Preliminary Taxonomic Study of Members of the Order *Actinomycetales* That Produce Antibiotics of the Aureolic Acid Group

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Fifteen strains of *Actinomycetales* alleged to produce antibiotics belonging to the aureolic acid group, some of which are useful as antitumor agents, were compared taxonomically with *Streptomyces cavourensis* subsp. *washingtonensis* strain AUW-83, a strain which produces chromomycin antibiotics. Of these strains, *Streptomyces aburaviensis* ATCC 23869, *Streptomyces minutiscleroticus* ATCC 17757, and *S. cavourensis* subsp. *washingtonensis* strain AUW-83 are type strains. None of the strains were regarded as similar enough to strain AUW-83 to be placed in *S. cavourensis* subsp. *washingtonensis*. Bristol's *Streptomyces* sp. C-14795, a chromomycin-producing strain which has not been previously reported in the literature, and *S. aburaviensis* ATCC 23869 appear to be different strains of the same species; the same is true of *Streptomyces griseus* no. 7 and *Streptomyces* sp. KCC S-1081 and of *Streptomyces argillaceus* F.D. no. 6590 and *Streptomyces* sp. M-198. However, to establish the identities and taxonomic relationships of these 15 strains, further studies involving direct comparisons with type and/or neotype strains of closely related nomenclatures will be required.

The aureolic acid group of antibiotics produced by members of the order *Actinomycetales* consists of aromatic glycosides of three recognized structural types: the aureolic acid, the chromomycin, and the olivomycin types (1). One member of each type, namely, mithramycin, chromomycin A₃, and olivomycin A, respectively, is of clinical usefulness as an antitumor agent, and these have recently been the subject of a review (25). Since 1953, the occurrence of over 20 antibiotic substances belonging to this group has been recorded. A chronological listing of these antibiotics, along with the strains which produce them, has been presented in our previous report describing the fermentation, isolation, and identification of chromomycin A₃ from *Streptomyces cavourensis* subsp. *washingtonensis* strain AUW-83 (23).

Reported here are the results of a direct taxonomic comparison of strain AUW-83 with 15 strains of *Actinomycetales* that have been claimed to produce antibiotics belonging to the aureolic acid group. The main purpose of this report is to present the taxonomic features of *S. cavourensis* subsp. *washingtonensis* strain AUW-83 that distinguish it from the other 15 study strains.

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bek in partial fulfillment of the requirements for the degree of Doctor of Philosophy.)

MATERIALS AND METHODS

Bacterial strains. A list of the strains used in this study is presented in Table 1. Cultures of these strains were acquired in 1972 and 1973. Strains Lilly M5-18903, Gruppo Lepetit L.A. 7017, and INA 3014 were maintained in the sporulating state on Difco ISP medium 4 (inorganic salts-starch agar); all remaining strains were maintained on Difco ISP medium 2 (yeast-malt extract agar).

ISP methods. Procedures recommended by collaborators of the International *Streptomyces* Project (ISP) (20) were employed for determinations of spore chain morphology, spore characteristics, aerial mass color, color of substrate mycelium and soluble pigments, melanoid pigmentation, and carbon utilization. ISP media 1, 2, 3, 4, 5, 6, 7, and 9 were obtained from Difco Laboratories.

Additional materials and methods. The procedure of Gyllenberg and Thelestam (6) was employed for the spectrophotometric evaluation of sporulating cultures for polyenes. Spores and aerial mycelia from cultures grown on both yeast-malt extract agar and oatmeal agar in screw-capped tubes were separately suspended with a loop in 10 ml of ethanol USP, and the suspensions were immediately decanted into test tubes. The suspensions were allowed to remain at room temperature for about 1 h and were then centrifuged at 10,000 rpm for 10 min. The supernatants were carefully decanted, and the spectra of these ethanolic extracts were recorded with a Beckman model

TABLE 1. *List of strains used*

Specific name of strain as received	Strain designation	Source
<i>Actinomyces atroolivaceus</i> (4)	INA 11294 ^a	G. F. Gause, Institute of New Antibiotics, Moscow, U.S.S.R.
<i>A. globisporus</i> subsp. <i>flavofuscus</i> (28)	INA 11296 ^b	G. F. Gause, Institute of New Antibiotics, Moscow, U.S.S.R.
<i>A. nigrificans</i> (28)	INA 3014 ^c	G. F. Gause, Institute of New Antibiotics, Moscow, U.S.S.R.
<i>A. variabilis</i> (28)	INA 232 ^d	G. F. Gause, Institute of New Antibiotics, Moscow, U.S.S.R.
<i>Streptomyces aburaviensis</i> Nishimura et al. (13, 21)	S-66 = ATCC 23869 (Type strain)	American Type Culture Collection, Rockville, Md.
<i>S. argillaceus</i> ^e	F. D. no. 6590 ^f	J. B. Routien, Pfizer Inc., Groton, Conn.
<i>S. cavourensis</i> subsp. <i>washingtonensis</i> Skarbek and Brady	AUW-83 = ATCC 27732 (Type strain) ^g	Laboratory of the authors
<i>S. griseus</i> ^h	No. 7 = ATCC 13273	American Type Culture Collection, Rockville, Md.
<i>S. lavendulae</i>	M5-18903 ⁱ	R. J. Hosley, Eli Lilly and Co., Indianapolis, Ind.
<i>S. minutiscleroticus</i> (Thirumalachar) Pridham (16, 22, 26)	HACC 147 = ATCC 17757 (Type strain)	American Type Culture Collection, Rockville, Md.
<i>S. plicatus</i> ^e	F. D. no. 13756 ^j	J. B. Routien, Pfizer Inc., Groton, Conn.
<i>Streptomyces</i> sp. (18)	C-1228 ^k	J. A. Bush, Bristol Laboratories, Syracuse, N.Y.
<i>Streptomyces</i> sp.	C-14795 ^l	J. A. Bush, Bristol Laboratories, Syracuse, N.Y.
<i>Streptomyces</i> sp.	KCC S-1081 ^m	A. Seino, Kaken Chemical Co., Tokyo, Japan
<i>Streptomyces</i> sp. (19)	L. A. 7017	F. Parenti, Gruppo Lepetit, Milan, Italy
<i>Streptomyces</i> sp.	NA 364-A24 ⁿ = M-198	W. E. Grundy, Abbott Laboratories, Chicago, Ill.

