ARIZONINS, A NEW COMPLEX OF ANTIBIOTICS RELATED TO KALAFUNGIN

I. TAXONOMY OF THE PRODUCING CULTURE, FERMENTATION AND BIOLOGICAL ACTIVITY

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(Received for publication March 7, 1988)

The arizonins, a novel complex of antibiotics related to kalafungin, were discovered in the fermentation broth of *Actinoplanes* sp. AB660D-122. Comparative taxonomic studies indicated that the culture is a new species and therefore has been designated *Actinoplanes arizonaensis* sp. nov. Two members of the complex, arizonins A1 and B1, exhibit moderate to potent *in vitro* antimicrobial activity against pathogenic strains of Gram-positive bacteria.

The arizonins were discovered while screening actinomycetes for new antimicrobial agents. Comparison of the producing culture to similar actinoplanetes revealed that it is a new member of the genus *Actinoplanes* and has been named *Actinoplanes arizonaensis* sp. nov. strain AB660D-122. This paper describes the taxonomy of the producing culture and the fermentation and biological activity of the antibiotic. Portions of this work were described previously[1]. A companion paper has been published detailing the isolation and characterization of the arizonins including their relationship to kalafungin[2].

Materials and Methods

Microorganisms

The culture that produces the arizonins was isolated from a soil sample collected near Tuba City, Arizona, U.S.A. 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 B-16399. Other *Actinoplanes* strains were obtained from the American Type Culture Collection (ATCC), the University of North Carolina Collection (UNCC) and our laboratory. The bacteria and fungi in the activity spectra of arizonins A1 and B1 were from the stock culture collection in our laboratory and from the ATCC.

Taxonomic Studies

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 AB660D-122. ATCC medium 172† and a dilute starch - yeast extract - salts (DSYS) agar[5] were added for morphological studies. Hydrolysis of starch and decomposition of 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††. Whole-cell sugars were identified

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

†† ISCC-NBS color-name charts illustrated with centroid colors. U.S. Dept. of Comm. supp. to NBS Cir. 553, Washington, D.C., U.S.A.

by the method of LECHEVALIER[7] and diaminopimelic acid isomers by the method of BECKER *et al.*[8].

Fermentation Studies

Slants of strain AB660D-122 grown on ATCC medium 172 were used to inoculate the seed medium which consisted of glucose monohydrate 0.1%, starch (Staley Staclipse JUB) 2.4%, yeast extract (Difco) 0.5%, Tryptone (Difco) 0.5%, beef extract (Scott) 0.3% and $CaCO_3$ 0.4%. The medium was prepared in distilled water and adjusted to pH 7.0 prior to sterilization. Inoculum for fermentation was prepared in three stages. The culture was incubated on a rotary shaker at 250 rpm (5.6 cm stroke) for 72 hours in 2-liter Erlenmeyer flasks containing 800 ml of seed medium. Then 5% inoculum was transferred to 55-liter fermentors charged with 30 liters of the same medium. After 72 hours, 5% inoculum was transferred to 1,000-liter fermentors charged to 300 liters of the medium noted above. All seed vessels were incubated at 30°C. Seed fermentors were aerated at 1.0 v/v/m. The 55-liter fermentor was agitated at 200 rpm and the 1,000-liter fermentor at 70 rpm. Seed medium in fermentors was supplemented with 0.01% DF 40P antifoam (Mazer Chemical Co.).

Five hundred liters from the last seed fermentors were then used to inoculate an 8,000-liter production fermentor containing 5,000 liters of medium consisting of sucrose 1%, Proflo cotton seed flour (Buckeye Cellulose Corporation) 1%, yeast extract (Difco) 0.5%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, $MnCl_2 \cdot 4H_2O$ 0.0005%, $CuSO_4 \cdot 5H_2O$ 0.0005%, $ZnSO_4 \cdot 7H_2O$ 0.0005%, $CoCl_2 \cdot 6H_2O$ 0.0001%, $CaCO_3$ 0.1% and DF 40P antifoam (Mazer Chemical Co.) 0.01%. This medium was prepared in tap water and adjusted to pH 7.3 prior to sterilization. The production fermentor was incubated at 30°C, aerated at 0.8 v/v/m and agitated at 60 rpm.

