ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 1973, p. 425-431

Vol. 3, No. .
Printed in U.S.A.

U-42, 126, a New Antimetabolite Antibiotic: Production, Biological Activity, and Taxonomy of the Producing Microorganism

L. J. HAŇKA AND A. DIETZ
Research Laboratories, The Upjohn Company, Kalamazoo, Michigan 49001

Received for publication 15 November 1972

A new antimetabolite antibiotic, U-42,126, was discovered by use of a specific in vitro screen. U-42,126 was produced by the fermentation of *Streptomyces sviceus*. Its antimicrobial activity in vitro was limited to fungi. Certain bacteria were inhibited only when cultivated in completely synthetic media. U-42,126 was active in vivo against L1210 leukemia in mice.

An in vitro screening system for antimetabolites was described by L. J. Haňka in 1967 (6). Several new drugs have been detected since that time in our laboratories by using this screen (7, 8, 9, 14). Furthermore, several other laboratories reported good results in their use of this screening technique (15; R. S. Gorder and T. F. Butler, Abst. Intersci. Conf. Antimicrob. Ag. Chemother., 11th, Atlantic City, p. 21, 1971 and J. P. Scannell et al., Abst. Intersci. Conf. Antimicrob. Ag. Chemother., 11th, Atlantic City, p. 23, 1971).

Recently, another new antimetabolite drug was discovered in our laboratories by this detection system: U-42,126.

In this communication, we present the taxonomy of the producing microorganism, fermentation conditions, and the in vitro and in vivo evaluation. Also presented are the microbiological assay and paper chromatography data.

The isolation and structure determination of U-42,126 will be described elsewhere (D. G. Martin et al., Tetrahedron Lett., in press).

MATERIALS AND METHODS

Culture. *Streptomyces sviceus* was characterized by the methods cited by Dietz (2) and by Shirling and Gottlieb (19).

Production. The organization of the screening procedure and the cultivation media utilized were described previously (6). The producing microorganism, *S. sviceus*, is deposited in the Upjohn culture collection as UC-5370. Inoculum for fermentation was maintained as plugs of agar, with the microogranism growing on top of it, and kept in a liquid nitrogen storage tank. The inoculum was cultivated for 48 h in a medium consisting of 25 g of dextrose and 25 g of Pharmamedia (Traders Oil Mill Co., Fort Worth, Tex.) per liter of tap water. The production medium contained per liter of tap water: starch, 10 g; mannitol, 10 g; Phytone, 10 g (BBL, Cockeysville, Md.); Kay soy, 10 g, 200 C (Archer Daniels, Midland Co., Decatur, Ill.); $CaCo_3$, 5 g; and NaCl, 2 g. The fermentation was carried out in 500-ml stippled flasks (with 100 ml of media) on a rotary shaker (250 rpm) at 32 C. Samples of the fermentation liquor were taken daily, and the titer of the drug was estimated by a microbiological disk plate assay.

Microbiological assay. The titers of U-42,126 in the fermentation liquors were monitored by a disk plate assay with *Bacillus subtilis* cultivated in a completely synthetic medium (6). The molten agar was innoculated with a spore suspension (1.5×10^{10} spores/ml) at a rate of 0.5 ml/liter. The fermentation liquors were applied to the 1.27-cm (0.5-inch) diameter paper disks (Carl Schleicher & Schuell Co., Keene, N. H.) at full strength and after being diluted with a pH 7.0 phosphate buffer to 0.5, 0.25, and 0.125. The assay plates were incubated overnight at 37 C, and the zones of inhibition were recorded.

Paper chromatography. The two best chromatography systems for differentiating U-42,126 were: (i) paratoluene sulfonic acid (2%) in water-saturated 1-butanol and (ii) 1-butanol-acetic acid (glacial)-water (2:1:1).