^a Deposited by J. D. Skarbek with the American Type Culture Collection (ATCC 27627) and with the Northern Regional Research Center (NRRL B-8031).

^b Deposited by J. D. Skarbek with the Northern Regional Research Center (NRRL B-8036).

^c Deposited by J. D. Skarbek with the Northern Regional Research Center (NRRL B-8032).

^d Deposited by J. D. Skarbek with the Northern Regional Research Center (NRRL B-8033).

^e Sobin et al., U.S. Patents 3,646,194 and 3,906,093.

^f Also available as ATCC 12956.

^g Also deposited by J. D. Skarbek with the Northern Regional Research Center (NRRL B-8030). Refer to the accompanying paper (24) for the taxonomic description of *S. cavourensis* subsp. *washingtonensis* strain AUW-83.

^h A. Miyake, K. Mizuno, K. Nakazawa, Y. Aramaki, and K. Kaziwara, U.S. Patent 3,313,691, 11 April 1967.

ⁱ No taxonomic information was to be found in the literature; identification of strain M5-18903 as *S. lavendulae* was through personal communication with R. J. Hosley.

^j Also available as ATCC 12957.

^k Produces the A-649 (olivomycin) complex of antibiotics.

^l A chromomycin-producing strain isolated by in-house screening and not previously reported in the literature (J. A. Bush, personal communication).

^m A reputed chromomycin-producing strain for which taxonomic information was not to be found in the literature. Strain KCC S-1081 was isolated by the Microbial Chemistry Research Laboratory (MCRL) of Tanabe Seiyaku Co., Saitama, Japan, from a soil sample collected in Nara Prefecture and deposited with A. Seino under the strain designation MCRL 0317 (personal communication from T. Okuda [MCRL] and from A. Seino).

ⁿ No taxonomic information was to be found in the literature. Two other aureolic acid-producing strains, NA 4-M40 and NA 4-X8, have been discarded and are no longer available (W. E. Grundy, personal communication).

DB spectrophotometer, with or without prior dilution, from 500 to 300 nm. Characteristic absorptions in this wavelength region (6) were taken as presumptive evidence for the presence of representative polyene metabolites extracted into the ethanol from the spores and aerial mycelia of the study strains.

For the determination of sodium chloride tolerance, the method of Tresner et al. (27) was followed. Yeast-malt extract agar was hydrated in sodium chloride solutions of 4, 7, 10, and 13%, dispensed into screw-

capped culture tubes, and autoclaved. One set of tubed media covering the entire range of sodium chloride concentrations and one tube of medium without sodium chloride supplementation were used for each culture. The slanted culture media were inoculated with spores suspended in sterilized water, incubated at 28°C for 10 days, and then observed for the maximal sodium chloride concentration allowing any growth.

To confirm that these strains produced antibiotics belonging to the aureolic acid group, a limited trial

FIG. 1. Light micrographs of sporulating aerial mycelium of available strains of Actinomycetales reported to produce antibiotics of the chromomycin or olivomycin type. Magnification: (1-4, 6, 8, 11, 12) $\times 217$; (5, 7, 9, 10) $\times 347$. (1) *Streptomyces aburaviensis* ATCC 23869. RF morphology on inorganic salts-starch agar, 14 days. (2) *S. griseus* no. 7. RF morphology on yeast-malt extract agar, 14 days. (3) *S. lavendulae* M5-18903. S morphology on oatmeal agar, 14 days. (4) *S. minutiscleroticus* ATCC 17757. S morphology on yeast-malt extract agar, 14 days. Note sclerotia. (5) *Actinomyces nigrificans* INA 3014. S morphology on yeast-malt extract agar, 14 days. (6) *S. cavourensis* subsp. *washingtonensis* AUW-83. RF morphology on glycerol-asparagine agar, 14 days. (7) Strain AUW-83 (single-spore isolate). RF morphology with occasional hook or loop (center of picture) on yeast-malt extract agar, 14 days. (8) Strain AUW-83 (single-spore isolate). RF morphology with incomplete spiral (lower left-hand corner of picture) on yeast-malt extract agar, 14 days. (9) Strain AUW-83 (single-spore isolate). RF morphology with incomplete spiral (slightly above center of picture) on yeast-malt extract agar, 14 days. (10) *Streptomyces* sp. C-1228. RF morphology on oatmeal agar, 14 days. (11) *Streptomyces* sp. C-14795. RF morphology on inorganic salts-starch agar, 14 days. (12) *Streptomyces* sp. KCC S-1081. RF morphology on yeast-malt extract agar, 14 days.

was made using culture and bioassay conditions, extraction procedures, and thin-layer chromatographic methods reported previously (23). Finally, medium 42 (tyrosine-yeast extract agar) of Waksman (31) was employed to complement the standard ISP media used for detection of melanoid pigments.

