ARIDICINS, NOVEL GLYCOPEPTIDE ANTIBIOTICS

I. TAXONOMY, PRODUCTION AND BIOLOGICAL ACTIVITY

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(Received for publication February 4, 1985)

A new species of a new genus of the *Actinomycetales* was discovered, *Kibdelosporangium aridum*. This strain produces a new family of glycopeptide antibiotics designated aridicins, that contain an unusual glycolipid constituent. They inhibit Gram-positive bacteria, including staphylococci, enterococci and *Clostridium* sp.

While studying organisms originating from unusual ecological niches, we isolated a new genus of the *Actinomycetales* that elaborated antibiotics which inhibited bacterial cell-wall synthesis. These were shown to be novel glycopeptide antibiotics named aridicins[1].

In this paper we will describe the new genus, *Kibdelosporangium*, cultural conditions for production of aridicins and the antibiotic activities of the major components of the complex.

Materials and Methods

Strain

The aridicin producer, SK&F-AAD-216, was isolated from a soil sample collected in a desert area of Pima County, Arizona. Stock cultures were grown on agar slants of thin potato - carrot agar or oatmeal agar (ISP 3) and aliquots were stored in the vapor phase of liquid nitrogen.

Physiological and Biochemical Tests

The physiological and biochemical tests used to characterize SK&F-AAD-216 were those of GORDON[2,3] and GORDON and MIHM[4]. Cell-wall preparations were analyzed by the method of BECKER *et al.*[5], and whole-cell hydrolysates were prepared and examined using the chemotaxonomic techniques of LECHEVALIER[6]. The lipid composition of cell-wall extracts was determined by the method of LECHEVALIER *et al.*[7].

Assay of Aridicins

Initially, microbiological assays were performed using *Bacillus subtilis* ATCC 6633. Subsequently, a HPLC procedure was developed and this was used to determine total antibiotics (aridicins A, B and C) in the experiments reported here.

Antimicrobial Activity

The minimum inhibitory concentrations (MICs, μg/ml) for aerobic bacteria were determined by microtiter broth dilution tests using Dynatech MIC-2000 equipment. The growth medium was Trypticase soy broth, pH 7.0, and the inoculum size was approximately 10^5 cfu/ml. The microtiter plates were incubated at 37°C overnight. The MICs for *Clostridium difficile* were determined by agar dilution tests in Wilkins-Chalgren agar. The inoculum was applied directly to the surface of the agar with a Steers replicator. The tests were incubated at 37°C for 48 hours in a Capco anaerobic chamber

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in an atmosphere of 88% N_2, 7.0% H_2 and 5.0% CO_2. Control compounds were commercial preparations.

In mouse protection studies, the growth from an 18-hour Trypticase soy agar slant of *Staphylococcus aureus* 127 was diluted in 5% hog gastric mucin to a level of 3.0×10^7 cfu/ml. This inoculum, 0.5 ml/mouse, was injected intraperitoneally to produce a uniformly lethal mouse infection in 18~21 g Webster-derived CD1 male mice (Charles River Laboratories). The test antibiotics were administered subcutaneously at 1 and 5 hours after infection. The final percentages of survival for groups of 10 mice each, obtained after 3 days of observation, were used to estimate the 50% effective dose (ED_{50}, mg/kg) and the 50% lethal dose (LD_{50}, cfu/mouse) values. The ED_{50} and the LD_{50} values were determined by the logit transformation analysis. Control compounds were commercial preparations.

Results

Morphological and Cultural Characteristics

The producing strain is a Gram-positive, non-acid-fast, filamentous organism that formed a mycelium differentiated into: 1) a substrate mycelium that penetrated the agar and formed a compact surface layer, and 2) an aerial mycelium that originated from the substrate mycelium.

No motile cells were produced in either the aerial or substrate mycelium.

The substrate mycelium was well developed and exhibited a tendency to fragment without hyphal displacement. The moderately branched hyphae were septate and approximately 0.4~1.0 μm in diameter. Present on the substrate hyphae, both deep within and at the surface of the agar, were specialized structures which consisted of dichotomously branched, septate hyphae radiating from a common stalk. These appeared to be "naked" sporangium-like structures analogous to the conidial structures which COUCH[8] described on the substrate hyphae of the *Actinoplanaceae*. Characteristic crystals were produced in the agar on many media.

The aerial hyphae (Fig. 1) bore straight or irregularly curved chains of spores and sporangium-like structures (Fig. 2). The spore chains were usually very long with greater than 50 spores per chain. The smooth-surfaced spores were rod-shaped and irregular in length (0.4 μm$\times$0.8~2.8 μm). The mature sporangium-like structures were usually round, approximately 9~22 μm in diameter, and surrounded by a well-defined wall. Despite extensive observation and experimentation, the development or release of spores was never seen.

