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Autoradiographic Study of Hyphal Growth during Aerial Mycelium Development in *Streptomyces antibioticus*

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The pattern of growth of aerial mycelium in *Streptomyces* species was investigated by autoradiography. Colonies of *Streptomyces antibioticus* were labeled with *N*-acetyl-D-[1-^{3}H]glucosamine to localize the sites of hyphal growth during the development of aerial mycelium. Autoradiographs obtained with sections of the colonies revealed that hyphal growth occurs not only at the top of the colony but also in the inner zones of the aerial mycelium.

Colonies of streptomycetes show a complex multicellular structure whose development involves the successive formation of two types of mycelia, each playing a different and specific role (3). One is a substrate or vegetative mycelium that grows on and into the culture medium; the other is an aerial or reproductive mycelium which will finally form the spores (8, 9). The substrate mycelium consists of a dense web of hyphae which develop in a radial pattern of growth, i.e., substrate hyphae elongate at the periphery of the colony, where fresh nutrients are continuously available, and then branch to start a new round of vegetative growth (1).

Little is known, however, of the growth and development of aerial mycelium. Aerial hyphae grow into the air and consequently develop in a nonaqueous environment. Under such conditions, nutrients required for growth are available only in the basal zone of the hyphae, where they maintain a cytoplasmic continuity with the substrate mycelium (4). Bearing this in mind, one would expect aerial mycelium to grow in a basal manner, that is, by a mechanism in which hyphae, initially formed near the substrate, would become displaced upwards, causing expansion of the colony. This possibility has not been investigated yet.

In order to address this question, colonies of *Streptomyces antibioticus* (ATCC 11891) were labeled with *N*-acetyl-D-[1-^{3}H] glucosamine ([^{3}H]GlcNAc) during aerial mycelium development and the sites of mycelial growth were localized by autoradiography. [^{3}H]GlcNAc was chosen as the labeled precursor since it is almost entirely incorporated into the cell wall of *S. antibioticus* (13). The microorganism was grown as lawns on cellophane disks (5 cm in diameter) which were placed on solid glucose-asparagine-yeast extract (GAE) medium (6). This cultivation procedure facilitates labeling and transfer of mycelium from one medium to another without disturbing mycelial differentiation (12). The disks were inoculated by spreading 100 μl of a spore suspension (10^8 spores per ml), obtained as described previously (7), and were incubated at 28°C until the development of aerial hyphae. This stage of development was reached after about 36 h of incubation, when the surface of the mycelium became white and powdery (16). Two cellophane disks with growing mycelium were then transferred onto GAE medium containing 50 μCi of [^{3}H]GlcNAc (specific activity, 2.84 Ci/mmol; Amersham) per ml. After a 20-min period of labeling at 28°C, the disks were removed from the medium. One disk was processed directly for microscopy. The other disk was floated on distilled water (containing 2 mM GlcNAc) and was then transferred to GAE medium containing 2 mM GlcNAc. After 12 h of incubation at 28°C, the disk was removed and further processed for microscopy. The procedure was as follows. Lawns of labeled mycelium (which were homogeneous with respect to the color of the aerial mycelium) were carefully removed from the disks, cut into small pieces, fixed in 1% (wt/vol) osmium tetroxide (15), dehydrated in acetone, and embedded in Epon 812. Before polymerization, samples were properly positioned to facilitate vertical sectioning of the whole mycelium. Samples (in triplicate) were cut with an LKB Ultratome III with glass knives. Only a ribbon containing no more than 15 successive thin sections (0.5 to 1 μm thick) was obtained from each sample. These sections were successively picked up and used alternately for autoradiography and for characterization of mycelial regions in the microscope. For autoradiography, sections were mounted on slides and dipped into a 0.5× Ilford L-4 emulsion. After 7 to 10 days of exposure, the slides were developed in Kodak D-19 developer (5 min at 20°C), fixed, washed with distilled water, and observed with a light microscope. For characterization of mycelial regions, sections were mounted on slides, stained with toluidine blue (0.1% toluidine blue in 0.1% aqueous sodium borate) for 30 s, and observed in a light microscope (11). Following the above procedure, we obtained sections in which the hyphal density and the overall thickness of the mycelium (measured in the microscope with a calibrated eyepiece) were similar. The boundary of the mycelium at the base of the colony (which in cellophane-grown cultures is very easy to recognize with a microscope [12]) was taken as the reference for the proper alignment of autoradiographs with toluidine blue-stained sections. On the other hand, the ultrastructure of the hyphae during colony development was monitored by electron microscopy. Ultrathin sections (with a pale grey interference color) were mounted on Formvar-coated grids, stained with uranyl acetate and lead citrate, and observed with a Philips EM-300 electron microscope.

