PHENELFAMYCINS, A NOVEL COMPLEX OF ELFAMYCIN-TYPE ANTIBIOTICS

I. DISCOVERY, TAXONOMY AND FERMENTATION

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Phenelfamycins A, B, C, E, F and unphenelfamycin have been discovered in the fermentation broth of two soil isolates, designated AB 999F-80 and AB 1047T-33. These isolates were identified as strains of *Streptomyces violaceoniger*. The antibiotics were selected for their activity against anaerobic bacteria.

Phenelfamycins, a complex of antibiotics related to the elfamycin group, have been found in the fermentation broths of two soil isolates. These isolates have been identified as *Streptomyces violaceoniger* AB 999F-80 and *S. violaceoniger* AB 1047T-33. This paper describes the discovery of the antibiotics, the producing organisms and the production of phenelfamycin A by fermentation of *Streptomyces violaceoniger* AB 999F-80. The isolation and structure determination of the individual members of the phenelfamycin complex and their biological properties are reported in companion papers[1,2].

Materials and Methods

Microorganisms

Strain AB 999F-80 was isolated from a soil collected at Mount Angel, Oregon and strain AB 1047T-33 from a soil collected near Niotaze, Kansas. Both cultures were isolated using media containing species restricting substrates[3]. For strain AB 999F-80 the carbon source in the isolation medium was inulin and for strain AB 1047T-33 it was arabitol. Cultures employed in the screen were from the stock culture collection in our laboratory or from the American Type Culture Collection (ATCC).

The Screen

For the discovery test, agar plugs were cut from cultures grown for 6 days on ATCC medium 172† (20 ml/petri dish). The plugs were placed on the surface of seeded agar plates, incubated overnight and scored for inhibition of bacterial growth surrounding the plug. *Bacteroides fragilis* was grown in Wilkins Chalgren agar and incubated anaerobically at 37°C. The other strains were grown in streptomycin assay agar with yeast extract (BBL) and incubated aerobically at 32°C.

Taxonomic Studies

Methods and media described by the International Streptomyces Project (ISP)[4], WAKSMAN[5] and GORDON *et al.*[6] were used to determine most of the morphological and physiological characteristics. ATCC medium 172 and starch - yeast extract - salts agar[7] were also employed for the cultural characteristics. Color names were assigned to the mycelial and diffusible pigments on the

† American Type Culture Collection. ATCC Media Handbook. First Ed. American Type Culture Collection, Rockville, 1984.

basis of the Inter-Society Color Council-National Bureau of Standards (ISCC-NBS) Color-Name Charts†. With the exception of the temperature growth study, incubation was at 28°C for 21 days. The diaminopimelic acid isomer was determined by the method of BECKER *et al.*[8].

Inoculum Preparation

S. violaceoniger AB 999F-80 was maintained on sporulation agar (ATCC medium 5). The seed medium consisted of glucose 1.5%, soybean flour 1.5%, yeast extract (Difco) 0.1%, NaCl 0.1% and $CaCO_3$ 0.1%. Seed preparation was carried out in two steps with incubation at 28°C on a rotary shaker at 250 rpm. In the first step, sporulated slants were used to inoculate 25×150 mm tubes containing 10 ml of seed medium. After 96 hours, vegetative growth from the tubes was inoculated at 5% into 2-liter Erlenmeyer flasks containing 600 ml of the same seed medium. The flasks were incubated for 72 hours.

Fermentation

A 150-liter New Brunswick fermentor was charged with 80 liters of a medium consisting of glucose monohydrate 2.0%, Lexein F-152 liquid peptone (Inolex) 1.0%, yeast extract (Difco) 0.1%, molasses (Delmonte) 0.5%, $CaCO_3$ 0.2% and antifoam XFO-371 (Ivanhoe) 0.01%. The medium was prepared in distilled water, the pH was not adjusted, and sterilization was at 121°C and 1.05 kg/cm² for 1 hour. The seed flask growth was used at 5% to inoculate the fermentor. The fermentation was carried out at 28°C for 162 hours. The agitation was 200 rpm, the aeration was 0.7 v/v/minute, and the head pressure was 0.35 kg/cm².

Packed Cell Volume

Culture growth was evaluated by centrifuging untreated fermentation broth in 15-ml conical tubes at 600×*g* for 30 minutes. The packed cell solids were reported as % of total broth volume.

