration 1 mg per ml dry weight) were suspended in 0.1 M potassium phosphate buffer, pH 7.2. Lysozyme (3X crystalline, Sigma Chemical Co.) at final concentrations from 50 to 500 μg per ml was added. The mixture was incubated at 37 C. Samples were removed periodically and examined by phase contrast and electron microscopy for morphological changes.

Myxobacter AL-1 protease digestion of peptidoglycan preparations. Preparations were treated by the method of Kolenbrander and Ensign (14). They were incubated at 37 C and changes in optical density were followed photometrically at 420 nm in a Beckman DU-2 spectrophotometer.

Penicillin treatment. Penicillin G (final concentration 5 mg/ml; potassium salt, approximately 1,400 U per mg, Nutritional Biochemicals Co) was added to *S. stenostrepta* cultures in the exponential phase of growth. After incubation for 4 h at 37 C, an additional 5 mg of penicillin G was added per milliliter of culture, and incubation was continued for 4 h more. Samples were removed periodically and examined for morphological changes by phase contrast and electron microscopy.

Effect of sodium deoxycholate on S. stenostrepta. Sodium deoxycholate (DOC) (2.4×10^{-2} M, final concentration) was added to test tubes each containing 10 ml of a suspension of unwashed actively motile cells (0.36 mg of cells per ml, dry weight) in 0.01 M potassium phosphate buffer, pH 7.2, containing 0.25 M sucrose. The mixtures were incubated at room temperature and the decrease in turbidity was followed by measuring the optical density at 425 nm. Samples were removed after 30 min for examination by phase contrast and electron microscopy.

Hydrolysis and amino acid analysis of peptidoglycan. Hydrolysis and amino acid analysis were performed as previously described (13), except that acid hydrolysis was carried out for 8 h.

Paper chromatography. Two to 20 μliters of peptidoglycan acid hydrolysate in 10% (vol/vol) isopropanol were spotted onto Whatman #1 or #4 filter paper. Ascending and descending, one- and two-dimensional paper chromatography were performed by using the three solvent systems described below. (i) *N*-butanol-acetic acid-water, 5:1:2 (vol/vol), (ii) water-saturated butanol; (iii) tertiary butyl alcohol-2-butanone-acetic acid-water, 4:3:2:1 (vol/vol).

Amino acids, amino sugars, and carbohydrates were detected as described by Wheat (24).

Electron microscopy. Methods were previously described by Joseph and Canale-Parola (13).

Other assays. See Table 1.

RESULTS

Location of the peptidoglycan. Thin sections of whole cells of *S. stenostrepta* (Fig. 1) and *S. litoralis*, fixed in osmium tetroxide without glutaraldehyde prefixation, displayed a cell wall-cytoplasmic membrane complex, approximately 11 to 12 nm thick, which appeared as two electron-dense layers separated by an electron-transparent layer. The outer electron-dense layer measured 6 nm in diameter and was approximately twice as thick as the inner electron-dense layer. In thin sections of cells pre-

TABLE 1. *Chemical and enzymatic assays*

Assay	Reference no.
Protein	15
Carbohydrate	6
Phosphorus	2
Ashing	1
Ornithine	3, 12
Citrulline	11
Free amino groups	21
N-terminal amino acids	8

fixed with glutaraldehyde (3%, vol/vol) the outer electron-dense layer appeared to be composed of two thinner electron-dense layers, each approximately 2.5 to 3 nm in diameter (Fig. 2, 3). The outermost of the latter two electron-dense layers represented the peptidoglycan layer, as described below.

Addition of penicillin G to exponential phase cultures of *S. stenostrepta* resulted in conversion of the helically shaped organisms into round or distorted cells, as observed by phase microscopy. Electron microscope examination showed that the protoplasmic cylinder was severely disrupted, but remained within the outer sheath (Fig. 4). Since it is known that penicillin inhibits specific steps in peptidoglycan synthesis, this observation suggested that the peptidoglycan layer was associated with the protoplasmic cylinder.

