Selective Filtration in the Isolation of Independent Clones of *Streptomyces*

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A single procedure for an efficient discrimination among the different size components of heterogeneous populations of *Streptomyces* spore suspensions is described. Conidia connected by substances of hydrophobic nature make obtaining all the spores in independent units difficult. For the isolation of pure clones, single spore suspensions were prepared by selective filtration through uniform and controlled pore size membranes. Single spore-enriched suspensions may be a useful tool for increasing the yield of auxotrophs, ensuring independent mutation during mutagenic treatments. This procedure can be extended to other sporulating species.

A clone is defined as a group of cells derived by vegetative reproduction from a single cell or spore. On some occasions, pure lines have been obtained with the aid of a micromanipulator (1), but for routine selection and purification of *Streptomyces* strains the dilution and plating technique is most commonly used. However, microscopy inspection of spore suspensions in water or lauryl sulfate solution (1:10,000) shows the presence of mycelium fragments and spore clusters (5). When these suspensions are strongly shaken and filtered through cotton or Whatman no. 2 filter paper which retain mycelium and the majority of large chains, a considerable amount of spore aggregates still appears in the filtrates. Therefore, cultures obtained from colonies isolated on agar plates seeded with these suspensions do not always represent a pure clone.

A colony that arises from a multipore chain may be considered as a combination of sub-strains, especially in the cases of spontaneous or induced changes in the genetic background of some of their spores. Although density gradient centrifugation was used by Tamir and Gilvard (7) to separate the spores of *Bacillus megaterium* from vegetative cells and by Prentice et al. (6) to separate germinated from nongerminated spores of *B. subtilis*, this time-consuming technique, so far as known, has not been used with the purpose of isolating monoclones.

This paper describes an easy procedure for obtaining single spores from a heterogeneous suspension of *Streptomyces* spores by means of selective filtration through adequate membranes of mixed esters of cellulose; it has been successfully applied to the isolation of pure clones.

**Materials and Methods**

Several species of *Streptomyces* were used throughout this work: *S. psammaticus*, *S. avellaneus*, *S. rimosus*, *S. aureofaciens*, and *S. coelicolor* A3-(2).

*S. rimosus* was cultivated on Emerson agar and the other *Streptomyces* on MC7 which has the following composition per liter: corn steep liquor (45% solid), 9.0 g; sucrose, 10.0 g; MgSO4·7H2O, 0.25 g; KH2PO4, 2.0 g; (NH4)2HPO4, 2.0 g; acid-hydrolyzed casein (Casamino Acids, Difco), 1.0 g; hydrolyzed ribonucleic acid solution, 4.0 ml; 1.0 ml of vitamin solution; and agar (Difco), 18.0 g. The pH was adjusted to 6.4.

The hydrolyzed ribonucleic acid and the vitamin solutions were prepared and used as described by Hopwood (3). Cultures were incubated 11 to 12 days at 28°C until full sporulation was observed.

**Preparation of spore suspensions.** Spore suspensions were prepared by adding an aqueous solution of 9% glycerol (wt/vol) to well-sporulated cultures and subjected to a vigorous agitation without scraping the surface, thus releasing spores with a minimal amount of mycelium. In some experiments, lauryl sulfate was used at a concentration of 1:10,000. The spore suspensions in 9% glycerol (wt/vol) were stored at −20°C (4).

**Filtration of spore suspensions.** In all cases, spore suspensions were first filtered by means of a cotton wool sterile filter assembly, to remove fragments of agar and vegetative mycelium, and then through the appropriate filter membrane of mixed esters of cellulose. Filters were washed after filtration with a volume of water equal to that of the filtered suspension.

Pore sizes of Millipore and Sartorius filters used were 0.22, 0.45, 0.6 to 0.65, 0.8, and 1.2 μm. According to the manufacturers, they are controlled by the mercury-intrusion method and by application of the Hagen Poiseuille principle (Sartorius Catalogue) and
have small variations in pore size, i.e., 0.45 ± 0.02 μm (Millipore Catalogue). Filter diameter normally was 47 mm, and when a larger area was required a 142-mm diameter filter mounted on a stainless-steel filter holder was used. Assemblies were sterilized 10 min at 120 C.

For preparation of suspensions with a high number of single spores per unit volume, filtrations through filters of 142 mm were used. Subsequent filtration through a 47-mm diameter filter of smaller pore size (0.22 or 0.45 μm) and resuspension in a small volume allowed the obtainment of more concentrated suspensions.