Total Reducing Sugars

Carbohydrate utilization was determined by analyzing for reducing sugars in hydrolyzed fermentation broth. Two ml of 2 N H_3PO_4 were added to 5 ml of fermentation broth supernatant. The mixture was heated for 30 minutes at 121°C, cooled and centrifuged. The supernatant from the hydrolysis was analyzed for reducing sugars by the method of HOFFMAN[9].

Assay for Phenelfamycins

The accumulation of phenelfamycins in the fermentation broth was monitored by HPLC. The broth was extracted with ethyl acetate, the solvent removed under vacuum and the residue suspended in methanol at a 50-fold concentration. The samples were injected with a U6K injector into a Waters Model 600A solvent delivery system with a 4.6×150 mm column packed with Adsorbosphere HS (Alltech). The eluting solvent was CH_3CN - 0.1% H_3PO_4 (50:50) at pH 3. The retention time of phenelfamycin A was 11 minutes.

Results

Discovery

The phenelfamycin producing cultures were selected from other soil isolates by their inhibition of the anaerobe *B. fragilis* and their lack of inhibitory activity against *Staphylococcus aureus* and several Gram-negative bacteria (Table 1).

Taxonomy

Morphological and Chemical Characteristics

The vegetative mycelium of strains AB 999F-80 and AB 1047T-33 is branched and does not fragment. Spores are produced on aerial hyphae in chains with a spiral conformation. Scanning elec-

† ISCC-NBS Color-Name Charts Illustrated with Centroid Colors. U.S. Dept. of Comm. Suppl. to Cir. 553, Washington, D.C., 1976.

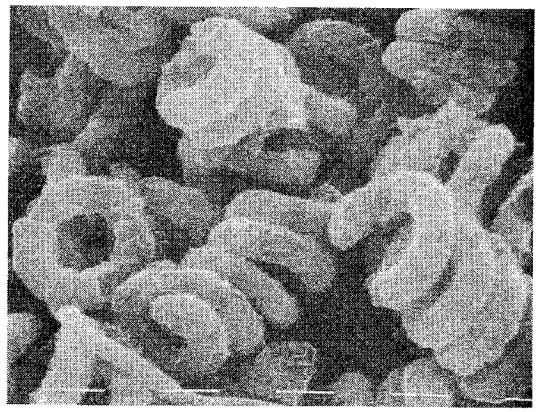
Table 1. Inhibition of bacterial growth by agar plugs of AB 999F-80 and AB 1047T-33.

	Inhibition of growth	
	AB 999F-80	AB 1047T-33
Bacteroides fragilis 784	+	+
Escherichia coli ATCC 26	−	−
Klebsiella pneumoniae ATCC 8045	−	ND
Pseudomonas aeruginosa BMH No. 1	−	−
P. aeruginosa 799/61*	ND	−
Staphylococcus aureus A5271	−	−

* Obtained from W. ZIMMERMANN (same as ATCC 35151).
ND: Not determined.

tron microscopy of AB 999F-80 revealed that the spore chains have a rugose surface. The arthrospores have average dimensions of 0.64×0.67 μm (Fig. 1). Analysis of whole cell hydrolysates of AB 999F-80 and AB 1047T-33 revealed the presence of LL-diaminopimelic acid in both cultures.

Fig. 1. Scanning electron micrograph of *Streptomyces violaceoniger* AB 999F-80 on starch - yeast extract - salts agar[7] incubated at 28°C for 14 days. Bars represent 1 μm.



Cultural and Physiological Characteristics

The appearance of strains AB 999F-80 and AB 1047T-33 on eleven media is given in Table 2. The ability of these microorganisms to utilize various carbon compounds in synthetic medium is given in Table 3. Both organisms are able to utilize most of the carbohydrates and polyols in the test. Some physiological characteristics are presented in Table 4. AB 1047T-33 has a slightly higher optimum growth temperature and an increased tolerance of NaCl. Strain AB 999F-80 was resistant to 50 μg/ml of erythromycin but was susceptible to neomycin, novobiocin, oxytetracycline, benzylpenicillin, rifampicin, streptomycin and vancomycin at 50 μg/ml.

Fermentation

Growth, pH, consumption of carbohydrate and accumulation of phenelfamycin A are plotted in a time course study of a fermentation with *S. violaceoniger* AB 999F-80 (Fig. 2). A maximum potency of 52 μg/ml was achieved at 114 hours, but the activity subsequently degraded rapidly. Both strains produce phenelfamycins A, B, C, E, F and unphenelfamycin, but fermentations with *S. violaceoniger* AB 1047T-33 were enriched with phenelfamycins E and F[1].

