HOPWOOD, D. A. (1960). *J. gen. Microbiol.* 22, 295–302

Phase-Contrast Observations on *Streptomyces coelicolor*

BY D. A. HOPWOOD

Botany School, University of Cambridge

SUMMARY: Observations on living colonies of *Streptomyces coelicolor* strain A3(2) growing on cellophan films were made by phase-contrast microscopy. Particular attention was paid to the mode of origin of the aerial mycelium. It was shown conclusively that aerial hyphae can originate as simple branches of the substrate mycelium, without any previous fusion of hyphae. The pattern of development of the substrate mycelium is markedly influenced by the composition of the medium.

The investigation described here was undertaken primarily to obtain information on the cycle of development of a strain of *Streptomyces coelicolor* in which genetic recombination is being studied (Hopwood, 1957, 1959); recombination is a rare event, but it was hoped nevertheless to observe any specialized structure that might exist at the site of recombination. Klieneberger-Nobel (1947) claimed that the aerial hyphae originate from fusion cells in the normal life-cycle of certain streptomycetes. This might be expected to have considerable genetical consequences, and so in this study attention was focused particularly on the mode of origin of the aerial hyphae. An additional object of this work was to help in the interpretation of the fine structure of the organism as observed in electron micrographs of thin sections (Hopwood & Glauert, 1958; Glauert & Hopwood, 1959; and to be published).

Most descriptions of the cycle of development of members of the genus *Streptomyces* have been based on stained preparations, often of smears or fragments of submerged growth. Although stained smears may give a satisfactory picture of the morphology of eubacteria, they are not ideal for Streptomyces since they give no information on the spatial organization of the colony. As a result, there is considerable confusion in the literature over the details of the developmental cycle. Phase-contrast studies of undisturbed cultures can give information on the organization of the complex colonies of the Actinomycetales that cannot be obtained in other ways. By using such methods, Brieger & Glauert (1952) were able to describe the development of mycelial strains of avian tubercle bacilli, and Erikson (1955) made observations on various streptomycetes, including the strain of *S. coelicolor* studied here.

METHODS

Organism. *Streptomyces coelicolor* strain A3(2) was obtained from Dr Dagny Erikson; this isolate was derived by her (Erikson, 1955) from one of the agar-decomposing strains (Waksman's 3443) studied by Stanier (1942). A nicotinamide-requiring mutant isolated from the survivors of ultra-violet irradiation of this strain was also examined.

Media. Minimal medium (%, w/v): $NaNO_3$, 0·3; KH_2PO_4, 0·1; KCl, 0·05;

$MgSO_4.7H_2O$, 0·05; $FeSO_4.7H_2O$, 0·001; glucose, 4·0; Difco Bacto-agar, 1·5 pH adjusted to 7·2 with N-NaOH. Minimal medium was also used with glucose omitted. The sparse growth of strain A3 (2) on this medium, with agar as carbon source (Erikson, 1955), allows the origin of the aerial mycelium to be observed clearly. Complete medium (% w/v): bacto-peptone, 0·2; yeast extract, 0·1; glucose, 2·5; K_2HPO_4, 0·5; NaCl, 0·05; $MgSO_4.7H_2O$, 0·05; agar, 1·5; (%, v/v, prepared as in Pontecorvo, 1953): nucleic acid hydrolysate, 0·5; casein hydrolysate, 0·5; vitamin solution, 0·1 pH adjusted to 7·2 with N-HCl.

Culture conditions. Ideally, a single growing colony would be observed throughout its development, so that there would be no subjective element in the construction of a developmental cycle. It was found, however, that *Streptomyces coelicolor* did not produce the sporulating aerial mycelium characteristic of the genus under conditions suitable for phase-contrast microscopy. Observations of all except the earliest stages of growth had therefore to be made on different colonies. These were mounted, without disturbance, after different periods of incubation at 30°. Two types of culture were grown. (1) Drops of a spore suspension of the organism were spread on small squares of cellophan placed on the surface of solid medium in Petri dishes. After incubation, the cellophan squares were mounted in water for microscopical examination. (2) A suspension of spores in molten agar medium at 45° was spread thinly on sterile coverslips. These were inverted and mounted on microscope slides, with strips of lens tissue as distance-pieces, the edges of the coverslips being sealed with wax. After incubation, water was admitted to the air-space between coverslip and slide to give good conditions for phase-contrast examination. The cellophan preparations gave the most complete picture of the cycle of development of the organism. Evidence of a developmental sequence was obtained by mounting cultures at intervals during the first 30 hr. of incubation, and by studying colonies in older cultures systematically from the margin towards the centre. Impression preparations, made by pressing coverslips on to the surface of sporing colonies, gave information about the final stages of spore delimitation.

