STREPTOMYCES VENEZUELAE, N. SP., THE SOURCE OF CHLOROMYCETIN

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The *Streptomyces* that produces "chloromycetin" differs from those described in *Bergey's Manual* (Breed *et al.*, 1948) and is therefore believed to be a new species for which the name *Streptomyces venezuelae* is proposed.

Two cultures have been studied, the first one isolated at New Haven (Burkholder no. A65) from a soil sample collected in a mulched field near Caracas, Venezuela, the second one isolated at Urbana (Gottlieb no. 8-44) from a compost soil on the horticultural farm of the Illinois Agricultural Experiment Station at Urbana (Ehrlich *et al.*, 1947; Carter *et al.*, 1948; Gottlieb *et al.*, 1948; Smith *et al.*, 1948). The first of these, which we regard as the type culture, has been placed in the Culture Bureau of Parke, Davis and Company at Detroit as no. 04745. The description of morphology is based on the type culture but both cultures were employed in the physiologic tests.

MORPHOLOGY

Primary mycelium growing in agar substrata is thin-walled, colorless, hyaline, monopodially branched (figure 1: C). Mature vegetative hyphae vary in diameter from 0.9 to 1.8 microns and the branches grow to about 150 microns in length. Sometimes the substratal mycelium forms oval spores by fragmentation (figure 1: B). The aerial mycelium is lavender under the microscope, thick-walled, generally not much branched, straight or slightly and irregularly curved, not forming loops or spirals, having individual filaments that appear stiff, and arising frequently from the primary mycelium at the surface of the substrate (figure 1: H, I, J). Individual filaments are rarely or not septate, 1.0 to 1.8 microns in diameter, and vary in length up to about 350 microns. In young colonies, the stiff aerial hyphae project outward radially over the surface of the colony and show a lavender color when examined microscopically. The color of colonies when viewed on agar without magnification is gray to light tan or pink, but not lavender. Distal portions of the aerial hyphae commonly subdivide into unbranched oidial spore chains (figure 1: A) which are readily fragmented into small groups or individual spores.

The spores are oval to oblong. Mature spores range from about 0.4 to 0.8 microns in diameter and from 0.7 to 1.6 microns in length. The spores formed by fragmentation of hyphae in the substrate are generally smaller than those

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formed from the aerial hyphae. Individual spores are colorless at maturity but in mass appear tan to gray when viewed without magnification. They may be stained readily with crystal violet and other bacteriological dyes. The

