COLORADOCIN, AN ANTIBIOTIC FROM A NEW *ACTINOPLANES*

I. TAXONOMY, FERMENTATION AND BIOLOGICAL PROPERTIES

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(Received for publication February 5, 1987)

Coloradocin was discovered in a screen for anti-anaerobe activity. The producing organism was determined to be a new species of *Actinoplanes*, designated *Actinoplanes coloradoensis* sp. nov. Coloradocin inhibits *Bacteroides*, *Clostridium* and other anaerobes. It does not inhibit most aerobic bacteria but is effective against *Neisseria gonorrhoeae* and *Haemophilus influenzae*. Coloradocin has low acute toxicity.

Coloradocin was first detected as an inhibitor of growth of *Bacteroides fragilis*. It was found to have activity against other anaerobes but is most potent against microaerophilic organisms such as *Neisseria gonorrhoeae* and *Haemophilus influenzae*. This paper reports the taxonomy of the producing organism, fermentation and biological properties. The structure elucidation of coloradocin and its identity with luminamicin are reported in a separate paper[1].

Materials and Methods

Isolation of the Organism

Strain AB 921J-26 was isolated from a soil collected near Cortez, Colorado. The isolation technique was a motile spore procedure for recovery of actinoplanetes (T. A. BOBIK and J. P. KARWOWSKI, unpublished).

Other Microorganisms

Actinoplanes deccanensis and *Actinoplanes missouriensis*, used for taxonomic comparison with AB 921J-26, were obtained from the American Type Culture Collection (ATCC). A mutant of *Escherichia coli* lacking lipopolysaccharide was sufficiently permeable to coloradocin when grown in a sparse medium with low inoculum to give a dose response curve and was used to monitor antibiotic production in fermentation broths. Strains employed for the evaluation of coloradocin are from the stock culture collection in our laboratory and the ATCC.

Taxonomic Studies

Methods and media described by the International Streptomyces Project (ISP)[2] and WAKSMAN[3] were used to determine most of the morphological and physiological characteristics. ATCC medium 172† was added for morphological studies. Hydrolyses of starch, tyrosine and casein were determined by the method of GORDON *et al.*[4]. 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-Science Color Council-National Bureau of Standards (ISCC-NBS) Centroid Color Charts††. Whole-cell

† 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., U.S.A.

sugars were identified by the procedure of LECHEVALIER[5]. The diaminopimelic acid isomer was determined by the method of BECKER *et al.*[6].

Inoculum for Fermentation

Vegetative seed growth was frozen at −75°C and used to inoculate the seed tubes of subsequent fermentations. The seed medium consisted of glucose monohydrate 0.1%, starch (Staley's Staclipse JUB) 2.4%, yeast extract (Difco) 0.1%, 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 prior to sterilization. Ten ml of medium in 25×150 mm culture tubes, covered with Bellco stainless caps, were inoculated with 0.5 ml of the frozen seed stock. The tubes were incubated for 96 hours. Two-liter Erlenmeyer flasks, containing 600 ml medium, were inoculated with the tube growth at 5% and incubated for 72 hours. The second passage flask growth was used to inoculate the fermentations at 5%. Both seed steps were incubated at 30°C on a rotary shaker at 250 rpm (3.2 cm stroke).

Fermentation

The fermentation medium consisted of glucose monohydrate 1%, starch (Staley's Staclipse JUB) 0.4%, molasses (Del Monte's Brer Rabbit green label) 0.5%, Lexein F-152 liquid peptone (Inolex) 0.4%, soybean flour 0.5%, NZ amine type A (Sheffield) 0.1%, yeast extract (Difco) 0.1%, $MgSO_4$ (anhydrous) 0.02%, $CaCO_3$ 0.1% and antifoam XFO-371 (Ivanhoe Chemical Co.) 0.01%. The fermentation was performed in a New Brunswick 150-liter fermentor charged to a volume of 80 liters. Incubation was at 30°C for 162 hours with an agitation rate of 200 rpm, an aeration rate of 0.7 vvm and a head pressure of 0.35 kg/cm^2.

Packed Cell Volume

Cell growth was evaluated by centrifugation in a 15-ml conical tube at 600×g for 30 minutes.

Total Reducing Sugars

Carbohydrate utilization was evaluated by analyzing for reducing sugars in hydrolyzed fermentation broth. Two ml of 2 N H_3PO_4 were added to 5 ml of fermentation broth supernatant. The mixture was heated for 30 minutes at 121°C, cooled and centrifuged. The supernatant from the hydrolysis was analyzed for reducing sugars by the method of HOFFMAN[7].

Assay for Coloradocin

Fermentation broth supernatant was adjusted to pH 4 and poured over a 1×10-cm Amberlite XAD-2 resin column (Rohm & Haas). The column was washed with distilled water and eluted with methanol. The solvent was removed under reduced pressure, and the residue was reconstituted in 98% methanolic water at a 250-fold concentration. Coloradocin was quantitated by a broth dilution assay in Antibiotic Assay medium 3 (BBL). A 1~4 dilution of an overnight culture of a lipopolysaccharide-deficient mutant *E. coli* was the inoculum. The assay was incubated at 37°C for 18 hours.