Observations were made with a Cooke, Troughton & Simms phase-contrast microscope, with a ×95 1·3 N.A. oil-immersion objective. Photographs were taken on Kodak Microfile 35 mm. film at a primary magnification of *c.* ×300.

RESULTS

The cycle of development

Development of the substrate mycelium and origin of the aerial hyphae. Soon after inoculation the spores increased in size and germinated, usually by one or two germ tubes (Pl. 1, fig. 1). These developed into the mycelium of the young colony. On minimal medium the colony at first consisted of radially-growing major hyphae (Pl. 1, fig. 2). Side branches arose at intervals along the major hyphae and began to colonize the areas between them, some turning to grow in a radial direction and so increasing the number of major hyphae as the circumference of the colony grew (Pl. 1, fig. 3). Thus the medium was uniformly

exploited by the mycelium. The hyphae varied somewhat in diameter, but all had essentially the same appearance. These substrate hyphae were characterized by a gently undulating course and a marked constriction at their point of origin (Carvajal, 1946). Later, finer, highly convoluted hyphae grew into the spaces between the existing hyphae, forming knotted, nest-like configurations (Pl. 1, fig. 4), somewhat reminiscent of those described by Klieneberger-Nobel (1947). By this time, there was considerable variation in the diameter of the hyphae of the substrate mycelium. Configurations suggestive of hyphal fusion were observed in the nest-like associations of hyphae, but not among the larger substrate hyphae. No conclusive evidence of hyphal fusion was obtained, however.

The substrate mycelium soon became so dense that it was difficult to observe the growth of the aerial hyphae, which appeared as branches of the substrate hyphae. On minimal medium without glucose, the pattern of development was similar to that described above, but colonization of the surface of the medium was even more regular (Pl. 1, fig. 5). There was little or no development of the nest-like configurations of hyphae (Pl. 2, fig. 6). Aerial hyphae appeared when the substrate mycelium was much less dense, so that their origin and development were clearly seen. The first aerial hyphae arose from the radially growing major hyphae of the substrate mycelium, and others later developed from the smaller side-branches. Because of the constriction at the base of the side-branches which grew into aerial hyphae, they at first appeared as small almost spherical structures (Pl. 2, fig. 7). Later they elongated (Pl. 2, fig. 8), usually remaining markedly constricted at the base (Pl. 2, fig. 9), and developed into typical aerial hyphae (Pl. 3, figs. 10, 11). These aerial hyphae had about the same diameter as the largest of the substrate hyphae, but were distinguished from them by being straighter and less highly branched. Large swollen structures (Pl. 3, fig. 12), similar to those described by Gregory (1956), were occasionally seen. They bore the same relationship to the substrate mycelium as did the young aerial branches, and were probably abnormal aerial hyphae.

In coverslip cultures, colonies on minimal medium without glucose produced aerial branches when the substrate mycelium was very slight in extent. The origin of the aerial hyphae as branches of the substrate mycelium was then seen very clearly (Pl. 3, fig. 14).

Production of spores. Stages in the delimitation of spores by the aerial hyphae were rarely seen clearly in cellophan preparations of whole colonies (Pl. 3, fig. 13) since the underlying substrate mycelium caused a deterioration in the quality of the phase-contrast image. Some features of the final stages in the process could be seen in impression preparations (Pl. 3, fig. 15). Further details, revealed by electron microscopy, will be reported elsewhere (Glauert & Hopwood, to be published). Spore delimitation usually proceeded more or less simultaneously along considerable lengths of an aerial hypha, but with a general basipetal trend. Certain regions, however, might lag slightly behind the rest of the hypha. Lengths of the hypha destined to form two or more spores might thus at first be flanked by nearly mature spores (Pl. 3, fig. 15);