RESULTS

Spore chain morphology. Light micrographs illustrating the sporophore morphologies of available cultures of *Actinomycetales* claimed to produce antibiotics of the chromomycin or olivomycin type and of the chromomycin-producing *S. cavourensis* subsp. *washingtonensis* strain AUW-83 are presented in Fig. 1. Light micrographs of some sporulating cultures of *Actinomycetales* claimed to produce antibiotics of the aureolic acid type are presented in Fig. 2. The study strains were observed to belong to the sections *Rectiflexibiles* or *Spirales*.

Spore characteristics. Electron micro-

graphs illustrating the spore types of available cultures of *Actinomycetales* claimed to produce antibiotics of the chromomycin or olivomycin type and of the chromomycin-producing *S. cavourensis* subsp. *washingtonensis* strain AUW-83 are presented in Fig. 3. Electron micrographs illustrating the spore types of some *Actinomycetales* claimed to produce antibiotics of the aureolic acid type are given in Fig. 4. The spore surfaces were found to be primarily of two types: smooth or spiny.

Color determinations. The mass colors of mature, sporulating aerial surface growth of all strains on standard media are presented in Table 2. The strains belonged to the Gray, White, Yellow, or Red color series of Tresner and Backus.

Distinctive pigments other than yellow-brown were observed in the reverse mycelium of only one strain, *S. lavendulae* M5-18903. The yellow-brown of the substrate mycelium was modified by a red pigment when the strain was grown on

FIG. 2. Light micrographs of sporulating aerial mycelium of available strains of *Actinomycetales* reported to produce antibiotics of the aureolic acid type. Magnification: (1, 4) $\times 226$; (2, 3, 5-7) $\times 360$. (1) *Streptomyces argillaceus* F. D.6590. S morphology on inorganic salts-starch agar, 14 days. (2) *Actinomyces atroolivaceus* INA 11294. S morphology on oatmeal agar, 14 days. (3) *A. globisporus* subsp. *flavofuscus* INA 11296. RF morphology, but crooked sporophores might suggest some S, on yeast-malt extract agar, 14 days. (4) *S. plicatus* F. D.13756. S morphology on yeast-malt extract agar, 14 days. (5) *Streptomyces* sp. L. A. 7017. S (tight terminal coils) morphology on oatmeal agar, 14 days. (6) *Streptomyces* sp. M-198. S morphology on inorganic salts-starch agar, 14 days. (7) *A. variabilis* INA 232. S (tight terminal coils) morphology on oatmeal agar, 14 days.

FIG. 3. Electron micrographs illustrating spore types of available strains of Actinomycetales reported to produce antibiotics of the chromomycin or olivomycin type. (1) *Streptomyces aburaviensis* ATCC 23869. Smooth spores; 14-day-old culture, inorganic salts-starch agar. (2) *S. aburaviensis* ATCC 23869. Higher magnification; smooth spores with rough surfaces. (3) *S. griseus* no. 7. Smooth spores; 14-day-old culture, yeast-malt extract agar. (4) *S. lavendulae* M5-18903. Smooth spores; 14-day-old culture, inorganic salts-starch agar. (5) *S. minutiscleroticus* ATCC 17757. Smooth spores; 14-day-old culture, glycerol-asparagine agar. (6) *Actinomyces nigrificans* INA 3014. Smooth spores with rough surfaces; 14-day-old culture, inorganic salts-starch agar. (7) *A. nigrificans* INA 3014. Rough spore surfaces appearing to be warty; 14-day-old culture, yeast-malt extract agar. (8) *S. cavourensis* subsp. *washingtonensis* AUV-83. Smooth spores; 14-day-old culture, yeast-malt extract agar. (9) *Streptomyces* sp. C-1228. Smooth spores; 14-day-old culture, yeast-malt extract agar. (10) *Streptomyces* sp. C-14795. Smooth spores; 14-day-old culture, yeast-malt extract agar. (11) *Streptomyces* sp. C-14795. Higher magnification; smooth spores with rough surfaces. (12) *Streptomyces* sp. KCC S-1081. Smooth spores; 14-day-old culture, yeast-malt extract agar.

inorganic salts-starch agar. The red pigment was not a pH indicator.

Diffusible pigments other than brown or black were observed with only four strains. *S. griseus* no. 7 and *Streptomyces* sp. KCC S-1081 exhibited a yellow-brown coloration modified by a green pigment on inorganic salts-starch agar. *S. lavendulae* M5-18903 was observed to secrete a red pigment into inorganic salts-starch agar, and *Streptomyces* sp. L.A. 7017 was found to have a grayish-green diffusible pigment when

grown on glycerol-asparagine agar. In all cases, the diffusible pigments were not sensitive to pH changes.