Packed cell volumes and total reducing sugars were measured as described by us previously[9].

Progress of the fermentation was monitored by placing paper disks holding 20 μl of broth extract on pH 8 streptomycin assay agar plates seeded with *Staphylococcus aureus* A5177. Extracts of 5,000-liter fermentation samples were prepared by adjusting broths to pH 5 with H_2SO_4 and mixing with 2 vol BuOH. The solvent layer was separated, evaporated under reduced pressure and then the residue was reconstituted in MeOH to a concentration 100 times that of the original fermentation broth. We also found that the arizonins could be obtained from the lower solvent layer when fermentation broths were adjusted to pH 3 and mixed with 1/2 vol MeOH and 1 vol methylene chloride.

In Vitro Activity

Bacterial MICs of arizonins A1 and B1 were determined by the agar dilution method†. Wilkins-Chalgren agar was used for anaerobes and brain heart infusion agar for aerobes. Fungal MICs were obtained by an analogous 2-fold agar dilution method using yeast nitrogen base agar buffered at pH 7 and containing 0.5% glucose[10].

Results and Discussion

Taxonomy

Morphological Characteristics

The vegetative mycelia of strain AB660D-122 are fine (0.5~0.7 μm i.d.) and irregularly branched. On both ISP No. 4 agar and DSYS agar the culture formed abundant spherical to oval shaped sporangia ranging in diameter from 5 to 8 μm (Fig. 1). When suspended in water, sporangia formed on DSYS agar released numerous spherical to subspherical actively motile spores that were 1.2~1.5 μm i.d. Electron microscopy of the sporangiospores showed that they are lophotrichously flagellated (Fig. 2). Sporangia formed on ISP No. 4 agar did not rupture even after overnight incubation in water or phosphate buffer. Purposely crushed sporangia released spores, but these were not motile.

† National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A. Reference agar dilution procedure for antimicrobic susceptibility testing of anaerobic bacteria. Approved standard M11-A. National Committee for Clinical Laboratory Standards, Villanova, 1985.

Fig. 1. Scanning electron micrograph of a sporangium of strain AB660D-122 from a 14-day old culture grown on ISP No. 4 agar (bar=5 μm).

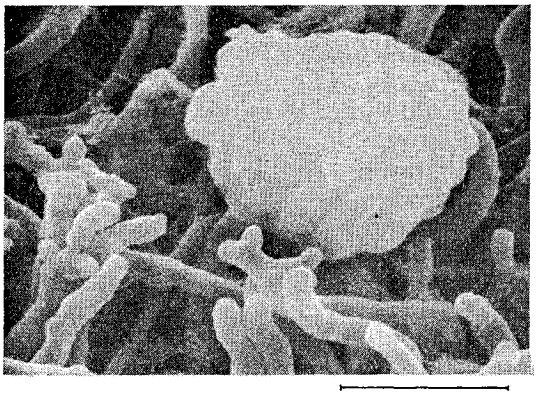

Fig. 2. Negatively stained transmission electron micrograph of a sporangiospore of strain AB660D-122 showing a tuft of polar flagella (bar=1 μm).

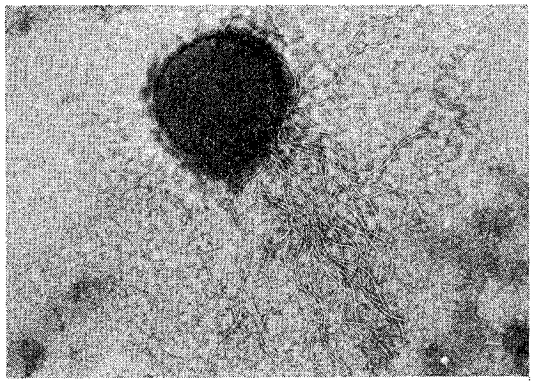

Table 1. Cultural characteristics of strain AB660D-122.

