PACIDAMYCINS, A NOVEL SERIES OF ANTIBIOTICS WITH ANTI-*PSEUDOMONAS AERUGINOSA* ACTIVITY

I. TAXONOMY OF THE PRODUCING ORGANISM AND FERMENTATION

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The pacidamycins are a new complex of nucleosidyl-peptide antibiotics with highly specific activity against *Pseudomonas aeruginosa*. They are produced by *Streptomyces coeruleorubidus* AB 1183F-64 which was isolated from a soil sample collected at Offenburg in the FRG. The mature spore masses of the producing organism are greenish gray to blue, and the spore chains are arranged in spirals. After the structures of the pacidamycins were determined, the fermentation medium was supplemented with component amino acids. This resulted in the directed biosynthesis of several members of the complex. The overall antibiotic recovered was increased from 1~2 mg/liter to more than 100 mg/liter through a combination of strain selection, medium manipulation and amino acid feeding experiments.

The pacidamycins are a new class of compounds discovered in a screening program designed to find antibiotics with activity against *Pseudomonas aeruginosa*.

This paper describes the taxonomy of the producing organism and the fermentation of the antibiotic complex. The isolation, structural elucidation and biological activity of the pacidamycins are described in accompanying publications[1,2].

Materials and Methods

Taxonomic Studies

The strain that produces the pacidamycins was isolated from a soil sample collected at Offenburg in the FRG. A subculture was deposited at the Northern Regional Research Center, United States Department of Agriculture, Peoria, Illinois, U.S.A. and assigned accession code NRRL 18370.

Methods and media described by the International Streptomyces Project (ISP)[3] and WAKSMAN[4] were used to determine most of the taxonomic characteristics of strain AB 1183F-64. ATCC medium 172† and a dilute starch yeast extract salts (DSYS)[5] agar were added for morphological studies. Hydrolysis of starch and decomposition of adenine, tyrosine and casein were determined by the method of GORDON *et al.*[6]. Observations were made after incubation at 28°C for 14 days. Color names were assigned to the mycelial and diffusible pigments on the basis of the Inter-Society Color Council-National Bureau of Standards (ISCC-NBS) Centroid Color Charts††. The diaminopimelic acid isomer was identified by the method of BECKER *et al.*[7].

Fermentation Studies

Sporulated slants of strain AB 1183F-64 grown on ISP 2 medium were used to inoculate seed

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

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

medium A which consisted of glucose monohydrate 1.0%, starch (Staley Staclipse JUB) 1.5%, yeast extract (Difco) 0.5%, NZ-Amine Type A (Sheffield) 0.5% and $CaCO_3$ 0.1%. The medium was prepared with distilled water and adjusted to pH 7.0 prior to sterilization. Inoculum for fermentation was prepared in two stages. Initially, the culture was inoculated into 25×150 mm culture tubes containing 10 ml of seed medium and incubated for 96 hours. At this time, 5% inoculum was transferred to 500-ml Erlenmeyer flasks containing 100 ml of the same medium. These flasks were incubated for 72 hours. All seed vessels were incubated on a rotary shaker at 250 rpm (5.6 cm stroke). Five % inoculum from the seed flasks was then transferred to production media. The production for screening was accomplished in medium A. Larger scale fermentations were conducted in an 150-liter fermentor charged with 80 liters of production medium B which consisted of glucose monohydrate 0.5%, starch (Staley Staclipse JUB) 2.5%, Lexein F-1000 liquid peptone (Inolex) 2.0%, molasses (Del Monte Brer Rabbit green label) 2.0%, dried whole yeast (Red Star) 0.5%, $CaCO_3$ 0.2% and DF100S antifoam (Mazur Chemical Co.) 0.01%. This medium was also prepared with distilled water and adjusted to pH 7 before sterilization. All seed and production vessels were incubated at 28°C. The fermentor was aerated at 0.7 vol/vol/minute and agitated at 200 rpm. Packed cell volumes were determined by centrifugation at $600 \times g$ for 30 minutes. Total reducing sugars were measured by a phenol-sulfuric acid method[8]. Antibiotic production during fermentation was monitored by a paper-disc agar-diffusion method using *P. aeruginosa* A5007 grown in Mueller-Hinton agar. Fermentation broth samples were prepared for assay by mixing with Amberlite XAD-2 resin, washing the recovered resin with water and then eluting successively with MeOH - water (1 : 1) and MeOH - water (3 : 1). Solvent was removed under reduced pressure, and the residue was reconstituted in EtOH - water (1 : 1) at a concentration 25 times that of the original fermentation broth.

