DUNAIMYCINS, A NEW COMPLEX OF SPIROKETAL 24-MEMBERED MACROLIDES WITH IMMUNOSUPPRESSIVE ACTIVITY

I. TAXONOMY OF THE PRODUCING ORGANISMS, FERMENTATION AND ANTIMICROBIAL ACTIVITY

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The dunaimycins are a new complex of spiroketal 24-membered macrolides discovered in the fermentation broth of two actinomycetes. Based on taxonomic studies these two cultures, which were isolated from soil, were identified as *Streptomyces diastatochromogenes* strains AB 1691Q-321 and AB 1711J-452. The dunaimycins possess both immunosuppressive and antimicrobial activity.

The dunaimycins are a new complex of compounds discovered in the fermentation broths of *Streptomyces diastatochromogenes* strains AB 1691Q-321 and AB 1711J-452. These compounds were discovered with a screen for antifungal activity. Additional testing showed that they had significant immunosuppressive effects. This paper describes the taxonomy of the producing microorganisms, the fermentation and the antibiotic activity of several members of the complex. The isolation, structural elucidation and immunosuppressive activity of the dunaimycins are described in accompanying publications[1,2].

Materials and Methods

Microorganisms

Strain AB 1691Q-321 was isolated from soil collected at Parsberg, Germany. A subculture of the microorganism was deposited at the Northern Regional Research Center, United States Department of Agriculture, Peoria, Illinois, U.S.A. and assigned accession code NRRL 18716. Strain AB 1711J-452 was obtained from soil collected on the banks of the Danube River near Belgrade, Yugoslavia. It was given accession code NRRL 18717 at the same repository. The bacteria and fungi used for bioassays 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] were used to determine most of the taxonomic characteristics of strains AB 1691Q-321 and AB 1711J-452. ATCC medium 172† and a dilute starch-yeast extract-salts (DSYS) agar[4] were added for morphological studies. Starch hydrolysis was determined by the method of GORDON *et al.*[5], the test for H_2S production was according to SMIBERT and KRIEG[6] and nitrate reduction was examined by KUTZNER's technique[7]. Morphological observations were made after incubation at 28°C for 21 days. Utilization of carbon sources was determined 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

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

Charts†. Analysis of the whole-cell diaminopimelic acid isomer was done by the method of BECKER *et al.*[8].

Fermentation Studies

The inocula for antibiotic production by strains AB 1691Q-321 and AB 1711J-452 were initiated by the following procedure. Vegetative mycelium, stored at −75°C, was used at 0.4% to inoculate 2-liter Erlenmeyer flasks containing 600 ml of medium. The seed medium consisted of glucose monohydrate 1.5%, soy flour 1.5%, yeast extract (Difco) 0.1%, NaCl 0.1% and $CaCO_3$ 0.1%. This medium was prepared with distilled water and adjusted to pH 7 before sterilization. The seed flasks were incubated for 72 hours at 28°C on a rotary shaker (5.6 cm stroke) at 225 rpm.

The fermentation medium for strain AB 1691Q-321 consisted of starch (Staley Staclipse JUB) 3.0%, molasses (Del Monte) 2.0%, Lexein F-159 liquid peptone (Inolex) 2.0%, whole yeast (Universal Foods) 0.5%, $CaCO_3$ 0.2% and XFO-371 antifoam. The antifoam was added initially at 0.01% and then was available on demand. This medium was also prepared with distilled water and adjusted to pH 7 before sterilization. Fermentation was carried out in a 150-liter vessel containing 80 liters of medium. The fermenter was inoculated with 4 liters of seed flask growth. The temperature was controlled at 28°C. The agitation rate was 250 rpm, and the air flow rate was 0.7 v/v/minute. The fermentation was terminated at 139 hours.