Discussion

Our interest in the phenelfamycin producing organisms originated with the observation that they synthesized a substance inhibitory for *B. fragilis*; however, the purified antibiotics are selectively active against *Neisseria gonorrhoeae* and Gram-positive anaerobes and have very poor activity against *Bacteroides* sp.[2].

The phenelfamycin producing organisms, AB 999F-80 and AB 1047T-33, can be assigned to the

Table 2. Cultural characteristics of strains AB 999F-80 and AB 1047T-33.

Medium		AB 999F-80	AB 1047T-33
Yeast extract - malt extract agar (ISP No. 2)	G:	Abundant	Abundant, mottled
	AM:	Sparse; white (263)[a] and medium gray (265)	Medium gray (265) with black coalescence (267)
	R:	Light grayish yellowish brown (79)	Strong yellowish brown (74)
	SP:	Absent	Absent
Oatmeal agar (ISP No. 3)	G:	Abundant	Moderate
	AM:	Brownish gray (64)	Light brownish gray (63) with black coalescence (267)
	R:	Grayish yellow (90)	Pale yellow (89) and medium gray (265)
	SP:	Absent	Absent
Inorganic salts - starch agar (ISP No. 4)	G:	Moderate	Moderate, mottled
	AM:	White (263) and medium gray (265)	Light brownish gray (63) with black coalescence (267)
	R:	Light yellowish brown (76)	Light grayish yellowish brown (79) and medium gray (265)
	SP:	Absent	Absent
Glycerol - asparagine agar (ISP No. 5)	G:	Moderate	Fair
	AM:	Sparse; white (263) and light gray (264)	Moderate; white (263)
	R:	Light yellowish brown (76)	Grayish yellow (90)
	SP:	Light grayish yellowish brown (79)	Absent
Peptone - yeast extract - iron agar (ISP No. 6)	G:	Poor	Poor
	AM:	Absent	Absent
	R:	Moderate orange (53)	Moderate orange (53)
	SP:	Absent	Absent
Tyrosine agar (ISP No. 7)	G:	Abundant	Moderate
	AM:	Mottled; white (263) and brownish gray (64)	Light gray (264) with black coalescence (267)
	R:	Dark reddish brown (44)	Deep yellowish brown (75)
	SP:	Grayish brown (61)	Slight; grayish yellowish brown (80)
Nutrient agar	G:	Moderate	Poor; flat
	AM:	Absent	Sparse; white (263)
	R:	Pale yellow (89)	Grayish yellow (90)
	SP:	Absent	Absent
Calcium malate agar	G:	Moderate	Fair
	AM:	Absent	White (263)
	R:	Grayish yellow (90)	Grayish yellow (90)
	SP:	Grayish yellow (90) Calcium malate is dissolved	Absent Calcium malate is dissolved
CZAPEK's agar	G:	Moderate	Moderate
	AM:	Absent	Sparse; white (263)
	R:	Yellowish white (92)	Light yellowish brown (76)
	SP:	Absent	Absent
Starch - yeast extract - salts agar, ref 7	G:	Poor	Moderate
	AM:	Brownish gray (64)	Light brownish gray (63) with brownish black coalescence (65)
	R:	Yellowish white (92) and brownish gray (64)	Medium gray (265)
	SP:	Absent	Absent
ATCC No. 172	G:	Moderate	Moderate
	AM:	Sparse; white (263)	Sparse; white (263)
	R:	Light yellowish brown (76) to moderate orange (53)	Light yellowish brown (76)
	SP:	Absent	Absent

[a] Color and number in parenthesis follow the color standard in K. L. KELLY and D. B. JUDD: ISCC-NBS Color-Name Charts Illustrated with Centroid Colors. U.S. Dept. of Comm. Suppl. to Cir. 553, Washington, D.C., 1976.

G: Growth, AM: aerial mycelium, R: reverse, SP: soluble pigment.

Table 3. Utilization of carbon sources by strains AB 999F-80 and AB 1047T-33.