Observations on the production of melanoid pigments on standard media and on Waksman medium 42 are presented in Table 3. Approximately 40% of these strains were found to be capable of forming melanoid pigments.

Carbon utilization. The utilization of standard carbon sources for growth by all of the strains is given in Table 4. The utilization pat-

FIG. 4. Electron micrographs illustrating spore types of available strains of Actinomycetales reported to produce antibiotics of the aureolic acid type. (1) *Streptomyces argillaceus* F. D.6590. Smooth spores; 14-day-old culture, inorganic salts-starch agar. (2) *Actinomyces atroolivaceus* INA 11294. Smooth spores; 14-day-old culture, yeast-malt extract agar. (3) *A. globisporus* subsp. *flavofuscus* INA 11296. Smooth spores; 14-day-old culture, yeast-malt extract agar. (4) *S. plicatus* F. D.13756. Smooth spores; 14-day-old culture, glycerol-asparagine agar. (5) *S. plicatus* F. D.13756. Smooth spores with only minor surface irregularities; 14-day-old culture, glycerol-asparagine agar. (6) *S. plicatus* F. D.13756. Spiny spores with short spines; 14-day-old culture, yeast-malt extract agar. (7) *S. plicatus* F. D.13756. Spiny spores with short spines; 14-day-old culture, glycerol-asparagine agar. (8) *Streptomyces* sp. L. A. 7017. Smooth spores; 14-day-old culture, oatmeal agar. (9) *Streptomyces* sp. M-198. Smooth spores; 14-day-old culture, yeast-malt extract agar. (10) *A. variabilis* INA 232. Smooth spores; 14-day-old culture, inorganic salts-starch agar.

terns were found to range from poor growth on nearly all carbon sources except glucose to good growth on all carbon sources.

Spectrophotometric analysis for polyenes. Of the strains studied, only the ethanolic extracts of sporulating cultures of *Streptomyces* sp. C-1228 exhibited absorption spectra characteristic of a polyene. The wavelength maxima observed at 340, 360, 380, and 402 nm are characteristic of a heptaene (6).

Tolerance to sodium chloride. Results of the sodium chloride tolerance test are presented in Table 5. Two strains, *S. aburaviensis* ATCC

23869 and *Streptomyces* sp. C-14795, were unable to tolerate 4% sodium chloride. None of the study strains were observed to tolerate 13% sodium chloride.

Bioassay of fermentation broths and thin-layer chromatographic determination of production of aureolic acid group antibiotics. Results of the bioassays and of the thin-layer chromatographic analyses of the fermentation liquors of all strains are presented in Table 6. It was observed that the liquors of several strains previously reported to produce aureolic acid group antibiotics were devoid of

TABLE 2. *Spore mass color of the strains studied*

Strain	Spore mass color on:			
	Yeast-malt extract agar	Oatmeal agar	Inorganic salts-starch agar	Glycerol-asparagine agar
<i>Actinomyces atroolivaceus</i> INA 11294	Gray ^a (3ih) ^b	Gray (3ih)	Gray (3ih)	Gray (e)
<i>A. globisporus</i> subsp. <i>flavofuscus</i> INA 11296	Gray (2dc)	White (b)	Poor sporulation	Poor sporulation
<i>A. nigrificans</i> INA 3014	Gray (d)	Poor sporulation	Gray (d)	White (b)
<i>A. variabilis</i> INA 232	Poor sporulation	Gray (g)	Gray (e)	Poor sporulation
<i>Streptomyces aburaviensis</i> ATCC 23869	Gray (2ih)	Gray (2ih)	Gray (2ih)	Gray (d)
<i>S. argillaceus</i> F.D.6590	Gray (2ih)	Gray (2ih)	Gray (3fe)	Poor sporulation
<i>S. cavourensis</i> subsp. <i>washingtonensis</i> AUW-83	Yellow (1½ ec)	Yellow (1½ ec)	Yellow (1½ ec)	Yellow (2ba)
<i>S. griseus</i> no. 7	Yellow (2db)	Yellow (1½ db)	Yellow (1½ db)	Yellow (2db)
<i>S. lavendulae</i> M5-18903	Red (3ec)	Red (4ec)	Red (4ge)	Red (3ec)
<i>S. minutiscleroticus</i> ATCC 17757	Yellow (24½ dc)	Poor sporulation	Yellow (24½ dc)	Gray (2fe)
<i>S. plicatus</i> F.D.13756	Gray (3ih)	Gray (2dc)	Gray (d)	Gray (2dc)
<i>Streptomyces</i> sp. C-1228	Gray (3ih)	Gray (3ih)	Gray (3ih)	Gray (3ih)
<i>Streptomyces</i> sp. C-14795	Gray (3fe)	Gray (e)	Gray (2ih)	Gray (2dc)
<i>Streptomyces</i> sp. KCC S-1081	Yellow (2db)	Yellow (2db)	Yellow (2db)	Yellow (2ba)
<i>Streptomyces</i> sp. L.A. 7017	Gray (2dc)	Gray (2dc)	White (b)	Gray (2fe)
<i>Streptomyces</i> sp. M-198	Gray (3ih)	Gray (3ih)	Gray (3fe)	Gray (3fe)

