Thurston, J. T.; Dudley, J. R.; Kaiser, D. W.; Hachenbleikner, I.; Schaefer, F. C.; Holm-Hansen, D. "Cyanuric Chloride Derivatives. 1. (Aminochloro)-s-triazines". *J. Am. Chem. Soc.* **1951**, *73*, 2981–2983.

Trebst, A. "The Topology of the Plastquinone and Herbicide Binding Peptides of Photosystem II in the Tylakoid Membrane". *Z. Naturforsch., C.*: Biosci. **1986**, *41C*, 240–245.

Verloop, A.; Hoogenstraaten, W.; Tipker, J. "Development and Application of New Steric Substituent Parameters in Drug Design". In *Drug Design*; Ariëns, E. J., Ed.; Academic: New York, 1976; Vol. VII.

Received for review November 23, 1987. Accepted April 20, 1988.

Production of Valinomycin, an Insecticidal Antibiotic, by *Streptomyces griseus* var. *flexipertum* var. *nov.*†

Rod M. Heisey,*[1] Jamin Huang,[2] Saroj K. Mishra,[3] James E. Keller, James R. Miller, Alan R. Putnam, and Themistocles D. J. D'Silva[2]

A screening program to discover microorganisms that produce novel pesticides has yielded a new streptomycete strain that produces valinomycin, an insecticidal and acaricidal antibiotic. Bioassays of the crude culture broth produced by this strain demonstrated an LC_{50} to mosquito larvae of 10^{-3}–10^{-4} dilution. Bioassays of the purified insecticide yielded LC_{50} values of 2–3 pm for mosquito larvae, 3 ppm for two-spotted spider mites, and 35 ppm for Mexican bean beetle larvae. Taxonomic studies indicated the valinomycin-producing microorganism was an atypical variant of *Streptomyces griseus*, which is hereby named var. *flexipertum* var. *nov.* Morphology and physiology of the new microorganism and production, isolation, and identification of the insecticidal metabolite are described.

Much interest currently exists in discovering metabolites of microorganisms that have potential for use as pesticides and plant growth regulators (American Chemical Society, 1987). We have been testing soil microorganisms for the production of such compounds (Heisey et al., 1985, 1988; Heisey and Putnam, 1986; Mishra et al., 1987a,b, 1988; Huang et al., 1988). One isolate, an atypical strain of *Streptomyces griseus*, produced culture broth that was strongly active against mosquito larvae. Chemical analyses revealed the presence of valinomycin, an insecticidal antibiotic (Figure 1). Valinomycin has previously been reported as a product of *Streptomyces fulvissimus* and a similar strain (Brockman and Schmidt-Kastner, 1955; Brown et al, 1962) and *Streptomyces roseochromogenes* (Patterson and Wright, 1970). It has been considered for insecticidal, nematocidal, and acaricidal use (Patterson and Wright, 1970; Pansa et al., 1973). Valinomycin has not heretofore been reported from *S. griseus* strains. This paper describes a new valinomycin-producing variant of *S. griseus* and the isolation, identification, and characterization of the insecticidal metabolite.

Pesticide Research Center, Michigan State University, East Lansing, Michigan 48824 (R.M.H., S.K.M., J.E.K., J.R.M., A.R.P.), and Union Carbide Agricultural Products Company, Box 12014, T. W. Alexander Drive, Research Triangle Park, North Carolina 27709 (J.H., T.D.J.D.).

[1] Present address: Department of Biological Sciences, Fordham University, Bronx, NY 10458.

[2] Present address: Rhone-Poulenc Ag Co., P.O. Box 12014, T. W. Alexander Dr., Research Triangle Park, NC 27709.

[3] Present address: Krug International, Suite 120, 1290 Hercules Dr., Houston, TX 77058.

† The nucleic acid sequence in this paper has been submitted to In Vitro International under Accession Numbers 10129 and 10130.

MATERIALS AND METHODS

Isolation of Microorganism. The valinomycin-producing strain was isolated from surface soil collected in 1982 in Ingham County, Michigan, near a manure pile in an outdoor cattle feeding area. The soil was mixed with calcium carbonate (1 g:1 g) and incubated 7–10 days at room temperature in a sterile Petri dish containing water-saturated filter paper above the mixture to maintain high humidity (El-Nakeeb and Lechevalier, 1963). Serial dilutions were plated onto arginine–glycerol–nutrient salts agar (El-Nakeeb and Lechevalier, 1963) and incubated at 28 °C. Actinomycete colonies that developed were transferred to other plates and used to inoculate liquid cultures for tests of insecticidal activity. The valinomycin-producing strain was distinguished on the basis of the potent insecticidal activity it produced in shaken broth culture. It is deposited with In Vitro International (611 P Hammondsferry Road, Linthicum, MD; accessions 10129, 10130).