Results and Discussion

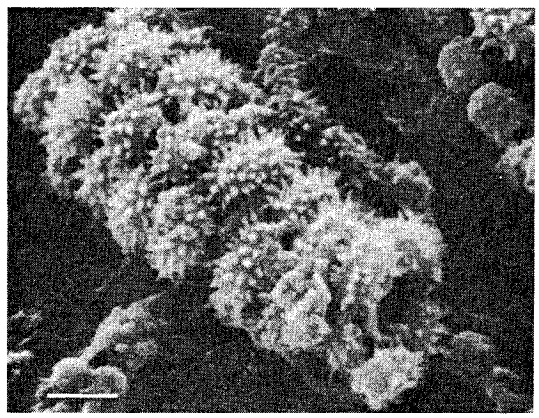
Taxonomy of Strain AB 1183F-64

The vegetative mycelium is well developed, branched and does not fragment. Spores occur on aerial hyphae in chains having a tight spiral configuration. Scanning electron microscopy showed that the spores have a spiny surface, are ellipsoidal and have an average size of 0.7~0.8 by 0.9~1.2 μm (Fig. 1). We did not observe sporangia, sclerotia or motile spores.

The cultural characteristics of strain AB 1183F-64 are shown in Table 1. The mature, sporulated, aerial surface growth is pale blue or greenish gray on most of the media tested. The reverse color is usually yellow to brown. A soluble melanoid pigment is produced on about half of the media examined.

Fig. 1. Sporophore morphology of strain AB 1183F-64 from a 10-day old culture grown on DSYS agar at 28°C.

Bar represents 1.0 μm.



The carbon source utilization pattern of this strain is shown in Table 2, and its physiological characteristics are indicated in Table 3. Whole-cell analysis of strain AB 1183F-64 revealed the presence of LL-diaminopimelic acid which indicates a Type I cell wall[9].

The taxonomic characteristics noted above clearly place this strain in the genus *Streptomyces*. Sporophore morphology places strain AB 1183F-64 in the *Spira* (S) morphology section of PRIDHAM *et al.*[10]. The blue to green color of the mature spore mass indicates that it belongs

Table 1. Cultural characteristics of strain AB 1183F-64.

Medium	Cultural characteristics
Yeast extract - malt extract agar (ISP 2)	G: Abundant AM: Greenish gray (155)[a] R: Dark grayish brown (62) SP: Dark grayish yellowish brown (81)
Oatmeal agar (ISP 3)	G: Moderate AM: Pale blue (185) R: Yellowish gray (93); dark grayish purple (229) and dark reddish brown (44) SP: Absent
Inorganic salts - starch agar (ISP 4)	G: Abundant AM: Greenish gray (155) R: Grayish reddish brown (46) SP: Absent
Glycerol - asparagine agar (ISP 5)	G: Moderate AM: Pale blue (185) R: Dark grayish reddish brown (47) SP: Absent
Peptone - yeast extract - iron agar (ISP 6)	G: Moderate AM: Absent R: Dark grayish yellowish brown (81) SP: Abundant; brownish black (65)
Tyrosine agar (ISP 7)	G: Moderate AM: Light greenish gray (154) R: Light yellowish brown (76) and dark grayish brown (62) SP: Grayish yellowish brown (80)
Nutrient agar	G: Moderate AM: Sparse; white (263) R: Grayish yellow (90) SP: Absent
CZAPEK's agar	G: Abundant AM: Absent R: Dark grayish purple (229) and grayish yellow (90) SP: Slight; grayish yellowish brown (80)
Calcium malate agar	G: Moderate; sporulation absent AM: Light grayish reddish brown (45) R: Very dark purplish red (260) SP: Light reddish brown (42) Calcium is solubilized
ATCC No. 172	G: Moderate AM: Sparse; light greenish gray (154) R: Moderate yellowish brown (77) SP: Absent
DSYS agar	G: Poor AM: Sparse; white (263) R: Yellowish gray (93) SP: Absent

[a] Color and number in parenthesis follow ISCC-NBS Centroid Color Charts.
G: Growth, AM: aerial mycelium, R: reverse, SP: soluble pigment.

to the blue series in the same classification scheme. TREJO and BENNETT[11] studied the blue-spored (Viridochromogenes) series in 1963. They found the series to be homogeneous in physiological characteristics and concluded that reverse color and spore morphology provide the basis for separation below the series level. Our strain fulfills the major characteristics of this series. It is chromogenic and has greenish gray to blue spores borne in spirals. Among the species described by TREJO and BENNETT[11], the occasional reddish-brown reverse color, spiny spore surface and long spiral chains of strain AB 1183F-64 best resemble *Streptomyces coeruleorubidus*. BERGEY's Manual and keys developed to identify strains in the ISP study also suggest *S. coeruleorubidus* as a possible identity for AB 1183F-64[12~16]. The numerical analysis of an extensive study of *Streptomyces* by WILLIAMS *et al.*[17] placed blue-spored streptomycetes in two clusters. Our strain is most compatible with cluster 18 which includes *S. coeruleorubidus*. LEE *et al.*[18], in a patent describing antibiotics related to the pacidamycins, identified their organism as *S. coeruleorubidus* and named it as subspecies *rubidus* on the basis of prolific production of red soluble pigments. While the reverse color of our strain was often reddish-brown, soluble pigments, if present, were usually yellowish-brown. We designate the pacidamycin-producing organism *Streptomyces coeruleorubidus* strain AB 1183F-64 (NRRL

Table 2. Utilization of carbon sources by strain AB 1183F-64.