^a Tresner-Backus color series.^b Tabs from *Color Harmony Manual*, 4th ed., 1958 (Container Corporation of America, Chicago): d, light gray; e, g, and 2fe, medium gray; 3ih, dark gray; 2dc, yellowish gray; 2ih, light olive gray; 3fe, light brownish gray; 3ec, grayish yellow; 4ec, grayish yellowish pink; 4ge, light grayish reddish brown; 2ba and 2db, pale yellow; 1½ db, pale greenish yellow; 1½ ec and 24½ dc, pale yellow green; b, white. Color names according to Intersociety Color Council (9).TABLE 3. *Production of melanoid pigments by the strains studied*

Strain	Production of melanoid pigments ^a on:			
	ISP medium			Waksman medium 42
	No. 1	No. 6	No. 7	
<i>Actinomyces atroolivaceus</i> INA 11294	—	—	—	—
<i>A. globisporus</i> subsp. <i>flavofuscus</i> INA 11296	S	S	—	—
<i>A. nigrificans</i> INA 3014	+	+	+	+
<i>A. variabilis</i> INA 232	—	—	S	—
<i>Streptomyces aburaviensis</i> ATCC 23869	—	—	—	—
<i>S. argillaceus</i> F.D.6590	+	+	+	+
<i>S. cavourensis</i> subsp. <i>washingtonensis</i> AUW-83	+	+	+ ^b	+
<i>S. griseus</i> no. 7	—	—	S	—
<i>S. lavendulae</i> M5-18903	+	+	+	+
<i>S. minutiscleroticus</i> ATCC 17757	S	—	— ^c	— ^d
<i>S. plicatus</i> F.D.13756	S	S	S	—
<i>Streptomyces</i> sp. C-1228	S	—	S	— ^c
<i>Streptomyces</i> sp. C-14795	S	—	S	—
<i>Streptomyces</i> sp. KCC S-1081	—	—	S	—
<i>Streptomyces</i> sp. L.A. 7017	+	+	+	+
<i>Streptomyces</i> sp. M-198	+	+	+	+

^a Symbols: +, positive; —, negative; S, only trace browning was observed, probably indicative of a negative melanin test, with some strains.^b Definite browning was observed beneath sporulating aerial mycelium, which had developed at the top of the test slants; only trace browning occurred where growth was present but no aerial mycelium had appeared.^c A blue-green diffusible pigment was observed.^d A red-yellow diffusible pigment was observed.

TABLE 4. *Utilization of carbon sources for growth by the strains studied^a*

Strain	Result of test for the utilization of:							
	L-Arabinose	D-Xylose	D-Fructose	D-Mannitol	meso-Inositol	D-Raffinose	L-Rhamnose	D-Sucrose
<i>Actinomyces atroolivaceus</i> INA 11294	+	+	++	++	—	+	—	+
<i>A. globisporus</i> subsp. <i>flavofuscus</i> INA 11296	—	—	++	—	++	—	—	++
<i>A. nigrificans</i> INA 3014	++	++	++	++	++	++	++	++
<i>A. variabilis</i> INA 232	—	+	++	+	+	+	—	++
<i>Streptomyces aburaviensis</i> ATCC 23869	±	—	—	—	—	±	—	—
<i>S. argillaceus</i> F.D.-6590	++	++	++	++	++	—	++	—
<i>S. cavourensis</i> subsp. <i>washingtonensis</i> AUW-83	++	++	++	++	—	—	—	—
<i>S. griseus</i> no. 7	±	++	++	++	—	—	++	—
<i>S. lavendulae</i> M5-18903	—	+	+	—	—	—	—	—
<i>S. minutiscleroticus</i> ATCC 17757	+	+	+	++	±	—	++	—
<i>S. plicatus</i> F.D.13756	+	+	+	++	++	—	++	—
<i>Streptomyces</i> sp. C-1228	++	++	++	—	—	—	—	+
<i>Streptomyces</i> sp. C-14795	+	±	—	—	—	—	—	±
<i>Streptomyces</i> sp. KCC S-1081	++	+	+	++	—	—	++	—
<i>Streptomyces</i> sp. L.A. 7017	++	++	++	++	++	+	++	—
<i>Streptomyces</i> sp. M-198	++	++	++	++	++	—	++	—

^a Symbols: —, negative response; ±, doubtful response; +, more growth than with carbon control but less than with glucose; ++, positive response equal to the amount of growth with glucose.

activity under the test conditions; among these were *Actinomyces nigrificans* INA 3014, *S. aburaviensis* ATCC 23869, *S. lavendulae* M5-18903, *S. minutiscleroticus* ATCC 17757, *Streptomyces* sp. C-1228, and *Streptomyces* sp. KCC S-1081. Except for the single olivomycin-producing strain in the study, *Streptomyces* sp. C-1228, all of the last-mentioned strains have been claimed to produce antibiotics of the chromomycin type. Other culture conditions or similarly important variables may be conducive for the production of detectable amounts of these antibiotics. However, it has been previously observed (23) that continuous subculturing of the chromomycin-producing strain AUW-83 resulted in a remarkably reduced capability of this strain to produce significant amounts of the chromomycins. Comparison of thin-layer chromatograms of ethyl acetate extracts of the fermentation broths of the remaining strains with authentic reference samples of mithramycin, chromomycin A₃, and

olivomycin A indicated that these strains were consistent with the available information concerning their production of these antibiotics.