this was also observed occasionally by Baldacci, Gilardi & Amici (1956). When such units were transferred to fresh medium before their development was complete, they failed to delimit spores, but germinated by numerous germ tubes, which grew into typical substrate mycelium (Pl. 4, fig. 16). The considerable variation in length and diameter of 'spores' in impression preparations as noted by Erikson (1955) and clearly seen in Pl. 3, fig. 15, is probably due to the fact that units may be sharply delimited from the rest of the hyphae when their further subdivision into spores is still to come. A spore suspension prepared from a mass culture may thus contain a rather heterogeneous collection of viable subunits of the aerial hyphae. This must be borne in mind in considering the results of recombination experiments in which spore suspensions are plated on selective media, since it might allow a colony arising from a single 'spore' to contain a mixture of genotypes (Hopwood, 1959). Impression preparations occasionally showed spores which had germinated *in situ* on the parent colony.

Effect of the composition of the medium on the pattern of development of the mycelium

The effect on the pattern of development of the substrate mycelium of omitting glucose from minimal medium has been mentioned. More extreme effects were seen when the growth of the wild-type organism on complete medium was compared with that of a nicotinamide-requiring mutant on minimal medium without glucose and no nicotinamide added. On complete medium, the growth of the wild-type organism was very vigorous, and the substrate hyphae tended to grow together in rope-like strands, without the uniform colonization of the surface shown on minimal medium (Pl. 4, fig. 17). The diameter of the hyphae was greater and less variable, and the hyphae tended to be straighter. Aerial hyphae arose only after a dense mat of substrate mycelium had been produced, so that their origin could not be studied microscopically. At the other extreme, substrate mycelium of the nicotinamide-requiring mutant growing on limiting medium colonized the surface very sparsely and uniformly, and aerial branches appeared at an early stage (Pl. 4, fig. 18). In the presence of a suitable concentration of nicotinamide, growth of the mutant was identical with that of the wild-type.

DISCUSSION

Studies of genetic recombination in *Streptomyces coelicolor* strain A3 (2) (Hopwood, 1957, 1959) have shown that a mechanism exists whereby large portions of genetic material of two parental types, and probably whole genomes, may become associated. It is probable, although not proven, that heterokaryosis is a preliminary to recombination. By means of experiments involving pairs of biochemically deficient mutants, heterokaryons have been shown to be formed in a number of streptomycetes (Bradley & Lederberg, 1956; Braendle & Szybalski, 1957). Occasional hyphal fusion in the substrate mycelium is the simplest hypothesis to account for the production

of heterokaryons. Hyphal fusion in Streptomyces is clearly less frequent than in most fungi, since many investigations have failed to reveal it (Erikson, 1949). Its cytological demonstration is therefore bound to be difficult. Gregory (1956) has come nearest to presenting convincing cytological evidence of anastomosis in Streptomyces. In his preparations (for example his fig. 3), the hyphae which appear to undergo fusion resemble the highly convoluted hyphae produced by *S. coelicolor* strain A3 (2) on minimal medium (Pl. 1, fig. 4). Fusions may be more frequent between these hyphae than between the larger substrate hyphae, where Erikson (1955) did not observe fusion. Unspecialized fusion of substrate hyphae might thus be a sufficient intermycelial event to account for heterokaryosis and eventually recombination. Recombination might possibly be the result of a more highly organized sequence of events, however. The site of recombination might, for example, be a fusion cell produced at a defined stage in the life cycle. Is there any evidence for such a structure?

Ørskov (1923) was among the first to recognize that colonies of organisms now placed in the genus *Streptomyces*, when growing on solid media, consist of two types of hyphae. He found the aerial hyphae to arise as simple branches of the substrate mycelium. Many later workers (Badian, 1936; Erikson, 1947, 1955; Giolitti, Graveri & Corti, 1955; Gregory, 1956) also found that the aerial hyphae originate in this way in a large number of streptomyces. Klieneberger-Nobel (1947), from an examination of stained preparations, was the first to suggest that the aerial mycelium arises by anything more complicated than simple branching from the substrate mycelium. She postulated an 'initial cell' as a discontinuity in the life cycle between substrate and aerial mycelia. It was thought to arise at a point of fusion of elements of the substrate mycelium, to survive as a discrete structure when the latter degenerated, and to germinate to produce the aerial mycelium. There was thus an alternation of two types of mycelium, separated in time by two isolated stages: initial cells and spores. In later papers (McGregor, 1954; Wilkin & Rhodes, 1955; Dickenson & MacDonald, 1955) illustrations were given of swollen bodies in intercalary or terminal positions in the substrate hyphae, and they were referred to as initial cells, although they did not correspond to Klieneberger-Nobel's description. As Gregory (1956) pointed out, the 'initial cells' of these authors are probably germinated spores, still attached to the substrate hyphae which they have produced. McGregor stated them to be about four times the diameter of spores, but his photographs show them to be of the same size and appearance as *germinating* spores.