Compound	Growth
Adonitol	++
Arabinose	++
Cellulose	−
Dulcitol	−
Fructose	++
Galactose	++
Glucose	++
Inositol	++
Lactose	++
Maltose	++
Mannitol	++
Mannose	++
Melezitose	−
Melibiose	++
Raffinose	++
Rhamnose	++
Ribose	++
Salicin	++
Sorbitol	+
Starch	−
Sucrose	++
Trehalose	++
Xylose	++

++: Well utilized, +: weakly utilized, −: not utilized.

Table 3. Physiological characteristics of strain AB 1183F-64.

Test	Reaction
Starch hydrolysis	+
H_2S production	+
Melanin formation in:	
Tryptone - yeast extract broth	+
Peptone - yeast extract - iron agar	+
Tyrosine agar	+
NaCl tolerance	Good growth at 4%
	Poor growth at 7%
Litmus milk	Digested
Nitrate reduction	−
Decomposition of:	
Adenine	+
Casein	+
L-Tyrosine	+
Growth temperature range	Growth at 21 to 37°C
	No aerial mycelium at 37°C

18370).

Fermentation of Strain AB 1183F-64

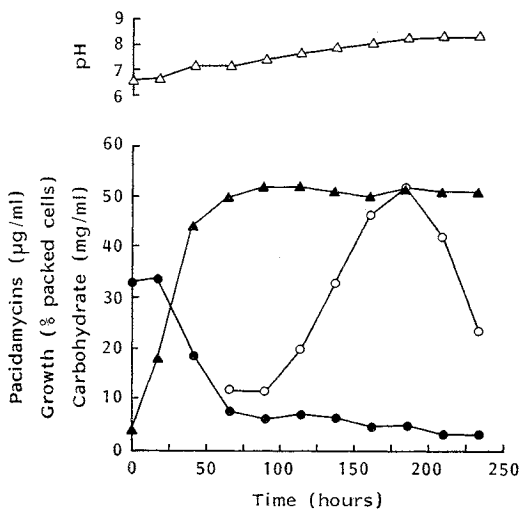
In initial studies, 1~2 mg/liter of antibiotic complex were recovered from medium A. The original soil isolate, however, quickly lost the ability to produce antibiotic. The culture was then plated on agar media, and 480 isolates were selected. Agar plugs of these cultures were placed on media seeded with *P. aeruginosa* BMH 1. The antibiotics were produced by sporulated but not by bald strains. The sporulated isolates producing the largest zones of inhibition were then studied in submerged fermentation. One isolate was found to consistently produce the antibiotic in medium A at the same levels first observed. The isolate was then grown in a variety of media to increase yields. This led to the selection of medium B from which as much as 106 mg/liter of the pacidamycin complex was recovered. Medium B served as the base medium for all amino acid supplementation work.

Once the structures of the pacidamycins were determined, feeding studies were initiated. Broth was supplemented with two of the terminal component amino acids. Table 4 shows the effect of adding 0.1% *meta*-tyrosine, 0.1% tryptophan and a mixture of 0.1% of each at 66 hours (harvest at 144 hours) to the fermentation broth. Tryptophan directed biosynthesis to the congener containing this amino acid (pacidamycin 1). The addition of *meta*-tyrosine resulted in a decrease in the level of pacidamycin 1 and shifted biosynthesis to pacidamycins 2 and/or 3†. A time course showing the production of the antibiotic complex by the fermentation supplemented with tryptophan is indicated in Fig. 2.

Table 4. Supplementation of fermentation broth with tryptophan and *meta*-tyrosine.

Amino acid	Pacidamycins (mg/liter antibiotic recovered)	
	1	2 and 3
Control	21	47
Tryptophan	57	23
meta-Tyrosine	8	60
Both	19	52

Fig. 2. Time course of pacidamycin fermentation.
Tryptophan (0.1%) added at 66 hours.
○ Pacidamycins, ● carbohydrate, ▲ growth, △ pH.



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† The analysis did not distinguish between pacidamycins 2 and 3.

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