In the double filtration experiments, 200 or 50 ml of spore suspension of S. aureofaciens was filtered through a 0.8-μm membrane and washed with 50 ml of water. The recovered volume was filtered again through a 0.8-μm membrane, followed by another filtration through a 0.45-μm filter. This last membrane retained all the spores on its surface and these were resuspended in 2 ml of water with the aid of a Vortex mixer. The number of viable units in the suspensions was determined by plating portions of appropriate dilutions or direct samples of 0.1 to 0.5 ml of filtrates on MC7 medium and incubating the plates for 4 days at 28 C.

**Microscopy examinations.** Spore suspensions were directly examined by phase contrast microscopy. Stained preparations were also used; they were made by incubating spores with crystal violet (0.1%) in a small tube for 30 min at 4 C and observed with a Zeiss optical microscope.

Spore size estimations were obtained and microscopy inspection was made throughout the procedure for controlling spore suspensions before and after filtrations.

**RESULTS**

**Obtainment of enriched single spore suspensions.** When Streptomyces colonies reached a certain stage of evolution, the aerial hyphae differentiated through successive protoplasmatic divisions forming chains of spores are connected by substances of hydrophobic nature. To facilitate the separation of the spores, a suspension of S. aureofaciens was treated with lauryl sulfonate solution (1:10,000), thoroughly mixed by Vortex, and filtered through cotton wool or Whatman no. 3 filter paper. The filtrates observed by phase contrast microscopy still showed substantial amounts of double, triple, and some short chains of spores, whereas fragments of vegetative mycelium and large groups of spores were eliminated. The use of a diluted detergent solution and filtration through paper or cotton wool certainly increased the percentage of simple spores in the suspensions, but this treatment is not selective enough to obtain from conidia chains all the spores as independent units.

The spore size of the Streptomyces species used in this study ranged from 0.5 to 1.2 μm as determined by microscopy. Filtration through adequate membrane filters allowed the obtainment of enriched suspensions of single spores.

Although the efficiency of the filters was high, in some cases only a small quantity of double or triple concatenated spores was eventually able to pass through the interconnected canals of the membranes during the first filtration, but with a second filtration this possibility was virtually eliminated. Double filtration was carried out with 50 ml of spore suspension of S. aureofaciens (2.4 × 10⁷ per ml) and results are shown in Table 1. From 1.2 × 10⁶ initial viable units, only 8.0 × 10⁴ spores were recovered after the first filtration, implying that a large amount of material was retained by the filter. The relative proportion of spore recovery from the second filtration was greater than that of the first one, indicating that a smaller amount of material was retained since it mainly consisted of single spores. Phase contrast microscopy showed single spores and no grouped spores.

The results in Table 2 show that an increase in the area does not improve the efficiency of filtration under the specified conditions. A slightly lower value on the 142-mm diameter can be noticed, but it could be attributed to a higher adsorption of single spores on this larger surface. Different volumes, from 5 to 50 ml of the cotton wool prefitered suspension, of S. aureofaciens spores were filtered as described above, and increase of recovered spores corresponded to increases in the filtered volumes; hence, spore passage through the membrane was not affected by the nonfilterable material present in large volumes of spore suspensions.

Repeated washing of membranes is important to flush the single spores that may be retained in the microscopic channels of the filter. When 50 ml of a spore suspension of S. aureofaciens (8 × 10⁴ viable units) was filtered through a 47-mm, 0.8-μm pore size membrane, no spores were detected in the filtrate; however, when it was washed with 50 ml of water in 10-ml portions, 10⁴ spores were recovered, as calculated from colony counting.

**Pore size limits for different species of Streptomyces.** Twenty milliliters of spore suspension of several species of Streptomyces, namely S. avermillaeus, S. aureofaciens, S. psammoticus, S. rimosus, and S. coelicolor, was filtered once through 0.22-, 0.45-, 0.6-, 0.8-, and 1.2-μm pore size filters to evaluate the capacity to discriminate among different size spores.

From results presented in Table 3, it is concluded that filters of 0.22 and 0.45 μm definitely retain the spores of all the tested strains. The limiting minimal pore size which allows the passage of spores was variable. Val-
ues were 0.8 μm for *S. aureofaciens*, *S. averellaneus*, and *S. psammoticus* and 0.6 μm for *S. rimosus* and *S. coelicolor*; the viable counts obviously rise with the increase in pore size, because this allows the passage of single spores plus some diplo and small chains of spores.

**DISCUSSION**

After repeated culture transfers, genetic segregation occurring during growth and spore formation is frequently associated with changes in the characteristics of strains. The use of uniform pore size filters of mixed esters of cellulose (2) allows a selective filtration of conidia and facilitates culture purification from the heterogeneous suspensions of *Streptomyces* spores. The double filtration procedure is more reliable for obtaining enriched suspensions of single spores, from which the isolation of pure clones may be achieved, than the direct dilution and plating technique and is much more feasible than the micromanipulation technique.