In Vitro Activity

Minimal inhibitory concentrations (MICs) of coloradocin were determined by the agar dilution method†. Wilkins-Chalgren agar was used for anaerobes, Proteose No. 3 agar (Difco) with 1% hemoglobin and 1% Kellogg supplement for *N. gonorrhoeae* and Mueller-Hinton agar supplemented with 5% lysed horse blood and 0.001% NAD for *H. influenzae*.

Acute Toxicity

Mice were injected intraperitoneally and observed for 6 days.

† 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.

Results

Taxonomy

Morphological Characteristics

The vegetative mycelia of strain AB 921J-26 are compact, fine (0.3~0.6 μm i.d.) and irregularly branched. Unbranched rudimentary aerial hyphae (0.8~1.0 μm i.d. and 4~14 μm long) are formed abundantly on ISP 5, nutrient and CZAPEK sucrose agars. Sporangia of irregular shape and ranging in diameter from 2.1 to 2.8 μm are formed in small numbers on ISP 5, CZAPEK sucrose and calcium malate agars (Fig. 1). Motile spores, observed in wet mounts, are released from the sporangia after suspension in water for about 1 hour. Examination by transmission electron microscopy revealed the sporangiospores are oval to spherical. The oval spores range in size from 0.8×1.0 μm to 1.1×1.3 μm. Spherical spores range in diameter from 0.9 to 1.2 μm.

Cultural and Physiological Characteristics

The cultural characteristics are recorded in Table 1, the physiological characteristics in Table 2

Table 1. Cultural characteristics of strain AB 921J-26.

Culture medium	Vegetative mycelium	Sporangia	Soluble pigment
ISP 2 (yeast extract - malt extract agar)	Abundant growth, wrinkled surface, moderate orange (53)	Absent	Absent
ISP 3 (oatmeal agar)	Poor growth, flat surface, pale orange yellow (73)	Absent	Absent
ISP 4 (inorganic salts - starch agar)	Abundant growth, raised surface, strong orange (50)	Absent	Absent
ISP 5 (glycerol - asparagine agar)	Moderate growth, flat surface, pale orange yellow (73) to moderate orange yellow (71)	Poor	Absent
ISP 6 (peptone - yeast extract - iron agar)	Few large plicate colonies, moderate orange (53) to grayish brown (61)	Absent	Dark brown (59)
ISP 7 (tyrosine agar)	Abundant growth, wrinkled surface, moderate orange (53)	Absent	Strong reddish orange (35)
CZAPEK sucrose agar	Moderate growth, flat surface, light orange (52)	Poor	Absent
Nutrient agar	Moderate growth, slightly raised surface, light orange (52)	Absent	Absent
Calcium malate agar	Moderate growth, slightly raised surface, pale orange yellow (73)	Moderate	Absent
ATCC 172 agar	Abundant growth, wrinkled surface, light orange (52)	Absent	Absent
Hickey-Tresner agar	Moderate growth, raised surface, grayish yellowish brown (80)	Absent	Absent
Bennett agar	Abundant growth, wrinkled surface, light orange (52)	Absent	Absent
Glucose - asparagine agar	Poor growth, flat surface, light orange (52)	Absent	Absent

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

Fig. 1. Scanning electron micrograph of sporangia of *Actinoplanes coloradoensis* strain AB 921J-26 on calcium malate agar incubated for 21 days at 30°C. Bar represents 5.0 μm.

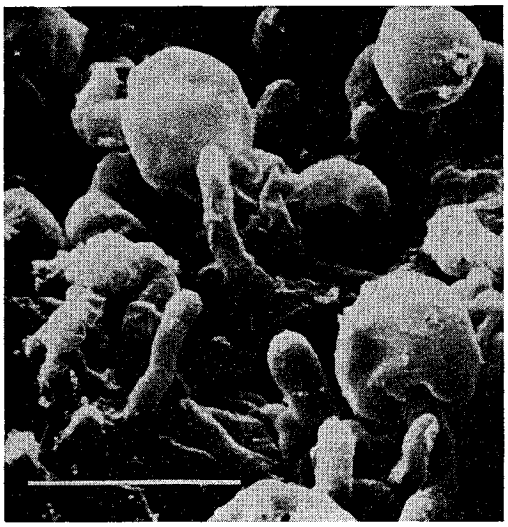

Fig. 2. Time course of fermentation in a New Brunswick 150-liter fermentor.

○ Coloradocin, ■ packed cell volume, □ total reducing sugar, ▲ pH.