Erikson (1949, 1955) criticized Klieneberger-Nobel's conclusions on the grounds that the tangled nests of hyphae in which initial cells were supposed to arise at points of fusion were a product of the cultural conditions used. In any case, there appear to be no objective reasons for Klieneberger-Nobel's interpretation of those of her photographs which are claimed to show initial cells. There is no apparent difference between the rows of rounded elements of her figs. 18 and 30, which are interpreted as initial cells and spores, respectively. Moreover, because of the marked constriction at the point of origin of aerial

branches, young aerial hyphae may appear as more or less discrete spherical structures (Pl. 2, fig. 7) until they begin to elongate, and in stained preparations might be mistaken for structures isolated from the parent hyphae. Aerial branches are often sharply curved as they begin to elongate (Pl. 2, fig. 8), and the tip may appear to make contact with the parent hypha. Thus an alternative interpretation of the loops in Klieneberger-Nobel's fig. 13 is possible, without assuming that a secondary hypha is attached at both ends to the substrate hypha from which it arose. Thus the existence of initial cells is not well authenticated even in the four strains studied by Klieneberger-Nobel, while, on the contrary, aerial hyphae have been observed to arise as simple branches of the substrate mycelium in many strains. The present investigation shows that this can occur in *Streptomyces coelicolor* strain A3 (2), confirming the observations of Erikson (1955). The production of a fusion cell is not a normal part of the cycle of development. There is, moreover, no evidence for the existence of a specialized structure of this type at the site of recombination.

The effects of the composition of the medium on the pattern of development of the mycelium illustrate the well-known plasticity of members of the genus *Streptomyces* and other actinomycetes in response to varied growth conditions (see, for example, Erikson, 1949, 1955). It is probable that the nutrient status of the medium ultimately controls the pattern of development of the substrate hyphae on a cellophan surface. When a growth factor is in short supply, as when a biochemically deficient mutant grows at the expense of limiting amounts of growth factor added with an inoculum, the first hyphae in some way influence the direction and extent of growth of the branches which arise later. These grow into the largest unoccupied spaces on the substrate surface (Pl. 4, fig. 18). It is unknown at present whether the controlling factor is mere exhaustion of nutrients by the first-formed hyphae, or whether a positive inhibition by them of later-formed branches occurs on a limiting medium.

It is a pleasure to thank Miss Audrey M. Glauert for much helpful advice and criticism. I am grateful to Dr Dagny Erikson for supplying me with the organism.

REFERENCES

Badian, J. (1936). Über die zytologische Struktur und den Entwicklungszyklus der Actinomyceten. *Acta Soc. Bot. Polon.* 13, 105.

Baldacci, E., Gilardi, E. & Amici, A. M. (1956). Il ciclo di vita degli attinomiceti osservato al microscopio elettronico. *Giorn. Microbiol.* 1, 512.

Bradley, S. G. & Lederberg, J. (1956). Heterokaryosis in *Streptomyces*. *J. Bact.* 72, 219.

Braendle, D. H. & Szybalski, W. (1957). Genetic interaction among Streptomycetes: heterokaryosis and synkaryosis. *Proc. nat. Acad. Sci., Wash.* 43, 947.

Brieger, E. M. & Glauert, A. M. (1952). A phase-contrast study of reproduction in mycelial strains of avian tubercle bacilli. *J. gen. Microbiol.* 7, 287.

Carvajal, F. (1946). Studies on the structure of *Streptomyces griseus*. *Mycologia*, 38, 587.

Dickenson, P. B. & MacDonald, K. D. (1955). An electron microscope examination of the initial cell stage in *Streptomyces* spp. *J. gen. Microbiol.* 13, 84.