TABLE 1. *Color pattern of Streptomyces sviceus on Ektachrome*

Agar medium	Surface	Reverse
Bennett's	Lavender-gray	Brown
Czapek's sucrose	Lavender-gray	Red-brown
Maltose-tryptone	Lavender-gray	Brown
Peptone-iron	No aerial growth	Brown
0.1% Tyrosine	Moderate lavender-gray	Red-brown
Casein-starch	Moderate lavender-gray	Light brown

TABLE 2. *Reference color characteristics of Streptomyces sviceus*[a]

Agar medium		Color harmony manual method (12)	U. S. Dept. of Commerce Circ. 553 (13)
Bennett's	S	3fe silver gray	63gm light brownish gray
	R	3ni clove brown	77m moderate yellowish brown 95g moderate olive brown
	P	3ig beige brown, mist brown	80m grayish yellowish brown 95g moderate olive brown
Czapek's sucrose	S	31c amber, butterscotch	71m moderate orange yellow
	R	2ge covert tan, griege	94m light olive brown 109 gm light grayish yellow
Maltose-tryptone	P	3ge beige, camel	79m light yellowish brown
	S	a white	263gm white
	R	31g adobe brown, cinnamon brown, light brown	77gm moderate yellowish brown
Yeast extract-malt extract (ISP-2)	P	3ig camel, maple sugar, tan	80m grayish yellowish brown 95g moderate olive brown
	S	3fe silver gray	63gm light brownish gray
	R	4nl chocolate, dark brown	64m brownish gray 81g dark grayish yellowish brown
Oatmeal (ISP-3)	P	31i beaver	80m grayish yellowish brown 95g moderate olive brown
	S	3fe silver gray	63gm light brownish gray
	R	2ig slate tan to 3ml beaver gray	110g grayish yellow 112m light olive gray to 96g dark olive brown 266m dark gray
Inorganic salts-starch (ISP-4)	P	2ih dark covert gray	112m light olive gray 265m medium gray
	S	3fe silver gray	63gm light brownish gray
	R	3ge beige, camel	79m light grayish yellowish brown 94m light olive brown
	P	3ge beige, camel	79m light grayish yellowish brown 94m light olive brown
Glycerol-asparagine (ISP-5)	S	C light gray	264gm light gray
	R	3ml beaver gray	96g dark olive brown 266m dark gray
	P	3fe silver gray	63gm light brownish gray

[a] S, Surface; R, reverse; P, pigment. All readings were made using the glossy surface of the chips.

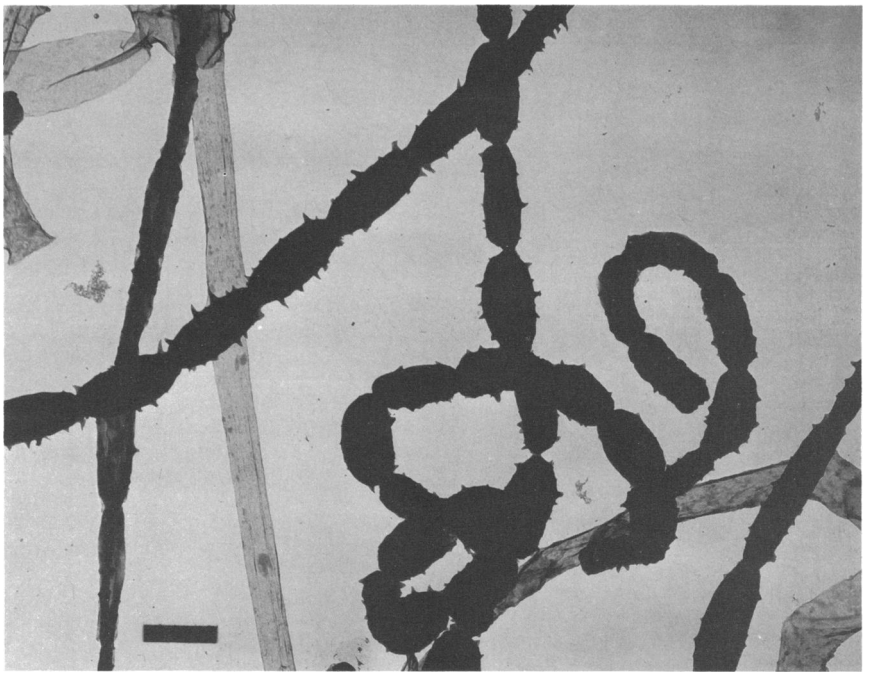

FIG. 1. *Streptomyces sviceus whole spores as seen by transmission electron microscopy.*

