

Composition and Ultrastructure of *Streptomyces venezuelae*

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Streptomyces venezuelae is a filamentous bacterium with branching vegetative hyphae embedded in the substrate and aerial hyphae bearing spores. The exterior of the spore is inlaid with myriads of tiny rods which can be removed with xylene. The spore wall is approximately 30 nanometers thick. Occasionally, it can be seen that the plasma membrane and the membranous bodies within a spore are connected. The spore's germ plasm is not separated from the cytoplasm by a nuclear envelope. The cell walls of the vegetative hyphae, which are about 15 nanometers thick, are structurally and chemically similar to those of gram-positive bacteria. The numerous internal membranous bodies, some of which arise from the plasma membrane of the vegetative hypha, may be vesicular, whirled, or convoluted. Membranous bodies are usually prominent at the hyphal apices and are associated with septum formation. The germ plasm is an elongate, contorted, centrally placed area of lower electron density than the hyphal cytoplasm. The spores differ from the vegetative hyphae, not only in fine structure, but also in the arginine and leucine contents of their total cellular proteins.

The only streptomycete whose fine structure has been adequately examined is *Streptomyces violaceoruber*. The excellent studies of Glauert and Hopwood (8, 12, 13) have established that the streptomycetes are typical prokaryotic organisms with extensive internal membranous bodies. Although *S. violaceoruber* has been the object of intensive genetic investigations (3), most of the research on actinophages has employed *S. venezuelae* as the host (4, 14). Accordingly, a detailed examination of the fine structure of *S. venezuelae* is needed as a prerequisite for further studies on intracellular multiplication of actinophages. Moreover, certain aspects of the streptomycete fine structure have not been resolved; for example, Hagedorn (11) concluded that the streptomycete spore is an endospore, whereas Glauert and Hopwood (10) concluded that it is merely a hyphal segment surrounded by a thickened wall.

In this study, the fine structure of the hyphae and spores has been examined; in addition, a few determinations of the chemical compositions of streptomycete components have been made. During sporulation, there are changes in both cellular fine structure and chemical composition.

MATERIALS AND METHODS

Fine structure studies. Stock cultures of *Streptomyces venezuelae* S13 were maintained at 30 C on

oatmeal-tomato paste-agar (5). The germinating spores and hyphal elements used for electron microscopic studies were obtained as follows. Spores were harvested from 4-day-old stock cultures of *S. venezuelae*; this material was suspended in peptone-yeast extract broth (16); the suspension was homogenized with a tissue grinder; and the dispersed spores were shaken at 30 C for the appropriate time. The desired samples were collected and fixed with 1% buffered osmium tetroxide (15), dehydrated by transfer through a graded ethyl alcohol series, and embedded in Epon (17). Thin sections were prepared with an LKB Ultratome by use of glass knives and were mounted on copper grids covered with a carbon-coated Parlodion film. The sections were stained with lead citrate (19) or with 2% (w/v) uranyl acetate and examined with a Siemens Elmiskop I electron microscope at initial magnifications between 10,000 X and 28,000 X. Samples for electron microscopic studies on sporogenesis were scraped from oatmeal-tomato paste-agar cultures, fixed in osmium tetroxide, and thereafter treated in the same manner as the hyphal preparations.

Carbon replicas of *S. venezuelae* spores were prepared by the method of Dietz and Mathews (6). The spores to be examined were picked up on a glass cover slide by gently touching it to the surface of sporogenous aerial growth on oatmeal-tomato paste-agar. The dried film of spores was first shadowed with chromium at an angle of 20° from the horizontal and then coated with carbon at an angle of 90°. The cover slide was immersed in 25% (w/v) aqueous KOH for 24 hr to destroy the spores per se. The replica-film was

teased from the cover slide and washed by floating it, first on 0.1 N HCl and then by several transfers to deionized water. The carbon replicas were mounted on copper grids and examined at an initial magnification of 10,000 \times .