ERIKSON, D. (1947). Differentiation of the vegetative and sporogenous phases of the actinomycetes. II. Factors affecting the development of the aerial mycelium. *J. gen. Microbiol.* **1**, 45.

ERIKSON, D. (1949). The morphology, cytology, and taxonomy of the actinomycetes. *Annu. Rev. Microbiol.* **3**, 23.

ERIKSON, D. (1955). Loss of aerial mycelium and other changes in streptomycete development due to physical variations of cultural conditions. *J. gen. Microbiol.* **13**, 136.

GIOLITTI, G., CRAVERI, R. & CORTI, G. (1955). Ricerche su alcuni aspetti morfologici di Attinomiceti. *Giorn. Microbiol.* **1**, 162.

GLAUERT, A. M. & HOPWOOD, D. A. (1959). A membranous component of the cytoplasm in *Streptomyces coelicolor*. *J. Biochem. Biophys. Cytol.* **6**. (In the Press.)

GREGORY, K. F. (1956). Hyphal anastamosis and cytological aspects of *Streptomyces scabies*. *Canad. J. Microbiol.* **2**, 649.

HOPWOOD, D. A. (1957). Genetic recombination in *Streptomyces coelicolor*. *J. gen. Microbiol.* **16**, ii.

HOPWOOD, D. A. (1959). Linkage and the mechanism of recombination in *Streptomyces coelicolor*. *Ann. N.Y. Acad. Sci.* **81**, 887.

HOPWOOD, D. A. & GLAUERT, A. M. (1958). The electron microscopy of *Streptomyces coelicolor*. *J. gen. Microbiol.* **18**, vi.

KLIENEBERGER-NOBEL, E. (1947). The life cycle of sporing *Actinomyces* as revealed by a study of their structure and septation. *J. gen. Microbiol.* **1**, 22.

MCGREGOR, J. F. (1954). Nuclear division and the life cycle in a *Streptomyces* sp. *J. gen. Microbiol.* **11**, 52.

ØRSKOV, J. (1923). *Investigations into the Morphology of the Ray Fungi*. Copenhagen: Levin & Munksgaard.

PONTECORVO, G. (1953). The genetics of *Aspergillus nidulans*. *Advanc. Genet.* **5**, 151.

STANIER, R. Y. (1942). Agar-decomposing strains of the *Actinomyces coelicolor* species-group. *J. Bact.* **44**, 555.

WILKIN, G. D. & RHODES, A. (1955). Observations on the morphology of *Streptomyces griseus* in submerged culture. *J. gen. Microbiol.* **12**, 259.

EXPLANATION OF PLATES

Phase-contrast photographs. Unless otherwise stated, the photographs are of *Streptomyces coelicolor* strain A3 (2) growing on cellophan over an agar surface; they are printed at a magnification of ×1590.

PLATE 1

Fig. 1. 8 hr. on minimal medium. Germinating spores.

Fig. 2. 17 hr. on minimal medium. Centre of a young colony with parent spore visible.

Fig. 3. 17 hr. on minimal medium. A side branch colonizing the area between two radially-growing major hyphae at top and bottom of picture, and turning to grow in a radial direction at arrow.

Fig. 4. 40 hr. on minimal medium. Late stage in the colonization of the medium by the substrate mycelium, showing highly convoluted hyphae growing into the spaces between the earlier-formed hyphae.

Fig. 5. 24 hr. on minimal medium without glucose. A major substrate hypha with side branches, near the edge of a young colony.

PLATES 2 AND 3

Figs. 6–13. 40 hr. on minimal medium without glucose. Different regions of the same colony.

Fig. 6. Substrate mycelium near the edge of the colony. Two radially-growing major hyphae (arrows), with systems of side branches.

Figs. 7–10. Portions of major substrate hyphae nearer the centre of the colony, showing stages in the development of aerial branches (arrows).

Fig. 11. A branched aerial hypha and, in lower focus, substrate mycelium with major hyphae running vertically in the photograph. The aerial hypha originates from the substrate mycelium in lower focus at arrow.

Fig. 12. A 'swollen body' (arrow) borne on the substrate mycelium.

Fig. 13. A sporulating aerial hypha near the centre of the colony.

Fig. 14. Coverslip preparation. 41 hr. on minimal medium without glucose. A spore (arrow) has produced a small amount of substrate mycelium bearing two aerial hyphae, one beginning to delimit spores.