Taxonomy of Microorganism. Growth of *S. griseus* var. *flexipertum* var. *nov.* was tested on glycerol–casitone (GC) agar (glycerol, 70 mL; Bacto casitone, 5 g; Bacto agar, 15 g; distilled water, 1 L; pH 7.0) and yeast extract–malt extract–glucose (YMG) agar (Mishra et al., 1980; Mishra and Gordon, 1986). Species identification was according to Mishra et al. (1980) and Mishra and Gordon (1986).

Spore chain morphology and spore surface texture were examined with scanning electron microscopy (Kutzner, 1982). The occurrence of diaminopimelic acid isomers was determined by paper chromatography according to Becker et al. (1964).

Culturing for Insecticide Production. The producer microorganism was grown in A-9 medium (Bacto peptone, 5 g; glucose, 10 g; Brer Rabbit green label molasses, 20 g) (Warren et al., 1955). Antifoam-A (Sigma Chemical Co.,

0021-8561/88/1436-1283$01.50/0

Table I. Eluting Solvents and Mosquitocidal Activity of Fractions from Flash Column Chromatography of *S. griseus* var. *flexipertum* var. *nov.* Crude Cell Extract Prepared as in Figure 2

	fraction							
	F1	F2	F3	F4	F5	F6	F7	F8
eluting solvent (MeOH/H_2O, %)	60	60	60	60	80	80, 90	90	100
LC_{50}, μg/mL (48 h)	>20	>20	>20	>20	5-10	5	5	>20

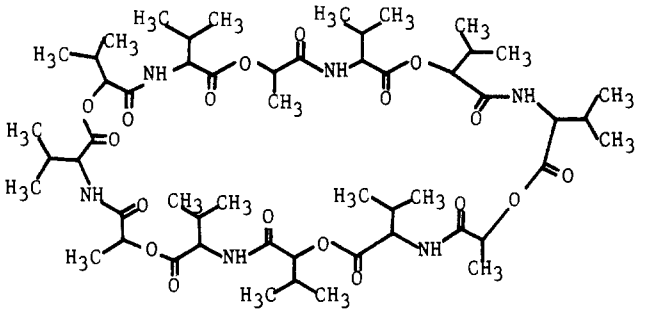

Figure 1. Valinomycin, an insecticidal antibiotic produced by *S. griseus* var. *flexipertum* var. *nov.*

St. Louis, MO), a silicone polymer, was added to reduce foaming. Two cultures were produced, one of 15 L in a 20-L glass carboy and a second of 75 L in a 100-L stainless steel fermentor. The carboy was inoculated with *S. griseus* var. *flexipertum* var. *nov.* growing on solid A-9 medium (containing 1.5% agar). Agitation and aeration in the carboy were carried out with a magnetic stirrer and filtered air, which was bubbled through the medium. Incubation was for 4 days at room temperature (about 22 °C). The stainless steel fermentor was inoculated with 5 L of 2-day-old liquid culture grown in 2-L flasks on an orbital shaker at 28 °C and 100 rpm. Incubation was for 5 days at 30 ± 2 °C, with stirring of 150–250 rpm and aeration of 100 L of air/min initially and 50 L/min after foaming began. The pH of the culture in the fermentor was adjusted to 7 with HCl or KOH twice during incubation.

Isolation and Identification of Valinomycin. Cells of *S. griseus* var. *flexipertum* var. *nov.* were centrifuged from the culture broth and frozen. After thawing, the cells (1507 g wet weight) were slurried in 3 L of methanol–dichloromethane (1:3) and extracted three times with 4 L of the same solvent, followed by a final extraction with 4 L of dichloromethane. The extracts were combined and taken to dryness in vacuo.