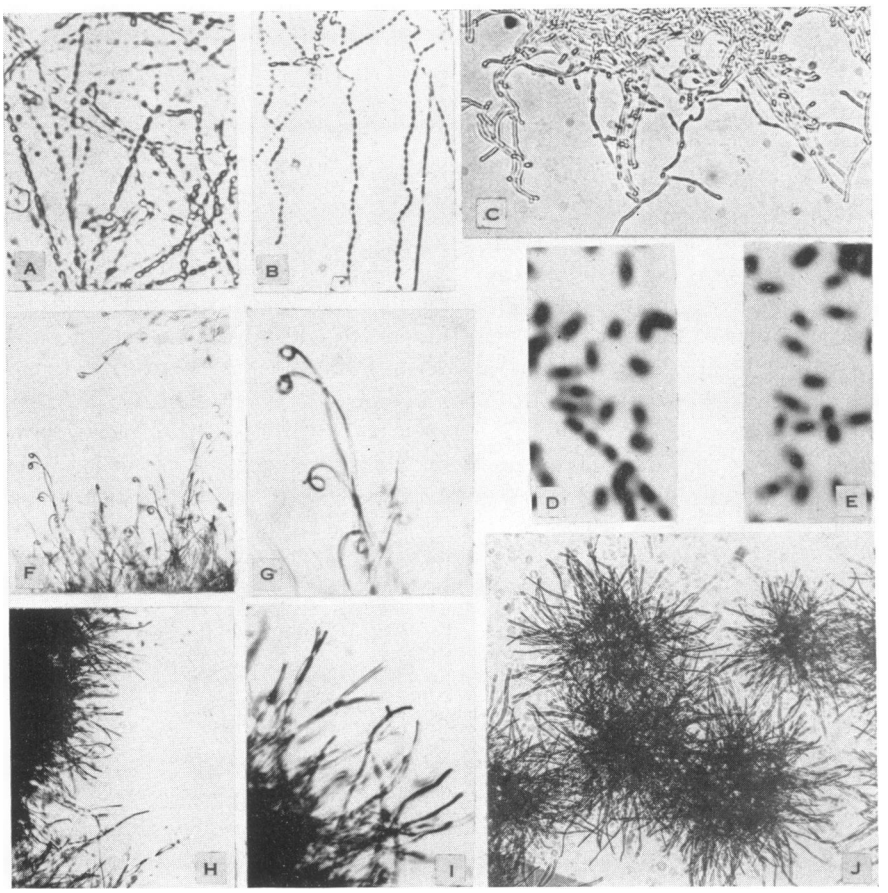

Figure 1. Streptomyces venezuelae: A, aerial hyphae fragmented into chains of spores B, substratal hyphae forming spores in chains; C, young primary mycelium developing many short branches; D, E, spores stained with Giemsa to show the nuclei and lightly counterstained with safranine Y; H, I, aerial hyphae about ready to form spores; J, young colonies forming stiff lavender aerial hyphae. *S. lavendulae:* F, G, aerial hyphae showing characteristic spirals.

Photographs A, B, C, G, I taken with 4-mm dry objective and × 10 ocular using living material growing on agar-covered glass microscope cover slips (magnification × 450). Photographs F, H, and J taken from living cultures on agar with 16-mm objective and × 10 ocular (magnification × 150). Photographs D and E taken with 1.8 mm oil immersion objective and × 10 ocular (magnification, × 3,000) using Giemsa-stained material mounted in "permount."

spores are uninucleate, as determined by Giemsa staining[4] (figure 1: D, E). Mycelium and spores are gram-positive.

[4] A spore suspension in distilled water is fixed on a slide by inverting a hanging droplet over 2 per cent osmic acid solution for 5 minutes and drying in air. After washing for 10 minutes in water, the slide is stained in Giemsa solution phosphate buffer pH 7.4, using 1

PHYSIOLOGY

Spores of *Streptomyces venezuelae* were sown on various media and incubated at room temperature or at 28 C in order to establish a spectrum of nutritional sources and of effects on certain differential media. The results are compiled in table 1.

STREPTOMYCES VENEZUELAE VS. STREPTOMYCES LAVENDULAE

Since *Streptomyces venezuelae* resembles *S. lavendulae* in certain respects (Carter *et al.*, 1948), cultures of the two species were compared in a variety of ways in order to obtain data on which to judge whether or not they might reasonably be regarded as separate species.

Morphology

Several conspicuous differences in morphology between cultures of *Streptomyces venezuelae* and *S. lavendulae* are apparent upon inspection of the characteristics shown in table 2.

Physiology

Nutritional spectrum and sporulation. Cultures of Waksman's strain 8 of *S. lavendulae* were compared with both cultures of *S. venezuelae* in the nutrition tests reported above. Although the three cultures gave essentially similar results in the majority of the test media, the two *S. venezuelae* strains differed from *S. lavendulae* in their ability to utilize a number of carbohydrates. These differences are shown in table 3 (A). The two species differed also in their requirements for sporulation, as shown in table 3 (B).

Antibiotic production. In order to determine whether *Streptomyces venezuelae* differs from *S. lavendulae* in the nature of the antibiotics produced in different media, the two cultures of *S. venezuelae* and the Parke, Davis subculture of Waksman's strain 8 *S. lavendulae* were grown in shaken flasks in media known to favor biosynthesis of chloromycetin and in others favorable to streptothricin formation. The results of one of several such tests are shown in table 4 and figure 2.[5]

When the flask cultures were assayed for streptothricin content against two test bacteria known to be sensitive to streptothricin but relatively insensitive to chloromycetin (table 4), the two strains of *S. venezuelae* were seen to have produced antibacterial material equivalent to from < 1 to 5.5 streptothricin units per ml in all three media, whereas *S. lavendulae* had produced close to 100 streptothricin units in a streptothricin medium, approximately 15 units in one chlor-

ml of dye solution (0.5 g National Aniline Division powder in 33 ml glycerol plus 33 ml methyl alcohol) in 30 ml buffer. The slide is allowed to stain until a colored spot becomes visible on the slide (about 15 minutes), then it is decolorized in acetone, counterstained in aqueous safranine, and dehydrated up through a series of acetone-xylol mixtures until in pure xylol. The preparation is mounted in "permount" or other similar material. The nuclei stain blue and the rest of the cell is red.—P.R.B.