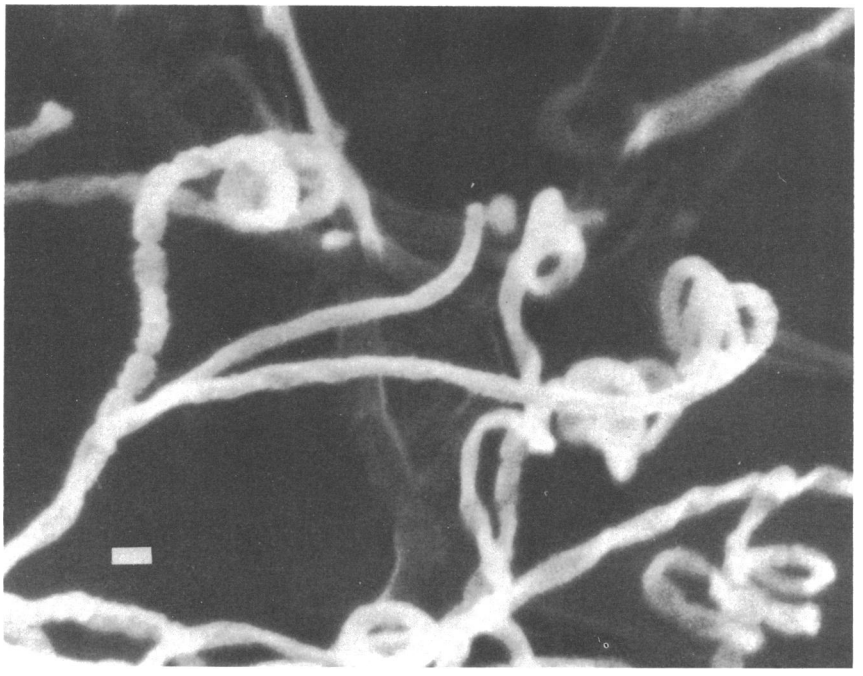

FIG. 2. *Streptomyces sviceus sporophores as seen by scanning electron microscopy.*

The paper strips developed for 16 h in these two systems were bioautographed against *B. subtilis* cultivated in synthetic agar, and the R_f values were recorded.

In vitro antimicrobial studies. The routine agar diffusion disk plate assay was used to evaluate the antimicrobial spectrum of U-42,126. The concentration used for testing was 500 μg/ml. The volumes of 0.08 ml were applied to paper disks of 13.6-mm diameter. After the incubation (18 h, 37 C), zones of inhibition were recorded.

In vivo antitumor testing. The first in vivo

TABLE 3. *Cultural and biochemical characteristics of Streptomyces sviceus*

Medium	Aerial growth		Other characteristics
	Surface	Reverse	
Agar			
Peptone-iron	Trace of gray	Brown	Brown pigment (melanin positive)
Calcium malate	Poor gray-white	White to cream	Malate solubilized around growth
Glucose-asparagine	Poor lavender-gray	Olive	
Skim milk	Very slight trace of gray	Yellow-tan-brown	Yellow-tan-brown pigment; casein not solubilized
Tyrosine	Poor gray	Tan brown	Brown pigment; tyrosine solubilized
Xanthine	Poor gray-white	Cream-tan	Cream-tan pigment; xanthine solubilized
Nutrient starch	Moderate gray-white	Cream-yellow	Yellow pigment; starch hydrolyzed
Yeast extract-malt extract	Good gray	Brown	Tan-brown pigment
Peptone-yeast extract-iron (ISP-6)		Brown	Brown pigment (melanin positive)
Tyrosine (ISP-7)	Gray	Brown	Brown pigment (melanin positive) 50% No pigment (melanin negative), 50%
Gelatin			
Plain	Trace of white on surface pellicle		Yellow to olive pigment; no liquefaction
Nutrient			Olive tan pigment; no liquefaction
Broth			
Synthetic nitrate	Colorless surface pellicle		Pale yellow pigment; compact bottom growth; nitrate not reduced to nitrite
Nutrient nitrate	Colorless surface pellicle		Brown pigment; compact bottom growth; nitrate not reduced to nitrite
Litmus milk	Brown surface ring		Litmus reduced in four of six tubes; no peptonization, pH 5.3

evaluations were done in L1210 and P-388 leukemias in mice. These were done at the Illinois Institute of Technology in Chicago, by the protocol of the National Cancer Institute. The first material tested was the lyophilized whole fermentation liquor. This was followed by a partially purified (~0.2%) preparation tested in the same laboratory.

RESULTS

Taxonomy. The microorganism was *S. sviceus* sp.n., Dietz (UC-5370, NRRL 5439).