When DOC was added to cells of *S.*

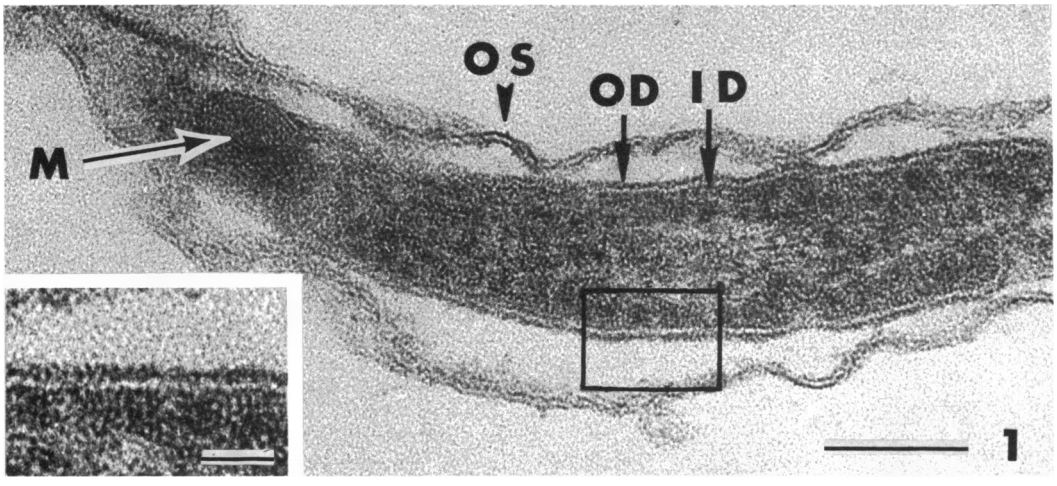

FIG. 1. *Electron micrograph of a thin section through part of a S. stenostrepta cell fixed with osmium tetroxide. Abbreviations: OS, outer sheath; OD, outer electron-dense layer of the cell wall-cytoplasmic membrane complex; ID, inner electron-dense layer of the cell wall-cytoplasmic membrane complex; M, mesosome. The bar represents a length of 100 nm. Inset: high magnification of a portion of the cell wall-cytoplasmic membrane complex. The inset bar represents a length of 25 nm.*

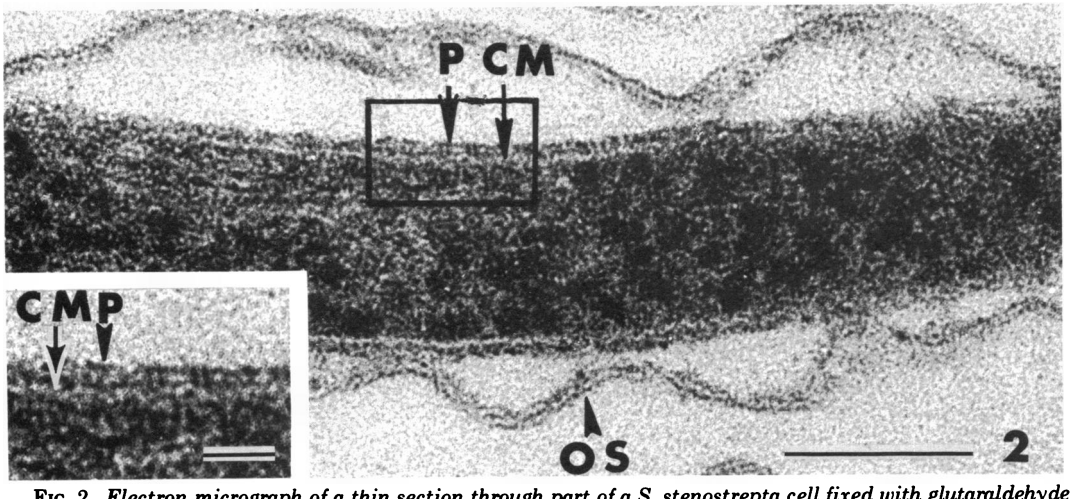

FIG. 2. *Electron micrograph of a thin section through part of a S. stenostrepta cell fixed with glutaraldehyde and osmium tetroxide. Abbreviations: OS, outer sheath, CM, cytoplasmic membrane; P, peptidoglycan. The bar represents a length of 100 nm. Inset: high magnification of a portion of the cell wall-cytoplasmic membrane. The peptidoglycan appears as an electron-dense layer surrounding the cytoplasmic membrane. The inset bar represents a length of 25 nm.*

