WILKIN, G. D. & RHODES, A. (1955). *J. gen. Microbiol.* **12**, 259-264

Observations on the Morphology of *Streptomyces griseus* in Submerged Culture

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SUMMARY: The effects are described of three different media, based on urea, glycine or meat casein digest as nitrogen sources, on the morphology of *Streptomyces griseus* when grown in submerged culture. The urea medium was characterized by an alternation of morphological generations similar to that described by Klieneberger-Nobel for surface cultures: 'primary mycelium' alternated with 'secondary mycelium', the two generations being separated by an 'initial cell' stage. 'Secondary mycelium' sporulated to give 'secondary mycelium spores'. The other two media did not support 'initial cell' formation, but led either to the development of intercalary 'chlamydospores' or lysis without sporulation. The media based on urea or glycine were used in convenient and satisfactory techniques for the production of ampoules containing large numbers of lyophilized spores which stored satisfactorily.

Klieneberger-Nobel (1947) described an alternation of morphological generations in the surface culture of several *Streptomyces* species. The spores on germination gave substrate or 'primary' mycelium, which underwent anastomoses resulting in the formation of 'initial cells'. Klieneberger-Nobel regarded these 'initial cells' as fusion cells which on germination gave aerial or 'secondary' mycelium. This 'secondary mycelium' eventually sporulated to yield spores which completed the life-cycle by developing into new cultures of 'primary mycelium' on transfer to fresh medium. Erikson (1949), however, criticized Klieneberger-Nobel's interpretation of these observations on the grounds that the 'initial cells' and the 'nests' in which they formed were artefacts produced by the technique employed (stained coverslip growths) and that, in fact, the aerial filaments developed by budding from any vegetative hypha. While working on the development of media for the submerged cultivation of *Streptomyces griseus* we observed (1953) in certain media morphological forms which resembled those described by Klieneberger-Nobel for surface culture. Since our conditions differed greatly from those of Klieneberger-Nobel's surface culture methods, it seemed improbable that the morphological forms observed could be dismissed as artefacts. More detailed observations were therefore made and are reported here.

METHODS

The three types of media used contained urea, glycine or meat extract casein digest as the chief source of nitrogen, and had the following compositions:

The urea medium contained the following ingredients (g./l.): glucose (AnalaR), 5; sucrose (brown sugar), 10; urea (B.P.), 2; KH_2PO_4 (AnalaR), 2. This medium was adjusted, when necessary, to pH 4.9-5.0 with 5% phos-

phoric acid and sterilized in 400 ml. amounts in 2 l. round flat-bottomed flasks by autoclaving at 15 lb./sq. in. for 20 min. The post-sterilization pH value was 6.8–7.1. The brown sugar was not utilized by *Streptomyces griseus* but was included to provide traces of minor elements and organic substances.

The glycine medium was modified from a medium described by Lumb (1949) so as to provide a lower pH value during growth, and contained the following ingredients (g./l.): glucose monohydrate (pure Manbré & Garton), 20; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (AnalaR), 10; sodium citrate (AnalaR), 1; NaCl (AnalaR), 2.5; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ (AnalaR), 0.87; KH_2PO_4 (AnalaR), 0.5; glycine (technical grade), 5; trace elements solution 10 ml./l. (The trace elements solution, used at its natural pH of about 5.4–5.5, consisted of (g./l.): $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.75; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.81; H_2MoO_4 , 0.17; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.10; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.44). The pH of the complete medium was adjusted to 7.0–7.1 with KOH before autoclaving 400 ml. volumes in 2 l. round flat-bottomed flasks at 15 lb./sq. in. for 20 min. The post-sterilization pH was 6.5–7.0.

The meat extract + casein digest medium was prepared from (g./l.): Lab-Lemco (Oxoid), 3; glucose (AnalaR), 10; casein digest (Benger's), 10. The pH value was adjusted to 7.0–7.2 with NaOH before filtering the medium through paper pulp, and 400 ml. volumes of the medium were then autoclaved at 15 lb./sq. in. for 20 min. in 2 l. round flat-bottomed flasks.

Microscopical examination. Material was smeared on a slide and fixed by gentle heat. Preparations were stained in basic fuchsin (1 % aqueous solution of Gurr's stain for 5–10 sec.), washed thoroughly with water and then destained in tannic acid (10 % aqueous solution of B.P. acid for 2 min.) to give a rose pink colour. The slides were finally washed thoroughly in water, dried and mounted in Canada balsam.

Lyophilization. When sporulated cultures were required to be lyophilized, 70 ml. spore suspension were diluted with 10 ml. sterile solution (4 %, w/v) of crude undegraded bacterial dextran so that the final dextran concentration was 0.5 % (w/v). This mixture was then filled into sterile ampoules in 2.5 ml. volumes. The ampoules were rapidly frozen by immersion in a mixture of light petroleum and 'Drikold' and dried in a desiccator under a vacuum of 50μ . Hg for 48 hr. Dry nitrogen was admitted to the desiccator, the cotton-wool plugs pushed down, a second drying effected over P_2O_5 under a vacuum of 50μ . Hg for 4 days and finally dry nitrogen was again admitted to the desiccator before sealing the ampoules.