Cultural medium	Vegetative mycelium	Sporangia	Soluble pigment
ISP No. 2 (yeast extract - malt extract agar)	Abundant growth, wrinkled surface, moderate reddish orange (37)	Absent	Strong brown (55)
ISP No. 3 (oatmeal agar)	Moderate growth, smooth surface, pale orange yellow (73), abundant digitate hyphae	Absent	Pale orange yellow (73)
ISP No. 4 (inorganic salts - starch agar)	Moderate growth, smooth surface, strong brown (55) to light orange (52), scant digitate hyphae	Abundant	Pale orange yellow (73)
ISP No. 5 (glycerol - asparagine agar)	Poor growth, wrinkled surface, strong orange yellow (68)	Moderate	Strong orange yellow (68)
ISP No. 6 (peptone - yeast extract - iron agar)	Moderate growth, wrinkled surface, grayish reddish brown (46)	Absent	Deep brown (56)
ISP No. 7 (tyrosine agar)	Moderate growth, smooth surface, moderate yellowish pink (29), abundant digitate hyphae	Absent	Strong reddish brown (40)
CZAPEK sucrose agar	Moderate growth, smooth surface, moderate orange (53), abundant digitate hyphae	Trace	Dark orange yellow (72)
Nutrient agar	Moderate growth, smooth surface, moderate orange (53)	Absent	Absent
Calcium malate agar	Moderate growth, smooth surface, moderate orange yellow (71), scant digitate hyphae	Moderate	Moderate orange yellow (71)
ATCC 172 agar	Abundant growth, raised, wrinkled surface, moderate reddish brown (43)	Absent	Strong brown (55)
Hickey - Tresner agar	Abundant growth, raised surface, light grayish yellowish brown (79)	Absent	Strong brown (55)
Glucose asparagine agar	Moderate growth, raised surface, yellowish white (92)	Absent	Absent
Bennett agar	Moderate growth, wrinkled surface, light yellowish brown (76)	Absent	Moderate orange yellow (71)
DSYS agar	Moderate growth, smooth surface, light yellowish brown (76)	Abundant	Absent

Color and number in parenthesis follow ISCC-NBS Centroid Color Charts.

Fig. 3. Scanning electron micrograph of digitate hyphae formed by strain AB660D-122 grown on ISP No. 4 agar for 14 days (bar=5 μm).

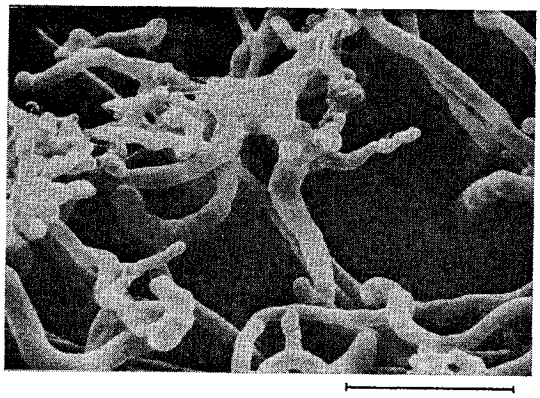

Table 2. Physiological characteristics of strain AB660D-122.

Test	Reaction
H_2S production	−
Gelatin liquefaction	+
Casein decomposition	+
Starch hydrolysis	+
Tyrosine decomposition	+
Nitrate reduction	+
Milk peptonization	−
Milk coagulation	−
Melanin formation[a]	+
Calcium malate solubilization	−
Growth temperature range[b]	15~37°C (no growth at 4 and 42°C)

[a] Peptone-yeast extract-iron agar (ISP No. 6) and tyrosine agar (ISP No. 7).
[b] ATCC medium 172.

