NOTES

Differentiation and Anaerobiosis in Standing Liquid Cultures of *Streptomyces coelicolor*

Geertje van Keulen,1 Henk M. Jonkers,2 Dennis Claessen,1 Lubbert Dijkhuizen,1* and Han A. B. Wösten1†

Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Haren, The Netherlands,1 and Microsensor Research Group, Max Planck Institute for Marine Microbiology, Bremen, Germany2

Received 19 July 2002/Accepted 15 October 2002

*Corresponding author. Mailing address: Microbial Physiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, P.O. Box 14, Kerklaan 30, 9750 AA Haren, The Netherlands. Phone: 31.50.3632150. Fax: 31.50.3632154. E-mail: L.Dijkhuizen@biol.rug.nl.
†Present address: Department of Microbiology, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands.

*Streptomyces coelicolor* is a filamentous gram-positive soil bacterium that undergoes a complex life cycle of morphological differentiation on solid agar medium. After a submerged feeding mycelium has been formed, hyphae escape the aqueous environment to grow into the air. This process is mediated by a surface-active peptide, SapB, that lowers the water surface tension (15). Aerial hyphae and spores possess several surface layers that make them hydrophobic. The rodlet layer is one of these layers. Its formation is mediated by the homologous rodlin proteins RdlA and RdlB (4) that are produced by hyphae in layers. The film could function as a fungal hydrophobin membrane by lowering the water surface tension (18). Colonies grown on gNMMP formed a confluent layer of aerial hyphae (Fig. 1C). The rigid film was rich in protein but did not contain antibodies raised against these polypeptides (data not shown). The film could function as a fungal hydrophobin membrane by lowering the water surface tension (18). Colonies grown on gNMMP formed few aerial hyphae often restricted to radial zones or rings (Fig. 1B). In contrast, colonies grown on mNMMP formed a confluent layer of aerial hyphae (Fig. 1C). The rigid film was rich in protein but did not contain antibodies raised against these polypeptides (data not shown). The rigid film could function as a fungal hydrophobin membrane by lowering the water surface tension (18). Colonies grown on gNMMP formed few aerial hyphae often restricted to radial zones or rings (Fig. 1B). In contrast, colonies grown on gNMMP formed a confluent layer of aerial hyphae (Fig. 1C). The rigid film was rich in protein but did not contain antibodies raised against these polypeptides (data not shown).

We here show that differentiation also occurs in standing liquid minimal media. After a period of submerged growth, hyphae migrate to the air interface, where they become fixed by a rigid reflecting film. Colonies that result from these hyphae form sporulating aerial hyphae. In addition, submerged hyphae in the liquid minimal medium may attach to the surface. Liquid standing cultures easily become anoxic only 1 to 2 mm below the surface. Yet, biomass increases, implying the existence of metabolic pathways supporting anaerobic growth.

*Streptomyces coelicolor* differentiates on solid agar media by forming aerial hyphae that septate into spores. We here show that differentiation also occurs in standing liquid minimal media. After a period of submerged growth, hyphae migrate to the air interface, where they become fixed by a rigid reflecting film. Colonies that result from these hyphae form sporulating aerial hyphae. In addition, submerged hyphae in the liquid minimal medium may attach to the surface. Liquid standing cultures easily become anoxic only 1 to 2 mm below the surface. Yet, biomass increases, implying the existence of metabolic pathways supporting anaerobic growth.

To investigate this, *S. coelicolor* M145 (9) was grown at 30°C in polystyrene microtiter plates (Costar 3370; Corning Inc.) or in petri dishes. Minimal NMMP medium (9) was used in the absence of polyethylene glycol 6000 and supplemented with 25 mM glucose and 0.25% Casamino Acids (gNMMP) or 25 mM mannitol (mNMMP) as the carbon source. Alternatively, *S. coelicolor* was grown in the complete medium tryptic soy broth, R2YE, or YEME (9). Spores or mycelium from a liquid shaken culture were used as inoculum.