RESULTS

Observations of morphology on the urea medium

Flasks of the urea medium were inoculated with a light scrape from a well-sporulated surface culture of a streptomycin-producing strain of *Streptomyces griseus*, or preferably with one ampoule of lyophilized spores (approximately 6×10^7 viable spores) prepared from the glycine medium in the manner described below. The cultures were incubated on a rotary shaker (2 in. throw

at 180 r.p.m.) at 28° for 10 days. Preparations for microscopical examination were made by sampling the cultures at frequent intervals.

The spores germinated within 48 hr. to give a profusely branched mycelium analogous to the 'primary mycelium' of Klieneberger-Nobel. After *c.* 96 hr. this 'primary mycelium' developed deeply staining goblet-shaped bodies, spaced along the hyphae (Pl. 1, fig. 1). Because of the subsequent development of these bodies we refer to them as 'initial cells'. The stimulus for the production of these initial cells appeared to be related to and preceded by anastomosis of the primary mycelium, but we have no unequivocal evidence that hyphal fusion actually occurred.

After *c.* 108 to 124 hr. the initial cells germinated to produce a weakly-branched septate mycelium analogous to the 'secondary mycelium' of Klieneberger-Nobel (Pl. 1, fig. 2). After *c.* 8 days the secondary mycelium rapidly developed chains of closely applied cylindrical spores. We do not know the true nature of these spores; having regard to their method of formation, we term them 'secondary mycelium spores'. Sporulation of the 'secondary mycelium' was not confined to special sporangiophores but proceeded from the tips of the secondary mycelium in an acropetal succession. The secondary mycelium spores were characterized by the fact that they readily separated to lie free in the medium. At this stage the sporulating secondary mycelium was seen to be deeply stained against a faintly stained background of lysing primary mycelium (Pl. 1, fig. 6).

Subsequently, the secondary mycelium also lysed, with the result that at 10 days the secondary mycelium spores lay free in a matrix of medium containing lysed mycelium. At this stage dextran was added and the cultures were lyophilized in 2.5 ml. amounts to provide ampoules containing approximately 6×10^7 viable secondary mycelium spores which on germination (Pl. 1, fig. 5) developed into primary mycelium. Such spores, on inoculation into fresh urea medium, repeated the cycle already described. When the lyophilization step was omitted and the liquid spore suspension was used as inoculum, it was found that mixed cultures of primary and secondary mycelium sometimes resulted. It is believed that this was due to the proliferation of some unlysed secondary mycelium that would have been killed under the normal conditions of lyophilization. In such mixed cultures, no clearly defined sequence of events was observed, presumably owing to the superimposition of two growth cycles which were similar but out of phase.

Some of the urea medium cultures, started from spores, were lyophilized at 96 hr. when initial cells had formed but had not yet germinated. Such initial cell ampoules, when inoculated one to each flask of urea medium, germinated to give substantial amounts of secondary mycelium, which subsequently sporulated to yield typical secondary mycelium spores. It was noted, however, that in cultures started from initial cells, sporulation began after *c.* 3 days and was complete by 5 days, thus preserving the time-scale of the full cycle but eliminating the proliferation stage of primary mycelium.

Several factors influenced the growth cycle in urea medium. Thus the incorporation of increasing amounts of casein hydrolysate (Pronutrin) into the

urea medium progressively favoured the growth of primary mycelium at the expense of initial cells, secondary mycelium and sporulation, until at a concentration of 1 % (w/v) of the hydrolysate, sporulation was almost completely prevented; primary mycelium alone proliferated and finally lysed. An increase in glucose concentration from 5 to 10 g./l. or increased aeration (by increase of the rotary shaker speed from 180 to 220 r.p.m.) similarly favoured primary mycelium and resulted in lower counts of viable secondary mycelium spores.

Observations of morphology on the glycine medium

Flasks of glycine medium were inoculated with a streptomycin-producing strain of *Streptomyces griseus*, in the manner described above for the urea medium. Cultures were incubated on a rotary shaker (2 in. throw and 220 r.p.m.) at 28° for 10 days, and microscopical preparations were made from samples taken at frequent intervals.

The spores germinated within 20 hr. and gave profusely branched primary mycelium. The young primary mycelium stained well and revealed large numbers of bodies which we consider to be nuclei. As the mycelium developed, it gradually lost this characteristic staining reaction until these nuclei could no longer be distinguished. The primary mycelium proliferated for several days. At c. 6–8 days the contents of the primary mycelium rounded-off to produce large numbers of spores. Since these spores were developed directly by the vegetative primary mycelium, and because they appeared to be formed in an intercalary manner, we term them 'chlamydospores'. When mature, these spores tended to remain in long chains of relatively widely spaced spores rather than to become free in the medium (Pl. 1, fig. 7). At this stage the cultures were lyophilized in 2.5 ml. amounts and gave ampoules containing approximately 6×10^7 viable chlamydospores. On germination, these produced typical primary mycelium.

