THE FINE STRUCTURE OF *STREPTOMYCES VIOLACEORUBER* (*S. COELICOLOR*)

III. The Walls of the Mycelium and Spores

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ABSTRACT

A study of thin sections of hyphae of *Streptomyces violaceoruber* in the electron microscope showed that the structure of the walls and the mode of formation of cross-walls are similar to those of Gram-positive bacteria. A beaded structure was seen in some regions of the wall, and the significance of this observation is discussed in relation to previous studies of the fine structure of bacterial cell walls. Elements of the intracytoplasmic membrane system appear to be involved in the process of cross-wall formation. The walls of the hyphae of the aerial mycelium divide into two layers before the spores are formed, and only the inner component of the wall grows inwards to form the cross-walls and so delimit the spores. The outer component remains intact for a time and acts as a sheath around the developing spores. Finally the sheath breaks and the spores are liberated. This process is contrasted with the formation of endospores in eubacteria. When the spores germinate, the walls of the germ tubes are continuous with those of the spores.

INTRODUCTION

This paper, which is one of a series on the fine structure of the actinomycete *Streptomyces violaceoruber*,[1] as seen in electron micrographs of thin sections (Glauert and Hopwood, 1959, 1960; Hopwood and Glauert, 1960), describes observations on the structure of the walls of the mycelium and spores, the mode of formation of cross-walls, and the delimitation and germination of the spores. These findings have helped to resolve some of the earlier controversies on the nature and mode of formation of the spores of streptomycetes.

Additional information on the surface layers of the organism, obtained by electron microscopy of carbon replicas and of isolated wall preparations, will be described in a later paper (Hopwood and Glauert, 1961).

[1] This organism was previously referred to as *Streptomyces coelicolor*, but it has recently been shown to have the characteristics of *S. violaceoruber* (Waksman and Curtis) Waksman as redefined by Kutzner and Waksman (1959).

MATERIALS AND METHODS

Whole colonies of *Streptomyces violaceoruber* were fixed and embedded by the methods described by Glauert and Hopwood (1960). To obtain stages in the germination of the spores, a spore suspension was first prepared as described by Glauert and Hopwood (1960). The suspension was centrifuged at 1200 *g* for 10 minutes to form a pellet which was resuspended in a small volume of molten agar medium (asparagine, 0.05 per cent; K_2HPO_4, 0.05 per cent; glucose, 1 per cent; agar, 1.5 per cent) kept fluid at 45°C. The medium was poured into a watch glass, allowed to solidify, and then incubated at 30°C. After various periods of incubation, cubes of about 1 mm^3 were cut out of the agar, fixed, dehydrated, and embedded. Details of the preparation, staining, and examination of the thin sections in the electron microscope have

already been described (Glauert and Hopwood, 1960).

RESULTS

I. *The Walls of the Hyphae of the Substrate Mycelium*

1. *The Structure of the Cell Wall:* In thin sections, the walls of the hyphae of the substrate mycelium are clearly visible as a dense layer bounding the cytoplasm (Fig. 1, *W*). There is an irregular coating of material (Fig. 1, *M*) on the outer surface of the wall, similar to that observed by Ryter and Kellenberger (1958) and by Glauert, Brieger, and Allen (1961) in sections of *Bacillus subtilis;* this probably consists of adhering particles of the growth medium. There is no morphological evidence for the existence of a capsule or slime layer in streptomycetes (Waksman, 1950), although Pfennig (1958) gave some chemical evidence for the presence of capsular material in a strain of *Streptomyces.*

The hyphal wall of *Streptomyces violaceoruber* has a total thickness of 15 to 20 mμ and consists of two dense layers, each about 5 mμ thick, separated by a less dense region (Fig. 2). In the thinner sections the two dense layers of the wall are not uniformly dense; in places there are rapid fluctuations in density and thickness along the length of the wall, while elsewhere the dense layers are regularly beaded (Fig. 2, arrow). These different appearances probably reflect differences in the plane of sectioning and in the thickness of the section. The beads have a diameter of about 5 mμ and are about 10 mμ apart, centre to centre, along the length of the wall. The beads in the outer and inner dense layers are often opposite one another, and their centres also are about 10 mμ apart.