Color characteristics. Aerial growth was gray, melanoid pigments were produced. The color pattern on Ektachrome (2) is given in Table 1. Reference color characteristics are given in Table 2. The culture may be placed in the gray (GY) and white (W) color series of Tresner and Backus (23).

Microscopy characteristics. Oval to rectangular spores were sparsely adorned with warts or short spines (Fig. 1) borne on candelabrum-like sporophores. Sporophores were long, straight, and coiled at the ends (Fig. 2). Sporophores may be placed in the spiral (S) group of Pridham et al. (18); spore surfaces may be placed in the warty group of Dietz and Mathews (4).

Cultural and biochemical characteristics. Cultural and biochemical characteristics are described in Table 3.

Carbon utilization. The growth of the culture on carbon compounds was determined by using the procedures of Pridham and Gottlieb (17) and Shirling and Gottlieb (19). In the procedure of Pridham and Gottlieb, *S. sviceus* showed moderate growth on the basal medium,

dulcitol, D-sorbitol, salicin, and sodium oxalate; good growth on D-xylose, L-arabinose, rhamnose, D-fructose, D-galactose, D-glucose, D-mannose, maltose, sucrose, lactose, cellobiose, raffinose, dextrin, inulin, soluble starch, glycerol, D-mannitol, inositol, sodium acetate, sodium citrate, and sodium succinate; poor growth on phenol, sodium formate, and sodium tartrate; and no growth on cresol and sodium salicylate. By the procedure of Shirling and Gottlieb, the culture did not grow on the negative control (basal medium only). Growth was good on the positive control (basal medium plus glucose). Growth was equal to or greater than the glucose control on the basal medium plus L-arabinose, sucrose, D-xylose, inositol, D-mannitol, D-fructose, rhamnose, and raffinose. Growth was doubtful with cellulose.

Temperature. The culture grew well at 18 to 28 C on Bennett and Czapek sucrose agars and at 18 to 37 C on maltose-tryptone agar. There was no growth at 45 and 55 C on Bennett's and Czapek's sucrose agar and maltose-tryptone agar.

Antibiotic-producing properties. The culture produced the antimetabolite antibiotic U-42,126.

Source of culture. The culture was isolated from soil.

Production. The fermentation studies were carried out in 500-ml flasks, and the titers of the drug were monitored by a microbiological assay with *B. subtilis* cultivated in completely synthetic agar. The results of a typical fermentation are presented in Table 4. The peak titers were usually reached after 48 h of incubation.

Paper chromatography. The R_f values of U-42,126, when bioautographed against *B. subtilis*, were 0.45 for system 1 and 0.6 for system 2.

TABLE 4. *Fermentation of Streptomyces sviceus in 500-ml flasks*

Time (h)	Drug titer (Biounits/ml)[a]	pH
48	41	7.7
72	28	8.2
96	25	7.5
120	17	8.3

[a] The biounit is defined as the concentration of the drug which gives a 20-mm zone of inhibition around a 13.6-mm paper disk under the standard conditions of an assay.

In vitro antimicrobial activity. The antimicrobial spectrum is presented in Table 5. The drug was inactive against the bacteria tested, except for *B. subtilis* and *Escherichia coli* cultivated in completely synthetic agar. However, it inhibited rather strongly all four fungi tested. The inhibition of *B. subtilis* and *E. coli* can be prevented by supplementing the synthetic cultivation media with histidine.

In vivo antitumor testing. The whole, lyophilized fermentation liquors were inactive against L1210 and P-388 leukemias at the highest nontoxic levels tested (200 mg and 400 mg/kg, respectively). However, the first partially purified preparation (~0.2% pure) was active in vivo in the same test systems. At a dose of 400 mg/kg, it extended the life of mice infected with L1210 leukemia by 40% over the untreated controls. The corresponding value for the P-388 leukemia was 37%. Data on the in vivo antitumor activity of pure U-42,126 will be presented in detail in a separate communication (L. J. Haňka et al., Cancer Chemother. Rep., in press).

