ISOLATION OF STREPTOMYCIN-PRODUCING STRAINS OF STREPTOMYCES GRISEUS[1,2]

SELMAN A. WAKSMAN, H. CHRISTINE REILLY, AND DONALD B. JOHNSTONE

New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, New Jersey

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In the study of the production of antibiotics by microorganisms, especially when a given substance receives recognition as a chemotherapeutic agent, it becomes necessary, in order to maintain the potency of the culture or to obtain more active cultures, to isolate fresh strains of the organism producing the particular antibiotic. This can usually be accomplished by two procedures: (1) Fresh cultures of the organism are isolated indiscriminately from various natural substrates, such as soils and composts, and tested for their potency. (2) New strains are obtained by plating the original culture and then isolating individual colonies. Before plating, the culture may be pretreated, as by exposure to different radiations, in order to kill a large number of sensitive spores. Both of these methods have been utilized with considerable success in the isolation of more potent strains of penicillin-producing fungi. Comparatively little progress has been made, however, in the case of streptomycin-producing strains of *Streptomyces griseus*.

It has been established that penicillin production is characteristic of the *Penicillium notatum* and *Penicillium chrysogenum* groups; the variation in potency of different strains is either quantitative or qualitative, according to the type of penicillin produced. The production of streptomycin, however, is characteristic of only a certain few strains of *S. griseus* (Waksman, Schatz, and Reynolds, 1946). This organism represents a distinctly heterogeneous group, especially in regard to the production of antibiotics. In a recent examination of 40 freshly isolated cultures of *S. griseus*, none was found to produce the typical streptomycin; only one of these cultures was found to form an interesting antibiotic. This antibiotic was active against certain gram-positive and gram-negative bacteria, in a manner comparable to streptothricin and streptomycin, but it was both chemically and biologically distinct from either of these two substances. These results pointed to the difficulty of obtaining fresh streptomycin-producing cultures from natural substrates. Numerous attempts to isolate streptomycin-producing strains of *S. griseus* from natural substrates have so far yielded, in addition to the two original strains obtained in this laboratory in 1943, namely, D-1 and 18-16 (Schatz, Bugie, and Waksman, 1944), only two cultures. One isolation was made in another laboratory, and the

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other isolation in our laboratory, as reported here. All the cultures, however, that are now being used in industrial organizations for the production of streptomycin have been isolated from one of our original strains, namely, 18-16 (Schatz and Waksman, 1945).

It was also established that the streptomycin-producing culture of *S. griseus* produces inactive mutants or strains (Schatz and Waksman, 1944). This necessitates continuous purification of the culture and use of the more active variants, not only to increase the yield of streptomycin, but also to prevent the continuous deterioration of the culture.

In order to facilitate the isolation of *S. griseus* from natural substrates, advantage was taken of the following principle: Microorganisms producing certain antibiotics are usually more resistant to the action of these antibiotics than are other organisms not capable of forming such antibiotics. This was found to hold true even of closely related forms, as shown for the inactive mutant of *S. griseus*, which is sensitive to streptomycin. In order to facilitate the isolation

TABLE 1

Inhibition of different actinomycetes by their respective antibiotics

ANTIBIOTIC	ORGANISM PRODUCING IT	ACTIVITY OF PREPARATION PER 1 MG	DILUTION UNITS PER MG, EXPRESSED AS ACTIVITY AGAINST		
			S. antibioticus	*S. lavendulae*	*S. griseus*
Actinomycin.....	*S. antibioticus*	100,000*	100	5,000	100
Streptothricin.....	*S. lavendulae*	100†	1,000	0.4	10
Streptomycin.....	*S. griseus*	125†	1,000	100	1.2

* *S. lutea* units; crystalline material.

† *E. coli* units; crude preparation.

of new and more potent strains of antibiotic-producing organisms, the particular antibiotic may be incorporated in the medium in concentrations sufficient to inhibit the growth of other organisms and of inactive strains of the same organisms. This, however, does not affect the growth of potent strains.

The principle underlying this method can be illustrated by the selective activity of three antibiotics produced by actinomycetes against the mother culture (table 1). Actinomycin, streptothricin, and streptomycin were selected because of their distinct bacteriostatic spectra and the ease with which differences in antibacterial action can be demonstrated. Their activity was expressed in dilution units per 1 mg of the respective preparations. Actinomycin was measured in terms of *Sarcina lutea* units, since *Escherichia coli* is resistant to it, whereas the potency of the other two antibiotics was expressed as *E. coli* units. Activity was measured by the agar streak method (Waksman and Reilly, 1945). *Streptomyces antibioticus* is fairly resistant to actinomycin, its own antibiotic, but is sensitive to streptothricin and streptomycin. *Streptomyces lavendulae* is also resistant to its own antibiotic, streptothricin, but it is sensitive to streptomycin, an antibiotic related to it, and it is especially sensitive to

actinomycin. *S. griseus* is also most resistant to its own antibiotic, streptomycin; it shows a certain degree of resistance to streptothricin, but it is far more sensitive to actinomycin.