Table 3. Utilization of carbon sources by strain AB660D-122.

Compound	Growth
Glucose	++
L-Arabinose	++
D-Xylose	++
Inositol	++
D-Fructose	++
Mannose	++
Mannitol	++
Rhamnose	++
Sucrose	+
Raffinose	−
Salicin	+
Lactose	+
Cellulose	−
None	−

++ Good growth, − no growth.

Table 4. Strains of *Actinoplanes* studied.

Species	Strain No.
Actinoplanes auranticolor	ATCC 31011
A. azureus	ATCC 31157
A. braziliensis	ATCC 25844
A. coloradoensis	AB921J-26[9]
A. deccanensis	ATCC 21983
A. ferrugineus	ATCC 29868
A. garbadinensis	ATCC 31049
A. ianthinogenes	ATCC 21884
A. liguriae	ATCC 31048
A. missouriensis	UNCC 431
A. philippinensis	ATCC 12427
A. teichomyceticus	ATCC 31121
A. utahensis	ATCC 14539

Unusual, short, digitate hyphae (0.3~1.0 μm i.d.) are formed abundantly on ISP No. 3, ISP No. 7 and CZAPEK sucrose agars and to a lesser degree on ISP No. 4 and calcium malate agars (Table 1). These structures could be abortive sporangia. Some do, in fact, resemble the immature sporogenous hyphae (without sporangial wall) depicted by VOBIS[11] for *Pilimelia*, a genus closely related to *Actinoplanes*. The spores of strain AB660D-122, however, are not arranged in parallel rows as are those of *Pilimelia*. A scanning electron micrograph of these hyphae is shown in Fig. 3.

Cultural and Physiological Characteristics

The cultural characteristics of strain AB660D-122 are shown in Table 1, the physiological characteristics in Table 2 and the carbon source utilization pattern in Table 3. The culture grew well on all media except ISP No. 5. Depending on the medium, the color of the substrate mycelium varies among hues of pink, yellow, orange and brown. Soluble pigment is produced on all media tested except

Table 5. Comparative cultural characteristics of *Actinoplanes arizonaensis*, *Actinoplanes garbadinensis* and *Actinoplanes utahensis*.

Medium		*A. arizonaensis* AB660D-122	*A. garbadinensis* ATCC 31049	*A. utahensis* ATCC 14539
ISP No. 5	G:	Poor, smooth surface	Moderate, smooth surface	Moderate, raised surface
	MC:	Strong orange yellow (68)	Light orange (52)	Strong orange (50)
	SP:	Strong orange yellow (68)	Absent	Absent
ISP No. 7	G:	Moderate, smooth surface	Abundant, raised surface	Abundant, raised surface
	MC:	Yellowish pink (29)	Light reddish brown (42)	Strong orange (50)
	SP:	Strong reddish brown (40)	Absent	Absent
CZAPEK	G:	Moderate, smooth surface	Moderate, raised surface	Abundant, flat surface
sucrose	MC:	Moderate orange yellow (71)	Light orange (52) to vivid orange (48)	Yellowish white (92)
	SP:	Moderate orange yellow (71)	Absent	Absent
Calcium	G:	Moderate, smooth surface	Poor, smooth surface	Poor, smooth surface
malate	MC:	Moderate orange yellow (71)	Pale orange yellow (73)	Yellowish white (92)
	SP:	Moderate orange yellow (71)	Absent	Absent

G: Growth, MC: mycelial color, SP: soluble pigment.

Table 6. Differentiating characteristics of *Actinoplanes arizonaensis*, *Actinoplanes garbadinensis* and *Actinoplanes utahensis*.