Fig. 15. Impression preparations from 48 hr. growth on minimal medium. Spore chains in different stages of maturation.

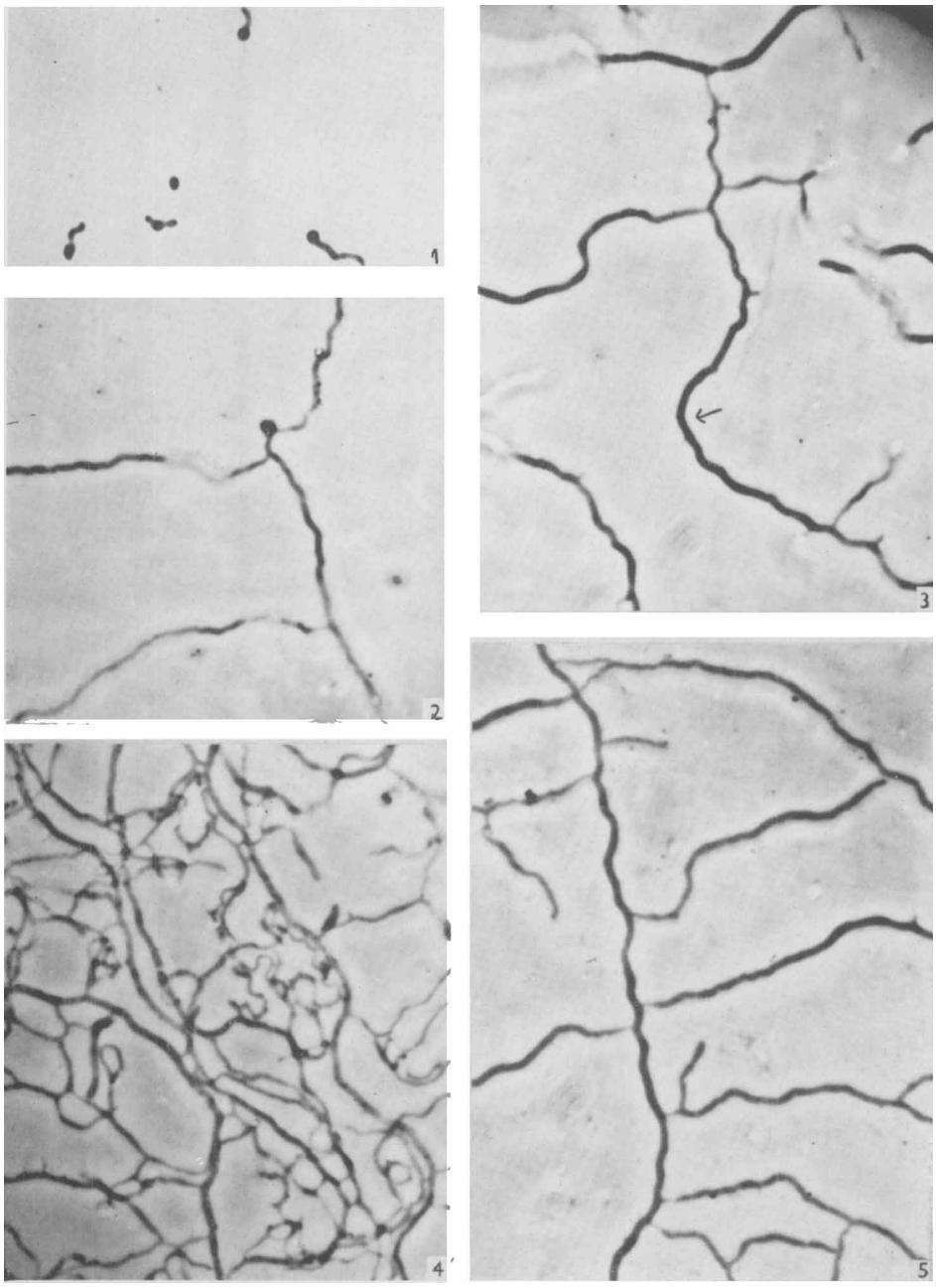
PLATE 4

Fig. 16. 17 hr. on minimal medium. A large unit of the aerial mycelium has produced four germ tubes, which are developing into substrate mycelium.