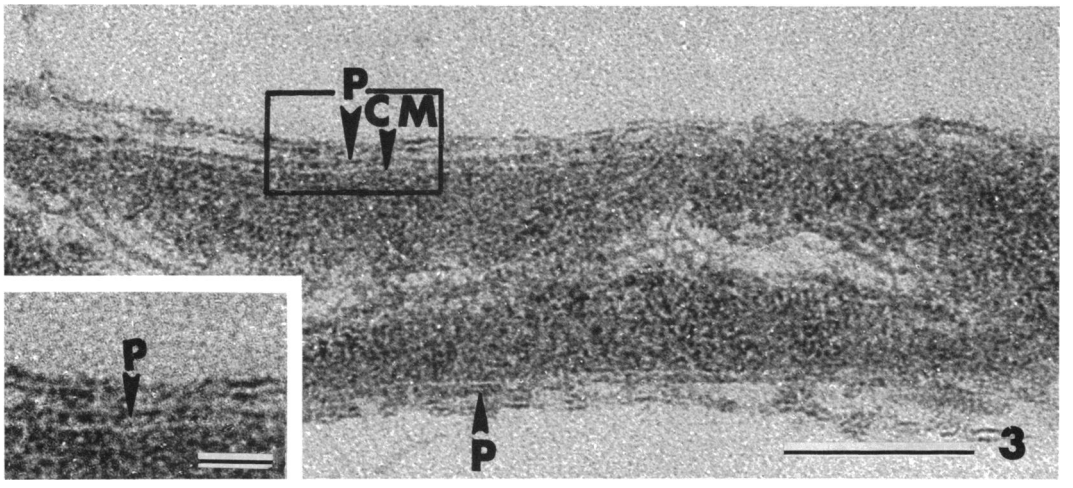

FIG. 3. *Electron micrograph of a thin section through part of a S. litoralis cell fixed with glutaraldehyde and osmium tetroxide. Abbreviations: P, outer electron-dense layer, presumably the peptidoglycan; CM, cytoplasmic membrane. The bar represents a length of 100 nm. Inset: high magnification of a portion of the cell wall-cytoplasmic membrane. The inset bar represents a length of 25 nm.*

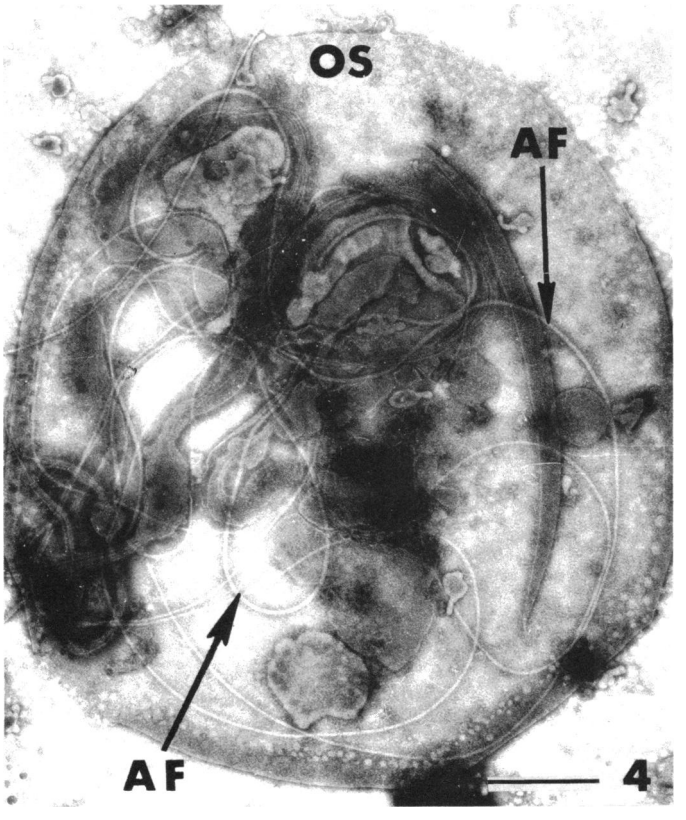

FIG. 4. *Electron micrograph of a negatively stained cell of S. stenostrepta treated with penicillin G. The protoplasmic cylinder is disrupted, but remains within the outer sheath (OS). AF, axial fibrils. The bar represents a length of 500 nm.*