Analytical determinations. Approximately 25 g (wet weight) of *S. venezuelae* spores or vegetative mycelia were extracted at 50 C for 2 hr with 250 ml of acetone, followed by 250 ml of diethyl ether at 30 C for 2 hr. The residue was suspended in 20 ml of deionized water and then exposed to ultrasonic vibrations (20,000 cycles/sec generated by an MSE ultrasonic disintegrator) while resting in an ice bath. The disrupted cellular material was centrifuged in the cold at 5,000 $\times g$ for 15 min to sediment intact cells and large cellular fragments. The residue was suspended in 20 ml of deionized water and shaken vigorously for 10 min at 4 C. This preparation was centrifuged in the cold at 5,000 $\times g$ for 15 min. The two supernatant fractions were pooled and centrifuged in the cold at 15,000 $\times g$ for 15 min to remove any remaining hyphal walls. The cellular proteins were precipitated and were leached for 30 min at 4 C with two 50-ml changes of 5% trichloroacetic acid. This residue was extracted twice with 50 ml of 5% trichloroacetic acid for 30 min at 100 C. The trichloroacetic acid remaining after these extractions was removed by washing the residue with 25 ml of acetone, followed by two washes with diethyl ether. This residue was acid hydrolyzed with 6 N HCl for 22 hr at 110 C. The resulting protein hydrolysate was analyzed with a Beckman Spinco model 120 automatic amino acid analyzer.

Vegetative mycelia from overnight, peptone-yeast extract broth cultures of *S. venezuelae* were collected by centrifugation and washed three times with deionized water. The hyphae were ruptured by ultrasonic treatment (20,000 cycles/sec). Whole cells were removed by centrifugation at 500 $\times g$, and the hyphal walls were collected by centrifugation at 15,000 $\times g$. The hyphal wall preparations were subjected to 20 to 25 washes with deionized water until the absorbance at 260 nanometers (nm) of the supernatant fluid was less than 0.001. The final, purified wall preparation was 5.8% nitrogen and 5.2% phosphorus; the A_{260}/A_{280} was less than 1.3, and the ratio A_{260}/A_{450} was less than 2 (W. J. Cooney, M.S. Thesis, Univ. of Minnesota, Minneapolis, 1964). The hyphal walls were hydrolyzed and analyzed by the same methods used for the total cellular proteins.

RESULTS AND DISCUSSION

Carbon replicas of *S. venezuelae* spores show that the spore surface is covered with a mosaic pattern formed by ordered arrangements of myriads of rods approximately 10 \times 100 nm. These tiny rods frequently become separated from the spore surface, indicating that they are superficially placed and loosely attached to the spore wall (Fig. 1). This surface material is not soluble in water or carbon tetrachloride, but is extracted by benzene, xylene, and ethyl alcohol (Fig. 2). This layer probably accounts for the hydrophobic

properties of *S. venezuelae* spores. Similar mosaics have been observed on the spore surface of a number of streptomycetes (6).

Spores of *S. venezuelae* are enclosed by a cell wall about 30 nm thick. Patches of the outer component of the cell wall of the sporogenous aerial hypha frequently remain attached to the mature spore surface. The plasma membrane is clearly evident in sections of spores, and an occasional membranous body can be found attached to the outer limiting membrane. The cytoplasm of the uranyl acetate-stained sections is dense and homogeneous. The electron-transparent germ plasma, with its delicate, electron-dense network is concentrated in the center of the spore. A membranous body is usually found within the nucleoid (Fig. 3). Similar structures in gram-positive bacteria have been variously designated as chondrioids, plasmalemmasomes, and mesosomes by other workers.

Spores placed in nutrient medium develop an extensive membranous system (Fig. 4). The cell wall of the germ tube is continuous with the inner component of the spore wall (Fig. 4-6). The cell wall of the germling is about 15 nm thick. During germination, the ribosomes, which cannot be distinctly seen in the spore sections, become progressively well defined (Fig. 4-6). The streptomycete ribosomes are about 12 nm in diameter, which is comparable to values obtained for other bacteria (22). In the vegetative hyphae, there are numerous membranous structures along the plasma membrane (Fig. 6-9). As the vegetative hyphae grow, the nucleoid becomes elongate and tortuous, and cross walls and branches develop (Fig. 5-8). Branch formation is neither positively nor negatively correlated with cross-wall formation (Fig. 7-9). The apices of vegetative hyphae usually contain prominent membranous bodies (Fig. 8, 9). Membranous bodies are also associated with septum development in both vegetative hyphae (Fig. 7-9) and sporogenous hyphae (Fig. 11, 12). These structures resembled those designated as peripheral bodies, chondrioids, or mesosomes in *Bacillus* (22). In contrast to the observation of Moore and Chapman (18) on an unidentified streptomycete, cross walls are relatively rare in *S. venezuelae* vegetative hyphae. In the stationary and death phases of growth, hyphal ghosts containing membranous vesicles are commonly seen (Fig. 10). Similar ghosts have been observed in degenerate sporophores of *S. noursei* (20) and disintegrating vegetative hyphae of *S. violaceoruber* (9). The plasma membrane in degenerating hyphae sometimes forms a partition across a filament independent of septum formation (Fig. 10). This may account for our observations with the light microscope in which discrete