As observed in toluidine blue-stained sections (Fig. 1a), white lawns of *S. antibioticus*, labeled for 20 min with [^{3}H]Glc NAc, contained two different mycelial regions: a lower region (about 50 μm thick) of tightly packed hyphae that had accumulated on the surface of the cellophane, and an upper region formed by a loose network of hyphae growing into the air and reaching a height of about 180 μm above the culture medium. As confirmed by electron microscopy (data not shown), the upper region of the colony contained hyphae

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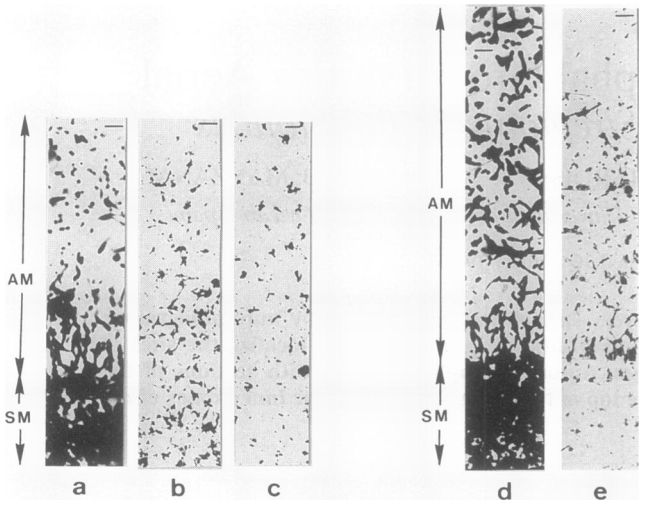

FIG. 1. Vertical sections of *S. antibioticus* colonies showing the sites of [^{3}H]GlcNAc incorporation during aerial mycelium development. Colonies 36 h of age were labeled with [^{3}H]GlcNAc for 20 min. Labeled colonies were processed directly for autoradiography (a through c) or incubated on GAE medium containing unlabeled GlcNAc for 12 h and then processed for autoradiography (d and e). (a and d) Control sections stained with toluidine blue; (b, c, and e) unstained sections; (b and e) untreated colonies; (c) trichloroacetic acid-treated colonies. Observations were carried out with a light microscope. The boundary of the mycelium at the base of the colony was taken as the reference for alignment of autoradiographs with toluidine blue-stained sections. AM, aerial mycelium; SM, substrate mycelium. Bar, 10 μm.

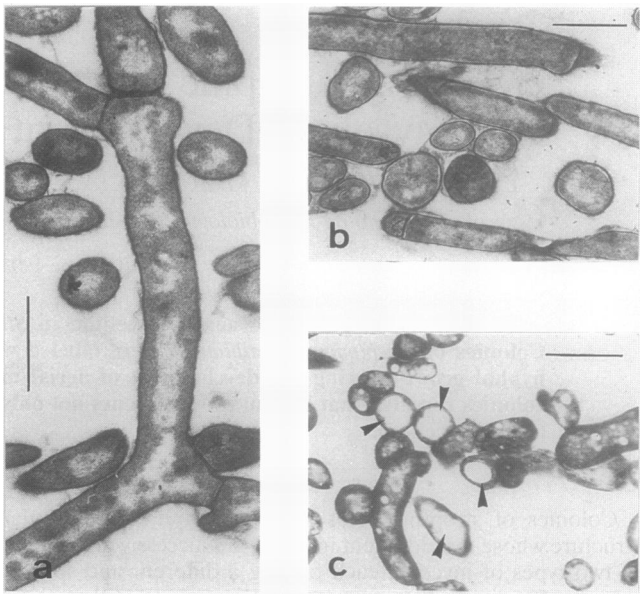

FIG. 2. Ultrathin sections of colonies of *S. antibioticus* grown for 36 h (a) or 48 h (b and c). (a) Branched hyphae in the inner region of the aerial mycelium; (b) aerial hyphae in the upper region of the colony; (c) lysed hyphae (arrows) in the substrate mycelium. Bar, 1 μm.