stenostrepta suspended in 0.01 M phosphate buffer, pH 7.0, containing 0.25 M sucrose, the turbidity of the suspension decreased by 85%. Phase-contrast microscopy indicated that the cells had become thinner, but had maintained their characteristic coiled shape. Electron microscope examination of the DOC-treated cells, after negative staining or thin sectioning, revealed the absence of the outer sheath and of portions of the cytoplasmic membrane (Fig. 5). In contrast, when lysozyme (1 mg per ml) was added to cells of *S. stenostrepta* (not previously treated with DOC) suspended in the above-mentioned phosphate buffer-sucrose solution, only clumps of rounded or distorted cells were observed by phase-contrast microscopy. In thin sections of these lysozyme-treated *S. stenostrepta* cells, the outer sheath appeared intact and enveloped a greatly swollen protoplasmic cylinder (Fig. 6). The outermost electron-dense layer (2.5 to 3 nm thick) of the cell wall-cytoplasmic membrane complex was extensively disrupted, as indicated by its jagged and irregular appearance (Fig. 6, inset). A triple-layered structure morphologically identifiable as the cytoplasmic membrane was observable (Fig. 6).

When *S. stenostrepta* cells treated with DOC, as described above, were subsequently incubated with lysozyme (1 mg per ml), only debris and cell fragments were observed in thin-sectioned and negatively stained preparations.

Since the outermost electron-dense layer of the cell wall-cytoplasmic membrane complex in *S. stenostrepta* was selectively attacked by lysozyme (as observed in thin sections), it was concluded that this layer corresponded to the peptidoglycan layer. The remaining triple-layered inner structure was morphologically comparable to the cytoplasmic membrane of other prokaryotic cells.

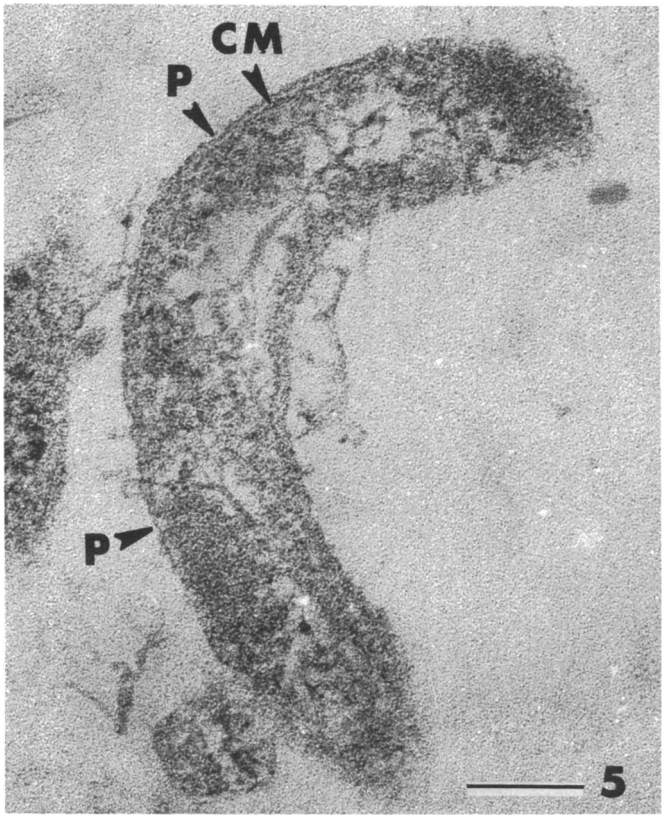

FIG. 5. *Electron micrograph of a thin section through part of a S. stenostrepta cell treated with sodium deoxycholate (1% wt/vol). The outer sheath has been removed by the treatment. The peptidoglycan layer (P) and portions of the cytoplasmic membrane (CM) remain intact. Prefixed with 3% glutaraldehyde. The bar represents a length of 100 nm.*