2. *The Formation of Cross-Walls:* The cross-walls that are discernible in the light microscope after appropriate staining (Klieneberger-Nobel, 1947) are clearly seen in thin sections. Stages in their formation are observed much less often than in thin sections of eubacteria, possibly because most of the hyphae in a colony of *Streptomyces* are mature and have ceased to form cross-walls; only at the margin of the growing colony would the hyphae be forming many new cross-walls. A very early stage in cross-wall formation is seen in Fig. 3. A triangular ingrowth of wall material extends towards the centre of the hypha; presumably an annular ingrowth has been formed around the periphery of the hypha. The plasma membrane (*PM*), which underlies the hyphal wall, extends inwards around this ingrowth of wall material and on one side of the hypha is continuous with a large body (*CM*) which is part of the intracytoplasmic membrane system (Glauert and Hopwood, 1960). Sometimes a cavity is observed within the tri-

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FIGURES 1 AND 2

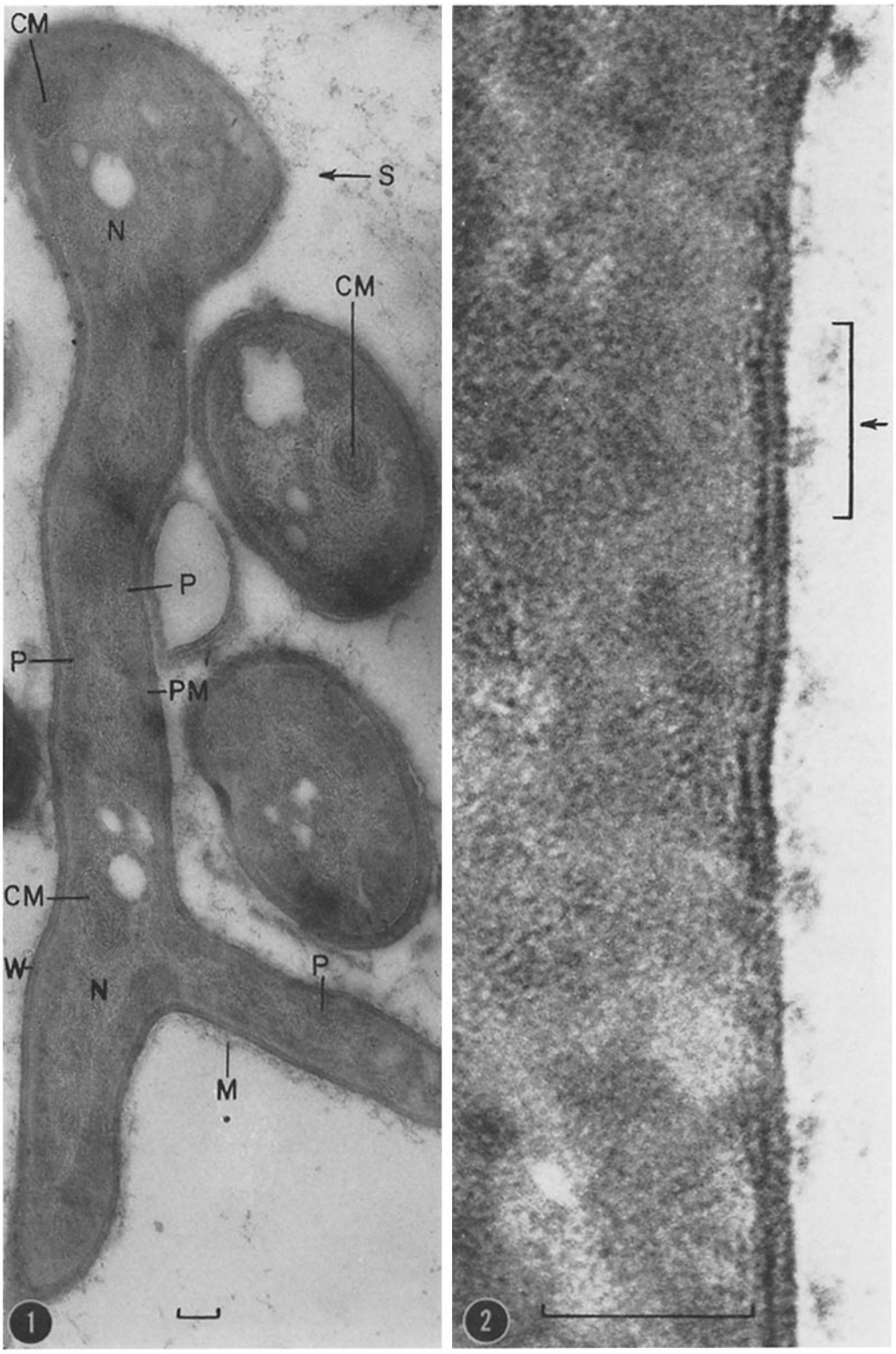
Electron micrographs of thin sections of *Streptomyces violaceoruber*. The scale marks represent 0.1 micron.

FIGURE 1

A longitudinal section through a spore (*S*) and germ tube. After 8 hours' incubation the germ tube has already produced a side branch. The hypha is bounded by the cell wall (*W*), under which lies the plasma membrane (*PM*), which is continuous with peripheral pockets of membranous material (*P*). The fibrillar nuclear material (*N*) extends from the spore into the germ tube, and from the hypha into the side branch. There is an irregular layer of material (*M*) on the outer surface of the wall. Two other spores, without visible germ tubes, are present. Membranous regions, which are part of the intracytoplasmic membrane system (Glauert and Hopwood, 1960), can be seen (*CM*). × 50,000.