Two methods were used to isolate and purify the insecticidal compound. In one, the insecticidal activity was first concentrated on the basis of solubility or ability to partition into several solvents (Figure 2). Subsequent fractionation was with flash column chromatography on reversed-phase Partisil Prep 40 ODS-3 (Whatman Inc., Clifton, NJ) with a stepwise methanol–water gradient increasing from 60 to 100% methanol (Table I). Final purification was with HPLC on reversed-phase octadecylsilane (Waters μBondapak in Z-module radial compression unit) with 87% methanol–water (3 mL/min) and UV detection (230 nm). In the second purification procedure, the crude cell extract was chromatographed on Florisil (100–200 mesh). The insecticide was eluted from the column with a hexane–ethyl acetate gradient.

Insecticidal activity was followed in broth culture, extraction, and purification with bioassays on third- and fourth-instar mosquito larvae (*Aedes aegyptii*, Rockefeller strain). This bioassay is a simple, sensitive indicator of toxicity (Ando, 1982). Culture broth was tested for activity in 226-mL polystyrene urine specimen cups in dilutions prepared with distilled water; controls were similar dilutions of cuture broth, which was kept sterile until use in

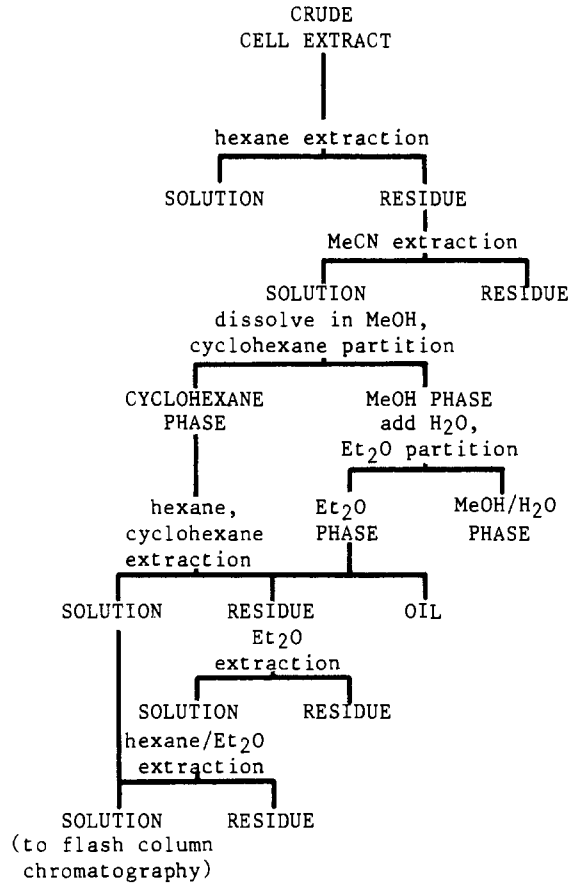

Figure 2. Preliminary purification of *S. griseus* var. *flexipertum* var. *nov.* crude cell extract for flash column chromatography.

these assays. Nonaqueous samples obtained during extraction and purification of the insecticide were assayed by dissolving them in methanol and adding appropriate volumes of the solutions to 2 mL of distilled water in 1.3 × 10 cm glass culture tubes. Additional methanol was added, as necessary, to equalize the methanol concentrations of all tubes in a particular bioassay. Controls contained an identical concentration of methanol, but no sample material. Methanol concentrations in these bioassays typically were less than or equal to 1% and never exceeded 2%. Toxicity resulting from the methanol alone was not observed at these concentrations. Approximately 10 mosquito larvae were added per tube. Two replicate tubes were used for each treatment and control.

The purified valinomycin was also tested on two-spotted spider mites (*Tetranychus urticae*) and Mexican bean beetle larvae (*Epilachna varivestis*). These bioassays were done according to Payne et al. (1966) except that foliage to which the bean beetle larvae were exposed was treated by spraying plants on a turntable (Hansberry, 1943) instead of dipping excised leaves.

^1H NMR spectra were obtained at 90 MHz and ^{13}C NMR spectra at 22.5 MHz on a JEOL FX-900 Fourier transform spectrometer with tetramethylsilane as an internal standard. Infrared spectra were recorded on a Perkin-Elmer 197 spectrophotometer. High-resolution electron impact mass spectra were obtained with an AEI

Table II. Comparison of Physiological and Morphological Characteristics of *Streptomyces* Strains Known To Produce Valinomycin

strain	acid production on						spore surface	spore chain morphology	pigmentation
	arabinose	xylose	inositol	mannitol	rhamnose	raffinose			
S. fulvissimus	+	+-	+	+	+-	+	smooth	rectus flexibilis	melanoid
S. roseochromogenes	-	+	-	+	-	-	spiny	spiral to rectus flexibilis	soluble
S. griseus var. *flexipertum* var. *nov.*	-	+	-	+	-	-	smooth	rectus flexibilis to rectinaculum apertum	no melanoid pigments

