METHODS FOR ISOLATION OF STREPTOVERTICILLIA FROM SOILS

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The genus *Streptoverticillium* was not explored much by laboratories screening for new antibiotics. Yet, interesting drugs, *e.g.*, mitomycin C[1], porfiromycin[2], and 5-azacytidine[3] were produced by members of this genus.

It appeared that it would be desirable to develop specific methods for isolation of Streptoverticillia from soils. Based on some published data[4~6],††, such study has been conducted and the results are presented herein.

A preliminary investigation was conducted with 21 known strains of *Streptoverticillium* and *Streptomyces* from Upjohn culture collection. It was established that the incorporation of 1,000 μg/ml of lysozyme and 25~30 μg/ml of oxytetracycline in cultivation media inhibited effectively the *Streptomyces*, but it had little effect on members of the genus *Streptoverticillium*. Soil samples from the following states were used in this study: Florida, Hawaii, Indiana and Tennessee. We kept separate records of each individual soil sample throughout the study.

The soils were air-dried, diluted (1 : 4) in water, and heat-treated (55°C, 6 minutes) to eliminate most bacteria[3]. The soil suspensions were spread onto the surface of 0.45 μm Millipore filters placed over the surface of the semi-defined agar medium in large plastic (Falcon, 135 mm) plates. The semi-defined agar consisted of; glucose 2.0 g, $(NH_4)_2SO_4$ 5.0 g, Na_2HPO_4 3.4 g, KH_2PO_4 0.5 g, NaCl 0.5 g, monosodium glutamate 0.1 g, yeast extract (Difco) 0.1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $Na_2MoO_4 \cdot 2H_2O$ 50 ng, $CuSO_4 \cdot 5H_2O$ 50 μg, $MnSO_4 \cdot 7H_2O$ 250 μg, $CaCl_2$ 5 μg, $ZnCl_2$ 0.5 μg, agar 15 g per 1 liter distilled water. Unadjusted pH before autoclaving was 7.0.

The agar was further supplemented with 50 μg/ml of cycloheximide and 50 μg/ml of nystatin to control the fungi. The control plates had just these two antifungal drugs. One set of plates had also 25 μg/ml of oxytetracycline in the agar and another set contained oxytetracycline at 25 μg/ml and lysozyme at 1,000 μg/ml. Each soil sample was plated on the three types of agar and the plates were incubated at 28°C. The Millipore filters were removed from the control plates after 2~3 days of incubation and from the antibiotic-containing plates after 5~7 days. After removal of the filters, the plates were incubated for an additional 7~10 days. At this time, well developed colonies were present. Randomly chosen colonies were selected using a stereomicroscope and subcultured with sterile toothpicks onto Petri dishes containing ISP-4 agar (Difco) or Czapek-Dox (CS) agar (Difco). These plates were incubated for 14~21 days at 28°C and then the growth was carefully examined under a light microscope (400X, wide angle objective) for the presence of verticils.

The results of the whole study are summarized in Table 1. The percent of Streptoverticillia isolated on control plates was 7.9, which is in fairly good agreement with the value of 5.9% found in our previous study[6]. And again, this percent was increased about 4-fold in the presence of oxytetracycline to 35.3%. In the presence of both oxytetracycline and lysozyme, the percent of Streptoverticillia increased to 58.9. The difference between the control and the group with oxytetracycline was highly significant ($P<0.0001$). The difference between values of 35.3 and 58.9 was also significant at $P=0.0155$. It appears that in soils from some locations (Hawaii and Indiana) this number can go even higher. The two soils from Tennessee contained very few Streptoverticillia.

We did not include in this study a group with lysozyme as the only drug in the isolation agar. In a previous investigation[6], we found that the difference in sensitivity to this drug alone between the genera *Streptomyces* and *Streptoverticillium* was too small.

The critical part of this study was the proper classification of the genus by stereomicroscope. It requires time, patience, and experience. The verticils are frequently found only in some parts of the surface growth. The best place to look

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Table 1. Effect of oxytetracycline and lysozyme on the proportion of *Streptoverticillium* species to other actinomycetes isolated from different soil samples.

Source of soil	Control agar (no antibacterial drugs)		Selective agar; oxytetracycline (25 μg/ml)		Selective agar; oxytetracycline (25 μg/ml) and lysozyme (1,000 μg/ml)	
	Total actino-mycetes examined	Number of *Strepto-verticillium*	Total actino-mycetes examined	Number of *Strepto-verticillium*	Total actino-mycetes examined	Number of *Strepto-verticillium*
Indiana (1)	22	3	17	7	22	16
Indiana (2)	8	1	1	1	8	8
Hawaii (1)	11	2	14	7	10	6
Hawaii (2)	16	1	10	6	15	13
Hawaii (3)	14	2	8	6	19	11
Hawaii (4)	11	2	11	7	10	6
Florida (1)	14	0	11	4	18	11
Florida (2)	12	0	12	0	6	0
Tennessee (1)	22	0	22	2	6	2
Tennessee (2)	21	1	13	2	10	0
Total Indiana	30	4 (13.3%)	18	8 (44.4%)	30	24 (80%)
Total Hawaii	52	7 (13.5%)	43	26 (60.5%)	54	36 (66.7%)
Total Florida	26	0 (0%)	23	4 (17.4%)	24	11 (45.8%)
Total Tennessee	43	1 (2.3%)	35	4 (11.4%)	16	2 (12.5%)

for them are the aerial hyphae growing in the groove made by the sharp point of the toothpick during the inoculation of agar. The growth on Czapek-Dox agar is usually less abundant, but it is easier to find the verticils. Of all the cultures classified as a *Streptoverticillium*, 107 were found on plates with Czapek-Dox agar and 79 with ISP-4 agar. It is true that the majority of Streptoverticillia were so classified on both types of agar. However, 17 (of the 186 total) would have been missed on Czapek-Dox agar, while 31 would have been missed if ISP-4 agar were utilized. Thus, it is recommended to utilize both these media at present.

Many cultures isolated from a given soil sample looked similar and might be identical. Thus, utilizing varying sources of soil is desirable to bring in variety. It appears that it is now possible to isolate large numbers of a genus which has been utilized little in the past by laboratories searching for new antibiotics.

To summarize, we have made use of both physical and chemical methods to effect necessary selective pressure. These included: (1) Heat treatment, to eliminate most non-spore-formers; (2) membrane filter technique, to eliminate most non-filamentous organisms; (3) addition of cycloheximide and nystatin, to inhibit fungal growth; and (4) addition of oxytetracycline and lysozyme to suppress several genera of actinomycetes, in particular the *Streptomyces*.

Utilizing this technique and soil samples from desirable locations, it appears possible that 60% or more of all the microorganisms isolated would belong to the genus *Streptoverticillium*. To the best of our knowledge, this is the highest rate reported so far.

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