Ultrastructural Studies on Sporulation in Wild-type and White Colony Mutants of *Streptomyces coelicolor*

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**SUMMARY**

Examination of thin sections of sporulating wild-type colonies revealed new structural details of the development of the sporulation-septum walls. Spores with very thick (about 75 nm) three-layered walls were seen in spore preparations.

Of the sporulation defective (whi) mutants examined, *whiD16* was defective in spore-wall thickening while *whiF99* was defective in rounding up and produced rod-shaped, thick-walled spores. A third mutant (*whi-92*) showed occasional abnormality in sporulation-septum spacing and produced immature as well as mature spores. One mutant (*whi-53*) produced only a few spores, all structurally normal. In two *whiE* mutants, structural abnormalities in spores were absent or rare.

**INTRODUCTION**

Fine-structural changes in the aerial mycelia during sporulation in wild-type *Streptomyces coelicolor* A3(2) were studied by Wildermuth & Hopwood (1970) who also summarized the results of earlier work. Mutants with defective sporulation were isolated on the basis of their white aerial mycelia which contrasted with the normal grey (Hopwood, Wildermuth & Palmer, 1970). Three of these white (whi) mutants were mapped and their phenotypes characterized by electron microscopy. They either failed to produce sporulation septa, the specialized cross-walls which divide the aerial hyphae into segments at the onset of sporulation, or produced abnormally spaced septa. A much larger number of white mutants was then classified phenotypically; examination by phase-contrast microscopy showed the existence of six distinct phenotypic classes and genetic mapping identified nine loci (Chater, 1972).

In the present study attention was concentrated mainly on mutants of phenotypic classes V and VI, which had not been examined previously in the electron microscope. Class V mutants produced rod-shaped spores somewhat longer and narrower than wild-type spores, while most mutants in class VI produced spores which looked normal under phase-contrast (Chater, 1972). Both classes of mutant could clearly form sporulation septa but there might be abnormalities in the later stages of spore formation. Some of these mutants were therefore examined in the electron microscope. The wild type was examined at the same time; in general the results of Wildermuth & Hopwood (1970) were confirmed and some new details of sporulation-septum formation and spore maturation were discovered.

**METHODS**

*Organisms and medium. Streptomyces coelicolor* (S. violaceoruber sensu Kutzner & Waksman, 1959) wild-type strain A3(2) and whi mutants (Chater, 1972) were grown at 30 °C on the minimal medium described by Hopwood (1967).

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Electron microscopy. Whole colonies were fixed after 5 to 8 days of incubation. Spore preparations were made by harvesting the aerial mycelia from slopes into distilled water after 7 days of incubation, as described by Hopwood (1967). A modification of the method of Wildermuth (1970), as described by McVittie, Wildermuth & Hopwood (1972), was used for fixation and embedding. Sections were cut with a diamond knife and stained with lead citrate. Silhouettes (impression preparations) were made as described by Wildermuth (1970) and examined without staining.

RESULTS

Wild type whole colonies

Sporulation in Streptomyces coelicolor was classified into a number of stages by Wildermuth & Hopwood (1970). The stages described below correspond to stage 2 (formation of sporulation septa and constriction of the nucleoplasm), stage 3 (thickening of the spore wall and beginning of spore separation), and stage 4 (mature spores).

Sporulation-septum formation began with evenly spaced wedge-shaped annular ingrowths of wall material, the plasma membrane extending inward at the same time to surround each wedge (Fig. 1a). Since Fig. 1(a) represents the only instance in which this early stage was seen, it is probably of very short duration; Wildermuth & Hopwood (1970) did not observe any example of such an early stage. The new wall material in the wedge was less electron dense than the hyphal wall and there was no sign of a division down the middle at this stage. Even at the earliest stage small mesosomes appeared to be associated with the ingrowing membrane (Fig. 1a), and larger mesosomes were clearly seen, invariably attached to the plasma membranes lining the septa at later stages (Fig. 1b, d, e).

As the septum extended inwards the originally undifferentiated wedge of wall material (Fig. 1a) appeared double, particularly at the inner edge (Fig. 1d, e, and Wildermuth & Hopwood, 1970). Later, narrow flanges of uniformly high electron density grew inwards from each half of the wedge with a gap between them (Fig. 1b). Eventually the flanges joined centrally, completely partitioning the hypha; the plasma membranes also joined centrally (Fig. 1c). Development of the septal wall involved changes in the appearance of the outer wedge. Fig. 1(c) also shows the development at the outer edge of the septal wall of short curved portions running underneath the hyphal wall along the corners of the developing spores. As shown diagrammatically in Fig. 2, there were three zones of septal wall: an outer corner zone resembling the hyphal wall in electron density and thickness, a thicker and less electron-dense intermediate zone next to this, and the narrow electron-dense zone spanning the centre. Only the intermediate wall zones of adjacent spores were in contact.