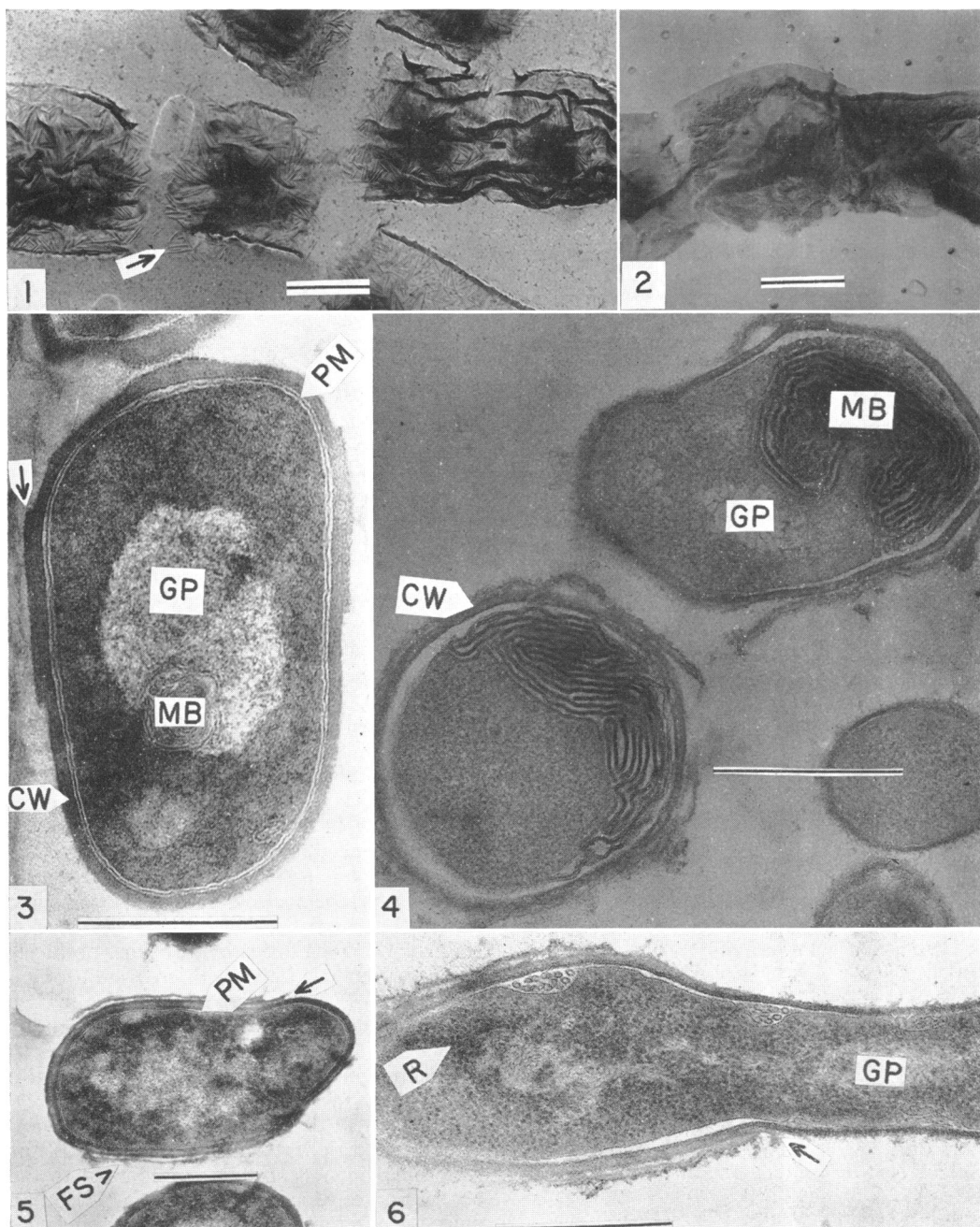


FIG. 1-14. Electron micrographs of sections of *Streptomyces venezuelae*. The marker bars denote 500 nanometers. Abbreviations used throughout: CW, cell wall; FS, fibrous sheath; GP, germ plasm; MB, membranous body; PM, plasma membrane; R, ribosome; and S, septum.

FIG. 1. Carbon replica of normal spores. Arrow indicates free crystals.

FIG. 2. Carbon replica of xylene-extracted spores.