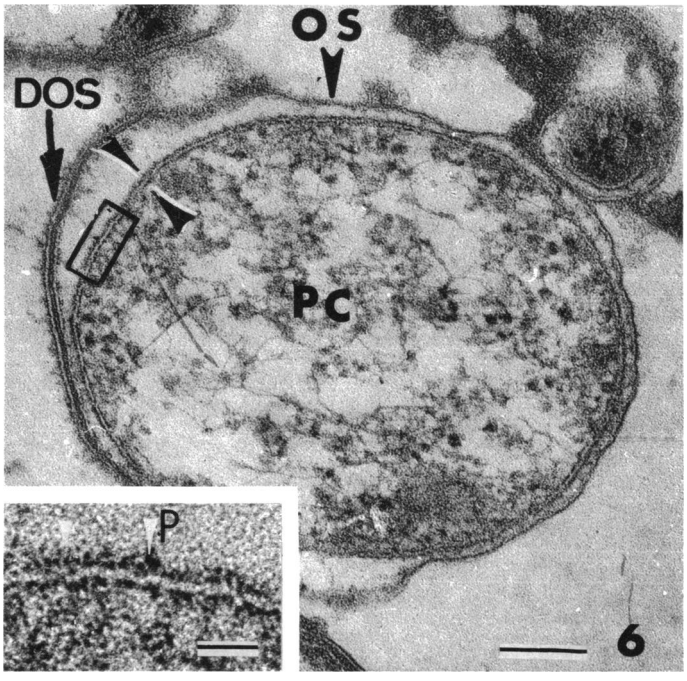

FIG. 6. *Electron micrograph of a thin section through a cell of S. stenostrepta treated with lysozyme (1 mg per ml) in a buffer-sucrose solution. The outer sheath (OS) is intact, but the peptidoglycan layer (P) is disrupted and extensively digested (inset). The protoplasmic cylinder (PC) is swollen to several times its original diameter. The section was prefixed with 3% glutaraldehyde. The bar represents a length of 100 nm. DOS: two adjacent outer sheaths, one from the cell in the center and the other from a second cell. Inset: high magnification of a portion of the cytoplasmic membrane. The inset bar represents a length of 25 nm.*

Isolation of the peptidoglycan. Purification methods involving preliminary mechanical disruption of the spirochetal cell, such as ballistic disintegration, sonic treatment, or homogenization (4, 16, 18, 20, 23) resulted in poor recovery of the peptidoglycan. When procedures described by Tinelli and Pillot (22) for peptidoglycan purification from the Reiter treponeme were used, the *S. stenostrepta* peptidoglycan obtained appeared, upon electron microscope examination, to be contaminated with amorphous material. On the other hand, the procedure for peptidoglycan purification used in this investigation (see Materials and Methods) yielded peptidoglycan preparations free of significant amounts of visible contamination. Electron microscopy of thin sections of purified peptidoglycan revealed a thin, electron-dense, single-layered structure (Fig. 7) which constituted approximately 1% of the cell dry weight of *S. stenostrepta* and *S. litoralis*. In negatively stained or platinum-carbon shadow-cast preparations (Fig. 8), the isolated peptidoglycan frequently retained its original helical shape, as previously described (13). The peptidoglycan was coiled and appeared thinner than the whole cell when examined by phase-contrast microscopy. Purified peptidoglycan appeared silky white when suspended in water and, after lyophilization, as a fluffy white powder.

Chemical analyses of the peptidoglycan. Paper chromatography and amino acid analyses of acid hydrolysates of *S. stenostrepta* peptidoglycan revealed the presence of five major amino-containing components: muramic acid, glucosamine, alanine, ornithine, and glutamic acid (Table 2). Peptidoglycan, prepared by Tinelli and Pillot's method (22), was grossly contaminated with other amino acids, whereas peptidoglycan prepared as described in the Materials and Methods section was essentially free from contaminating amino acids. The ratio of alanine to ornithine in *S. stenostrepta* peptidoglycan varied from 1.34 to 1.81 in several preparations, whereas the ratio of other amino acids to ornithine was essentially constant.