Fig. 17. 19 hr. on complete medium. Edge of a young colony showing rope-like strands of substrate hyphae. (×1190.)

Fig. 18. Nicotinamide-requiring mutant of *S. coelicolor* A3 (2). 37 hr. on minimal medium without glucose. Approximately 1/4 of a young colony. Germinated spore at (*a*). Very regular colonization of the medium by the substrate mycelium. Young aerial hypha at (*b*). (×1190.)

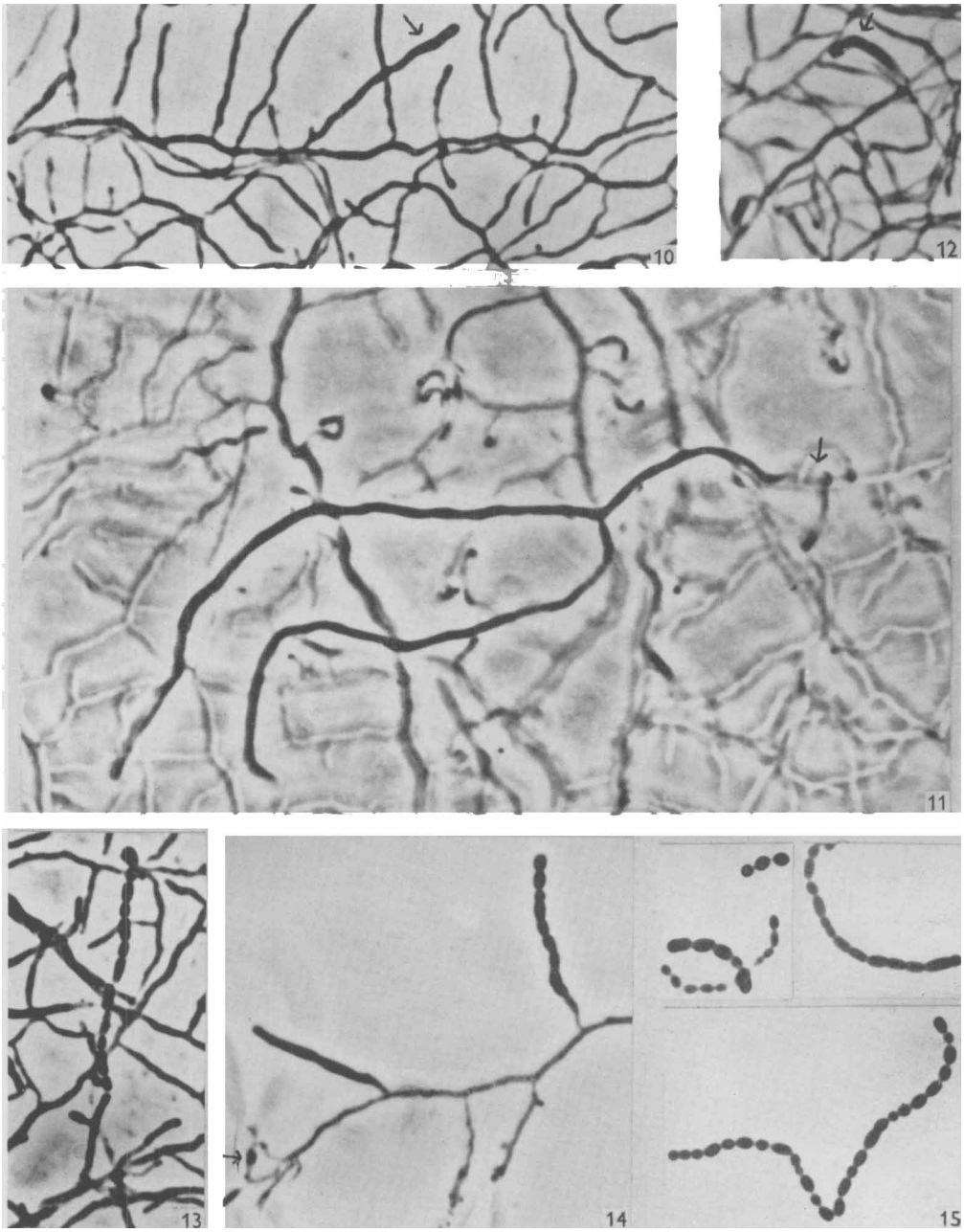
(*Received* 3 *September* 1959)