FIG. 3. Section of a normal spore, stained with uranyl acetate. Arrow indicates an attached strip of the outer layer of the cell wall of the aerial hypha.

FIG. 4. Section of a germinating spore, stained with lead citrate.

FIG. 5-6. Sections of germinating spores, stained with uranyl acetate and lead citrate (Fig. 5) or uranyl acetate alone (Fig. 6). Arrows indicate the points where the outer layer of the spore separates from the inner layer which is continuous with the cell wall of the germling.

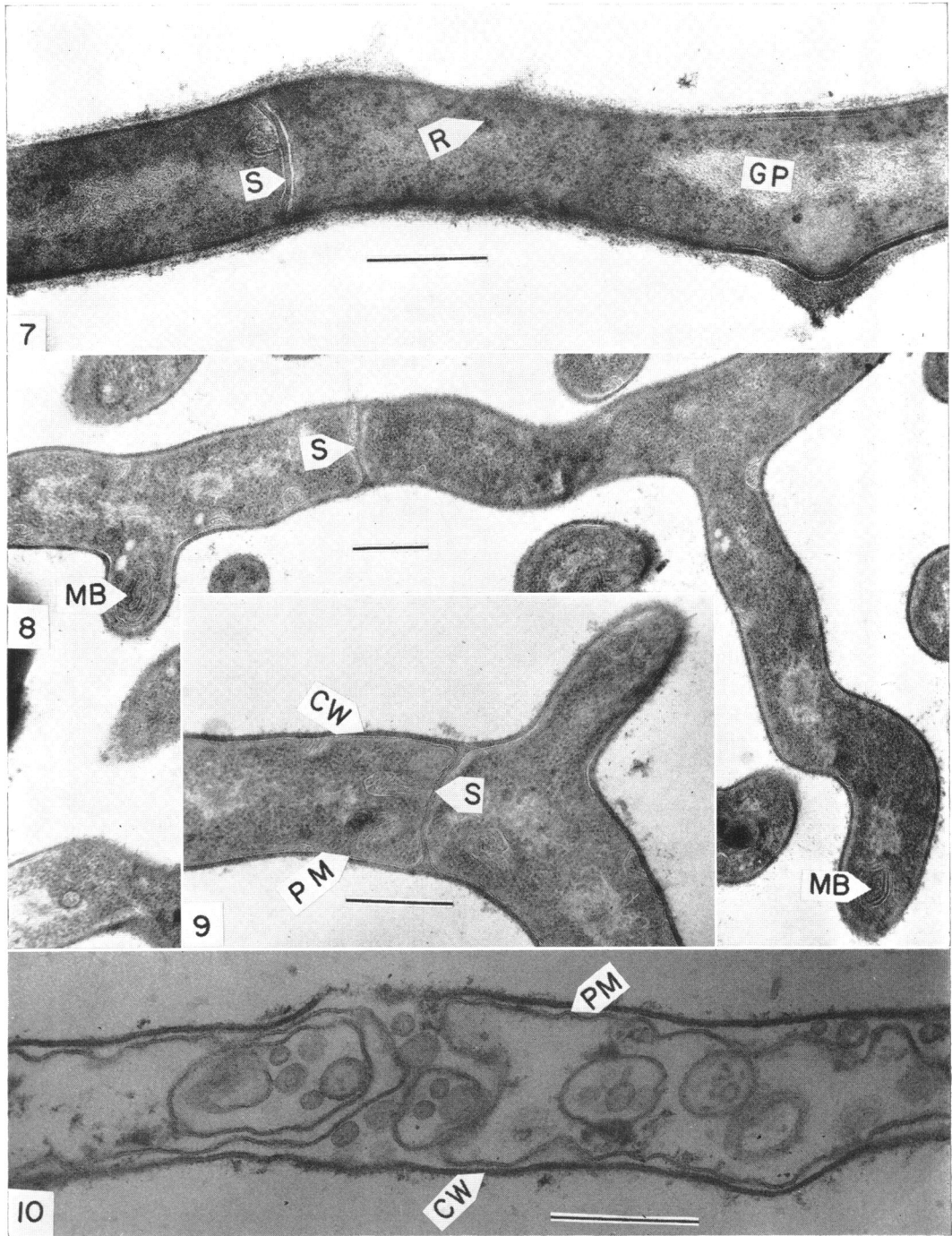


FIG. 7-9. Sections of vegetative hyphae, stained with uranyl acetate.
FIG. 10. Section of a degenerate vegetative hypha, stained with uranyl acetate.