DISCUSSION

A summary of the types of aureolic acid group antibiotics produced, the primary characters, and the carbon utilization patterns of the strains studied is presented in Table 7. Two of these strains, *S. aburaviensis* ATCC 23869 and *S. minutiscleroticus* ATCC 17757, are type strains and were previously evaluated in the ISP (21, 22). Similarities in taxonomic characters suggest that both Bristol's strain C-14795 and *S. aburaviensis* ATCC 23869, which produce chromomycins, are different strains of the same species. Sporophore morphology, spore surface, color of sporulating aerial mycelium, and a negative melanin test, features regarded by Gottlieb and Shirling (5) as primary taxonomic characters, are the same for both strains. The carbon utili-

TABLE 5. *Sodium chloride tolerances of the strains studied*

Strain	Sodium chloride tolerance
<i>Actinomyces atroolivaceus</i> INA 11294	≥7% but <10%
<i>A. globisporus</i> subsp. <i>flavofuscus</i> INA 11296	≥7% but <10%
<i>A. nigrificans</i> INA 3014	≥7% but <10%
<i>A. variabilis</i> INA 232	≥4% but <7%
<i>Streptomyces aburaviensis</i> ATCC 23869	<4%
<i>S. argillaceus</i> F.D.6590	≥7% but <10%
<i>S. cavourensis</i> subsp. <i>washingtonensis</i> AUW-83	≥7% but <10%
<i>S. griseus</i> no. 7	≥7% but <10%
<i>S. lavendulae</i> M5-18903	≥7% but <10%
<i>S. minutiscleroticus</i> ATCC 17757	≥7% but <10%
<i>S. plicatus</i> F.D.13756	≥10% but <13%
<i>Streptomyces</i> sp. C-1228	≥4% but <7%
<i>Streptomyces</i> sp. C-14795	<4%
<i>Streptomyces</i> sp. KCC S-1081	≥10% but <13%
<i>Streptomyces</i> sp. L.A. 7017	≥7% but <10%
<i>Streptomyces</i> sp. M-198	≥7% but <10%

zation patterns are distinctively similar in that cultures of both strains grew poorly on almost all of the carbon sources except glucose. Of the additional taxonomic characters evaluated, the inability of both strains to tolerate 4% sodium chloride is noteworthy. These were the only two strains in the study which were unable to tolerate this low concentration of sodium chloride. The results of a study by Tresner et al. (27), who proposed the use of tolerance of sodium chloride for the taxonomy of streptomycetes, indicated that about 5% of the 313 species studied could not tolerate 4% sodium chloride.

Another pair of chromomycin-producing strains which may be considered different strains of the same species are *S. griseus* no. 7 and *Streptomyces* sp. KCC S-1081. The primary characters of these two strains are the same, and the carbon utilization patterns differ only with respect to arabinose, but members of the "*griseus*" group are known to be variable in the utilization of this carbon source (14). The production by these strains of a soluble green pigment in inorganic salts-starch agar is a distinctive characteristic. *Streptomyces* sp. KCC S-1081 tolerated 10% sodium chloride better than did strain no. 7, but the study by Tresner et al. (27) indicated that nearly as many species of the yellow color series could tolerate 10% sodium chloride as could tolerate 7%, and therefore this difference may not be important. A deoxyribonucleic acid-deoxyribonucleic acid homology test would be useful in assessing the subspecific relationship of these two strains.

TABLE 6. *Results of the bioassays and of the thin-layer chromatographic analyses of broths fermented by the strains studied*

Strain	Bioassay ^a of fermentation broth (μg/ml)	Type ^b of aureolic acid group antibiotic produced
<i>Actinomyces atroolivaceus</i> INA 11294	5	Aureolic acid
<i>A. globisporus</i> subsp. <i>flavofuscus</i> INA 11296	69	Aureolic acid
<i>A. nigrificans</i> INA 3014	ND ^c	N ^c
<i>A. variabilis</i> INA 232	69	Aureolic acid
<i>Streptomyces aburaviensis</i> ATCC 23869	ND	N
<i>S. argillaceus</i> F.D.6590	49	Aureolic acid
<i>S. cavourensis</i> subsp. <i>washingtonensis</i> AUW-83	260	Chromomycin
<i>S. griseus</i> no. 7	35	Chromomycin
<i>S. lavendulae</i> M5-18903	ND	N
<i>S. minutiscleroticus</i> ATCC 17757	ND	N
<i>S. plicatus</i> F.D.13756	34	Aureolic acid
<i>Streptomyces</i> sp. C-1228	ND	N
<i>Streptomyces</i> sp. C-14795	142	Chromomycin
<i>Streptomyces</i> sp. KCC S-1081	ND	N
<i>Streptomyces</i> sp. L.A. 7017	14	Aureolic acid
<i>Streptomyces</i> sp. M-198	7	Aureolic acid

^a A paper disk agar diffusion method with *Staphylococcus aureus* NRRL B-313 as the test organism was employed; chromomycin A₃, lot no. AUW-83-A₃-A#1 (23), was used as a reference standard to which the antibiotic activities of the study broths were compared.