Fig. 1. Micrograph of spore chains of strain SK&F-AAD-216 (22-day-old culture on oatmeal agar).
Bar=10 μm.

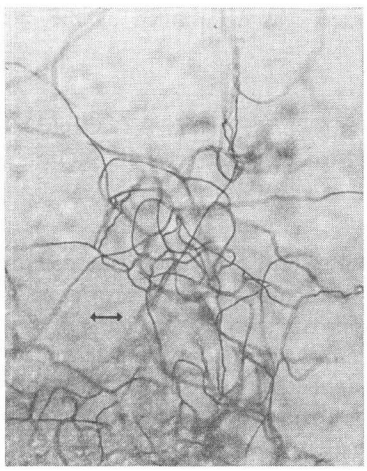


Fig. 2. Micrograph of sporangium-like structures of strain SK&F-AAD-216 (8-week-old culture on water agar).
Bar=10 μm.

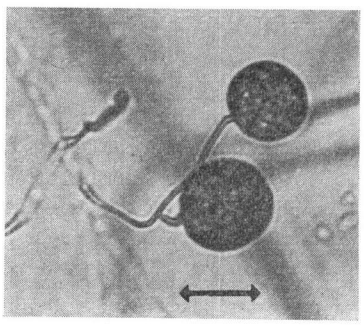


Table 1. Growth characteristics of SK&F-AAD-216 on different media.

Medium	Growth and morphology	Medium	Growth and morphology
Yeast extract - malt extract agar (ISP 2)	G: Excellent, raised, grayish yellow-brown AM: None to very sparse, white SP: Light yellow-brown SPL: Sparse SC: Moderate C: Present	Thin potato - carrot agar	G: Fair, relatively flat, off-white to pale yellow-brown AM: Sparse to moderate, white SP: None SPL: Numerous SC: Numerous C: None detected
Oatmeal agar (ISP 3)	G: Good, slightly raised, off-white to yellow-brown AM: Sparse, white SP: None SPL: Moderate to numerous SC: Moderate to numerous C: Present	Soil extract agar	G: Fair, relatively flat, pale yellow-brown AM: Sparse, white SP: None SPL: Numerous SC: Moderate C: Variably present
Inorganic salts - starch agar (ISP 4)	G: Good, slightly raised, off-white to yellow-brown AM: Sparse, white SP: None SPL: Moderate SC: Moderate C: Present	Nutrient agar	G: Fair to good, raised, yellow-brown AM: Sparse to moderate, white SP: Yellow-brown SPL: None to sparse SC: Sparse C: Variably present
Glycerol - asparagine agar (ISP 5)	G: Fair to good, raised, pale yellow-brown AM: Sparse to moderate, white SP: Pale grayish yellow-brown SPL: None SC: Sparse C: Present	Bennett agar	G: Good to excellent, raised, grayish yellow-brown AM: None visible to sparse, white SP: Grayish yellow-brown SPL: Numerous SC: Moderate C: None detected
Peptone - yeast extract - iron agar (ISP 6)	G: Good, raised, gray to bronze-brown AM: None SP: Dark brownish black SPL: None SC: None C: None detected	Water agar	G: Poor, relatively flat, translucent to yellow-brown AM: Sparse to moderate, white SP: None SPL: Moderate to numerous SC: Moderate to numerous C: None detected

G: Growth of substrate mycelium, AM: aerial mycelium, SP: soluble pigment, SPL: sporangium-like structures, SC: spore chains, C: crystals.

When placed on a nutrient agar, these structures germinated by the direct production of germ tubes.

Plates for the determination of cultural characteristics of SK&F-AAD-216 were incubated at 28°C in closed Petri dish cans and observed at intervals up to 21 days. The growth characteristics of SK&F-AAD-216 on various media are presented in Table 1. On all media tested, the vegetative mycelium was off-white to grayish yellow-brown. The aerial mycelium was white, sparse to moderate in amount, and tended to be thin. No pigments, other than melanin or yellow-brown soluble pigments, were produced.

Chemotaxonomy, Physiological and Biochemical Characteristics

The cell-wall contained *meso*-diaminopimelic acid, alanine, glutamic acid, glucosamine, muramic acid, galactose and a minor amount of arabinose. Whole-cell hydrolysates contained galactose,

glucose, mannose, ribose, rhamnose, arabinose and a trace of madurose. No mycolic acids were found. Thus, this organism has a Type IV cell - wall and a Type A sugar pattern plus a trace of madurose[9].