TABLE 5. *Antimicrobial spectrum of U-42,126 (500 μg/ml)*

Microorganism	Upjohn UC no.	Zone of inhibition (mm) around a 13-mm paper disk
Bacillus subtilis (in synthetic agar)	564	78
B. subtilis (in nutrient agar)	564	0
Lactobacillus casei	60	0
Sarcina lutea	130	0
Staphylococcus aureus	80	0
Mycobacterium avium	159	0
Escherichia coli (in nutrient agar)	51	0
E. coli (in synthetic agar)	51	36 hazy
Salmonella schottmuelleri	126	0
Proteus vulgaris	93	0
Klebsiella pneumoniae	57	0
Saccharomyces pastorianus	1342	53
Penicillium oxalicum	1268	32
Candida albicans (tested at 50 μg/ml)	1392	20
Saccharomyces cerevisiae (tested at 50 μg/ml)	1606	38

TABLE 6. *Differentiation of Streptomyces sviceus UC-5370 and S. hawaiiensis ATCC 12236, UC-2504*

Microorganism	Reference color (23)	Sporophores (12)	Spore surface (4)	Gelatin (plain and nutrient)	Litmus milk	Tyrosine-agar (ISP-7)	Antibiotic production
S. sviceus UC-5370	White to gray	Long, straight, and coiled at tip; many candelabrum-like	Warty to spiny (spines short and sparse)	No liquefaction	pH 5.3	Melanin positive	U-42, 126
S. hawaiiensis ATCC 12236, UC-2504	White to yellow to red	Moderately short; open spiral to spiral	Spiny (spines short to long)	Complete liquefaction	pH 6.1	Melanin negative	Bryamycin

DISCUSSION

S. sviceus is an actinomycete with characteristics of the genus *Streptomyces* as set forth in *Bergey's Manual of Determinative Bacteriology*, 7th ed. The culture, which was isolated from soil, is distinctly different from *Streptomyces* species in the Upjohn culture collection and, insofar as can be determined, from those in the literature descriptions in *Bergey's Manual* and in references 5, 10, 16, 20–22, and 24.

S. sviceus showed some similarity to *S. hawaiiensis* (1) ATCC 12236. Both cultures are melanin positive and have similar carbon utilization patterns in the synthetic medium of Pridham and Gottlieb. *S. hawaiiensis* has open spiral sporophores which are shorter and less distinctive than those of the new culture, which are long and coiled at the tip. The spores, as observed by transmission electron microscopy, are round to oval and covered with fine spines for *S. hawaiiensis* and oval to rectangular with a sparsely warty to spiny surface for *S. sviceus*. Distinguishing characteristics are cited in Table 6.

S. sviceus is easily distinguished by its distinctive color pattern and microscopy characteristics from characterized species of *Streptomyces* in the Upjohn culture collection and, as far as can be determined, from those cultures characterized in the literature. The cultural characteristics cited in the tables reinforce the distinctive features of *S. sviceus*. A unique property of this organism is its ability to produce the antitumor, antimetabolite U-42,126. The organism characterized in this paper is considered a new type species of *Streptomyces* and is designated *S. sviceus* sp.n., Dietz (from svíc or svícen, Czech for candle or candle holder; sporophores of culture are candelabrum-like). The type culture *S. sviceus* is maintained in the Upjohn collection as UC-5370 and in the NRRL collection as NRRL 5439. The type species is designated the type variety *S. sviceus* var. *sviceus* in accordance with Rule 7 of the International Code of Nomenclature of Bacteria (11).

The in vitro antimicrobial activity of U-42,126 was limited to fungi. Bacteria inhibited by U-42,126 were *E. coli* and *B. subtilis* when cultivated in a completely synthetic medium. Such response is fairly typical for an antimetabolite (6, 7, 9).

ACKNOWLEDGMENTS

This investigation was supported by the Public Health Service contract no. PH43-68-1023 from the National Cancer Institute.

The technical assistance of J. M. Swanson and W. E. Titus is acknowledged. The chromatography was done by L. M. Reineke, J. L. Fox, and M. S. Barnett. Transmission electron micrograph was courtesy of John

Mathews. The scanning electron micrograph was taken at the John A. Brown SEM Service.