Amino acid analyses indicated that ornithine was the only diamino-amino acid present in the peptidoglycan of *S. stenostrepta* and *S. litoralis*. Ornithine in peptidoglycan hydroly-

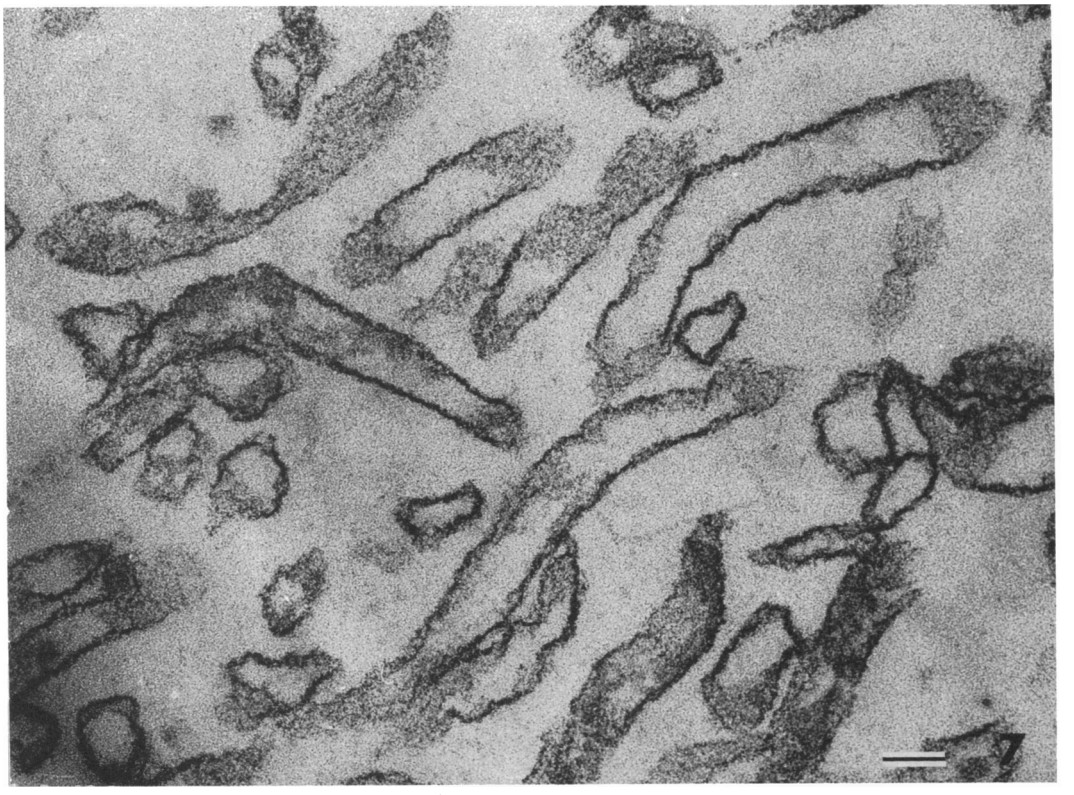

FIG. 7. *Electron micrograph of a thin section of a preparation of purified S. stenostrepta peptidoglycan fixed with osmium tetroxide. The bar represents a length of 100 nm.*

sates was further identified by separating it from the amino acid mixture by paper chromatography, and by comparing its spectrum with that of a known ornithine standard after reaction with acidic ninhydrin (3). Furthermore, ornithine separated from the hydrolysate acted as a substrate for ornithine carbamoyltransferase (EC 2.1.3.3), which specifically converts L-ornithine to L-citrulline. More than 96% of the ornithine in the peptidoglycan of both *S. stenostrepta* and *S. litoralis* was L-ornithine. The peptidoglycans were examined for the extent of peptide cross-linkage by determining the free N-terminal amino acids (3, 8). δ-Dinitrophenyl-ornithine (δ-DNP-ornithine) was the only dinitrophenylated amino acid recovered by thin-layer chromatography. The amount of δ-DNP-ornithine recovered corresponded to the number of free amino groups detected with ninhydrin. Approximately one-half of the total δ amino groups of ornithine in *S. stenostrepta* or *S. litoralis* peptidoglycan were free to react with either fluorodinitrobenzene or with ninhydrin. This suggests that nearly 50% of the ornithine in the peptidoglycan of these spirochetes is involved in peptide cross-linkage.