surrounded by the fibrous sheath characteristic of the aerial mycelium (18), whereas the lower region of the colony, since it contained only unsheathed hyphae, corresponded to the substrate mycelium. On the other hand, most aerial hyphae were branched at the inner zones of the aerial mycelium (Fig. 2a), but branching decreased towards the upper region. A random observation of 10 autoradiographs obtained from sections of these colonies did not reveal preferential accumulation of silver grains at the base of the aerial mycelium. As shown in Fig. 1b, silver grains appeared on the substrate mycelium but were also uniformly distributed throughout the region of the colony occupied by the aerial mycelium. The possibility that this pattern of labeling was caused by soluble radioactive compounds not incorporated into the cell wall was investigated. Colonies of *S. antibioticus*, grown and labeled as described above, were treated with ice-cold 5% (wt/vol) trichloroacetic acid for 10 min and were processed for autoradiography. Autoradiographs obtained from sections of trichloroacetic acid-treated colonies showed no changes in the labeling of the aerial mycelium, but labeling decreased significantly throughout the substrate mycelium (Fig. 1c). Our data indicate that irrespective of their location, aerial hyphae incorporate [^{3}H]GlcNAc into the wall, and the data also seem to exclude the possibility that only those hyphae or hyphal segments close to the substrate mycelium contribute to aerial mycelium development. On the other hand, labeling of the substrate mycelium was apparently due to trichloroacetic acid-soluble radioactive compounds.

Further experiments corroborated these observations. White lawns of *S. antibioticus* were labeled with [^{3}H]GlcNAc as described above and allowed to grow for a further 12 h on GAE medium containing unlabeled GlcNAc before being processed for autoradiography. As observed under a light microscope (Fig. 1d), the whole organization of the lawn remained unchanged after the additional period of incubation, but the aerial mycelium increased in both height and hyphal density (Fig. 1a and d). The autoradiographs obtained with sections of these lawns (a total of 10 sections were examined) showed the upper zone of the aerial mycelium almost devoid of labeling (Fig. 1e), thus indicating hyphal growth at this region of the colony during incubation in cold medium. Neither sporulating hyphae nor symptoms of lysis were observed in the upper region of the colony (Fig. 2b). As Fig. 1e also shows, the amount of label in the substrate mycelium decreased significantly after the chase. Although lysis in the substrate mycelium may account for the loss of label (Fig. 2c), it is also possible that cell wall synthesis occurred in this region of the colony or that some of the radioactivity in the substrate mycelium had moved upwards during the chase.

The data reported indicate that during development of the aerial mycelium, hyphal growth occurs not only at the top but also in the inner zones of the aerial mycelium, where new hyphae are formed by branching of the preexisting ones. This pattern of development resembles that reported for the substrate mycelium (1), but it raises questions about the mechanism by which hyphae, at the top of the aerial mycelium, obtain nutrients for growth. This implies that nutrients must be transported intracellularly over considerable distances, from the substrate mycelium to the growing hyphae at the top of the colony. However, in streptomycetes the cytoplasm is not continuous and is divided into long segments, or hyphal compartments, separated by septa (17). As in other gram-positive bacteria, these septa consist of a single electron-dense layer of wall material and two membranes that, as a consequence of the invagination process, have their outer surfaces facing each other (10). Wall material does not seem to be a barrier to nutrient passage, since it frequently appears to be perforated by fine pores (2, 10). However, it is not clear how

nutrients could move through two membranes of opposite polarities.

Although discussion of this problem lies beyond the aim of this work, some possible mechanisms should be considered. For example, nutrient passage through hyphal compartments could be facilitated by changes in membrane permeability. Similar changes were proposed to explain nutrient passage to the forespore in *Bacillus* species (5, 14), a process that in some aspects resembles that described above for the septa of *Streptomyces* species, since after engulfment the forespore becomes surrounded by two membranes in opposite orientations and nutrients and/or metabolites can enter the forespore through the mother cell cytoplasm. In streptomycetes, these changes might arise as a consequence of their adaptation to aerial environments, where systems for the selective incorporation of nutrients from the external medium are no longer required.

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