Size differences in the single spores of the different tested strains were noticed in microscopy estimations. Furthermore, not all the single spores in a single-species suspension are uniform in their diameters, and the passage of single spores may be affected by this parameter and others, such as cultural conditions, adsorption of particles in the microscopic channels, membrane washing, etc. However, in general, results from filtration experiments are in agreement with microscopy information. Pore occlusion by nonfiltrate material does not occur with the cotton wool prefiltered suspensions; the numbers of recovered spores in the filtrates rise with volume increments without evident signs of filter saturation. At non-saturating volumes, the efficiency of filtration is not increased by the enlargement of the filtering area, demonstrating that small chains on the upper surface do not interfere with the passage of single spores.

**TABLE 1. Double filtration of *S. aureofaciens* spore suspension**

| Stage                   | Total no. of viable units | Recovery*       |
|-------------------------|--------------------------|----------------|
| Before filtration       | 1.2 × 10⁶                |                |
| After first filtration  | 8.0 × 10⁶                | 6.6 × 10⁻⁴     |
| After second filtration | 1.1 × 10⁶                | 9.2 × 10⁻⁴     |

*Recovery, number of viable units after filtration/number of viable units before filtration.

**TABLE 2. Influence of the membrane area on filtration of *S. aureofaciens* spore suspension**

| Membrane diameter (mm) | Total no. of viable units | Before filtration | After filtration |
|------------------------|---------------------------|-------------------|------------------|
|                        |                           |                   |                  |
| 47                     | 8.0 × 10⁶                 | 10 × 10⁴          |
| 142                    | 8.0 × 10⁶                 | 5.2 × 10⁴         |

*In 0.8-μm filter.

**TABLE 3. Pore size limits for spores of some *Streptomyces* species**

| Strain             | Filter pore size (μm) | Total no. of viable units | Recovery (per million)* |
|--------------------|-----------------------|---------------------------|-------------------------|
|                    |                       | Before filtration | After filtration |         |
| *S. aureofaciens*  | 0.6                   | 1.1 × 10⁹         | 0                  | 0         |
|                    | 0.8                   | 1.1 × 10⁹         | 4.0 × 10⁴         | 3.6       |
|                    | 1.2                   | 1.1 × 10⁹         | 7.2 × 10⁴         | 6.5 × 10³ |
| *S. psammoticus*   | 0.6                   | 2.0 × 10⁹         | 0                  | 0         |
|                    | 0.8                   | 2.0 × 10⁹         | 4.8 × 10⁴         | 2.4 × 10⁻¹|
|                    | 1.2                   | 2.0 × 10⁹         | 5.0 × 10⁴         | 2.5 × 10³ |
| *S. averellaneus*  | 0.6                   | 1.6 × 10⁹         | 0                  | 0         |
|                    | 0.8                   | 1.6 × 10⁹         | 3.0 × 10⁴         | 1.9 × 10⁴ |
|                    | 1.2                   | 1.6 × 10⁹         | 7.6 × 10⁴         | 4.7 × 10⁴ |
| *S. rimosus*       | 0.45                  | 2.8 × 10⁹         | 0                  | 0         |
|                    | 0.6                   | 2.8 × 10⁹         | 4.6 × 10⁴         | 1.6 × 10⁴ |
|                    | 0.8                   | 2.8 × 10⁹         | 1.2 × 10⁴         | 4.3 × 10⁴ |
| *S. coelicolor*    | 0.45                  | 1.0 × 10⁹         | 0                  | 0         |
|                    | 0.6                   | 1.0 × 10⁹         | 1.0 × 10⁴         | 1.0 × 10³ |
|                    | 0.8                   | 1.0 × 10⁹         | 5.0 × 10⁴         | 5.0 × 10³ |
|                    | 1.2                   | 1.0 × 10⁹         | 5.2 × 10⁴         | 5.2 × 10³ |

*Recovery, number of viable units after filtration/number of viable units before filtration. For simplicity, recovery is expressed in all cases as the number of units obtainable on filtering 10⁶ viable units.
Isolation of auxotrophic mutants which originate from a mutated spore could be masked by the concomitant growth of the other nonmutated spores linked to it in a multisporule chain. In replica plating of these multiclonal colonies grown in complete media, they will behave as prototrophs and will make those mutants with nutritional requirements undetectable. Therefore, the use of single spore suspensions in mutagenic treatments is advisable to increase the yield of auxotrophs, ensuring independent mutation. The available range of filter pore size allows the obtaining of spore suspensions of concentration adequate for mutagenic purposes.

This procedure has been useful in the isolation of independent clones in Streptomyces and it may be extended to Penicillium, Aspergillus, and other sporulating species.

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