The organism did not grow under anaerobic conditions. The temperature range for growth was 15~42°C. The following tests were positive: hydrogen sulfide production; peptonization of milk; gelatin hydrolysis and liquefaction; melanin production; hydrolysis of casein, L-tyrosine, hypoxanthine, guanine, elastin, urea, esculin and hippurate; catalase and phosphatase production. Tests for allantoin decomposition were weakly positive. Negative results were obtained for: reduction of nitrate; hydrolysis of starch, adenine, xanthine and Avicel. No growth occurred in lysozyme broth. Growth in 4% NaCl was consistent; growth in 5% to 7% NaCl was inconsistent. No growth occurred at 8% NaCl.

Acid was produced from L-arabinose, D-cellobiose, dextrin, dextrose, D-fructose, glycerol, glycogen, D-galactose, *i*-inositol, lactose, D-mannitol, D-mannose, α-methyl-D-mannoside, α-methyl-D-glucoside, melibiose, D-melezitose, raffinose, rhamnose, D-ribose, sucrose, trehalose, D-xylose and maltose. No acid was produced from dulcitol, *i*-erythritol, inulin, D-sorbitol or L-sorbose. Citrate, malate, succinate, oxalate, lactate, acetate, pyruvate, propionate and formate were utilized; benzoate and tartrate were not utilized.

Identification and Classification

A comparison of the description of SK&F-AAD-216 with the actinomycetes listed in the Approved Lists of Bacterial Names[10], BERGEY's Manual of Determinative Bacteriology[11], and recent taxonomic literature indicated that this organism could not be accommodated in any of the previously described genera of the actinomycetes. Sporangia with well-defined walls, where present in other actinomycete genera, are true spore vesicles; at maturity they contain aplanospores or zoospores which are eventually released by rupture or dissolution of the sporangial wall. The sporangium-like structures of the aridicin producer did not contain spores and germinated by the production of one or more germ tubes directly from the sporangium-like structure. Therefore, we regard this organism as a new species of a new genus for which we propose the name *Kibdelosporangium aridum* (kibdelos, Gr. adj., false, ambiguous; spora, Gr. n., a seed; angium, Gr. n., a vessel); the specific epithet (aridus,

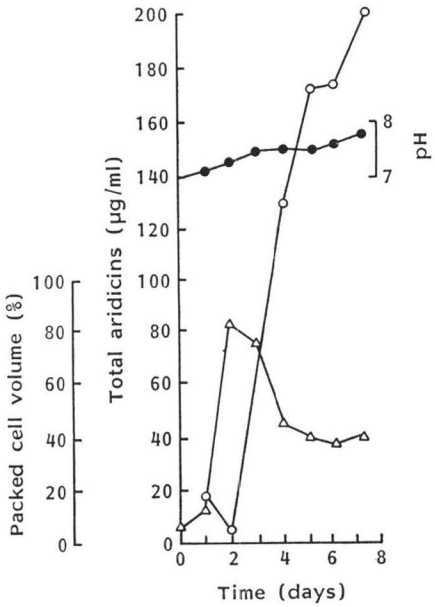
Table 2. Maintenance, seed and production media for aridicins.

Maintenance agar (M172) (g/liter)		Seed medium (13H) (g/liter)		Production medium (V-2) (g/liter)	
Glucose	10	Starch	15.0	Soybean meal	15.0
Starch	20	Sucrose	5.0	Beet molasses	10.0
Yeast extract	5	Dextrose	5.0	Estrasan 4[b]	10.0
N-Z Amine	5	HySoy	7.5	Glucose	10.0
$CaCO_3$	1	Corn steep liquor	5.0	NaCl	0.3
Agar	20	K_2HPO_4	1.5	pH adjusted to 7.0 with NaOH	
		NaCl	0.5		
		$CaCO_3$	1.5		
		Mineral supplement	5 ml/liter[a]		
		pH=7.0			

[a] Mineral supplement (g/liter): $ZnSO_4 \cdot 7H_2O$ 2.8, $Fe(NH_4)_2HC_6H_5O_7$ 2.7, $CuSO_4 \cdot 5H_2O$ 0.125, $MnSO_4 \cdot H_2O$ 1.0, $CoCl_2 \cdot 6H_2O$ 0.1, $Na_2B_4O_7 \cdot 10H_2O$ 0.1, $Na_2MoO_4 \cdot 2H_2O$ 0.05.

[b] Commercial methyl oleate, approximately 55% v/v.

Fig. 3. Production of aridicins.
○ Concentrations (μg/ml) of total glycopeptide (aridicins A, B and C). △ Biomass (%, v/v). ● pH.