[5] Robert M. Smith of Parke, Davis and Company grew the shaken cultures.

TABLE 1

Response of S. venezuelae to various media

A. Miscellaneous

SUBSTRATE	DARKENING	OTHER EFFECTS
Gelatin	+	Liquefaction
Litmus milk	+	Peptonization, basic reaction
Nitrate broth	+	Reduction to nitrite
Kligler iron agar	+	H_2S production
Tryptone broth	+	No indole production
Dorset egg agar	+	—
Potato plug	+	—
Tyrosine broth	+	—
Glucose nutrient agar	+	—

B. Nitrogen sources

Medium: Synthetic agar* + 1% starch + N source at conc. of 0.106 g N per liter

N SOURCE	GROWTH
Ammonium sulfate	+
Sodium nitrate	+
Sodium nitrite	+†
Acetamide	Slight
Asparagine	+
L-Tyrosine	+
DL-Tryptophan	+

C. Carbohydrate sources

Medium: Synthetic agar* + 0.264% $(NH_4)_2SO_4$ + CHO source at concentrations indicated below

CHO SOURCE	CONCENTRATION	GROWTH
	per cent	
Pentoses	1.0	
Arabinose		+
Rhamnose		+
Ribose		Slight
Xylose		+
Hexoses	1.0	
Glucose		+
Galactose		+
Fructose		+
Mannose		+

* KH_2PO_4 2.38 g; K_2HPO_4 5.65 "; $MgSO_4 \cdot 7H_2O$ 1.00 "; $CuSO_4 \cdot 5H_2O$ 0.0064 "; $FeSO_4 \cdot 7H_2O$ 0.0011 "; $MnCl_2 \cdot 4H_2O$ 0.0079 g; $ZnSO_4 \cdot 7H_2O$ 0.0015 "; Difco agar 15.0 "; Distilled water 1 liter; Medium adjusted to pH 6.8–7.0

† The cultures failed to grow in the presence of sodium nitrite at a concentration of 2.64 grams per liter.

TABLE 1—*Continued*

CHO SOURCE	CONCENTRATION	GROWTH
	per cent	
Disaccharides	1.0	
Cellobiose		+
Lactose		+
Maltose		+
Sucrose		Slight
Polysaccharides	1.0	
Dextrin		+
Inulin		Slight
Raffinose		Slight
Starch		+
Polyhydric alcohols	1.0	
Dulcitol		Slight
Erythritol		Slight
Glycerol		+
Inositol		Slight
Mannitol		−
Sorbitol		Slight
Sodium salts of organic acids	0.15	
Acetate		+
Citrate		+
Formate		−
Malate		Slight
Oxalate		−
Salicylate		−
Succinate		+
Tartrate		−
Miscellaneous		
o-Cresol	0.1	−
m-Cresol	0.1	−
p-Cresol	0.1	−
Phenol	0.1	−
Salicin	1.0	+

TABLE 2

Some morphological characteristics of S. venezuelae and S. lavendulae

CHARACTERISTIC	S. VENEZUELAE*	S. LAVENDULAE†
Colony color before sporulation	Gray to light tan or pink	Lavender
Spore color in mass	Tan to gray	Pink to lavender
Aerial hyphae	Stiff and straight or slightly curved (Fig. 1: J, H, I)	Markedly curved or spirals (Fig. 1: F, G)
Spore size in microns	0.7–1.6 × 0.4–0.9	1.6–2.0 × 1.0–1.2

* P.D. 04745 and Ill. 8-44.