Characteristic	*A. arizonaensis* AB660D-122	*A. garbadinensis* ATCC 31049	*A. utahensis* ATCC 14539
Utilization of:			
Raffinose	−	−	+
Inositol	+	−	−
H_2S formation	−	+	+
Calcium malate solubilization	−	+	+
Soluble pigment	Yellow to brown on most media	Brown on some media	Absent
Diameter of sporangia (μm)	5~8	7~12	8~18
ref		13)	13)

nutrient, glucose asparagine and DSYS agars.

Whole-cell Chemical Analyses

Analysis of whole-cell hydrolysates of strain AB660D-122 demonstrated the presence of a major amount of *meso*-diaminopimelic acid and a trace of the hydroxy analog. Moderate levels of the diagnostic sugars arabinose and xylose were also detected along with large amounts of galactose, mannose and glucose.

Species Determination

The morphological and cultural characteristics of strain AB660D-122 placed it in the genus *Actinoplanes* as first described by COUCH[12]. Strain AB660D-122 was compared in our laboratory to the *Actinoplanes* species listed in Table 4. The results of this study plus a review of published descriptions of other *Actinoplanes* strains indicate that our isolate is different from all those that are known. The most striking feature that distinguishes strain AB660D-122 from other members of this genus is the formation of abundant, digitate hyphae on several media. The cultures that most closely resembled AB660D-122 morphologically are *Actinoplanes garbadinensis* and *Actinoplanes utahensis*[13]. Our

Fig. 4. Time course of arizonins production.

▲ pH, ○ *Staphylococcus aureus* zone size, ■ packed cell volume, △ total reducing sugar.

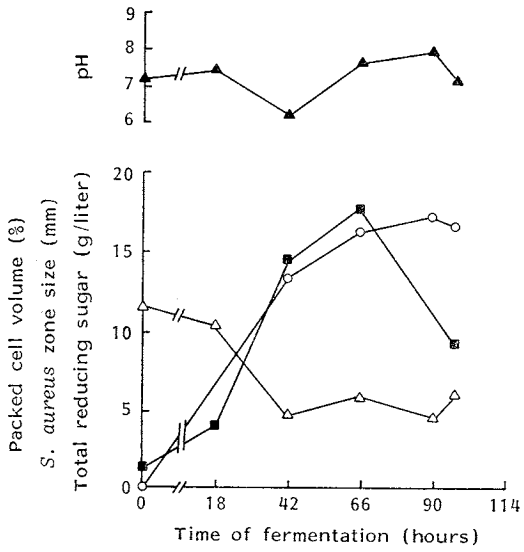

Table 7. *In vitro* antimicrobial activity of arizonins A1 and B1.

Test microorganism	MIC (μg/ml)	
	A1	B1
Staphylococcus aureus ATCC 6538P	6.2	3.1
S. aureus CMX686B	6.2	1.56
S. aureus A5177	6.2	0.2
S. aureus 45	6.2	0.39
S. aureus 45 RAR2	6.2	1.56
S. epidermidis 3519	12.5	1.56
Micrococcus luteus ATCC 9341	25	3.1
M. luteus ATCC 4698	3.1	0.2
Enterococcus faecium ATCC 8043	50	12.5
Streptococcus bovis A5169	25	3.1
S. agalactiae CMX508	6.2	1.56
S. pyogenes EES 61	12.5	3.1
S. pyogenes 930	3.1	0.78
Escherichia coli Juhl	>25	>25
Pseudomonas aeruginosa A5007	>25	>25
Bacteroides fragilis ATCC 25285	50	25
Clostridium perfringens ATCC 13124	>50	>25

Table 8. *In vitro* antifungal activity of arizonins A1, B1 and kalafungin.

Test microorganism	MIC (μg/ml)		
	A1	B1	Kalafungin
Candida albicans ATCC 10231	100	50	6.25
C. albicans 579A	100	50	6.25
C. albicans CCH 442	100	50	6.25
C. tropicalis NRRL-Y-112	100	50	6.25
Torulopsis glabrata ATCC 15545	50	25	6.25

culture, however, could be differentiated from these two species in a number of features as indicated in Tables 5 and 6. We are, therefore, designating strain AB660D-122 a new species of the genus *Actinoplanes* and propose the name *Actinoplanes arizonaensis* sp. nov. (M.L. adj. pertaining to Arizona).