It was observed that chlamydospore formation was depressed by decreased aeration, by incubation on a slower rotary shaker (2 in. throw and 180 r.p.m.) or by the addition of 1 % (w/v) peptone to the medium. With the incorporation of 2 % (w/v) peptone sporulation was completely inhibited and the primary mycelium finally lysed. Proliferation of primary mycelium, culminating in the direct production of large numbers of apparently intercalary spores, was observed in a rather wide range of media, with fairly low concentrations of available nitrogen. Thus, for example, essentially the same process occurred in a medium based on glucose and soya-bean meal.

Media which supported chlamydospore formation when inoculated with primary mycelium also supported the formation of secondary mycelium spores when inoculated with secondary mycelium or lyophilized initial cells. Such media, however, did not provide the stimulus for initial cell formation, and therefore no cycle involving an alternation of two morphologically different types of mycelia was observed.

Observations of morphology on the meat extract + casein-digest medium

Flasks of meat extract + casein-digest medium were inoculated with a streptomycin-producing strain of *Streptomyces griseus* as previously described. The cultures were incubated on a rotary shaker (2 in. throw and 220 r.p.m.) at 28° for 10 days; microscopical preparations were made from samples taken at frequent intervals. The spores developed into primary mycelium which proliferated in much the same manner as that from the glycine medium already described. Growth of primary mycelium was very vigorous but essentially complete within 60 hr. No initial cells or secondary mycelium were formed, the primary mycelium lysing after *c.* 120 hr. Occasionally a few chlamydospores appeared, but sporulation was generally completely suppressed.

DISCUSSION

The nature and origin of the initial cells is of interest, because they appear to arise as a result of hyphal fusion. Some indications as to how they may be formed can be surmised from an examination of their structure and morphology on germination. Typical initial cells of *Streptomyces griseus* (Pl. 1, fig. 1) are formed at intervals along a strand of primary mycelium at points where anastomoses seem to have occurred. Each initial cell usually has at least one filamentous appendage of rather short but variable length. The primary mycelium between initial cells increases in girth, but subsequently loses its contents; at such points the mycelium readily fragments. Fragmentation may or may not precede the germination of the initial cells. Thus free initial cells may be seen to develop into septate secondary mycelium (Pl. 1, figs. 2, 3) or alternatively germination may occur *in situ* (Pl. 1, fig. 4). The filamentous appendages are usually longest when the initial cells are first recognized (Pl. 1, fig. 1), and remain as mere fragments by the time germination of the initial cells is under way (Pl. 1, fig. 3). It may be that these appendages are the remains of primary mycelium hyphae that have taken part in anastomoses and then become moribund. Rigid proof of anastomosis before initial cell formation, however, is unlikely to be obtained from visual observations alone; genetical evidence would probably be required.

There is no proof that the morphological forms observed in our submerged cultures in urea medium are the same as those found by Klieneberger-Nobel for surface cultures, but there is a marked similarity. These morphological forms cannot be explained as artefacts, since they only occur under certain cultural conditions. Moreover, free initial cells have been observed in all stages of germination. It may therefore be concluded that *Streptomyces griseus* exhibits an alternation of morphological generations in submerged culture in urea medium similar to that described by Klieneberger-Nobel for surface cultures, primary mycelium alternating with secondary mycelium and the two generations being separated by an initial cell stage. The secondary mycelium sporulates to yield secondary mycelium spores. The stimulus for initial cell formation is unknown, but the requisite conditions in submerged

culture are believed to be rather narrow and to be conditioned by aeration and the type of nitrogen supply. Media which do not support initial cell formation may permit the development of intercalary chlamydospores directly from primary mycelium.

The use of dextran for lyophilization was developed jointly with Miss J. O. R. Day, while the lyophilization procedure was determined in co-operation with Dr N. Blakebrough and Mr H. M. S. Kitching. The photomicrographs were obtained by Mrs K. M. King, Dr P. B. Dickenson and Mr R. McWilliam. Valuable technical assistance was afforded by Miss E. Theakston throughout.

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EXPLANATION OF PLATE

All figures show cultures of a streptomycin-producing strain of *Streptomyces griseus* stained with basic fuchsin; $\times 6000$.

- Fig. 1. Young initial cells.
- Fig. 2. Free initial cells and young septate secondary mycelium derived from them on germination.
- Fig. 3. Free initial cells and secondary mycelium at a later stage than that shown in fig. 2.
- Fig. 4. Initial cells which have germinated to form secondary mycelium before separation from the site of their formation in primary mycelium.
- Fig. 5. Germ tubes of primary mycelium from secondary mycelium spores.
- Fig. 6. Typical appearance of a sporulated culture in the urea medium showing secondary mycelium spores.
- Fig. 7. Typical appearance of a sporulated culture in the glycine medium showing chlamydospores.

(Received 7 October 1954)