Effects of enzymes on S. stenostrepta peptidoglycan. Incubation of suspensions of purified *S. stenostrepta* peptidoglycan in the presence of lysozyme (50 μg per mg of peptidoglycan) resulted in clumping of the peptidoglycan. Electron microscope examination of the lysozyme-treated peptidoglycan showed extensive disruption of the peptidoglycan. Clumping and degradation of cell wall material under similar conditions has been reported for other bacteria (7).

Incubation of suspensions of *S. stenostrepta* peptidoglycan in the presence of a preparation of myxobacter AL-1 protease (7 μg of AL-1 protease per mg of peptidoglycan), which hydrolyzes muramyl-alanine bonds present in peptidoglycan (14), resulted in a loss of 45% of the initial turbidity within 10 min. Phase-contrast and electron microscope examination of the preparations revealed only debris.

DISCUSSION

Murray et al. (17) and de Petris (5) reported that the peptidoglycan of *Escherichia coli* is present as a thin layer surrounding the cytoplasmic membrane. Our results indicate that the peptidoglycan layer of *S. stenostrepta*, like that of *E. coli*, occupies a position adjacent and external to the cytoplasmic membrane. Treatments of spirochetes with agents affecting the integrity of the peptidoglycan resulted in a loss of the typical cell shape. The latter observation complements the previous finding that the peptidoglycan of spirochetes serves to maintain the coiled configuration of these organisms (13).

Species of the genus *Spirochaeta* give a gram-negative reaction. The occurrence of the peptidoglycan as a thin layer surrounding the cytoplasmic membrane of *S. stenostrepta* is in agreement with the gram-negative nature of the cell wall indicated by the Gram stain. Furthermore, as in other gram-negative bacteria (17), a lipoprotein layer external to the peptidoglycan was detected in *S. stenostrepta* (Joseph, Holt, and Canale-Parola, Bacteriol. Proc., p. 57, 1970). Thus, the evidence indicates that the cell wall of *S. stenostrepta*, and possibly that of different species of spirochetes, is structurally similar to the cell wall of other gram-negative bacteria.

The peptidoglycans of *S. stenostrepta* and *S. litoralis* contain amino acids and amino sugars commonly found in other bacterial peptidoglycans. However, L-ornithine is the only diamino-amino acid detected in the peptidoglycans of both of these spirochetes, whereas the peptidoglycans of most other bacteria studied contain lysine or diaminopimelic acid. As previously mentioned, ornithine was found in the peptidoglycan of the Reiter treponeme (22). The presence of ornithine in the peptidoglycans of the three species of spirochetes examined to date suggests that this amino acid may occur in all spirochetal peptidoglycans.

The purified *S. stenostrepta* peptidoglycan contained ornithine, glutamic acid, glucosamine, muramic acid, and alanine in the approximate molar ratios of 1:1:1:1:1.4. These molar ratios account for the occurrence of one peptide