† Waksman 8.

omycetin medium, and only 5 in another. Both *S. venezuelae* cultures differed from *S. lavendulae* also in their relative activity against *Bacillus subtilis* and *Es-*

cherichia coli, their coli:subtilis ratios being 3.3 to 4.3 and 0.7 to 1.2, respectively, in all three media.

When the flask culture filtrates were tested for antibacterial titer and assayed for chloromycetin content against a test bacterium known to be sensitive to chloromycetin (figure 2), the two *S. venezuelae* cultures were seen to have produced similar amounts of antibacterial material in all three media, whereas *S. lavendulae* had produced little measurable activity in the streptothricin medium and less in the two chloromycetin media. One of the two cultures of *S. venezuelae* was slightly more productive than the other in all three media. The two cultures did not vary in their response to the different media: both gave lower yields in medium B than in A and, oddly, both were most productive in C, the streptothricin medium.

TABLE 3

Some physiological characteristics of S. venezuelae and S. lavendulae

CULTURE MEDIUM	S. VENEZUELAE	S. LAVENDULAE
A. Carbohydrate utilization		
Pentoses		
Arabinose	+	? to −
Rhamnose	+	? to −
Ribose	Slight	−
Xylose	+	? to −
Others		
Lactose	+	Slight
Fructose	+	?
Sodium acetate	+	+ to −
B. Sporulation		
Moyer's penicillium sporulation agar	+	± to −
Synthetic agar (table 1)		
+ glucose and asparagine	± to −	+
+ B vitamins	± to −	+
+ casein hydrolyzate	± to −	+
+ yeast extract	± to −	+
+ tyrosine	± to −	+
Yeast-beef agar	± to −	+
Glucose-tryptone agar	± to −	+

+ = positive; ± = sparse; − = negative; ? = doubtful.

Thus, *S. lavendulae* in a streptothricin medium showed high activity by a streptothricin assay and low activity by a chloromycetin assay, whereas in two chloromycetin media it showed low activity by a streptothricin assay and none by a chloromycetin assay. *S. venezuelae*, on the other hand, showed low activity in all three media by a streptothricin assay and relatively high activity in all media by a chloromycetin assay.

In order to compare the antibiotic substances produced by the three strains, the shaken flask cultures of *S. venezuelae* grown in medium A and *S. lavendulae* grown in medium C (table 4, figure 2) were fractionated[6] as shown in figure 3.

TABLE 4

Antibacterial activity (streptothricin units per ml) vs E. coli and B. subtilis of S. venezuelae and S. lavendulae in three shaken culture media

CULTURE MEDIUM FAVORABLE FOR	ASSAY SPECIES USED	ANTIBACTERIAL ACTIVITY OF SHAKEN FLASK CULTURES OF					
		S. venezuelae				*S. lavendulae*	
		P.D. 04745		Ill. 8-44		Waksman 8	
		Potency	Ratio	Potency	Ratio	Potency	Ratio
		*u/ml**	*E.c./B.s.*	*u/ml**	*E.c./B.s.*	*u/ml**	*E.C./B.S.*
Chloromycetin							
A	*E. coli*	5.5	4.2	4.3	>4.3	16	1.1
	B. subt.	1.3		<1.0		14	
B	*E. coli*	4.1	>4.1	3.3	>3.3	5.8	1.2
	B. subt.	<1.0		<1.0		4.7	
Streptothricin	*E. coli*	3.7	>3.7	3.9	>3.9	74	0.7
C	*B. subt.*	<1.0		<1.0		105	

Composition of media (percentages)

	A	B	C
Carbohydrate	Glycerol..... 1.0	Cerelose..... 1.0	Maltose..... 2.0
Nitrogen	Hog stomach residue 0.5	Soy bean oil meal... 1.0	Corn steep liquor. 6.0
Supplement	B-Y fermentation solubles (CSC).... 0.5	Curbay BG (USIC). 0.05	
Salt	NaCl..... 0.5 $CaCO_3$..... 0.1	NaCl..... 0.5 $CaCO_3$..... 0.1	NaCl..... 0.2 K_2HPO_4..... 0.2

* Potency is expressed as streptothricin units when assayed by the paper disk agar plate method with a standard curve plotted from dilutions of a streptothricin sulfate preparation kindly supplied by Dr. George F. Cartland of The Upjohn Company, Prep. no. 239-WGJ-4, assumed to contain 400 streptothricin units per milligram. Each potency figure in the table represents the mean of triplicate flasks shaken for 5 days at 22 to 24 C. These assays were performed by Mrs. Frances E. Guest, Dorothy E. Kohberger, and Blanche M. Duckworth of Parke, Davis and Company.