FIGURE 2

A longitudinal section of part of a hypha of the substrate mycelium at high magnification. The wall consists of two dense layers separated by a less dense region. The dense layers appear beaded in the region indicated by the arrow. × 320,000.


CM
S
N
CM
P
P
PM
CM
W
N
P
M
1
2

angular ingrowth of wall material and remains visible in some of the completed cross-walls (Fig. 6, *CW*). In Fig. 6 small membranous elements extend from the plasma membranes which bound the completed cross-walls into the neighbouring cytoplasm; probably these extensions connect with elements of the intracytoplasmic membrane system not seen in this plane of section. Sometimes the young cross-wall is initially quite thin at the centre of the hypha (Fig. 4); when completed it is about 20 mμ thick and consists of three dense layers separated by less dense layers (Fig. 5). Normally the wall of the hypha remains intact at the site of the cross-wall so that the two parts of the hypha remain joined; only during the delimitation of the spores in the aerial hyphae is the formation of cross-walls followed immediately by separation.

II. *The Formation of Spores*

1. *The Delimitation of Spores in the Aerial Hyphae:* The walls of the mature aerial hyphae, in which spores will be delimited, differ in structure from those of the substrate hyphae and consist of three dense layers separated by less dense layers (Figs. 7 and 10 *b*), the total thickness of the wall being about 25 mμ; thus the wall appears to have an inner and an outer component. It seems that the the walls of the aerial hyphae do not a first differ in structure from those of the substrate mycelium but that the wall thickens and acquires the extra dense layer prior to sporulation; in Fig. 8 a local thickening of the wall is observed and the extra dense line (*L*) is seen in the thickened region. During the formation of the spores many cross-walls appear simultaneously in the aerial hyphae

FIGURES 3 TO 6

Electron micrographs of thin sections of *Streptomyces violaceoruber*. The sections were stained with uranyl acetate. The scale marks represent 0.1 micron.

FIGURE 3

A germinating spore. At an early stage in cross-wall formation a triangular ingrowth of wall material is seen at the periphery of the hypha. The plasma membrane (*PM*) extends inwards around this ingrowth and is continuous with a large membranous body (*CM*). The thick, double-layered spore wall (*SW*) is continuous with the thinner cell wall (*W*) of the germ tube. The superficial fibrous layer (*F*) has separated from the spore. Vacuoles (*V*) in the nuclear region (*N*) of the spore probably represent the site of storage material. The small dense granules (*R*) in the cytoplasm of the germinating spore are probably ribonucleoprotein particles, and the very dense granule (*D*) a volutin granule. × 83,000.

FIGURE 4

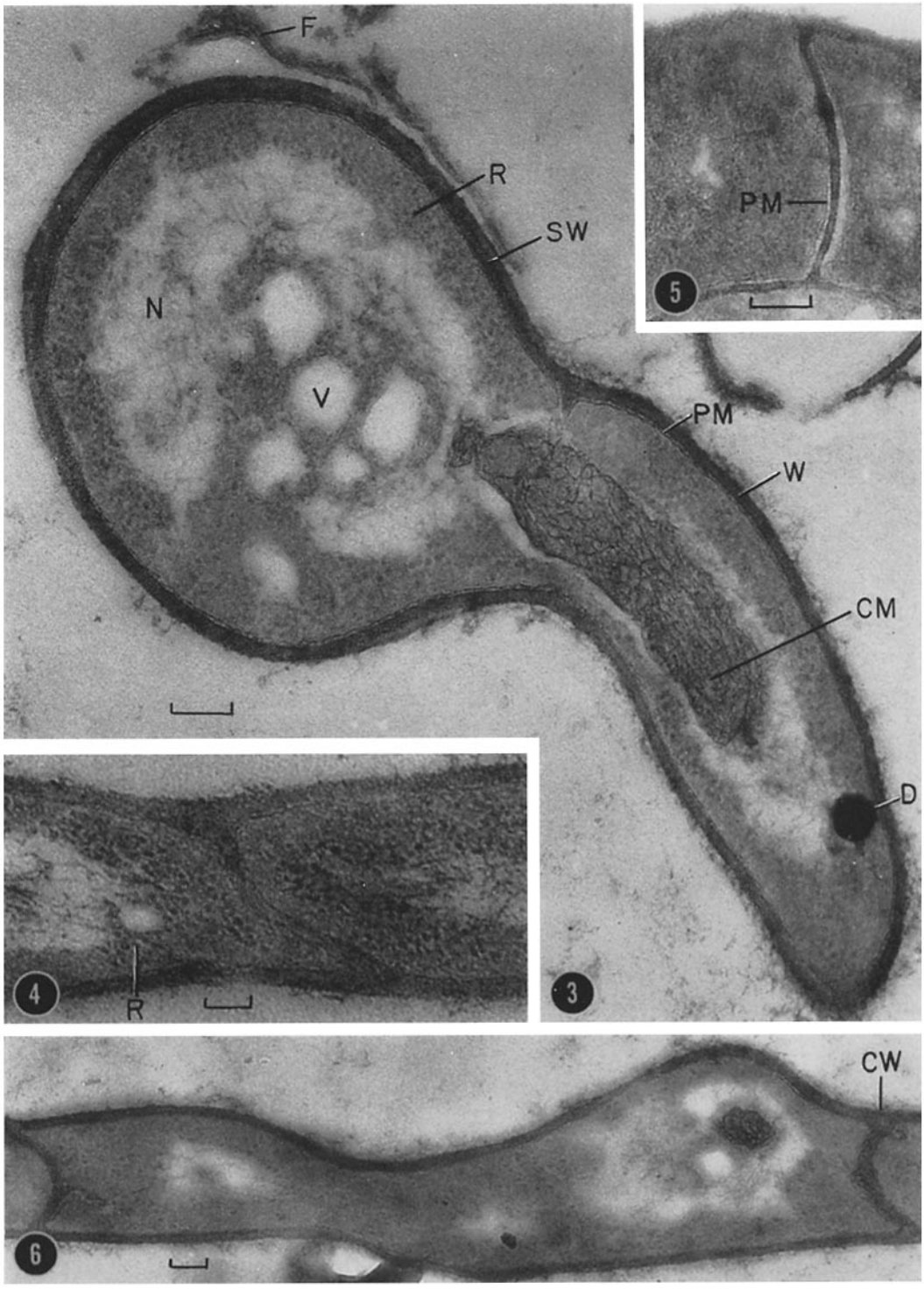
A young cross-wall in a hypha of the substrate mycelium. Many small dense granules (*R*) are visible in the cytoplasm and are probably ribonucleoprotein particles. × 68,000.