^b Ethyl acetate extracts of fermentation broths were concentrated in vacuo, and thin-layer chromatographic comparisons were made with authentic reference samples of mithramycin, chromomycin A₃, and olivomycin A.

^c Symbols: N, no aureolic acid group antibiotics detected; ND, no detectable activity.

A third pair of strains believed to belong to the same species are Pfizer's *S. argillaceus* F.D.-6590 and Abbott's *Streptomyces* sp. M-198, both producers of the antibiotic aureolic acid (= mithramycin). The four primary characters and the carbon utilization patterns of these strains are identical. *S. argillaceus* was proposed as a new species in the patent literature (B. A. Sobin, J. B. Routien, K. V. Rao, W. S. Marsh, and A. L. Garretson, U.S. Patent 3,646,194, 29 February 1972, and U.S. Patent 3,906,093, 16 September 1975); however, patent publications contravene Rule 25b of the *Bacteriological Code*, 1976 revision (10), and therefore the name

TABLE 7. Summary of the types of aureolic acid group antibiotics produced, the primary characters, and the utilizations of carbon sources for growth of the strains studied

Specific name of strain as received	Strain designation	Type ^a of aureolic acid group antibiotic produced	Primary characters ^b				Utilization ^c of:							
			SM	SS	CM	MR	Ab	Xy	Fr	Mt	Is	Rf	Rh	Su
<i>Streptomyces griseus</i>	No. 7	Chromomycin	RF	Sm	Y	—	±	+	+	+	—	—	+	—
<i>Streptomyces</i> sp.	KCC S-1081	NC	RF	Sm	Y	—	+	+	+	+	—	—	+	—
<i>S. cavourensis</i> subsp. <i>washingtonensis</i>	AUW-83	Chromomycin	RF	Sm	Y	+	+	+	+	+	—	—	—	—
<i>S. aburaviensis</i>	ATCC 23869	Chromomycin	RF	Sm	Gy	—	±	—	—	—	—	±	—	—
<i>Streptomyces</i> sp.	C-14795	Chromomycin	RF	Sm	Gy	—	+	±	—	—	—	—	—	±
<i>Streptomyces</i> sp.	C-1228	Olivomycin	RF	Sm	Gy	—	+	+	+	—	—	—	—	+
<i>Actinomyces globisporus</i> subsp. <i>flavofuscus</i>	INA 11296	Aureolic acid	RF ^d	Sm	Gy, Wh	—	—	—	+	—	+	—	—	+
<i>A. variabilis</i>	INA 232	Aureolic acid	S	Sm	Gy	—	—	+	+	+	+	+	—	+
<i>A. atroolivaceus</i>	INA 11294	Aureolic acid	S	Sm	Gy	—	+	+	+	+	—	+	—	+
<i>S. plicatus</i>	F.D.13756	Aureolic acid	S	Sp, Sm	Gy	—	+	+	+	+	+	—	+	—
<i>S. minutisclerotiscus</i>	ATCC 17757	NC	S	Sm	Gy, Y	—	+	+	+	+	±	—	+	—
<i>S. argillaceus</i>	F.D.6590	Aureolic acid	S	Sm	Gy	+	+	+	+	+	+	—	+	—
<i>Streptomyces</i> sp.	M-198	Aureolic acid	S	Sm	Gy	+	+	+	+	+	+	—	+	—
<i>Streptomyces</i> sp.	L.A. 7017	Aureolic acid	S	Sm	Gy, Wh	+	+	+	+	+	+	+	+	—
<i>A. nigrificans</i>	INA 3014	NC	S	Sm, Wa	Gy, Wh	+	+	+	+	+	+	+	+	+
<i>S. lavendulae</i>	M5-18903	Chromomycin	S	Sm	R	+	—	+	+	—	—	—	—	—

^a See Skarbek and Brady (23) for a chronological listing, including antibiotic names and references to the chemical literature, of reported antibiotic substances belonging to the aureolic group. NC, Confirmation by thin-layer chromatographic analysis in a limited fermentation study was lacking, and the reported experimental data for the antibiotic substances do not allow their absolute placement within a structural type (aureolic acid, chromomycin, or olivomycin type) of the aureolic acid group; the antibiotics produced by strains KCC S-1081, ATCC 17757, and INA 3014 possibly may have been of the chromomycin type.

^b Abbreviations: SM, spore-chain morphology (*S. Spirales*; RF, *Rectiflexibiles*); SS, spore characteristics (Sm, smooth; Wa, warty; Sp, spiny); CM, aerial mass color (Gy, gray; R, red; Y, yellow; Wh, white); MR, melanoid pigmentation (—, negative; +, positive).

^c Symbols: —, No utilization; ±, doubtful utilization; +, positive utilization for growth. Abbreviations: Ab, arabinose; Xy, xylose; Fr, fructose; Mt, mannitol; Is, inositol; Rf, raffinose; Rh, rhamnose; and Su, sucrose.

^d The presence of crooked sporophores, in addition to RF morphology, observed in the microscopic examinations of strain INA 11296 might suggest placement in the category *Spirales*. All other strains in this study alleged to produce antibiotics of the aureolic acid type were observed to have *S* morphologies.

