ALTROMYCINS, NOVEL PLURAMYCIN-LIKE ANTIBIOTICS

I. TAXONOMY OF THE PRODUCING ORGANISM, FERMENTATION AND ANTIBACTERIAL ACTIVITY

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The altromycins are novel anthraquinone-derived antibiotics related to the pluramycins. They are produced by an actinomycete, AB 1246E-26, which was isolated from a South African bushveld soil. The altromycins have Gram-positive antibacterial activity with MICs of 0.2 to 3.12 μg/ml against Streptococci and Staphylococci.

The altromycins are a complex of novel anthraquinone-derived antibiotics found in the culture broth of an actinomycete designated strain AB 1246E-26. They are structurally related to the pluramycins[1]. This paper describes the discovery and fermentation of the altromycins, the taxonomy of the producing organism and the antibacterial properties of altromycin B. The isolation and structure elucidation of the altromycins are reported in a companion paper[2].

Materials and Methods

Microorganisms

Strain AB 1246E-26 was isolated from a soil collected from a South African bushveld. The isolation procedure was intended to enrich for nocardioform actinomycetes. The soil was suspended in water and heated to 55°C for 5 minutes. Dilutions of the heated suspension were plated on a dilute nutrient medium consisting of Tryptone 0.15%, peptone 0.025%, NaCl 0.025%, glucose monohydrate 0.05% and agar 1.5%. This medium was amended with cycloheximide (50 μg/ml), nystatin (20 μg/ml), nalidixic acid (30 μg/ml) and novobiocin (20 μg/ml). The plates were incubated for 21 days at 30°C. *Pseudomonas aeruginosa* K799/61 was obtained from Zimmermann[3]. Other strains were from the American Type Culture Collection (ATCC) or from the stock culture collection in our laboratory.

Discovery Screen

The altromycins were found by a two stage screen for substances with activity against *P. aeruginosa* which we described previously for the discovery of the coumamidines[4]. Essentially, strain AB 1246E-26 was selected because plugs of the culture grown on agar had activity against K799/61, a highly antibiotic-sensitive strain of *P. aeruginosa*.

Taxonomic Studies

Methods and media described by the International Streptomyces Project (ISP)[5], Waksman[6] and Gordon *et al.*[7] were used to determine most of the morphological and physiological characteristics. ATCC medium 172† and modified Gause No. 1 agar were also employed for the cultural characteristics. Color

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

names were assigned to the mycelial and diffusible pigments on the basis of the Inter-Society Color Council-National Bureau of Standards (ISCC-NBS) Color-Name Charts. With the exception of the temperature study, incubation was at 28°C. The diaminopimelic acid isomer was determined by the method of BECKER *et al.*[8]. Whole cell sugars were characterized by the procedure of LECHEVALIER[9]. Menaquinones were extracted as described by ATHALYE *et al.*[10] and analyzed by mass spectroscopy.

Fermentation

The seed medium consisted of glucose monohydrate 1.0%, Staclipse JUB starch (Staley) 1.5%, yeast extract (Difco) 0.5%, NZ amine type A (Humko Sheffield) 0.5% and $CaCO_3$ 0.1%. The pH was adjusted to 7.0 before sterilization. Inoculum for fermentations was maintained as frozen (−75°C) vegetative mycelium. It was used at 0.7% to inoculate 25 × 150 mm tubes containing 10 ml of the seed medium, which were incubated for 96 hours at 28°C. Vegetative growth from the seed tubes was transferred at 5% to 2-liter Erlenmeyer flasks containing 600 ml of the seed medium. The flasks were incubated for 72 hours at 28°C. For production of the antibiotic, a 150-liter New Brunswick fermenter was charged with 80 liters of a medium consisting of glucose monohydrate 2%, Lexein F-152 liquid peptone (Inolex) 1%, molasses (Del Monte) 0.5%, yeast extract (Difco) 0.1% and $CaCO_3$ 0.2%. The medium was prepared in distilled water and the pH was not adjusted. Sterilization was at 121°C and 1.05 kg/cm^2 for 1 hour. The fermentation was carried out at 28°C for 162 hours. The agitation was 200 rpm, the aeration was 0.7 v/v/m and the head pressure was 0.35 kg/cm^2.