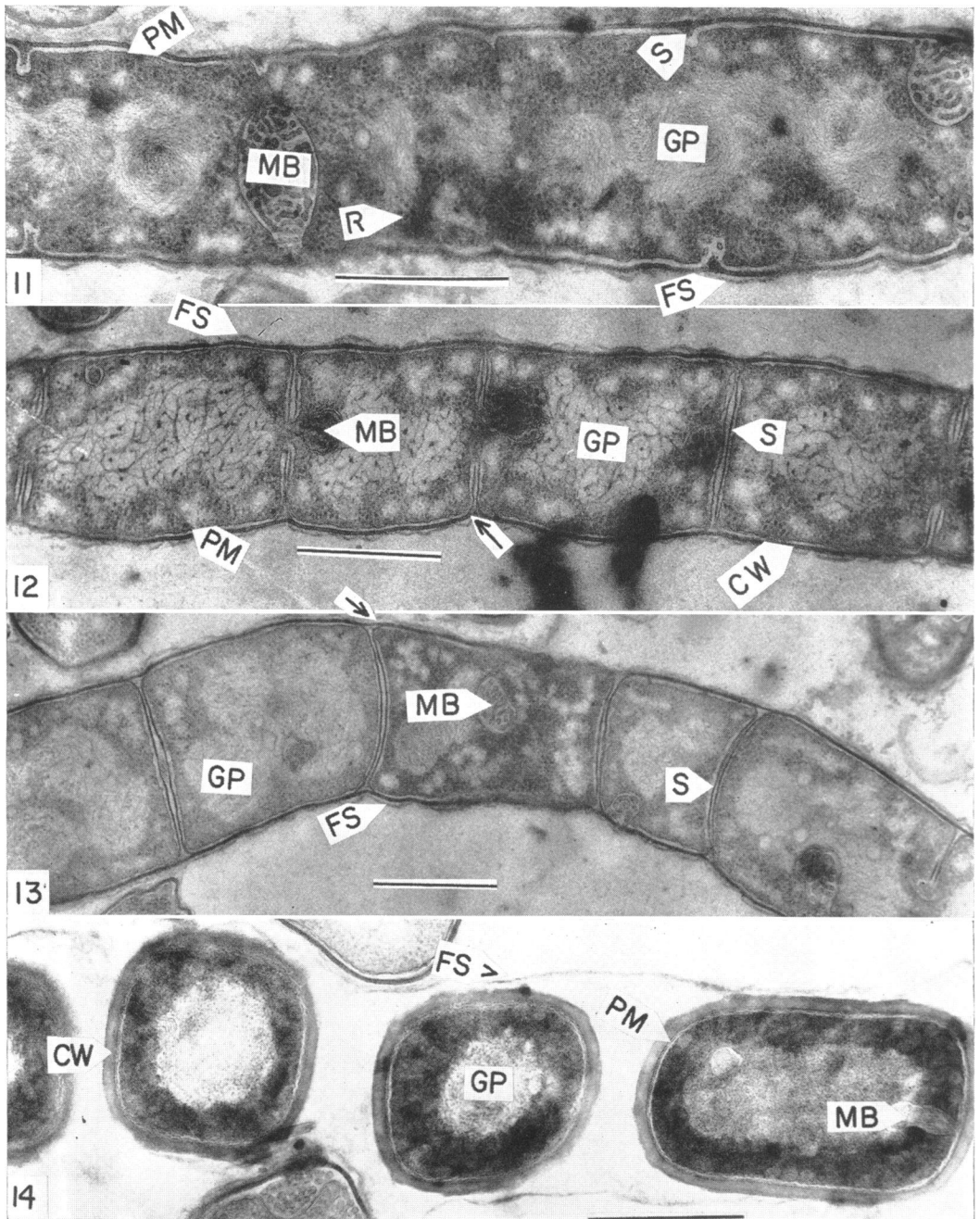


FIG. 11-14. Sections of aerial hyphae stained with uranyl acetate. Arrows indicate the points where the inner layer of the cell wall turns in to form septa.

regions of a hypha behaved as cells, although they were not separated by cross walls.