The fermentation medium for strain AB 1711J-452 consisted of glucose monohydrate 2.0%, molasses (Del Monte) 0.5%, Lexein F-1000 liquid peptone (Inolex) 1.0%, whole yeast (Universal Foods) 0.5%, $CaCO_3$ 0.2% and XFO-371 antifoam. The antifoam was added initially at 0.01% and then was available on demand. This medium was also prepared with distilled water with no pH adjustment. Fermentation was carried out in a 22-liter vessel containing 15 liters of medium. The fermenter was inoculated with 750 ml of seed flask growth. The temperature was controlled at 28°C. The agitation rate was 250 rpm, and the air flow rate was 0.7 v/v/minute. The fermentation was terminated at 116 hours.

Fermentation Analyses

Cell growth was evaluated as packed cell volume by centrifuging the fermentation broth in a graduated conical tube at $600 \times g$ for 20 minutes. Residual carbohydrates were monitored by DUBOIS' phenol-sulfuric acid method[9]. Dunaimycins were determined by an agar diffusion disk assay with *Aspergillus niger* ATCC 16404. Fermentation samples were prepared for bioassay by treating the whole culture broth with an equal volume of acetone.

In Vitro Activity

MICs of dunaimycins A1, C2, D2, D2S, D3 and D4S were determined by microtiter broth dilution methodology. Aerobic bacteria were tested in Brain Heart Infusion broth (Difco), anaerobes in Wilkins-Chalgren broth (Difco) and fungi in Yeast Nitrogen Base broth (Difco) containing 0.05% glucose. Tests were conducted with initial inocula of approximately 10^4 to 10^5 cfu/ml. Aerobic bacteria and fungi were incubated 24 hours. Anaerobes were incubated for 48 hours.

Results and Discussion

Taxonomy

Morphological and Chemical Characteristics

Both strains produce branched vegetative hyphae typical of the order *Actinomycetales*. Each culture forms spores on flexuous chains which terminate in open spirals. Scanning electron microscopy of strain AB 1691Q-321 revealed that the spore surface is slightly wrinkled, and the spores have an average size of 1.2×0.5 μm (Fig. 1). Scanning electron micrograph of strain AB 1711J-452 showed that the spore surface is smooth, and the spores have an average size of 1.0×0.8 μm (Fig. 2). Analysis of whole cell hydrolysates of AB 1691Q-321 and AB 1711J-452 revealed the presence of LL-diaminopimelic acid in both cultures which indicates a type I cell wall[10].

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

Fig. 1. Sporophore morphology of strain AB 1691Q-321 from a 15-day old culture grown on 1/20 strength ISP 4 medium (1.5% agar) at 28°C.

Bar represents 1 μm.

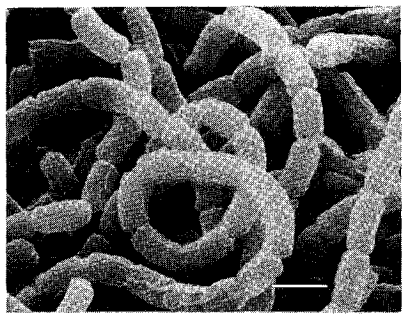

Fig. 2. Sporophore morphology of strain AB 1711J-452 from a 10-day old culture grown on ISP 4 medium at 28°C.

Bar represents 1 μm.

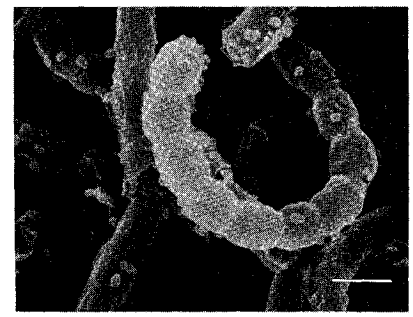

Table 1. Cultural characteristics.