Fermentation Analyses

Cell growth was evaluated as packed cell volume by centrifuging the fermentation broth in a graduated conical tube at 600 × *g* for 20 minutes. Reducing sugar was determined by the dinitrosalicylic acid method described by BERNFELD[11]. An agar diffusion assay using *Staphylococcus aureus* ATCC 6538P in streptomycin assay agar with yeast extract (BBL) was performed to monitor the accumulation of antibiotic during the fermentation.

In Vitro Activity

The MICs for altromycin B were determined by a 2-fold agar dilution method† in brain heart infusion agar.

Results

Discovery

The altromycin producing culture was selected from other soil isolates for its activity against *P. aeruginosa* K799/61. Concentrates of the fermentation broth were sufficiently active against other *Pseudomonas* strains to sustain interest in the antibiotic, and the isolation of the active components was accomplished using *P. aeruginosa* BMH 10 as the indicator.

Taxonomy

The vegetative mycelium of AB 1246E-26 is branched and has a tendency to fragment. Spores have not been observed. The appearance of strain AB 1246E-26 on eleven media is given in Table 1. Aerial mycelium is formed on yeast extract - malt extract, tyrosine and peptone - yeast extract - iron agars but only sparsely on other media. Soluble pigments are formed on media containing peptones and amino acids. The ability of this strain to utilize various carbohydrates and polyols as the sole source of carbon in synthetic medium is shown in Table 2. Some physiological properties are given in Table 3.

meso-Diaminopimelic acid, arabinose and galactose were found in whole-cell hydrolysates indicating

† National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A. National Committee for Clinical Laboratory Standards, Villanova, 1985.

Table 1. Cultural characteristics of strain AB 1246E-26.

Medium	Cultural characteristics
Yeast extract - malt extract agar (ISP 2)	G: Abundant AM: White to light gray (264)[a] R: Moderate reddish brown (43) SP: Moderate brown (58)
Oatmeal agar (ISP 3)	G: Moderate AM: Sparse, white R: Yellowish gray (93) SP: Absent
Inorganic salts - starch agar (ISP 4)	G: Poor AM: Sparse, white SP: Absent
Glycerol - asparagine agar (ISP 5)	G: Abundant AM: Sparse, white R: Grayish reddish orange (39); moderate orange (53); grayish yellow (90) SP: Light grayish reddish brown (45)
Peptone - yeast extract - iron agar (ISP 6)	G: Moderate AM: White R: Grayish reddish orange (39) SP: Light reddish brown (42)
Tyrosine agar (ISP 7)	G: Abundant AM: Yellowish white (92) R: Moderate reddish brown (43) SP: Grayish reddish brown (46)
Nutrient agar	G: Moderate AM: Sparse, white R: Light orange (52) SP: Light grayish reddish brown (45)
CZAPEK's agar	G: Moderate AM: Sparse, white R: Yellowish gray (93) SP: Absent
Calcium malate agar	G: Poor AM: Sparse, white SP: Absent
ATCC No. 172	G: Moderate AM: Sparse, light gray (264) R: Grayish reddish orange (39) SP: Absent
Gause No. 1 modified[b]	G: Moderate AM: Sparse, white R: Light gray (264) SP: Absent

[a] Color names and number in parentheses follow the color standard in KELLY, K. L. 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.

[b] KNO_3 0.1%, K_2HPO_4 0.05%, $MgSO_4$ 0.05%, NaCl 0.05%, $FeSO_4$ 0.001%, starch 0.1%, yeast extract 0.01% and agar 1.5%.

Observations after incubation for 14 days at 28°C.

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

a cell wall of type IVA[12]. The major menaquinone has MW 720 by MS and is, therefore, tetrahydrogenated with 8 isoprenoid units.

Fermentation

Growth, pH, consumption of carbohydrate and accumulation of altromycins are plotted in a time

Table 2. Utilization of various compounds as the sole source of carbon[5] by strain AB 1246E-26.

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

Incubation was at 28°C for 30 days.
++: Good utilization, +: poor utilization, −: did not utilize.

Table 3. Physiological characteristics of strain AB 1246E-26.