FIGURE 5

A completed cross-wall in an older substrate hypha consists of three dense layers separated by less dense layers. The plasma membrane (*PM*) on one side of the cross-wall is continuous with vesicular elements in the cytoplasm. × 85,000.

FIGURE 6

Two cross-walls are visible in a longitudinal section of a young germ tube. Triangular cavities are seen at the junctions of the right-hand cross-wall (*CW*) with the hyphal wall. Small membranous elements extend from the plasma membranes lining the cross-walls into the neighbouring cytoplasm. × 53,000.


F
R
SW
N
V
PM
W
CM
D
5
PM
4
R
3
6
CW

and eventually each compartment becomes a spore. The cross-walls seem to be formed in the same way as those in the hyphae of the substrate mycelium, but only the inner component of the hyphal wall grows inwards to form the cross-wall (Fig. 10 *c*). Subsequently the cross-walls in the aerial hypha become thicker until they are about 30 mμ thick and then they separate into two layers. This separation begins at the junction of the cross-wall with the hyphal wall (Fig. 9) and extends towards the centre of the cross-wall to form the end walls of the young spores. At this stage these new walls of the developing spores appear to be continuous with the inner component alone of the hyphal wall; the outer component at first remains intact across the place at which the spores are separating from one another (Figs. 9 and 10 *c*), but later it ruptures, traces of it persisting as projections on the outside of the developing spores (Figs. 10 *d* and 11).

In thin sections of sporulating aerial hyphae the superficial layer of material, which in electron micrographs of carbon replicas of aerial hyphae and spores (Hopwood and Glauert, 1961) is seen to consist of a "basket-work" of intersecting fibres, is visible as a discontinuous layer (Fig. 11, *F*).

During spore formation and germination the appearance of the plasma membrane changes. Normally it appears as a "unit" membrane, consisting of two dense layers, each 2 to 3 mμ thick, separated by a less dense layer about 3 mμ thick (Fig. 1, *PM*), but in maturing spores (Fig. 9, *PM*) and germ tubes (Fig. 3, *PM*) the outer dense layer is thicker. It is possible that this change in appearance of the plasma membrane indicates a change in functional activity while new wall material is being synthesised.

2. *The Structure of the Spore Wall:* The wall of the mature spores usually seems to consist of two parts (Figs. 10 *e* and 13). The outer layer, which is about 12 mμ thick and probably represents the outer component of the original hyphal wall, is separated from the inner layer by a space, and discontinuities in it are sometimes observed; presumably it does not cover those regions of the spore wall that are formed from the cross-walls in the parent hypha. The inner layer, which alone probably represents the spore wall proper, is about 30 mμ thick and is presumably derived from the thickened inner component of the parent hyphal wall. In some sections this spore wall can be seen to be subdivided into a number of layers (Fig. 13, *SW*).

The superficial fibrous layer is still present outside the spore (Fig. 13, *F*), and the mature spores and aerial hyphae are often surrounded by a matrix of amorphous material of unknown nature (Fig. 12, *M*).