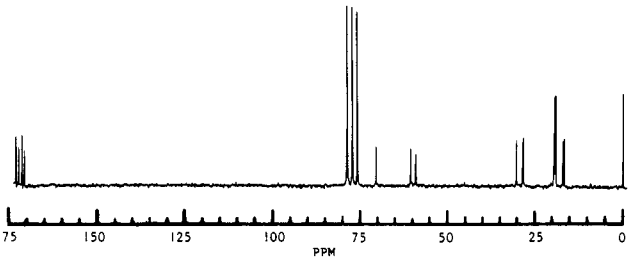

Figure 3. ^{13}C NMR spectrum of the insecticidal compound produced by *S. griseus* var. *flexipertum* var. *nov.* Chemical shifts ($CDCl_3$): 172.4 (s), 171.5 (s), 170.8 (s), 170.0 (s), 78.6 (d), 70.3 (d), 60.4 (d), 58.9 (d), 30.3 (d), 28.6 (q), 28.4 (q), 19.7 (q), 19.5 (q), 19.1 (q), 17.1 (q), 16.9 (q) ppm.

Model MS 902 double-focusing spectrometer having static resolution of approximately 10 000 amu.

RESULTS AND DICUSSION

Taxonomy. *S. griseus* var. *flexipertum* var. *nov.* colonies were powdery or dusty in texture on GC agar and were leathery on YMG agar. Aerial mycelia, which were white to cream, were abundant on GC agar but were scanty on YMG agar. Abundant aerial mycelia were also produced on synthetic media containing either maltose, mannitol, trehalose, or xylose as the sole carbon source (Mishra et al., 1980; Mishra and Gordon, 1986). Brown diffusible pigment was produced on GC and A-9 agar and became intense after 3–4 weeks. Dark brown pigmentation also developed after several days of growth in A-9 broth shaken at 100 rpm and 28 °C.

The isolate decomposed adenine, casein, hypoxanthine, L-tyrosine, and xanthine. It produced acid on cellobiose, methyl α-D-glucoside, D-(+)-galactose, glucose, D-(+)-lactose, D-(+)-maltose, D-(−)-mannitol, D-(+)-trehalose, and D-(+)-xylose. Acid production was not observed with adonitol, L-(+)-arabinose, L-erythritol, L-inositol, D-(+)-melibiose, D-sorbitol, D-(+)-melezitose, D-(+)-raffinose, and D-(+)-rhamnose. No growth was observed in lysozyme broth. Paper chromatography of the whole-cell hydrolysate revealed the presence of large amounts of LL-diaminopimelic acid.

A comparison of the available data on taxonomic characteristics of valinomycin-producing microorganisms suggests our isolate is physiologically, as well as morphologically, different (Table II). The spore chain morphology of our isolate was very unusual, varying from retinaculum apertum to rectus flexibilis. Arthrospores were rare and, when present, irregular in size and shape. Scanning electron microscopy showed a smooth spore surface. The gross morphology of colonies, texture of the spore surface, and physiological properties of our organism demonstrated it belonged to the *S. griseus* complex. No *S. griseus* strains have been reported, however, to have rectus flexibilis to retinaculum apertum spore chains (Hutter, 1967; Pridham, 1976). The uniqueness of this important taxonomic attribute enables us to designate our isolate as a new variety, *S. griseus* var. *flexipertum* var. *nov.* The powdery growth on GC agar, the white to cream spore mass, the smooth spore surface with irregular arthrospores, and the aforementioned spore chain morphology and physiological characteristics constitute, in concert, the diagnostic features of this new variety.