LITERATURE CITED

1. Cron, M. J., D. F. Whithead, I. R. Hooper, B. Heinemann, and J. Lein. 1956. Bryamycin, a new antibiotic. Antibiot. Chemother. **6**:63–67.
2. Dietz, A. 1954. Ektachrome transparencies as aids in actinomycete classification. Ann. N.Y. Acad. Sci. **60**:152–154.
3. Dietz, A. 1967. *Streptomyces steffisburgensis* sp.n. J. Bacteriol. **94**:2022–2026.
4. Dietz, A., and J. Mathews. 1971. Classification of *Streptomyces* spore surfaces into five groups. Appl. Microbiol. **21**:527–533.
5. Gauze, G. F., T. P. Preobrazenskaya, E. S. Kudrina, N. O. Blinov, I. D. Ryabova, and M. A. Iveshnikova. 1957. Problems in the classification of antagonistic actinomycetes. State Publishing House for Medical Literature, Moscow. (English ed. translated by Fritz Danga, David Gottlieb [ed.].) The American Institute of Biological Sciences, Washington, D. C.
6. Hańka, L. J. 1967. *In vitro* screen for antimetabolites. Proc. 5th Int. Congr. Chemother. **B9,2**: 351–357.
7. Hańka, L. J., M. E. Bergy, and R. B. Kelly. 1966. Naturally occurring antimetabolite antibiotic related to biotin. Science **154**:1667–1668.
8. Hańka, L. J., J. S. Evans, D. J. Mason, and A. Dietz. 1967. Microbial production of 5-azacytidine. I. Production and biological activity. Antimicrob. Ag. Chemother. 1966, p. 619–624.
9. Hańka, L. J., D. G. Martin, and L. M. Reineke. 1972. Two new antimetabolites of biotin: α-methyldethiobiotin and α-methylbiotin. Antimicrob. Ag. Chemother. **1**:135–138.
10. Hütter, R. 1967. Systematik der Streptomyceten unter besondere Berücksichtigung der von ihnen gebildeten Antibiotica. S. Karger, Basel.
11. International Code of Nomenclature of Bacteria. 1966. Edited by the Editorial Board of the Judicial Commission of the International Committee on Nomenclature of Bacteria. Int. J. Syst. Bacteriol. **16**:459–490.
12. Jacobson, E., W. C. Granville, and C. E. Foss. 1948. Color harmony manual, 3rd ed. Container Corp. of America, Chicago, Ill.
13. Kelly, K. L., and D. B. Judd. 1955. The ISCC-NBS method of designating colors and a dictionary of color names. U.S. Dep. Comm. Circ. 553.
14. Kelly, R. B., D. G. Martin, and L. J. Hanka. 1969. 2-Amino-4-methyl-5-hexenoic acid, a naturally occurring antimetabolite antibiotic. Can. J. Chem. **47**:2504–2506.
15. Korobkova, T. P., T. S. Maksimova, I. N. Kovsharova, and L. P. Terekhova. 1971. Production of antibiotic antimetabolites of amino acids and vitamins by actinomycetes. Antibiotiki **1**:36–39.
16. Krassil'nikov, N. A. 1949. *Actinomycetes*. *In* Guide to the identification of bacteria and *Actinomycetes*. Academy of Sciences, U.S.S.R., Moscow. English Edition translated by J. B. Routien, Chas. Pfizer and Co., Inc., 1957.
17. Pridham, T. G., and D. Gottlieb. 1948. The utilization of carbon compounds by some actinomycetales as an aid for species determination. J. Bacteriol. **56**:107–114.
18. Pridham, T. G., C. W. Hesseltine, and R. G. Benedict. 1958. A guide for the classification of streptomycetes according to selected groups. Placement of strains in morphological sections. Appl. Microbiol. **6**:52–79.
19. Shirling, E. B., and D. Gottlieb. 1966. Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. **16**:313–340.
20. Shirling, E. B., and D. Gottlieb. 1968. Cooperative description of type cultures of *Streptomyces*. II. Species descriptions from first study. Int. J. Syst. Bacteriol. **18**:69–189.
21. Shirling, E. B., and D. Gottlieb. 1968. Cooperative description of type cultures of *Streptomyces*. III. Additional species descriptions from first and second studies. Int. J. Syst. Bacteriol. **18**:279–392.
22. Shirling, E. B., and D. Gottlieb. 1969. Cooperative description of type cultures of *Streptomyces*. IV. Species descriptions from the second, third and fourth studies. Int. J. Syst. Bacteriol. **19**:391–512.
23. Tresner, H. D., and E. J. Backus. 1963. System of color wheels for streptomycete taxonomy. Appl. Microbiol. **11**:335–338.
24. Waksman, S. A. 1961. The actinomycetes: classification, identification, and descriptions of genera and species, vol. 2. The Williams & Wilkins Co., Baltimore.