III. *The Germination of the Spores*

During germination the double-layered spore wall appears to be continuous with the wall of

FIGURES 7 TO 9

Electron micrographs of thin sections of *Streptomyces violaceoruber*. The scale marks represent 0.1 micron.

FIGURE 7

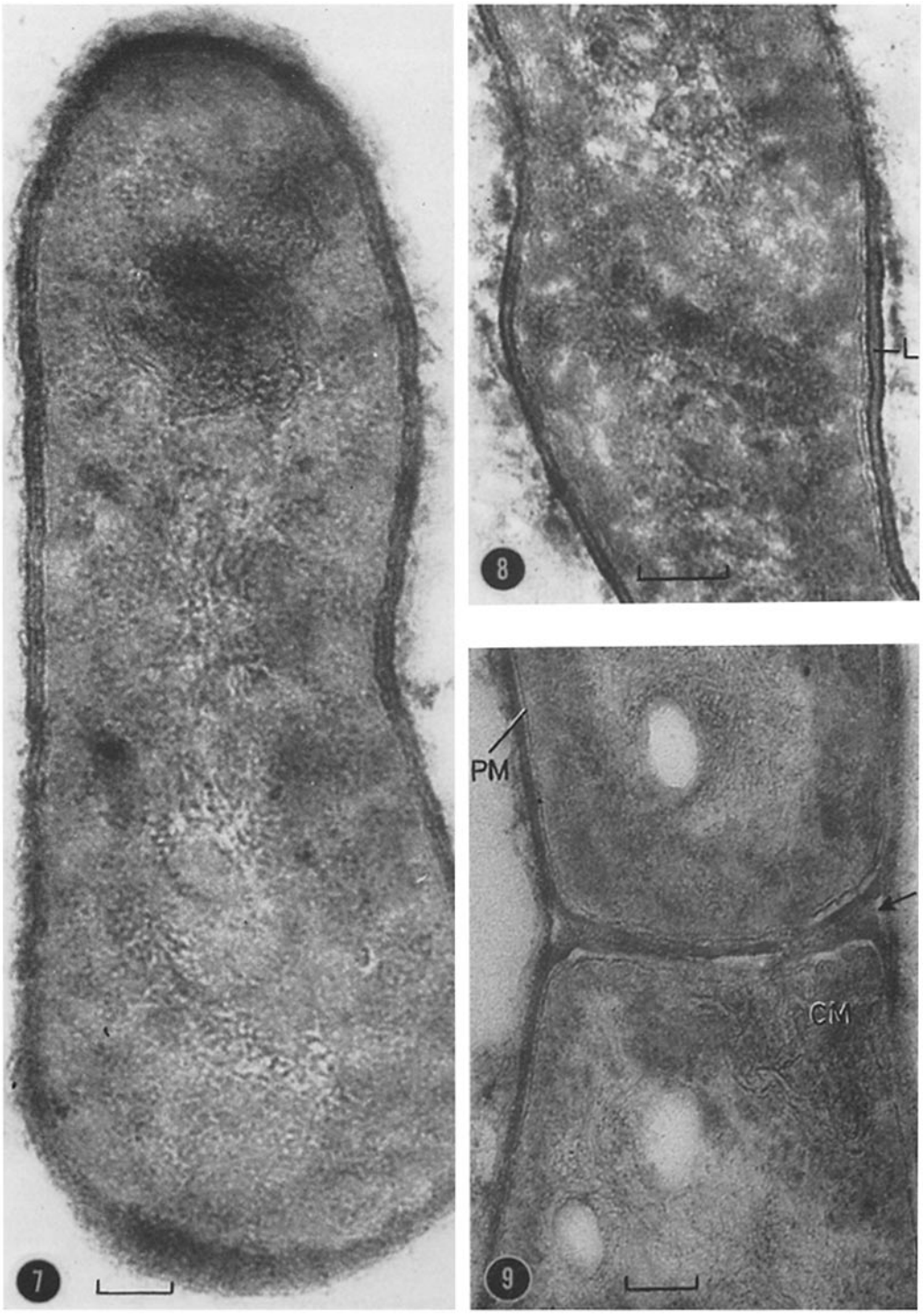
Part of a hypha of the aerial mycelium. The wall consists of three dense layers separated by less dense layers. Section stained with uranyl acetate. × 107,000.

FIGURE 8

Part of a hypha of the aerial mycelium. There is a local thickening of the wall, and an extra dense line (*L*) is visible in the thickened region. × 135,000.

FIGURE 9

A completed cross-wall between two developing spores in an aerial hypha is beginning to separate into two layers at the junction of the cross-wall and the hyphal wall (arrow). The outer layer of the plasma membrane (*PM*) is unusually dense. The cross-wall is associated with a membranous region of the cytoplasm (*CM*) in the lower spore. × 100,000.