Because chloromycetin is more soluble in ethyl acetate than in water and streptothricin is relatively insoluble in ethyl acetate, it was reasoned that assayable activity in the aqueous layers could be streptothricin, whereas that in the solvent-extracted residues could be regarded as chloromycetin but not streptothricin.

[6] Dr. Quentin R. Bartz of Parke, Davis and Company directed the fractionations.

The experimental data compiled in table 5 lead inescapably to the conclusion that the two strains of *S. venezuelae* produced no streptothricin and that *S. lavendulae* produced no chloromycetin. The aqueous layers from the solvent-extracted *S. venezuelae* filtrates exhibited practically no activity against *B. subtilis*, whereas the solvent-extracted residues contained all or nearly all of the activity of the filtrates when assayed against *Shigella*. In contrast, the aqueous layer

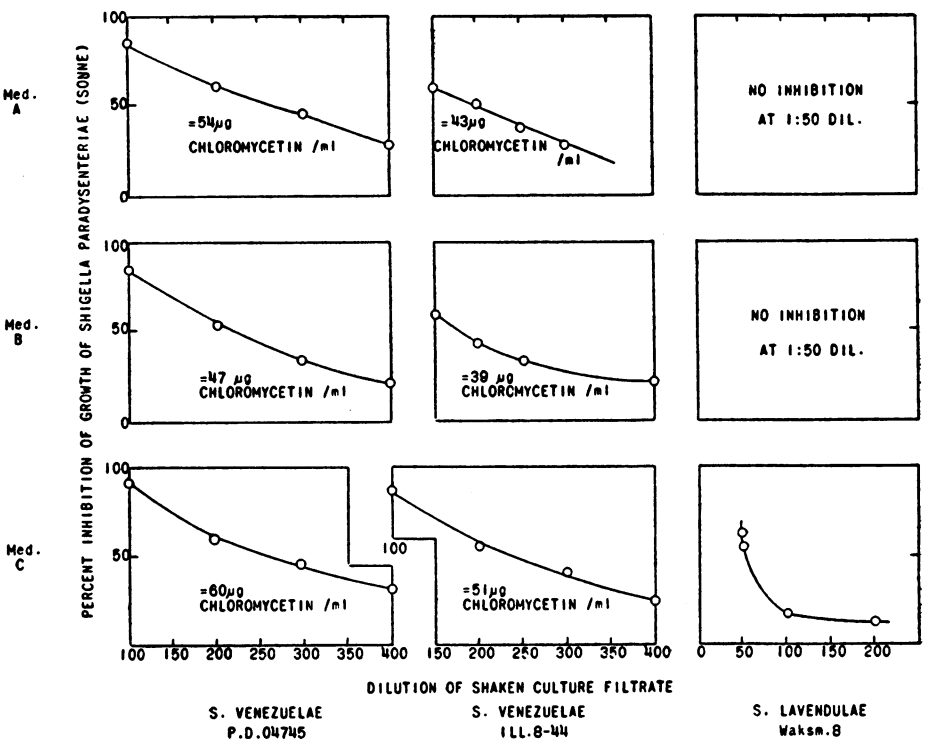

Figure 2. Antibacterial activity (per cent of inhibition over dilution, and equivalent micrograms chloromycetin per ml) vs. *Shigella paradysenteriae* (Sonne) of *S. venezuelae* and *S. lavendulae* in three shaken culture media.