The aerial mycelium generally resembles the vegetative mycelium except that the former has a

larger diameter and is usually unbranched. The outermost layer of an aerial hypha is a sheath about 5 nm thick; the cell wall per se is about 15 to 20 nm thick. The aerial hyphae contain a large

number of electron-transparent foci (Fig. 11–13) which, in *S. violaceoruber*, were interpreted as storage vacuoles by Glauert and Hopwood (10). The first step in sporogenesis is the simultaneous formation of many septa that eventually divide the aerial hyphae into compartments, each of which becomes a spore (Fig. 11). Initially, the plasma membrane invaginates at regular intervals to form annular folds. At these sites, the inner layer of the hyphal wall breaks around its entire circumference; the free edges turn inward (Fig. 11, 12). Both margins of the in-turned, delicate cell wall layer are extended centripetally at the same rate. Each advancing cross wall is only 2.5 nm thick at first (Fig. 12). It is significant that the cross walls of developing contiguous spores are separate entities at all stages (Fig. 12, 13). After the cross wall is completed, the outer and inner layers of the hyphal wall separate laterally as the spore rounds up (Fig. 13). Ultimately, the outer layer of the hyphal wall ruptures leaving a belt of this material firmly attached to each developing spore surface (Figs. 3, 14). The inner layer, which now constitutes the spore wall per se, thickens to about 30 nm. The mature spores are held in the chains typical of streptomycetes by a thin outer sheath (Fig. 14). It is significant that the outer sheath, also observed by Vernon (23), Enghusen (7), and Baldacci, Gilardi, and Amici (1), is distinct from the outer layer of the cell wall of the aerial hyphae, in contrast to the conclusion reached by Glauert and Hopwood (10) for *S. violaceoruber* (Fig. 13, 14). The origin and composition of the sheath, however, is still unclear. The outer sheath is probably not the continuation of the parental cell wall of the vegetative hyphae as proposed by Hagedorn (11) for *S. griseus*. The little rods on the spore surface (Fig. 1) apparently are an integral component of this superficial fibrous layer.

The total proteins of vegetative hyphae and spores were extracted and their amino acid compositions determined. The spore protein preparation contained cell wall material, as indicated by the presence of diaminopimelic acid in the hydrolysate. In general, the spore protein and protein from the vegetative mycelium had the same amino acid composition; two exceptions were noted. Both the arginine and leucine contents of spore protein were less than that of protein from the vegetative mycelium (Table 1). In addition, Tewfik and Bradley (21) observed that the deoxyribonucleic acid (DNA) from *S. venezuelae* spores displays a buoyant density appreciably less than that of the mycelial DNA. The cause for the lightness of the spore DNA has not been established, but it is presumably attributable to a change in secondary structure or to binding with

TABLE 1. *Amino acid and amino sugar composition of Streptomyces venezuelae*

Amino acids and amino sugars	Amt of amino acids or amino sugar/mg of total cell protein from		
	Cell wall	Vegetative mycelium	Spores
	μmoles	μmoles	μmoles
Alanine.....	0.53	1.26	1.18
Arginine.....	— ^a	0.44	0.28
Aspartate ^b	—	0.66	0.68
Cystine (1/2).....	—	0.16	0.09
Diaminopimelic acid.....	0.43	—	0.16
Glutamate ^b	0.33	1.03	0.93
Glycine.....	0.36	0.91	0.96
Histidine.....	—	0.12	0.11
Isoleucine.....	—	0.32	0.28
Leucine.....	—	0.70	0.55
Lysine.....	—	0.28	0.21
Methionine.....	—	0.16	0.09
Phenylalanine.....	—	0.29	0.31
Proline.....	—	0.31	0.31
Serine.....	—	0.31	0.28
Threonine.....	—	0.41	0.35
Tyrosine.....	—	0.20	0.17
Valine.....	—	0.59	0.65
Glucosamine.....	0.27	—	0.10
Muramic acid.....	0.25	—	0.09

^a None detected.

^b Includes the respective amine.

some other cellular component. The cell walls from vegetative hyphae were purified and analyzed. The mucopeptide contained, in addition to glucosamine and muramic acid, alanine, diaminopimelic acid, glutamate, and glycine. These results agree with an earlier report (2).

The fine structure and cell wall composition of the streptomycetes are similar to those of *Bacillus megaterium* and other gram-positive bacteria (22). Although membranous bodies are extensive in this group of microbes, it has not been possible to correlate definitively specific physiological functions with particular cytological configurations. The membranous bodies at the hyphal apices and in germinating spores (Fig. 4, 8) are, generally speaking, convoluted, whereas those associated with septa are vesicular (Fig. 7, 9, 11, 12). The membranous body associated with the germ plasma seems to be made of concentric membranes (Fig. 3, 13). Conclusive correlations between fine structure and metabolic activity await the results of concurrent physiological, histochemical, biochemical, and cytological investigations.

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