Medium		Strain AB 1691Q-321	Strain AB 1711J-452
Yeast extract-malt extract agar (ISP 2)	G:	Abundant, sporulated	Abundant
	AM:	Light brownish gray (63)[a]	Light gray (264) and black (267)
	R:	Dark brown (59)	Dark orange yellow (72)
	SP:	Dark grayish yellowish brown (81)	Grayish yellowish brown (80)
Oatmeal agar (ISP 3)	G:	Abundant, sporulated	Moderate, partial aerial
	AM:	Light brownish gray (63)	Medium gray (265) and black (267) coalescence
	R:	Dark grayish yellow (91)	Dark gray (266)
	SP:	Absent	Absent
Inorganic salts-starch agar (ISP 4)	G:	Abundant	Abundant
	AM:	Light gray (264) and medium gray (265)	Light gray (264) and medium gray (265)
	R:	Grayish reddish brown (46)	Grayish reddish brown (46)
	SP:	Absent	Absent
Peptone-yeast extract-iron agar (ISP 6)	G:	Moderate	Moderate
	AM:	Absent	Absent
	R:	Dark yellowish brown (78)	Dark grayish brown (62)
	SP:	Brownish black (65)	Brownish black (65)
Tyrosine agar (ISP 7)	G:	Abundant	Abundant; pocks in aerial mycelium
	AM:	Light brownish gray (63)	Light brownish gray (63) and yellowish gray (93)
	R:	Brownish black (65)	Brownish black (65)
	SP:	Dark grayish yellowish brown (81)	Brownish black (65)
ATCC 172 agar	G:	Abundant	Abundant
	AM:	Yellowish gray (93)	Absent
	R:	Deep yellowish brown (75)	Moderate yellowish brown (77)
	SP:	Dark yellowish brown (78)	Slight; dark yellowish brown (78)
DSYS agar	G:	Moderate	Moderate
	AM:	Light brownish gray (63)	Light gray (264)
	R:	Yellowish gray (93)	Yellowish gray (93); colony centers are dark gray (266)
	SP:	Absent	Absent

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

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

Cultural and Physiological Characteristics

The cultural characteristics of strains AB 1691Q-321 and AB 1711J-452 are shown in Table 1. The mature, sporulated, aerial surface growth of both isolates is gray on all media tested. The cultures also produce soluble melanoid pigment on rich media. The carbon source utilization pattern of each is indicated in Table 2, and their physiological characteristics are shown in Table 3. The physiological characteristics are identical, but strain AB 1691Q-321 can utilize starch and sorbitol while AB 1711J-452 can not.

Species Determination

The morphology and type I cell wall of the dunaimycin producers place them in the genus *Streptomyces*. The 8th Edition of BERGEY's Manual[11] and keys published by NONOMURA[12] and KURYŁOWICZ *et al.*[13] were used to generate a list of species with similar characteristics. A comparison of our cultures with descriptions of these species suggested *S. diastatochromogenes* as the most appropriate assignment. The description of

Table 2. Utilization of carbon sources.

Carbon source	Growth	
	AB 1691Q-321	AB 1711J-452
Arabinose	++	++
Cellulose	−	−
Fructose	++	++
Glucose	++	++
Inositol	++	++
Mannitol	++	++
Raffinose	++	++
Rhamnose	++	++
Sorbitol	+	−
Starch	+	−
Sucrose	++	++
Xylose	++	++

++: Good utilization, +: poor utilization, −: did not utilize.

Fig. 3. Time course of the dunaimycin fermentation by *Streptomyces diastatochromogenes* AB 1691Q-321 in a 150-liter fermenter.

△ Growth, ▲ pH, ○ total carbohydrate, ● dunaimycins.

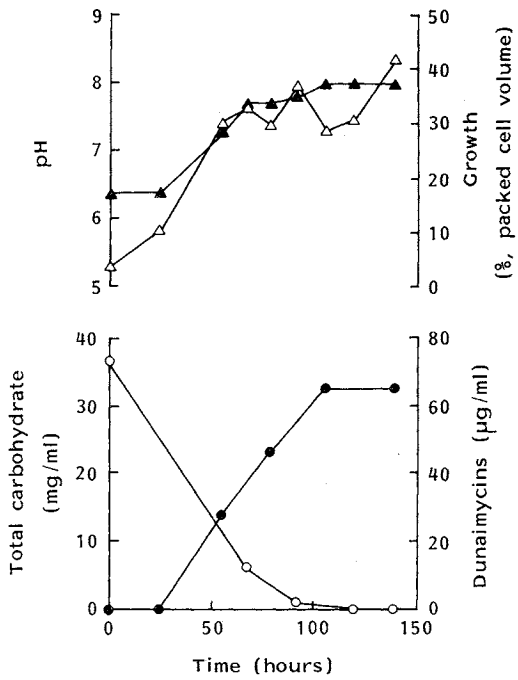

Table 3. Physiological characteristics.