A submerged mycelium was produced in all media. In contrast, aerial hyphae were only formed in minimal medium (gNMMP and mNMMP). After 3 days of submerged growth in these media, colonies formed at the water-air interface from which aerial hyphae developed (Fig. 1A). These colonies were fixed at this interface by a rigid light-reflecting film that surrounded the colony. Scanning electron microscopy revealed that the film had no clear ultrastructure and was therefore not an extension of the rodlet layer that coats the aerial hyphae (Fig. 1F and G). The rigid film was rich in protein but did not contain SapB or rodlin proteins as determined by immunodetections with antibodies raised against these polypeptides (data not shown). A submerged mycelium was produced in all media. In contrast, aerial hyphae were only formed in minimal medium (gNMMP and mNMMP). After 3 days of submerged growth in these media, colonies formed at the water-air interface from which aerial hyphae developed (Fig. 1A). These colonies were fixed at this interface by a rigid light-reflecting film that surrounded the colony. Scanning electron microscopy revealed that the film had no clear ultrastructure and was therefore not an extension of the rodlet layer that coats the aerial hyphae (Fig. 1F and G). The rigid film was rich in protein but did not contain SapB or rodlin proteins as determined by immunodetections with antibodies raised against these polypeptides (data not shown).
[J2450] [1], whiJ [J2452] [J. A. Ainsa, John Innes Center]) did not form floating air-interface colonies on gNMMP and mNMMP, and consequently aerial hyphae could not be formed. However, submerged growth was unaffected, as was shown by total protein determinations using the DC protein determination kit (Bio-Rad).

The standing cultures studied may resemble flooded soil, a condition S. coelicolor may escape from by forming floating sporulating colonies. How the hyphae move from the lower zone in the liquid to the air interface to form a sporulating colony is intriguing since S. coelicolor has no means of motility by, e.g., flagella. Within the genome sequence of S. coelicolor two gene clusters, SCO0649-SCO0658 and SCO6499-SCO6508, are present with homology to gas vesicle gene clusters from halophilic archaea and cyanobacteria (2). It is therefore not unlikely that S. coelicolor forms gas vesicles to become buoyant to reach the air interface.

In minimal medium, not only were aerial hyphae formed but submerged hyphae also attached to the hydrophobic surface of the microtiter plates. In contrast, hyphae did not adhere in the nutrient-rich media YEME, tryptic soy broth, and R2YE. Attachment of hyphae grown in gNMMP and mNMMP was quantified using a staining assay with crystal violet, which was measured at an optical density at 590 nm (11, 13). The number of attaching hyphae increased with culture age (Fig. 2) and correlated in a linear way with the increase in total biomass as measured by total protein determination (data not shown). Cultures grown on glucose formed more biomass than those grown on mannitol, and more hyphae attached. The stationary phase was reached after 7 and 10 days of growth in gNMMP and mNMMP, respectively, after which the number of attaching hyphae no longer increased. Recently, it was shown that hyphae in contact with air or a hydrophobic solid produce rodlin. These proteins are involved in formation of the rodlet layer of aerial hyphae and mediate attachment to a hydrophobic surface (4). The latter may be instrumental in the establishment of symbiotic or pathogenic interactions. For instance, streptomycetes have been shown to associate in a beneficial way to the cuticle of leaf-cutting ants, which grow fungi as their main source of food (5, 6).

Growing standing liquid cultures are expected to develop oxygen gradients. Indeed, using a sensitive Clark-type oxygen microsensor (10) it was shown that 7-day-old YEME and gNMMP cultures were anoxic at a depth of 0.7 and 2 mm, respectively (Fig. 3). Yet, biomass still increased as measured by total protein determinations (data not shown), indicating an active metabolism. Standing mNMMP cultures also developed oxygen gradients, but they were less steep and cultures did not become anoxic (Fig. 3). Apparently, diffusion matches the requirement for oxygen, as can be explained by the reduced growth rate in this medium (Fig. 2). Because steep oxygen gradients are present in soil (14), S. coelicolor should be able to overcome or even grow under conditions with no or low amounts of oxygen. The genome sequence of S. coelicolor revealed gene clusters, e.g., genes SCO0216-SCO0219, SCO4947-SCO4950, and SCO6532-SCO6535, that putatively encode a typical four-subunit respiratory nitrate reductase (2). This protein is involved in anaerobic metabolism, indicating that S. coelicolor possesses enzymes to accommodate metabolism under anoxic or microaerobic conditions. Because S. coeli-
color did not form colonies at the air interface in anoxic nutrient-rich standing cultures, oxygen availability may not be the signal for their formation.

In conclusion, standing cultures of \textit{S. coelicolor} are an attractive model system to study a wide range of phenomena not studied thus far, such as attachment of streptomycetes to solids, differentiation at a liquid interface, and anaerobic metabolism.

We thank Ietsie Stokroos for performing the scanning electron microscopy, Joanne Willey for supplying the \textit{bld} strains, and Keith Chater (JIC, the Biotechnology and Biological Sciences Research Council [BBSRC]) for supplying the \textit{wbi} strains.

G. van Keulen and D. Claessen are supported by a grant of the Dutch Program EET (Economy, Ecology and Technology), a joint initiative of the Ministries of Economic Affairs, Education, Culture, and Sciences and of Housing, Spatial Planning, and the Environment (EETK98020).

REFERENCES