After completion of the sporulation septa the hyphal wall between the spores broke down but this did not involve the outermost layer, the delicate hyphal sheath (Hopwood & Glaueart, 1961; Wildermuth, Wehrli & Horne, 1971). This showed in sections as a thin line running continuously along the spore chain except where it had been broken during processing (Fig. 3). Fig. 3(a) shows part of a chain of immature spores. At this stage the corners had rounded off and the walls had thickened to about 20 to 25 nm compared with about 12 to 15 nm at the beginning of septum formation. The central portion of the septal wall now resembled the corner zone in thickness and appearance but the less electron-dense intermediate zone next to it could still be distinguished and here adjacent spores remained in contact.

The spore wall continued to thicken and the more mature spores shown in Fig. 3(b) had
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Fig. 1. Longitudinal sections of wild-type sporulating hyphae. (a) Very early stage of formation of two adjacent sporulation septa showing ingrowths of wall material and a possible mesosome (M). N, nucleoplasm. (b) Intermediate stage of septum formation showing narrow flanges of ingrowing wall (F) and a mesosome (M) attached to the septum membrane. (c) Newly completed septum. The septum wall contains three distinct zones (see Fig. 2). (d) and (e) Partially and newly completed septa. Note the even spacing and synchrony of development. M, mesosomes.
walls about 50 nm thick, with a uniform appearance over the whole spore surface. Adjacent spores were no longer in contact, being held together only by the hyphal sheath. The spores had rounded up and were now oval rather than almost rectangular in section. Internal structural changes in spores at this stage included gradual loss of a distinct nucleoplasm and clearly visible mesosomes. A considerable proportion of the spore interior was now occupied by white patches which, by analogy with similar patches in *Bacillus megaterium* spores (Freer & Levinson, 1967), may have been due to poly-β-hydroxybutyrate granules.

**Spore preparations**

Nearly all the most mature spore chains in colony preparations resembled the one shown in Fig. 3(b). Additional spore types with thicker walls and different internal fine structure were seen in spore preparations, and these probably represented mature spores largely lost from colony preparations during washing. In one of the three preparations examined there appeared to be two distinct types of thick-walled spore but in the other two preparations there was a range of different types of spore morphology; the reason for the difference between preparations is not known. The two morphological types are shown in Fig. 4. The first type was characterized by extensive electron-transparent regions giving the cytoplasm a blotchy appearance; no mesosomes were visible and the walls were about 50 nm thick (Fig. 4a). The second type, presumably representing a later stage of development, had very thick walls (about 75 nm) which appeared to be differentiated into three areas, a narrow outer dense zone, a broad middle light zone and a narrow inner dense zone (Fig. 4b). The fairly dense cytoplasm had a granular appearance and contained small mesosome-like structures at the outer edge but these were very indistinct. In neither type was it possible to distinguish the nucleoplasm from the cytoplasm, or to recognize the plasma membrane. These spores were probably more mature than any seen by Wildermuth & Hopwood (1970).
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Fig. 3. Longitudinal sections of immature (a) and mature (b) spore chains in wild-type colony preparations. H, hyphal sheath.

Fig. 4. Two types of very thick-walled spores seen in spore preparations. The spore walls in (b) show an inner (I), middle (M) and outer (O) layer.
Fig. 5. Spore chains in colony preparations of whi-16; (d) and (e) show abnormal sporulation septa (arrows).

Examination of whi mutants in classes V and VI

whi-16 (locus D). Phase-contrast microscopy indicated the possibility of morphological abnormality in spores of this mutant (Chater, 1972). Electron microscopy revealed defective spore wall thickening, irregularity of spore size (presumably due to irregularity of sporulation-septum spacing) and shape, and considerable lysis of spores. Although the spore walls sometimes became abnormally thickened at the interface between spores (Fig. 5a) the wall thickness over the rest of the surface was only about 20 to 25 nm (Fig. 5a, b, c), i.e. the same thickness as in immature wild-type spores (Fig. 3a). In one colony fixed after 7 days of incubation some spores appeared swollen and were sometimes almost spherical (Fig. 5a). Fig. 5(a) shows part of a very irregular spore chain with spores in varying stages of lysis, including the formation of membrane bounded vesicles (Glauert & Hopwood, 1960).
Ultrastructure of sporulation in *Streptomyces*