7
8
L
PM
CM
9

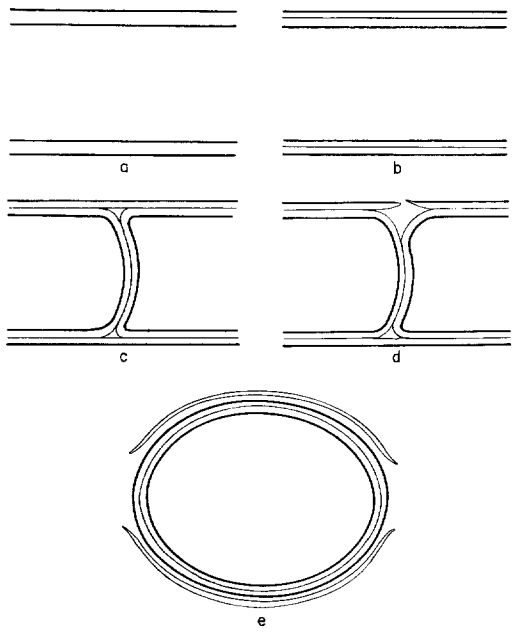


FIGURE 10

Stages in the formation of spores in an aerial hypha of *Streptomyces violaceoruber*. (*a*) Normal hyphal wall. (*b*) Multi-layered hyphal wall. (*c*) Cross-wall formed from inner component of hyphal wall. (*d*) Splitting of outer component of hyphal wall. (*e*) Spore with multi-layered spore wall and remains of outer component of hyphal wall.

the germ tube which grows into the hyphae of the new substrate mycelium (Figs. 1 and 3). The wall of the germ tube adjacent to the spore is thicker than that of a normal substrate hypha, but gradually thins out until the typical dimensions of a substrate hypha are reached some distance from the spore.

The vacuoles that appear in the central regions of the spores during their delimitation in the aerial hyphae (Figs. 11 *V*, and 12, *V*) remain for some time after germination (Fig. 3, *V*); later they become less numerous, and finally disappear. They probably represent the site of granules of some storage material similar to the "granulose" found in the cytoplasm of some anaerobic bacilli during spore formation (Robinow, 1960).

Many small dense granules, about 15 mμ in diameter, are present in the cytoplasm of germinating spores and young substrate hyphae (Figs. 3, *R*, and 4, *R*), in addition to the more finely granular ground substance which is seen in more mature hyphae (Glauert and Hopwood, 1960). These 15 mμ granules are probably ribonucleoprotein particles (Palade, 1955); their distribution in the cytoplasm does not appear to be random, and they frequently occur in groups separated by more finely granular regions (Fig. 3). Larger,

FIGURES 11 TO 13

Electron micrographs of thin sections of *Streptomyces violaceoruber*. The scale marks represent 0.1 micron.

FIGURE 11

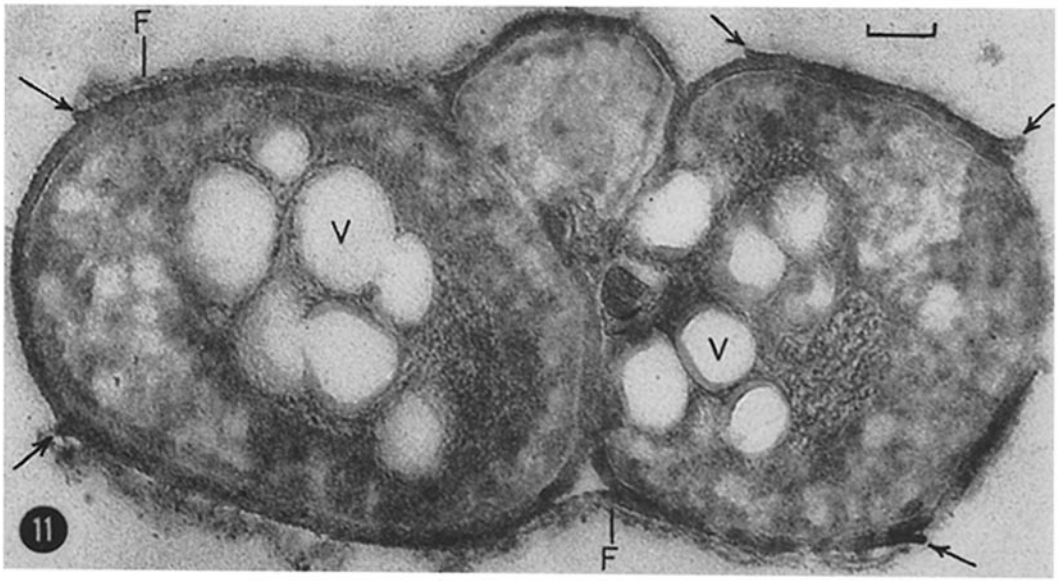
A chain of spores in which the middle spore is imperfectly formed. The broken ends of the outer component of the parent hyphal wall are visible (arrows) as projections on the surface of the spores. The superficial fibrous layer (*F*) is visible, and the vacuoles (*V*) probably represent the site of storage material. × 83,000.