These assays were made on Seitz filtrates of the same shaken flask cultures as those in table 4, but after 6 days. They were performed by a turbidimetric broth dilution method, employing a crystalline chloromycetin standard for the gravimetric estimates of potency (Joslyn and Galbraith, 1947; Smith *et al.*, 1948). The assays were run by Dwight A. Joslyn and Mrs. Margaret Galbraith of Parke, Davis and Company.

from the *S. lavendulae* filtrate exhibited considerable activity against *B. subtilis*, but the solvent-extracted residue showed practically none. Also noteworthy is the relative stability of the *S. venezuelae* culture filtrates at pH 9.5 and 100 C (characteristic of chloromycetin but not of streptothricin) in contrast to the expected instability of the *S. lavendulae* filtrate under these conditions.

Susceptibility to actinophages. The appearance of an actinophage in a subculture of the P.D. 04745 strain of *S. venezuelae* provided the opportunity to ascertain whether or not the available strains of *S. venezuelae*, *Streptomyces*

griseus, and *S. lavendulae* were all susceptible to the same actinophages. The two strains of *S. venezuelae* and two strains of *S. lavendulae* were seeded on the surfaces of agar plates and streaked with Seitz filtrates of actinophage-containing shaken cultures of the P.D. strain of *S. venezuelae* and two strains of *S. griseus*. Actinophages were not available from the Ill. 8-44 strain of *S. venezuelae* or from *S. lavendulae*. Plates seeded with *S. griseus* strains were also included in order to check on the susceptibility of these strains to the available actinophage-containing filtrates. Table 6 shows the results of these experiments. Both strains

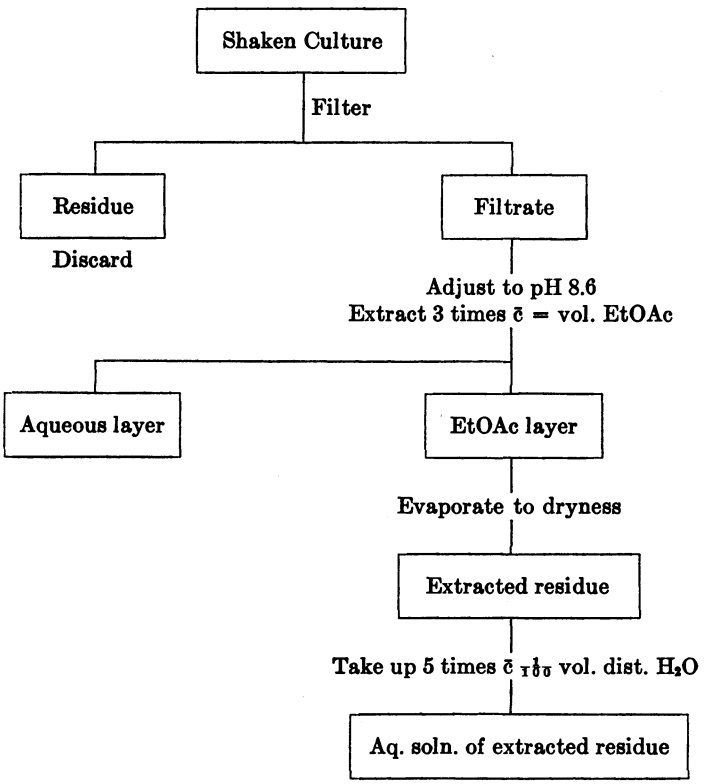

Figure 3. Fractionation of shaken cultures of *S. venezuelae* in medium A and *S. lavendulae* in medium C.

of *S. venezuelae* but neither of the two strains of *S. lavendulae* were lysed by the actinophage from P.D. 04745, thus contributing another point of difference between the two species. The four *S. griseus* strains were unaffected by the *S. venezulae* phage but all—insofar as tested—were susceptible to at least one of the *S. griseus* phages. Excepting one unexplained result, no strain of *S. venezuelae* or *S. lavendulae* was susceptible to any of the *S. griseus* phages against which it was tested. It may be noted that these limited data constitute examples of specificity among these actinophages; that is, each of these phages proved able to lyse all the tested antibiotic-producing strains of the actinomycete species from

TABLE 5

Antibacterial activity of fractions from shaken cultures of S. venezuelae in medium A and S. lavendulae in medium C