Fig. 6. Electron micrographs of impression preparations of sporulating cultures. (a) Wild-type spore chain. (b) *whi-99* spore chain. The spores are longer and thinner than wild type. (c) *whi-92* spore chain. Some spores are longer than wild type (arrows). (d) *whi-53* spore chains tend to be shorter than wild type; some non-sporulating hyphae.
Fig. 7. Thin sections of *whi* mutant colony preparations. (a) and (b) *whi*-99. Long, thick-walled spores. (c) *whi*-92. Long spore next to spore of normal length. (d) *whi*-107. Walls show abnormal thickening (arrows) at interface between adjacent spores. (e) *whi*-73. Portion of aerial hypha showing abnormal wall thickening.
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Some chains, however, consisted of thin-walled spores of uniform size and shape (Fig. 5c). In another colony fixed after 5 days of incubation some spores appeared to have shrunk (Fig. 5b). This variation in shape between the two preparations and the fairly high proportion of lysed spores seen in both probably implies that the thinner walls of whi-16 spores compared with those of the wild-type make them osmotically sensitive. Spore preparations also lacked thick-walled spores.

A second aberration of spore wall development was occasionally seen when spore-sized compartments were partitioned into smaller irregularly shaped units by septation in different planes (Fig. 5d, e). Their formation may have been the result of the initiation of sporulation septa at abnormal sites.

whi-99 (locus F) and whi-79. whi-99 produced rod-shaped spores longer and narrower than those of the wild type, and was defined as a class V mutant (Chater, 1972 and Fig. 6b; compare with the wild type, Fig. 6a). It was suggested that this might be attributable to failure of the spores to round up; alternatively, the sporulation septa might be spaced at greater intervals than in the wild type. Examination of thin sections by electron microscopy showed that the spacing was normal. Chains of spores resembling wild type in every feature other than shape were seen: the spores developed thick walls and rounded off at the corners but remained long relative to their width, confirming that they were defective in rounding up (Fig. 7a, b). Measurement of the lengths of 49 spores in silhouette preparations gave a mean length of 1.38 \( \mu m \) compared with a mean of 1.10 \( \mu m \) for 79 wild type spores. A second feature of whi-99 was that it produced far fewer spores than wild type as judged by the extinction of aerial-mycelia suspensions harvested from slopes both before and after filtering to remove all material other than spores. Silhouettes also showed non-sporulating as well as sporulating hyphae whereas in the wild type only sporulating hyphae were seen.

whi-79, which produced as many spores as wild type, also had a tendency to produce rod-shaped, thick-walled spores. However, during the course of this investigation whi-79 lost the capacity to produce spores under the growth conditions used and it was impossible to measure spore dimensions.

whi-92. From silhouettes it was evident that the incidence of long spores was higher than in wild type. Fig. 6(c) shows four such spores among a total of 12 in the same chain; eight such spores were seen in a total of 86, compared with one in 79 for wild type. The average spore length of 1.07 \( \mu m \) was normal. Fig. 7(c) shows a long spore next to a shorter one; it is clear that in this case the long spore arose from widely spaced septa rather than failure to round up. In whi-92 the nucleoplasm was less electron dense than in wild type. There tended to be fewer of the more mature rounded-up and thick-walled spores than in wild type. Thus the whi-92 phenotype was complex, with both sporulation-septum spacing and spore maturation being partially affected.

whi-53. This mutant was oligosporogenous; few spores could be harvested from spore cultures and a minor proportion of the aerial hyphae formed spores. Fig. 6(d) shows a mixture of sporulating and non-sporulating hyphae in an impression preparation made after 7 days of growth. Some hyphae showed sporulation over only part of their length. In wild type impression preparations, only spores and spore chains were seen and the chains were longer than in whi-53. The spores were morphologically identical to those of wild type, both of the thick-walled types being present, and it would have been reasonable to assume that the white colour of the colonies was the result of the small number of spores rather than of any defect in the sporulation process. However, there must have been a colour difference in the spores themselves since spore pellets of whi-53 were beige in contrast to the grey-brown colour of wild type.
**whi-107 and -124 (both locus E).** These are class VI mutants. The only fine-structural abnormality seen in **whi-107** was the presence in a few spores of thickened ingrowths of the wall at the middle region of the interface between adjacent spores (Fig. 7d); other spores were quite normal. **whi-124** spores appeared entirely normal and both of the thick-walled types were seen. Thus the aerial-mycelium colour difference between **whi-124** and wild type may have been due to an absent or modified pigment or to a structural defect that could not be identified by these studies.

**Examination of whi mutants in classes I and II**

Impression preparations of class I mutants showed long non-helical hyphae while class II mutants showed long tight helices; sporulation septa could not be seen by phase-contrast examination (Chater, 1972).