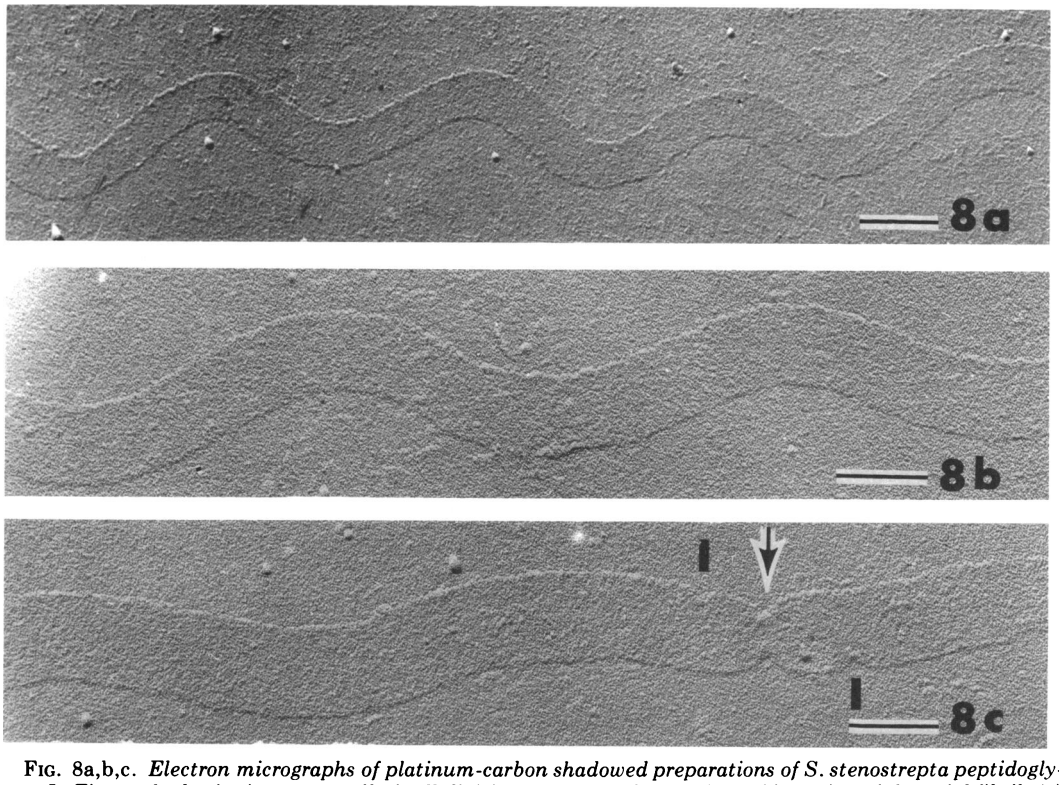

FIG. 8a,b,c. *Electron micrographs of platinum-carbon shadowed preparations of S. stenostrepta peptidoglycan. In Fig. 8c the beginning cross wall of cell division (arrow) and the points of insertion of the axial fibrils (I) are visible. The bar represents a length of 250 nm.*

TABLE 2. *Amino acid and amino sugar composition of peptidoglycan preparations*[a]

Component	Molar ratios[b]	
	S. stenostrepta	*S. litoralis*
Ornithine	1.00	1.00
Glutamic acid	1.00	0.99
Glucosamine	0.90	0.63
Muramic acid	0.85	0.63
Alanine	1.40[c]	1.41
Ammonia	0.26	0.45
Glycine	0.07	0.04
Serine	ND	0.02
Aspartic acid	0.01	0.02
Lysine	ND	ND
Leucine	0.01	ND
Threonine	0.002	0.01
Valine	ND	ND
Proline	ND	ND
Isoleucine	0.001	ND
Tyrosine	ND	ND
Methionine	0.01	0.01
Arginine	ND	ND
Histidine	ND	ND
Cysteic acid	NA	0.15

[a] Peptidoglycan preparations were purified by a modification of Kolenbrander and Ensign's method (14) and were hydrolyzed with 4 N HCl for 8 h at 110 C.

[b] Ratios to ornithine. ND, not present in detectable amounts. NA, not assayed for.

[c] In two other *S. stenostrepta* peptidoglycan preparations analyzed, the ratio of alanine to ornithine was 1.34 and 1.81, respectively.

subunit composed of alanine, glutamic acid, and ornithine per disaccharide unit in the glycan chain. The number of alanine residues greater than necessary for peptide subunits composed of alanine-glutamic acid-ornithine (1:1:1) may represent terminal alanine residues available for cross-linkage. It is possible that these terminal alanine residues are cross-linked to ornithine of adjacent peptide subunits. This possibility is supported by the finding that the number of alanine residues which are available for cross-linkage approaches the estimated degree of cross-linkage in the peptidoglycan of the spirochetes.

The lower molar ratios of glucosamine and muramic acid to amino acids in *S. litoralis* peptidoglycan hydrolysates are probably due to loss of amino sugar residues during hydrolysis. The peptidoglycans of *S. litoralis* and *S. stenostrepta* may have identical peptide subunits and the same type of cross-linkage.

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