Fermentation

A time course in an 8,000-liter fermentor showing the production of the arizonin antibiotic mixture is given in Fig. 4. After our initial studies we observed a significant decrease in yield in the arizonin fermentation. Additional work showed that antibiotic production could be restored to former levels by supplementing the fermentation medium with 0.1% citric acid indicating that the problem might be trace metal toxicity.

In Vitro Activity

As shown in Table 7, arizonins A1 and B1 show moderate to potent activity against pathogenic strains of Staphylococci and Streptococci. The antifungal activity of both arizonins and kalafungin is indicated in Table 8. Arizonin B1 is the more potent of the two compounds. Neither antibiotic has significant activity against fungi, anaerobic bacteria or Gram-negative aerobic bacteria.

Acknowledgments

The authors are grateful to Dr. M. F. MILLER and Ms. F. A. DURUM for electron microscopy.

References

1) KARWOWSKI, J. P.; M. JACKSON, R. J. THERIAULT, J. F. PROKOP, M. L. MAUS & D. M. HENSEY: Arizonins, a new complex of antibiotics related to kalafungin. I. Taxonomy, fermentation and biological activity. Program and Abstracts of the 26th Intersci. Conf. on Antimicrob. Agents Chemother., No. 921, p. 265, New Orleans, Sept. 28~Oct. 1, 1986
2) HOCHLOWSKI, J. E.; G. M. BRILL, W. W. ANDRES, S. G. SPANTON & J. B. MCALPINE: Arizonins, a new complex of antibiotics related to kalafungin. II. Isolation and characterization. J. Antibiotics 40: 401~407, 1987
3) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
4) WAKSMAN, S. A. (*Ed.*): The Actinomycetes. Vol. 2. Classification, Identification and Descriptions of Genera and Species. Williams & Wilkins Co., Baltimore, 1961
5) KARWOWSKI, J. P.: The selective isolation of *Micromonospora* from soil by cesium chloride gradient density ultracentrifugation. J. Indust. Microbiol. 3: 181~186, 1986
6) GORDON, R. E.; D. A. BARNETT, J. E. HANDERHAN & C. H. PANG: *Nocardia coeliaca*, *Nocardia autotrophica*, and the nocardin strain. Int. J. Syst. Bacteriol. 24: 54~63, 1974
7) LECHEVALIER, M. P.: Identification of aerobic actinomycetes of clinical importance. J. Lab. Clin. Med. 71: 934~944, 1968
8) BECKER, B.; M. P. LECHEVALIER, R. E. GORDON & H. A. LECHEVALIER: Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. Appl. Microbiol. 12: 421~423, 1964
9) JACKSON, M.; J. P. KARWOWSKI, R. J. THERIAULT, P. B. FERNANDES, R. C. SEMON & W. L. KOHL: Coloradocin, an antibiotic from a new *Actinoplanes*. I. Taxonomy, fermentation and biological properties. J. Antibiotics 40: 1375~1382, 1987
10) HOLT, R. J.: Laboratory tests of antifungal agents. J. Clin. Pathol. 28: 767~774, 1975
11) VOBIS, G.: Sporogenesis in the *Pilimelia* species. *In* Biological, Biochemical and Biomedical Aspects of Actinomycetes. *Ed.*, L. ORTIZ-ORTIZ *et al.*, pp. 423~439, Academic Press, Inc., Orlando, 1984
12) COUCH, J. N.: *Actinoplanes*, a new genus of *Actinomycetales*. J. Elisha Mitchell Sci. Soc. 66: 87~92, 1950
13) PARENTI, F. & C. CORONELLI: Members of the genus *Actinoplanes* and their antibiotics. Ann. Rev. Microbiol. 33: 389~411, 1979