Characterization of the Insecticide. The LC_{50} of the *S. griseus* var. *flexipertum* var. *nov.* culture broth to mosquito larvae was 10^{-3}–10^{-4} dilution after 4 h. Extraction of 1507 g (wet weight) of the cell cake with methanol–dichloromethane yielded 17.7 g of crude extract having an LC_{50} of approximately 50 μg/mL on mosquito larvae after 4 h and 16 ppm on two-spotted spider mites. Sample cleanup via the procedure of Figure 2, in preparation for flash column chromatography, yielded an extract having an LC_{50} to mosquito larvae of 4–8 μg/mL after 24 h. The most toxic fractions produced in flash chromatography (F6 and F7, Table I) eluted with 80 and 90% methanol–water and had LC_{50} values of 5 μ/mL on mosquito larvae after 48 h. Further separation of F7 with HPLC yielded a purified compound having a retention time of 10 min. This compound had LC_{50} values of 2–3 μg/mL on mosquito larvae after 36 h, 3 ppm on two-spotted spider mites, and 35 ppm on Mexican bean beetle larvae.

High-resolution mass spectrometry of the purified insecticide established an empirical formula of $C_{54}H_{90}N_6O_{18}$ (at *m/e* 1110 for M^+, measured mass 1110.6311, calculated mass 1110.6311). The presence of only 16 nonequivalent carbon peaks on the ^{13}C NMR spectrum (Figure 3) suggested the material was the trimer of a subunit containing 16 nonequivalent carbon atoms. This early recognition was helpful in the final elucidation of structure. The mass spectral fragmentation pattern indicated the presence of $(CH_3)_2CHCH{=}O$ [at *m/e* 72, 72.0576; calculated for C_4H_8O, 72.0575], $(CH_3)_2CHCH_2NH$ [at *m/e* 72, 72.0814; calculated for $C_4H_{10}N$, 72.0813], and $(CH_3)_2CHCH_2NH(C{=}O)CHCH(CH_3)_2$ [at *m/e* 155, 155.1325; calculated for $C_9H_{17}NO$, 155.1310] in the molecule. The IR spectrum also indicated the presence of an ester group (1755 cm^{-1}) and a primary amide group (1660, 1540 cm^{-1}). These data were consistent with the structure of the known antibiotic valinomycin. The structure was unequivocally confirmed by comparison of the ^{13}C NMR, ^1H NMR, and IR spectra with those of an authentic sample of valinomycin (Sigma).

The microbial strain reported here is, therefore, an atypical variant of *S. griseus* that produces valinomycin. It may have potential, following strain improvement and culture optimization, for commercial production of this insecticidal antibiotic. The possibility of growing *S. griseus* var. *flexipertum* var. *nov.* in broth culture and using the cells directly in insecticidal preparations, thus eliminating expensive chemical extraction and purification, merits further investigation.

ACKNOWLEDGMENT

We thank E. C. Bailey for producing the NMR spectra; L. Hall for help with HPLC; K. A. Kukorowsky and members of his group, especially P. R. Timmons, for assaying insecticidal and acaricidal activity; D. Grant and F. Matsumura for *A. aegyptii* eggs; H. H. Moorefield for his enthusiasm and help in characterizing acaricidal ac-

tivity; H. Sadoff for the use of his fermentor; and S. Singhawangcha for aid in structural elucidation.

Registry No. Valinomycin, 2001-95-8.

LITERATURE CITED

American Chemical Society. "Biologically Active Natural Products for Potential Use in Agriculture". Symposium at the 194th National Meeting of the American Chemical Society, New Orleans, LA, 1987.

Ando, K. "How to Discover Antibiotics for Insecticidal Use". In *Pesticide Chemistry: Human Welfare and the Environment*; Miyamoto, J., Kearney, P. C., Eds.; Pergamon: New York, 1982; Vol. 2, pp 253–260.

Becker, B.; Lechevalier, M. P.; Gordon, R. E.; Lechevalier, H. A. "Rapid Differentiation between *Nocardia* and *Streptomyces* by Paper Chromatography of Whole-Cell Hydrolysates". *Appl. Microbiol.* **1964**, *12*, 421–423.

Brockman, H.; Schmidt-Kastner, G. "Valinomycin I, XXVII. Mitteilung uber Antibiotica aus Actinomyceten". *Chem. Ber.* **1955**, *88*, 57–61.

Brown, R.; Brennan, J.; Kelley, C. "An Antifungal Agent Identical with Valinomycin". *Antibiot. Chemother.* **1962**, *12*, 482–487.

El-Nakeeb, M. A.; Lechevalier, H. A. "Selective Isolation of Aerobic Actinomycetes". *Appl. Microbiol.* **1963**, *11*, 75–77.

Hansberry, R. In *Laboratory Procedures in Studies of the Chemical Control of Insects*; Campbell, F. G., Monlton, F. R., Eds.; American Association for the Advancement of Science: Boulder, CO, 1943.