Test	Reaction	Test	Reaction
Starch hydrolysis	−	Casein	+
H_2S production	+	Xanthine	−
Melanin formation		Hypoxanthine	+
Peptone-yeast extract-iron agar	−	Resistance to lysozyme	+
Tyrosine agar	−	Resistance to antibiotics (50μg/ml)	
NaCl tolerance	Growth at 4%	Erythromycin	+
	but not at 7%	Gentamicin	−
Temperature range	Growth at 21	Kanamycin	+
	to 42°C	Novobiocin	+
	No growth at 54°C	Oxytetracycline	±
Litmus milk	Alkaline digestion	Rifampicin	+
Decomposition of:		Streptomycin	+
Adenine	+	Vancomycin	+

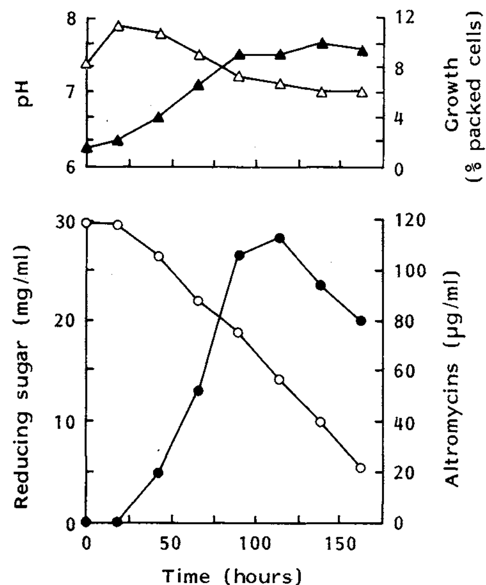
Table 4. Antibacterial activity of altromycin B.

Organism	MIC (μg/ml)	Organism	MIC (μg/ml)
Staphylococcus aureus ATCC 6538P	0.39	*S. pyogenes* 2548	0.39
S. aureus CMX 686B	1.56	*Escherichia coli* Juhl	>100
S. aureus A5177	1.56	*E. coli* SS	0.2
S. aureus 45	0.39	*E. coli* DC-2	>100
S. aureus 45 RAR2	3.12	*E. coli* H560	>100
S. aureus CMX 503A	1.56	*E. coli* KNK 437	25
S. aureus CMX 553	3.12	*Enterobacter aerogenes* ATCC 13048	>100
S. epidermidis 3519	1.56	*Klebsiella pneumoniae* ATCC 8045	>100
Micrococcus luteus ATCC 9341	0.1	*Providencia stuartii* CMX 640	>100
M. luteus ATCC 4698	0.39	*Pseudomonas aeruginosa* BMH10	50
Enterococcus hirae ATCC 8043	3.12	*P. aeruginosa* A5007	>100
Streptococcus bovis A5169	3.12	*P. aeruginosa* K799/WT	3.12
S. agalactiae CMX 508	0.39	*P. aeruginosa* K799/61	0.1
S. pyogenes EES61	0.39	*P. cepacia* 2961	>100
S. pyogenes 930	0.2	*Acinetobacter* sp. CMX 669	>100

course study of a fermentation with strain AB 1246E-26 (Fig. 1). A maximum potency of 112 μg/ml was achieved in 114 hours.

Fig. 1. Time course of the altromycin fermentation.

△ pH, ▲ growth, ● altromycins, ○ reducing sugar.



Antibacterial Activity

The MICs of altromycin B are given in Table 4. MICs for clinical isolates of Staphylococci range from 0.39 to 3.12 μg/ml. MICs for Streptococci are 0.2 to 3.12 μg/ml. With the exception of strains with compromised outer membranes, the MICs for Gram-negative bacteria are 25 to >100 μg/ml.

Discussion

The altromycin-producing strain is an actinomycete with a cell-wall of type IVA as expected by the soil isolation technique. The lack of sporulation and any remarkable morphological features requires that the identification of this culture rely on chemotaxonomy. The accumulated data narrow the taxonomic assignment of this culture to *Nocardia* or one of the genera grouped as Micropolysporas[13]. More extensive lipid analysis is needed to definitively assign this strain to a specific genus. The culture has been deposited at the Northern Regional Research Center in Peoria, Illinois, U.S.A., where it was assigned the accession No. NRRL 18371.

Although the altromycins were originally selected and isolated for their activity against a sensitive strain of *Pseudomonas*, their antibacterial activity is restricted to Gram-positive organisms. The MICs for Streptococci and Staphylococci suggest that the altromycins may have clinical utility.

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