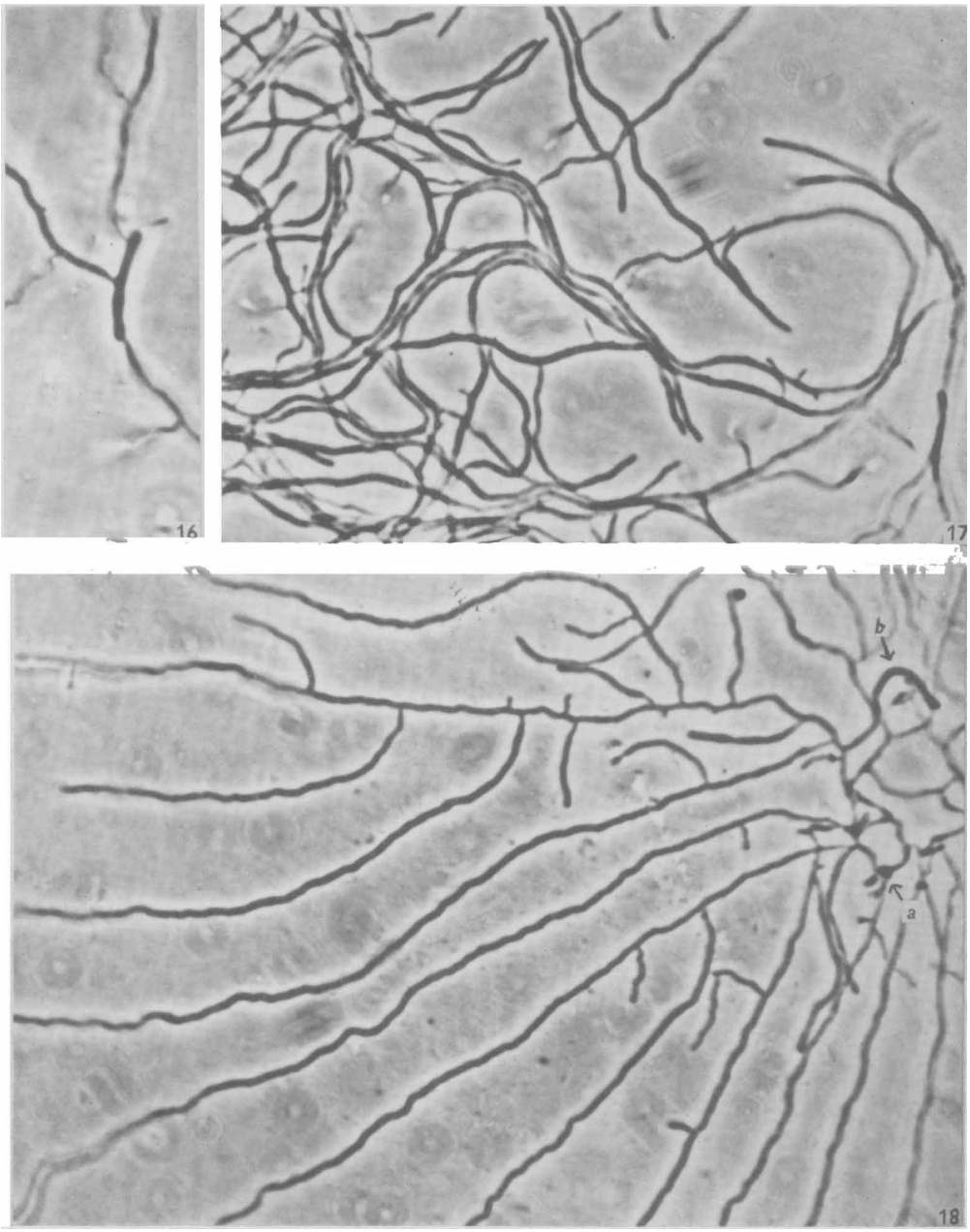
D. A. HOPWOOD—*STREPTOMYCES COELICOLOR*. PLATE 1

(*Facing p.* 302)

D. A. Hopwood—*Streptomyces coelicolor*. Plate 2



D. A. Hopwood—*Streptomyces coelicolor*. Plate 3



D. A. HOPWOOD—*STREPTOMYCES COELICOLOR*. PLATE 4