S. argillaceus was not effectively published and hence has no standing in bacteriological nomenclature.

The remaining strains can be differentiated from the other strains in the study and from each other with regard to primary characters, carbon utilization patterns, and additional features, and therefore are probably members of different species.

S. cavourensis subsp. *washingtonensis* strain AUW-83 is a member of the *griseus* series, but it differs from strains no. 7 and KCC S-1081 regarding soluble pigment production in inorganic salts-starch agar, melanin production, and the utilization of rhamnose for growth. Strains no. 7 and KCC S-1081, but not strain AUW-83, were observed to form a soluble green pigment in inorganic salts-starch agar. Strain AUW-83 was capable of forming melanin pigments in

several tyrosine-containing media; strains no. 7 and KCC S-1081 were not observed to form melanoid pigments in these media. Strains no. 7 and KCC S-1081 were able to utilize rhamnose for growth, but strain AUW-83 was not.

There have been several reports in the literature claiming the discovery of additional strains producing antibiotics belonging to the aureolic acid group; however, these strains were not available for this study, and the taxonomic information to be found in the literature on these strains was sometimes very sketchy. A list of additional strains reported to produce antibiotics of the aureolic acid group and available taxonomic information on these strains, together with pertinent references, is presented in Table 8. *S. cavourensis* subsp. *washingtonensis* strain AUW-83 appears to be quite distinct not only from the strains included in this study but also

TABLE 8. *Information regarding additional strains of Actinomycetales reported to produce antibiotics of the aureolic acid group*

Strain	Antibiotic(s) ^a produced	Primary characters ^b				Carbon utilization	Source of information (literature references)
		SM	SS	CM	MR		
<i>Actinomyces abura-viensis</i> subsp. <i>verrucosus</i> 144-3	Chromomycins		Wa				29, 30
<i>A. halstedii</i> INA 7193	7193	S		Gy	—		28
<i>A. iverini</i> (strain not mentioned)	Mithramycin ^c						— ^d
<i>A. olivovariabilis</i> 6604-9	Variamycin ^e	S	Sm	Gy	—	Glucose, arabinose, xylose, and inositol are utilized for growth; no growth with mannitol, raffinose, and rhamnose, and growth on sucrose is doubtful	17, — ^f
<i>Streptomyces fulvoretus</i> B-599	Chromomycins	RF	Sm	Gy			11, 12
<i>S. griseus</i> SK-229	Chromomycins	RF	Sm	Y	—		— ^g
<i>S. katsunumaensis</i> A468 = FERM-P 2071	Chromomycins ^h (= SR1768)						15, — ⁱ
<i>S. olivochromogenes</i> 69895	Chromomycins	S	Sm	Gy	+	Glucose, arabinose, xylose, fructose, mannitol, inositol, raffinose, and rhamnose are utilized for growth; growth with sucrose is doubtful	7 ^j
<i>S. olivoreticuli</i> INA 16749	Olivomycins	V					3, 28
<i>Streptomyces</i> sp. 24-10	24-10	S		Gy		Glucose, arabinose, and raffinose are utilized for growth; utilization of mannitol and sucrose is doubtful	2
<i>Streptomyces</i> sp. C-2449	C-2449						8

^a The experimental data reported for some of these antibiotics do not allow for their absolute placement within a structural type (aureolic acid, chromomycin, or olivomycin type) of the aureolic acid group; the antibiotics produced by strains C-2449 and INA 7193 may possibly be of the aureolic acid type, and by strain 24-10, of the chromomycin type.

^b Abbreviations: SM, spore-chain morphology (*S*, *Spirales*; *RF*, *Rectiflexibiles*; *V*, *Verticillati*); SS, spore characteristics (Sm, smooth; Wa, warty); CM, aerial mass color (Gy, gray; Y, yellow); MR, melanoid pigmentation (—, negative; +, positive).

^c Mithramycin is structurally identical to aureolic acid.

^d I. Gado, J. Gyimesi, E. Gal, I. Horvath, K. Magyar, B. Doczi, and M. Halasz, Hungarian Patent 155,679, February 1969.

^e Variamycin is a structural analog of aureolic acid, differing by the substitution of the sugar, varirose, for mycarose.

^f J. V. Zhdanovich, G. B. Lokshin, A. D. Kuzovkov, S. M. Rudaya, and N. K. Solovieva, U.S. Patent 3,821,085, June 1974.

^g Shionogi and Co., Japanese Patent 11,047, 1964. Taxonomic information on *S. griseus* SK-229 was received through personal communication with Y. K. Sawa, Shionogi and Co., Osaka, Japan.

^h SR1768A = cytrimycin, a structural analog of chromomycin A₂; SR1768B = chromomycin A₂; SR1768E = chromomycin A₃.

ⁱ T. Oki, Y. Matsuzawa, K. Numata, K. Kono, and A. Takamatsu, Japanese Patent 13,593, 1975.

^j Strain 69895 coproduces the macrolide antibiotic oleandomycin.

from those listed in Table 8. The identification at the species level and the taxonomic relationships of these strains will have to await studies involving their direct comparison with type and/or neotype strains of closely related nomenclature species.

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