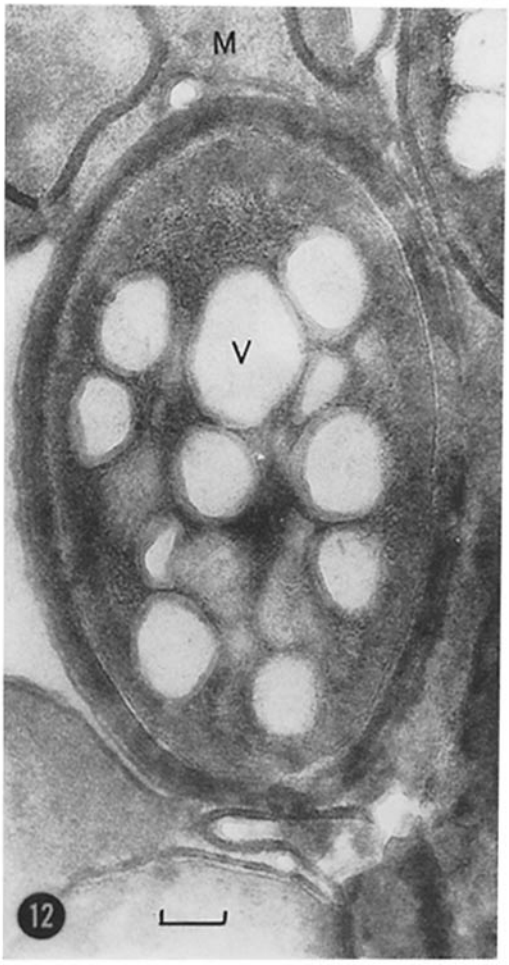
FIGURE 12

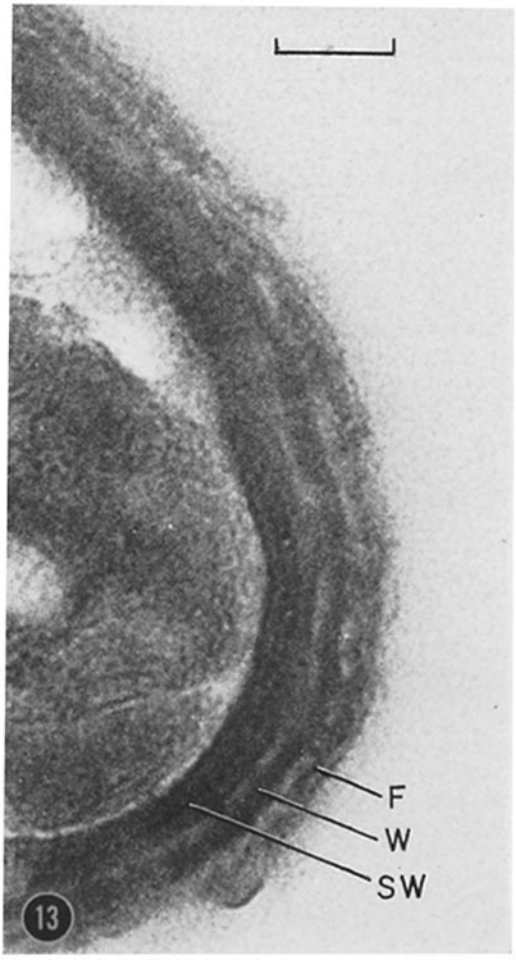
A mature spore has a thick spore wall, and vacuoles (*V*) are present in the nuclear region. The spore is surrounded by a matrix (*M*) of amorphous material of unknown nature. Section stained with uranyl acetate. × 80,000.

FIGURE 13

Part of the wall of a mature spore at high magnification. The spore wall (*SW*) is multilayered and is surrounded by the remains of the parent hyphal wall (*W*) and a superficial layer of fibrous material (*F*). × 150,000.


F
V
V
F
11


M
V
12


F
W
SW
13

very dense granules (Fig. 3, *D*) are seen more frequently in the germ tubes than in the rest of the mycelium and are probably similar to the metachromatic, polyphosphate granules observed in other bacteria (Winkler, 1953; Glauert and Brieger, 1955).

DISCUSSION

The walls of the hyphae of *Streptomyces violaceoruber*, as seen in thin sections, consist of two dense layers separated by a less dense region, with a total thickness of 15 to 20 mμ; the exact thickness of the wall may not be significant, as it appears to differ in different regions of the same hypha. Hagedorn (1960) described a similar structure for the wall of *S. griseus*, but the strains of streptomycetes studied by Stuart (1959) and Petras (1959) appear to have thinner walls only 10 to 12 mμ thick. The wall of the unidentified streptomycete described by Moore and Chapman (1959) appeared uniformly dense, probably as a result of the thickness of the sections. The walls of *S. violaceoruber* have a fine structure similar to that of the walls of Gram-positive bacteria (Chapman and Hillier, 1953; Piekarski and Giesbrecht, 1956; Ryter and Kellenberger, 1958; Glauert, Brieger, and Allen, 1961) and the walls of the two groups also have a similar chemical composition (Cummins and Harris, 1958). The different chemical composition of the walls of Gram-negative bacteria (Salton, 1956) is associated with a different fine structure, the walls appearing only 9 to 12 mμ thick in thin sections Chapman and Kroll, 1957; Kellenberger and Ryter, 1958; Hickman and Frenkel, 1959).