L. adj., dry, arid) refers to the desert soil from which the culture was isolated. Strain SK&F-AAD-216, the type strain of *K. aridum*, has been deposited in the American Type Culture Collection, Rockville, Md., as strain ATCC 39323.

Production of Aridicins

An agar slant culture of *K. aridum* was grown on medium M172 (Table 2) at 28°C for 14 days. The slant contents were dispersed and suspended in 10 ml of sterile distilled water and inoculated into 500 ml of seed medium 13H (Table 2) contained in a 4-liter aspirator bottle. This seed culture was incubated at 28°C for 3 days on a shaker at 250 rpm and 5-cm throw. After 3 days the entire seed was transferred to 10 liters of medium V-2 (Table 2) in a 14-liter New Brunswick Fermentor M-19. The fermentor was maintained at 26°C with agitation at 400 rpm and aeration of 4 liters/minute.

Under the above conditions the maximum concentration of the complex (aridicins A, B and C) was 200 μg/ml, after 7.5 days (Fig. 3). The nature of the medium ingredients automatically controlled the pH to its optimum; the pH gradually increased to 8.0 by the time of harvest. The production of aridicins followed the termination of biomass accumulation, presumably due to exhaustion of a growth limiting nutrient. Antibiotic produc-

Table 3. Antibacterial activity of aridicins and comparative compounds.

Strains	MIC (μg/ml)						
	Aridicin A	Aridicin B	Aridicin C	Vanco-mycin	Genta-micin	Erythro-mycin	Clinda-mycin
Staphylococcus aureus 127	1.6	3.1	3.1	1.6	3.1	0.4	—
S. aureus 910	3.1	3.1	3.1	1.6	6.3	>200	—
S. epidermidis 2479	6.3	6.3	6.3	1.6	0.4	0.4	—
S. haemolyticus 651	50	25	25	1.6	0.2	200	—
Streptococcus faecalis 34358	0.8	0.4	0.8	3.1	50	>200	—
Listeria monocytogenes 2255	0.8	0.4	0.4	1.6	0.8	≤0.2	—
Proteus mirabilis 444	>100	>100	>100	100	3.1	200	—
Escherichia coli 12140	>100	>100	>100	100	6.3	50	—
Klebsiella pneumoniae 4200	>100	>100	>100	>100	0.4	100	—
Pseudomonas aeruginosa 63	>100	>100	>100	>100	6.3	200	—
Serratia marcescens ATCC 13880	>100	>100	>100	>100	6.3	200	—
Morganella morganii 179	>100	>100	>100	>100	0.8	50	—
Providencia sp. 276	>100	>100	>100	>100	12.5	>200	—
Enterobacter cloacae 31254	>100	>100	>100	>100	1.6	200	—
Salmonella gallinarum 595	>100	>100	>100	25	1.6	12.5	—
Bacteroides sp.	16	16	16	32	—	—	0.25
Clostridium difficile	0.125	0.25	0.25	2	—	—	>32

tion was inhibited when media contained KH_2PO_4 in excess of 1.5 mg/ml.

Antimicrobial Activity

Studies were carried out to determine the antimicrobial spectrum of purified products of aridicins A, B and C. These compounds were compared with vancomycin and resembled other members of the vancomycin-class in having only Gram-positive activity (Table 3). Aridicins A, B and C had similar activities. They were equivalent to vancomycin against strains of *S. aureus* but less active against coagulase negative staphylococci. They were superior to vancomycin against *Streptococcus faecalis* and were far superior to vancomycin against *C. difficile*.

In mouse protection tests aridicins A, B and C protected against strains of *Staphylococcus* sp. which were sensitive *in vitro*. The ED_{50}'s against *S. aureus* 127 were 5, 5 and 8 mg/kg, respectively, compared to 2 mg/kg for vancomycin.

Discussion

K. aridum ATCC 39323 following thorough investigation of its morphology, chemotaxonomy and physiology was identified as a new species of a new genus of the *Actinomycetales*. Other cultures belonging to this new genus have, subsequently, been isolated from widely distributed geographical areas. Many of these isolates, as well as *K. aridum*, produce novel glycopeptide antibiotics related to the vancomycin-class, but containing a unique glycolipid component[1]. Interestingly, these compounds are markedly more active than vancomycin against *S. faecalis* and *C. difficile* and we are investigating the possible structure activity functions of glycopeptides in this respect. The antibacterial activity indicates that these compounds may be useful in treating Gram-positive infections and may also have applications as growth promotants[12].

Acknowledgments

We would like to gratefully acknowledge the expert technical assistance of P. COLMAN, R. FERRIN, R. GERBER, M. POLANSKY, S. VOGT-SPETH, A. GIOVENELLA, R. REID and L. PHILLIPS during the course of this work.

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