Heisey, R. M.; Putnam, A. R. "Herbicidal Effects of Geldanamycin and Nigericin, Antibiotics from *Streptomyces hygroscopicus*". *J. Nat.* Prod. **1986**, *49*, 859–865.

Heisey, R. M.; DeFrank, J.; Putnam, A. R. *A Survey of Soil Microorganisms for Herbicidal Activity*; Thompson, A. C., Ed.; The Chemistry of Allelopathy, ACS Symposium Series 268; American Chemical Society: Washington, DC, 1985; pp 337–349.

Heisey, R. M.; Mishra, S. K.; Putnam, A. R.; Miller, J. R.; Whitenack, C. J.; Keller, J. E.; Huang, J. *Production of Herbicidal and Insecticidal Metabolites by Soil Microorganisms*; Cutler, H. G., Ed.; American Chemical Society: Washington, DC, 1988, in press.

Huang, J.; Putnam, A. R.; Werner, G. M.; Mishra, S. K.; Whitenack, C. J. "Herbicidal Metabolites from a Soil-Dwelling Fungus, *Scopulariopsis brumptii*". *Weed Sci.* **1988**, in press.

Hutter, R. *Systematik der Streptomyceten*; Karger: New York, 1967.

Kutzner, H. J. "The Family Streptomycetaceae". In *The Procaryotes*; Starr, M. P., Stolp, H., Truper, H. G., Barlows, A., Schlegel, H. G., Eds.; Springer-Verlag: New York, 1982; pp 2028–2090.

Mishra, S. K.; Gordon, R. E. "*Nocardia* and *Streptomyces*". In *Infectious Diseases and Medical Microbiology*; Braude, A. I., Davies, C. E., Fierer, J., Eds.; Saunders: Philadelphia, 1986; pp 371–381.

Mishra, S. K.; Gordon, R. E.; Barnett, D. "Identifiation of Nocardia and Streptomycetes of Medical Importance". *J. Clin. Microbiol.* **1980**, *11*, 728–736.

Mishra, S. K.; Taft, W. H.; Putnam, A. R.; Ries, S. K. "Plant Growth *Regulatory* Metabolites from Novel Actinomycetes". *J. Plant Growth Regul.* **1987a**, *6*, 75–84.

Mishra, S. K.; Keller, J. E.; Miller, J. R.; Heisey, R. M.; Nair, M. G.; Putnam, A. R. "Insecticidal and Nematocidal Properties of Microbial Metabolites". *J. Indust. Microbiol.* **1987b**, *2*, 267–276.

Mishra, S. K.; Whitenack, C. J.; Putnam, A. R. "Herbicidal Properties of Metabolites from Several Genera of Soil Microorganisms". *Weed Sci.* **1988**, *36*, 122–126.

Nonomura, H. "Key for Classification and Identification of 458 Species of the Streptomycetes Included in ISP". *J. Ferment. Technol.* **1974**, *2*, 78–92.

Pansa, M. C.; Natalizi, G. M.; Bettini, S. "Toxicity of Valinomycin on Insects". *J. Invert. Pathol.* **1973**, *22*, 148–152.

Patterson, E. L.; Wright, P. U.S. Patent No. 3520973, July 21, 1970.

Payne, L. K., Jr.; Stansbury, H. A., Jr.; Weiden, M. H. J. "The Synthesis and Insecticidal Properties of Some Cholinergic Trisubstituted Acetaldehyde *O*-(Methylcarbamoyl) Oximes". *J. Agric. Food Chem.* **1966**, *14*, 356–365.

Pridham, T. G. "Identification of Streptomycetes and Streptoverticillia at the Species Level". In *Actinomycetes: the Boundary Organisms*; Arai, T., Ed.; Toppan: Tokyo, 1976; pp 175–182.

Shirling, E. B.; Gottlieb, D. "Methods for Characterization of *Streptomyces* Species". *Int. J. Syst. Bacteriol.* **1966**, *16*, 313–340.

Warren, H. B., Jr.; Prokop, J. F.; Grundy, W. E. "Nonsynthetic Media for Antibiotic Producing Actinomycetes". *Antibiot. Chemother.* **1955**, *5*, 6–12.

Received for review November 9, 1987. Accepted May 16, 1988. Journal article No. 12574 from the Michigan Agricultural Experiment Station.