Compound	AB 999F-80	AB 1047T-33
None (control)	−	−
Adonitol	++	++
Arabinose	++	++
Cellulose	−	−
Dulcitol	+	−
Fructose	++	++
Galactose	++	++
Glucose	++	++
Inositol	++	++
Lactose	++	++
Mannitol	++	++
Melezitose	ND	−
Melibiose	++	++
Raffinose	++	++
Rhamnose	++	++
Salicin	++	+
Sorbitol	ND	−
Starch	+	−
Sucrose	++	++
Trehalose	++	++
Xylose	++	++

++: Good growth, +: poor growth, −: no growth, ND: not determined.

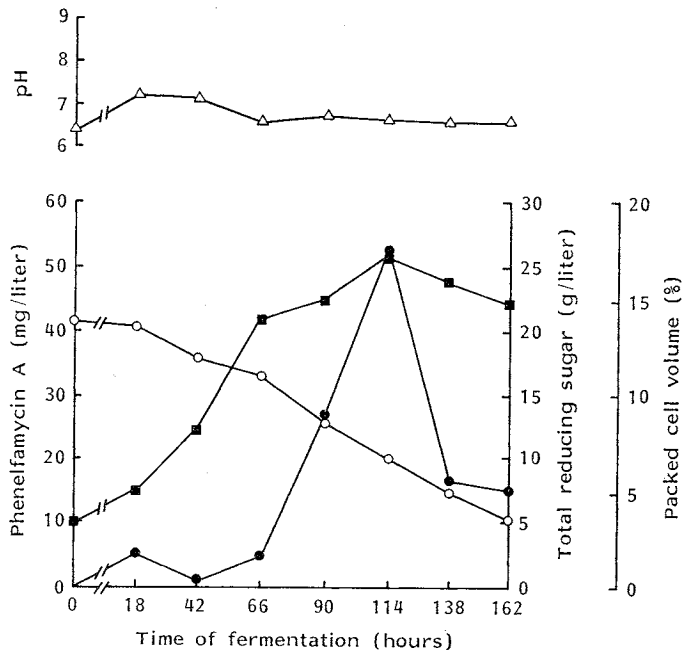
Table 4. Physiological characteristics of strains AB 999F-80 and AB 1047T-33.

Test	AB 999F-80	AB 1047T-33
Gelatin liquefaction	+ (weak)	ND
Starch hydrolysis	+	+
Nitrate reduction	−	+
Melanin formation	−	−
H_2S production	+	+
NaCl tolerance (%)	0~4	0~7
Litmus milk	Alkaline	Alkaline digestion
Temperature range (°C)[a]	15~37	21~37
Optimum (°C)	28	32
Decomposition		
Adenine	+	+
Casein	+	+
Hypoxanthine	+	+
L-Tyrosine	+ (weak)	+ (weak)
Xanthine	−	−

[a] Yeast extract - malt extract agar and ATCC medium 172.
ND: Not determined.

Fig. 2. Time course of the phenelfamycin fermentation by *Streptomyces violaceoniger* AB 999F-80 in a New Brunswick 150-liter fermentor.

● Phenelfamycin A, ■ packed cell volume, ○ total reducing sugar, △ pH.



genus *Streptomyces* on the basis of their morphology and cell wall composition. The key tables and species descriptions for the genus *Streptomyces* presented by PRIDHAM and TRESNER[10] in BERGEY's Manual suggest that strains AB 999F-80 and AB 1047T-33 are most closely related, if not identical, to *S. violaceoniger*. In addition the characteristics we determined for AB 999F-80 and AB 1047T-33 are in agreement with those reported by WILLIAMS *et al.*[11] for the *S. violaceoniger* cluster group, and the description of *S. violaceoniger* prepared by the ISP study group[12] is appropriate for AB 999F-80 and AB 1047T-33. Therefore, we have identified the phenelfamycin producing cultures, AB 999F-80 and AB 1047T-33, as strains of *S. violaceoniger*. While AB 999F-80 and AB 1047T-33 are sufficiently similar to be members of the same species, they differ in several cultural and physiological characteristics as well as in their production ratio of phenelfamycin components. We consider AB 999F-80 and AB 1047T-33 to be different strains of the same species rather than duplicate cultures. *S. violaceoniger* AB 999F-80 and *S. violaceoniger* AB 1047T-33 have been deposited at the Northern Regional Research Center in Peoria, Illinois where they have the accession numbers NRRL 18084 and NRRL 18920, respectively. The production of elfamycin-type antibiotics is distributed among several taxonomically distinct actinomycetes[13~20]. *S. violaceoniger* AB 999F-80 and *S. violaceoniger* AB 1047T-33 can be added to the list of organisms producing unique members of this antibiotic class.

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