FRACTION			DISK PLATE ASSAY VS. B. SUBTILIS, A.T.C.C. 6633			TURBIDIMETRIC ASSAY VS. SHIGELLA PARADYSENTERIAE (SONNE), P.D. 04628				
			S. venezuelae		*S. lavendulae*	*S. venezuelae*		*S. lavendulae*	*S. venezuelae*	
			P.D. 04745	Ill. 8-44	Waksman 8	P.D. 04745	Ill. 8-44	Waksman 8	P.D. 04745	Ill. 8-44
			streptothricin units/ ml			*dilution causing 50% inhib.*			*μg chloromycetin/ml*	
Culture filtrate			3	2	72	265	182	36	49	38
Stability tests										
pH	*°C*	*min*								
2.0	20	30	—	—	72	240	180	—	45	37
2.0	100	15	—	—	72	235	170	—	44	34
9.5	20	30	—	—	76	245	220	24	48	44
9.5	100	15	—	—	15	195	170	<1*	38	34
Aqueous layer			<1	0	56	—†	—†	—†	—†	—†
Aqueous solution of residue from ethyl-acetate-extracted fraction			60	57	<2	4,260	3,760	<1‡	920	770§
Yield: Total activity of residue + total activity of filtrate						80%	103%	—	94%	101%

* 1–10 dilution caused only 20 per cent inhibition.

† These values are low and have no significance because the ethyl acetate dissolved in the water possesses activity against *Shigella paradysenteriae* (Sonne) in this test.

‡ 1–10 dilution caused less than 20 per cent inhibition.

§ Estimated without standard curve for day tested.

TABLE 6

*Susceptibility of strains of S. venezuelae, S. griseus, and S. lavendulae to actinophage-containing culture filtrates of some of these strains**

ACTINOPHAGE CONTAINING CULTURE FILTRATES OF	LYSIS OF AGAR PLATE SEEDINGS OF							
	S. venezuelae		*S. lavendulae*		*S. griseus*			
	P.D. 04745	Ill. 8-44	W.† 8	W. 14	W. 4	W. 9	W. 10	W. 19
S. venezuelae P.D. 04745	+	sl. +	0	0	0	0	0	0
S. griseus								
W. 4 (Upjohn subcult.)	—	0‡	—	—	+	—	—	—
W. 9 (P.D. ")	0	0	0	0	+	+	+	+
W. 9 (Lilly ")	—	0§	—	0§	—	+§	—	—

\+ = lysis. 0 = no lysis. — = not tested.

* Except as otherwise noted, these tests were made by Robert M. Smith of Parke, Davis and Company.

† W. = Waksman.

‡ Gottlieb's result; but Colingsworth (Gottlieb *et al.*, 1948) obtained lysis.

§ Lilly results, kindly communicated by Dr. J. M. McGuire of the Lilly Research Laboratories.

which it had been isolated, but none of the tested strains of other actinomycete species.

Serological comparison of cultures.[7] Rabbits were immunized for 3.5 months with weekly intravenous injections of saline suspensions of living mycelium and spores of the cultures. The sera were then tested for precipitins against antigens prepared as saline extracts of the fungi. Strong precipitation occurred between the sera and the homologous antigens of each strain and between the sera and heterologous antigens of the *S. venezuelae* strains. Weak reactions occurred between the sera and heterologous antigens of *S. lavendulae* and the *S. venezuelae* strains.

The demonstration of strong cross reactions between the *S. venezuelae* strains is interpreted as further evidence of their specific identity, and the absence of cross reactions between either of them and *S. lavendulae* as further evidence that they are specifically distinct from *S. lavendulae*.

SUMMARY

The actinomycete that produces "chloromycetin" is described as a new species for which the name *Streptomyces venezuelae* is proposed.

The decision to regard *S. venezuelae* as a species distinct from the somewhat similar *Streptomyces lavendulae* is based on differences in morphology, nutrition, antibiotic production, susceptibility to actinophages, and serological reactions.

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[7] These tests were performed in the Research Laboratories of Parke, Davis and Company by Dr. A. B. Hillegas and Miss Marion McCracken, who will report their work in detail at a later date.