The beaded structure that is sometimes seen in the inner and outer dense layers of the hyphal walls of *Streptomyces violaceoruber* is remarkable since no similar structures have previously been described in thin sections of bacterial cell walls. Since no corresponding structure is observed on the surfaces of the walls in electron micrographs of carbon replicas or in wall preparations examined by the negative staining technique (Hopwood and Glauert, 1961), it seems likely that the beaded elements are associated with some other material so that they are not seen in a surface view. Alternatively the units may be hemispherical with flat outer surfaces, as suggested by Houwink (1953) for a *Spirillum*. It remains to be seen whether these units can be made visible by suitable treatment of the wall. It is interesting that the beads have approximately the same size and arrangement as the spherical or hemispherical units observed by Labaw and Mosely (1954) in the wall of an unidentified Gram-positive bacterium. In this organism also the outside of the wall showed only a faint suggestion of periodicity, the units being clearly visible on the inside.

The cross-walls in the substrate hyphae of *Streptomyces violaceoruber* appear to form in a way similar to that of the cross-walls of the Gram-positive bacterium *Bacillus subtilis* (Glauert, Brieger, and Allen, 1961), in which invagination of the plasma membrane is followed closely by ingrowth of wall material. In *B. subtilis* the invagination of the plasma membrane is preceded by the development of an annular membranous "peripheral body" which remains attached to the plasma membrane throughout the division process and is still seen attached to the sides of completed cross-walls. There is no direct evidence for the formation of a peripheral body in *S. violaceoruber* although some of the many pockets of membranous material at the periphery of young hyphae (Fig. 1, *P*) may represent similar structures. The plasma membrane of the ingrowing cross-wall in Fig. 3 is continuous with a large membranous body, and frequently the plasma membrane which lines completed cross-walls has connections with neighbouring elements of the intracytoplasmic membrane system (Figs. 5 and 6; Glauert and Hopwood, 1960). These observations suggest that some elements of the membrane system are involved in the process of cross-wall formation, and that they may be sites of synthesis of some of the components of the cell wall. Moore and Chapman (1959) showed one micrograph of an incomplete cross-wall in an unidentified streptomycete and concluded that peripheral bodies were absent. Elements of the intracytoplasmic membrane system were observed near the cross-walls of this organism but were described by the authors as "superfluous membranes."

The formation and separation of the spores in the aerial hyphae of *Streptomyces violaceoruber* have many features that have not been encountered before in thin sections of bacteria, although there are some similarities with the process of cross-wall formation and separation of the daughter cells of bacilli. Previous studies of unsectioned hyphae with the electron microscope (Enghusen, 1955; Vernon, 1955; Baldacci, Gilardi, and Amici, 1956)

showed that the spores of streptomycetes are formed within a sheath, but the origin, nature, and fate of this sheath were not clear. The thin sections of *S. violaceoruber* show that the sheath is the outer component of the thickened hyphal wall, which remains continuous while the cross-walls are formed by the inner component alone (Fig. 10). The sheath remains intact while the spores are delimited (Moore and Chapman, 1959) and is still attached to the spores when they finally separate from one another, the broken ends of the sheath being visible as projections on the outsides of the spores. The spore wall proper, which develops from the inner component of the hyphal wall, now thickens, and the sheath is still visible in some sections, but presumably only covers part of the spore. During germination the sheath is finally lost, together with the superficial fibrous layer (Hopwood and Glauert, 1961).

The structure of the spore walls of *S. griseus* was interpreted differently by Hagedorn (1960), who considered that the outermost layer, regarded by us as a superficial fibrous layer, was the parent hyphal wall and that the underlying structures represented a multilayered spore wall. He concluded that the spores were normally produced as endospores in a way similar to that of endospores of eubacteria, by the formation of an entirely new wall within the cytoplasm of the parent hypha. It is well known, however, that the spores of streptomycetes differ in many ways from the endospores of bacilli; they are formed in chains, instead of singly within a vegetative cell, they possess readily stainable nuclear regions, and they are only mildly resistant to heat (Waksman, 1950). These differences are reflected in differences in fine structure. A spore of *S. violaceoruber* can be considered to be merely a segment of an aerial hypha, surrounded by a thickened hyphal wall; it lacks the multilayered spore coats and thick "cortex" which are characteristic of eubacterial endospores (Robinow, 1953).

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