On the basis of these results one would be justified in concluding that the addition of streptomycin to nutrient media would tend to eliminate non-streptomycin-producing organisms, namely, various bacteria, as well as the majority of actinomycetes, but not fungi which are resistant to streptomycin. This medium should thus favor the development of organisms which either produce streptomycin or are resistant to it. This medium should also prove favorable, not only to the isolation of fresh cultures from natural substrates, but also to the elimination of inactive strains from a streptomycin-producing culture. The results (table 2) of a comparative study of the sensitivity of different strains of *S. griseus* to streptomycin, on the one hand, and to the living culture of an

TABLE 2

Sensitivity of 8 different strains of S. griseus to streptomycin and to the antagonistic action of S. griseus 3463-4

STRAIN OF *S. griseus* NO.	GROWTH IN PRESENCE OF STREPTOMYCIN (μG/ML) IN NUTRIENT AGAR*			ZONE OF INHIBITION PRODUCED BY NO. 4, MM†
	50	10	1	
3326	0	0	+	25
3378	0	0	+	24
3463	+++	+++	+++	6
3464	+++	+++	+++	5
3463-4	+++	+++	+++	6
3475	+++	+++	+++	6
3478	trace	++	+++	16
3481	+++	+++	+++	7

* 0 = no growth; trace, +, ++, +++ = relative amounts of growth.
† Cross streak tests on nutrient agar.

active streptomycin-producing strain of *S. griseus*, on the other, further serve to illustrate this principle.

Eight strains of *S. griseus* were used:

3326—Original type culture of the organism, isolated from soil in 1915 and kept in the collection. Inactive.

3378—Isolated by Dr. M. B. Morrow of Texas, from a soil in Yucatan. Inactive.

3463—Original 18-16 culture which produced streptomycin.

3464—Original D-1 culture which produced streptomycin.

3463-4—Active isolate from 18-16, obtained in our laboratory.

3475—Active isolate (42.1) obtained by Dr. H. W. Anderson of the University of Illinois from our strain 3863-4.

3478—A culture designated as G-25, which produced an antibiotic of the nonstreptomycin type.

3481—A streptomycin-producing culture of *S. griseus* freshly isolated from soil by the streptomycin-enriched medium.

The results show that strains 3326 and 3378, the non-streptomycin-producing strains, are very sensitive to streptomycin. The two original streptomycin-producing cultures (18-16, and D-1) and their isolates (3863-4 and 3475) are very resistant, a fact brought out particularly by the streak method. The freshly isolated culture (3481) is also resistant to streptomycin, but somewhat less so than the others. The sensitivity of G-25 fell between that of the inactive and the streptomycin-producing strains.

In order to demonstrate growth on the streptomycin-enriched medium of streptomycin-producing and non-producing strains found in the same culture, the results of the following experiment may be reported. A suspension of spores of strain 3463-4 was plated out on ordinary nutrient agar as well as on the same agar plus 100 μg of streptomycin per ml. The corresponding numbers of colonies obtained on the two media were 20.4 and 5.8 millions per ml of spore suspension,

TABLE 3

Production of streptomycin by freshly picked colonies from S. griseus plates

(Shaken cultures)

COLONY NO.	ACTIVITY, μG PER ML AFTER INCUBATION OF	
	3 days	6 days
NA 1*	<5	<5
NA 2	24	28
NA 4	<5	<5
NA 6	28	28
SNA 2†	14	38
SNA 6	13	46

* Strains NA 1, 2, 4, and 6 were isolated from nutrient agar plates, 1 and 4 being atypical colonies, since they produced on glucose-asparagine agar slants a red vegetative mycelium.

† Colonies SNA 2 and SNA 6 were isolated from nutrient agar enriched with 100 μg per ml of streptomycin.

indicating that nearly 80 per cent of the colonies are sensitive to 100 μg of streptomycin per ml. Ten colonies were picked at random from the nutrient agar plates and grown on glucose-asparagine agar. Six of these colonies produced an atypical, reddish, vegetative growth and a typical, greenish-gray mycelium. The remaining four colonies, as well as all colonies picked from the streptomycin-enriched agar plates, produced the typical growth of *S. griseus*. The atypical colonies produced no streptomycin, as shown in table 3.

In order to establish again whether streptomycin-enriched agar can be used for the isolation of fresh streptomyin-producing cultures, agar media containing 25 to 100 μg of streptomycin per ml were used for plating out various natural materials, such as soil, peat, and compost. These media depressed the development of nearly all the bacteria and actinomycetes, but had virtually no effect upon the growth of the fungi. A few actinomycetes developed on these media, but they grew only very slowly. Many of them proved to belong to the *S. griseus* group. They were isolated and cultivated on media favorable

for the production of streptomycin. Some of them produced antibiotic agents. Most of these antibiotics were found, however, to be not of the streptomycin type. Three of the resistant cultures were grown on different media, both in stationary and in submerged culture, and the culture solutions were tested for their activity against several bacteria. Only one showed fairly high activity against *Bacillus subtilis*, when grown on all media, and limited activity against *E. coli* and *Bacillus mycoides*. The properties of this agent did not seem to fit in with those of streptomycin.

One culture (3481) isolated during one of these surveys, however, appeared to be definitely of the streptomycin type. The substrate from which this culture was isolated was a soil adjoining a dairy barn and was thus heavily manured. The culture filtrate gave 30 units of activity against *E. coli*, and 100 against *B. subtilis*, *B. mycoides*, *Mycobacterium avium*, and *Mycobacterium phlei*. On isolation and purification it proved to give the typical streptomycin spectrum.

The results of this study permit the following conclusions: (1) not all strains of *S. griseus* are capable of producing streptomycin; (2) streptomycin-producing strains of *S. griseus* form active and inactive variants; (3) the inactive variants comprise two types, one being free from aerial mycelium and the other producing a pink tinge in the vegetative growth, the aerial mycelium being typical of *S. griseus*; (4) a medium enriched with streptomycin can be utilized for the isolation of fresh strains of *S. griseus* from natural substrates; and (5) a streptomycin-enriched medium can also be utilized for purifying active cultures of *S. griseus* from inactive variants.

Although these investigations did not result in obtaining superior strains of streptomycin-producing cultures of *S. griseus*, they established that streptomycin production is associated only with a certain few strains of *S. griseus* and that these strains may continuously form inactive substrains, and that by the use of suitable procedures it may be possible in time to isolate new and more potent strains of streptomycin-producing organisms.

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