Test	AB 1691Q-321	AB 1711J-452
Melanin formation:		
Peptone-yeast extract-iron agar	+	+
Tyrosine agar	+	+
Starch hydrolysis	+	+
H_2S production	+	+
NaCl tolerance (%) (yeast extract-malt extract agar)	<4	<4
Temperature range (yeast extract-malt extract agar)	Growth at 22 to 32°C, no growth at 42°C	Growth at 22 to 32°C, no growth at 42°C
Nitrate reduction	+	+

S. diastatochromogenes prepared by the International Streptomyces Project[14] is valid for AB 1691Q-321 and AB 1711J-452. Although quite similar, the two dunaimycin cultures vary in their gross appearance on some agar media and deviate slightly in their carbohydrate utilization patterns. These variations as well as differences in component production of the dunaimycins during fermentation encouraged us to regard them as separate strains of the same species. The latest edition of BERGEY's Manual[15] treats *S. diastatochromogenes* as a subjective synonym of *S. diastaticus*. We prefer to retain *S. diastatochromogenes* to recognize the melanin production of these cultures. Therefore, we have designated the dunaimycin producers *S. diastatochromogenes* AB 1691Q-321 and *S. diastatochromogenes* AB 1711J-452.

Fermentation

The time course of the production of the dunaimycins by strain AB 1691Q-321 is shown in Fig. 3. A maximum yield of 65 μg/ml of the dunaimycins was reached at 105 hours. *S. diastatochromogenes* strain AB 1691Q-321 produces dunaimycins C1, C2, D2, D2S, D3 and D4S in fermentation. Strain AB 1711J-452 produces dunaimycins A1, D2S and D3S.

Antimicrobial Activity

Table 4 indicates that the dunaimycins show only slight activity against bacteria. Table 5 indicates that the dunaimycins have modest activity against *A. niger* but are much less active against *Candida albicans* and other yeasts.

Table 4. *In vitro* antibacterial activity of dunaimycins A1, C2, D2, D2S, D3 and D4S.

Test microorganism	MIC (μg/ml)					
	A1	C2	D2	D2S	D3	D4S
Staphylococcus aureus ATCC 6538P	>8	16	32	32	>8	32
S. aureus A5177	8	16	32	64	>8	64
Enterococcus faecium ATCC 8043	8	>128	128	128	>8	128
Streptococcus pyogenes EES61	4	128	64	128	>8	64
Micrococcus luteus ATCC 4698	4	1	1	4	4	1
Escherichia coli Juhl	>64	>128	>128	>128	>64	>128
Enterobacter aerogenes ATCC 13048	>64	>128	>128	>128	>64	>128
Klebsiella pneumoniae ATCC 8045	>64	>128	128	>128	>64	>128
Pseudomonas aeruginosa A5007	>64	128	64	128	64	128
Acinetobacter calcoaceticus CMX 669	64	128	128	>128	64	128
Bacteroides fragilis ATCC 25285	>128	>128	>128	64	>128	64
Clostridium perfringens ATCC 13124	16	>128	64	64	32	128
C. difficile ATCC 9689	32	>128	32	64	>128	64

Table 5. *In vitro* antifungal activity of dunaimycins A1, C2, D2, D2S, D3 and D4S.

Test microorganism	MIC (μg/ml)					
	A1	C2	D2	D2S	D3	D4S
Candida albicans ATCC 10231	100	>100	>100	100	50	100
C. albicans 579A	100	>100	>100	100	50	>100
C. albicans ATCC 38247	100	>100	>100	100	50	>100
C. tropicalis NRRL-Y-112	100	100	100	100	100	100
C. kefyr ATCC 28838	>100	>100	>100	>100	50	>100
C. glabrata ATCC 15545	>100	100	100	>100	>100	>100
Cryptococcus albidus ATCC 34140	>100	>100	>100	100	50	100
Aspergillus niger ATCC 16404	25	6.25	12.5	6.25	25	6.25

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