Examinations by electron microscopy of thin sections of **whi-75** (class I, locus G) and of **whi-70** (class II, locus B) also failed to show any sporulation septa.

A second class II mutant, **whi-73** (locus A), was examined in the electron microscope since under phase-contrast some portions of the aerial hyphae showed the high refractility characteristic of spores. Although this mutant resembled **whi-70** in having no sporulation septa there were some regions of the hyphae with very thick walls (Fig. 7e) which presumably corresponded to the refractile regions seen in the light microscope. The thickening was irregular and in some places was greater than in the normal spore walls. Other class II mutants at locus A did not have these regions of high refractility.

**DISCUSSION**

Although sporulation in the genus *Streptomyces* is not as complex as in the bacilli or in *Thermoactinomyces vulgaris* (Cross, Davies & Walker, 1971; McVittie et al. 1972) it is, nevertheless, accompanied by changes in most of the major structural components of the aerial hypha.

There have recently been electron-microscope studies of sporulation in several streptomycetes which have been reviewed by Williams, Sharples & Bradshaw (1972). The present studies of the wild type *Streptomyces coelicolor* A3(2) have added some details to the description of sporulation-septum formation of Wildermuth & Hopwood (1970) and have confirmed the specialized double inner edge of the growing septum (Fig. 1b). This was not included in the description of ‘type 2’ septum formation by Williams, Sharples & Bradshaw (1972); however, in view of the tenuous nature of the double flange it seems possible that it could have been overlooked, and it is therefore premature to conclude that *S. coelicolor* is unusual amongst streptomycetes in its sporulation septa.

The number of distinct processes occurring during sporulation, as deduced by electron microscopy and genetic evidence (Chater, 1972), suggests that there are rather more genes involved than was at first supposed (Hopwood et al. 1970). Thus even when attention was restricted mainly to mutants producing spore-like units (class V and VI mutants) almost every one of the few mutants examined had a different phenotype. Four of these mutants had been assigned to definite map locations: locus *D* (**whi-16**), locus *E* (**whi-107** and **-124**) and locus *F* (**whi-99**) (Chater, 1972). Although **whi-79** resembled **whi-99** to some extent in having rod-shaped spores, the phenotype was not identical and moreover it has been shown to map close to *strA* some distance from locus *F* (K. F. Chater & D. A. Hopwood, unpublished). **whi-53** and **whi-92** also mapped close to *strA* (K. F. Chater & D. A. Hopwood, unpublished) but differed phenotypically from each other and also from **whi-79**. Thus
these mutants may represent three other loci. Isolation and characterization of further class V and VI mutants would probably reveal additional loci.

The most striking defect in whi-16 is failure of spore walls to reach the wild type thickness of 50 nm or more. It is interesting that wall thickening was not completely blocked but was arrested at a stage corresponding to immature spore chains in wild type. Conceivably this could indicate that the first part of wall thickening involves the deposition of material of the same composition as the pre-sporulation hyphal wall while the further thickening is due to addition of material of a different composition. Locus D is clearly involved in synthesis or incorporation of spore wall material. The varying shape (rounded or shrunken) and tendency to lyse are probably a consequence of the defective spore walls.

A stage V mutant of Bacillus subtilis blocked in the formation of the spore coat, and thus to some extent comparable to whi-16, also lysed readily (Ryter, Schaeffer & Ionesco, 1966). The abnormal septation seen in whi-16 (Fig. 5d, e) also has its counterpart in a B. subtilis mutant. Van Alstyne & Simon (1971) described a temperature-sensitive mutant which, although producing some normal septa, also produced some which were very closely spaced or which grew out from the cell surface at an angle.

The basis for failure to round up in whi-99 and at least some spores of whi-79 is not immediately apparent. Presumably the shape change in wild-type spores as they mature is partly dependent on the way in which the spore wall is laid down and the molecules cross-linked. If the wall became rigid before rounding up had occurred this would probably result in the longer, narrower, thick-walled spores of these mutants. The actual lengths reported here are subject to error owing to shrinkage in the electron microscope but the ratio of the average length of whi-99 spores to wild-type spores agrees closely with the value of Chater (1972) based on phase-contrast observations.

It is not easy to account for the phenotype of whi-92 in which more than one aspect of sporulation was defective but some normal spores were also produced. The same applies to whi-53 in which the defect was in the number of spores produced rather than in the spores themselves.

From examination of these mutants it was apparent that spore wall thickening could sometimes occur in an uncontrolled manner, particularly at the interface between spores as in whi-16 and, less frequently, in whi-107. A class II mutant lacking spores, whi-73, showed occasional patches of wall even thicker than that of normal spores.

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REFERENCES


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