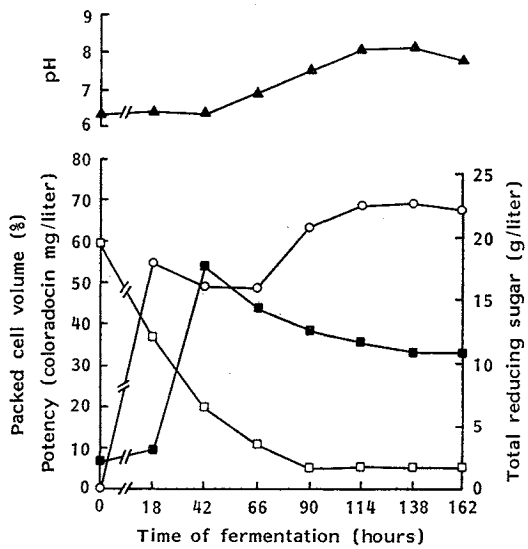

Table 2. Physiological characteristics of strain AB 921J-26.

Test	Reaction
H_2S production	+
Gelatin liquefaction	−
Casein hydrolysis	+
Starch hydrolysis	+
Tyrosine hydrolysis	+
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 6) and tyrosine agar (ISP 7).
[b] ATCC medium 172.

Table 3. Utilization of carbon sources by strain AB 921J-26.

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

++: Good growth, +: moderate growth, −: no growth.

and the carbon source utilization pattern in Table 3. The culture grew well on all media except ISP 3 and glucose - asparagine agars. The mycelial color was orange to yellowish orange on most media. Soluble pigment was produced on ISP 6 and ISP 7 agars.

Whole-cell Chemical Analyses

Analysis of whole-cell hydrolysates of strain AB 921J-26 demonstrated the presence of hydroxy-diaminopimelic acid. No LL or *meso* isomers of diaminopimelic acid were found. Galactose was

Table 4. Differentiating characteristics of *Actinoplanes coloradoensis*, *Actinoplanes deccanensis* and *Actinoplanes missouriensis*.

Characteristic	*A. coloradoensis* AB 921J-26	*A. deccanensis* ATCC 21983	*A. missouriensis* ATCC 14538
H_2S production	+	−	−
Melanin formation	+	+	−
Tyrosine hydrolysis	+	+	−
Casein hydrolysis	+	−	+
Calcium malate solubilization	−	+	+
Gelatin liquefaction	−	+	−
Utilization of:			
D-Fructose	+	−	+
Mannitol	+	−	+
Salicin	+	−	−
Whole-cell sugars	Trace of xylose and galactose	Xylose, arabinose and galactose	
Cell-wall diamino acid	Hydroxy-DAP	*meso*-DAP	Hydroxy- and *meso*-DAP
Growth temperature range	15~37°C	27~42°C	
Rudimentary aerial mycelium	+	−	−
refs		13, 14	14, 15

DAP: Diaminopimelic acid.

Table 5. Antibacterial activity of coloradocin against anaerobes.

Organism	MIC (μg/ml)
Bacteroides bivius B 6140	32
B. disiens ATCC 29426	32
B. fragilis ATCC 25285	32
B. fragilis 784	32
B. fragilis UC-2	32
B. fragilis SFM 2906A	16
B. fragilis SFM 2975-7	16
B. fragilis SFM 2929-1	64
B. loescheii ATCC 15930	64
B. melaninogenicus ATCC 25845	32
B. thetaiotaomicron ATCC 29741	0.5
B. thetaiotaomicron ATCC 29742	32
B. thetaiotaomicron 106	16
B. vulgatus 792	8
B. vulgatus SFBC 2375	64
Clostridium difficile ATCC 9689	16
C. difficile ATCC 17857	16
C. perfringens ATCC 13124	32
C. perfringens SFBC 2026	4
C. perfringens 788	16
C. ramosum 7	32
Fusobacterium nucleatum ATCC 25586	16
Peptococcus asaccharolyticus ATCC 14963	0.5
P. magnus ATCC 29328	0.5
Peptostreptococcus anaerobius ATCC 27337	64
P. micros ATCC 33270	2
Propionibacterium acnes 132	8
Veillonella parvula ATCC 10790	0.5

Table 6. Antibacterial activity of coloradocin against aerobes.

Organism	MIC (μg/ml)
Staphylococcus aureus ATCC 6538P	>100
S. aureus CMX 686B	>100
S. aureus A5177	>100
S. epidermidis 3519	>100
Streptococcus agalactiae CMX 508	100
S. bovis A5169	>100
S. faecium ATCC 8043	>100
S. pyogenes EES61	>100
Acinetobacter sp. CMX 669	>100
Enterobacter aerogenes ATCC 13048	>100
Escherichia coli Juhl	>100
E. coli DC-2	>100
Klebsiella pneumoniae ATCC 8045	>100
Providencia stuartii CMX 640	>100
Pseudomonas aeruginosa A5007	>100
P. aeruginosa BMH 10	>100
P. cepacia 2961	>100

the major sugar detected. A trace of xylose was also detected, but no arabinose.

Species Comparison

The morphological and cultural characteristics of strain AB 921J-26 placed it in the genus *Actinoplanes* as first described by COUCH[8]. A laboratory comparison of strain AB 921J-26 with

Table 7. Antibacterial activity of coloradocin against *Neisseria gonorrhoeae*.

Organism	MIC (μg/ml)
Neisseria gonorrhoeae CMX 556	4
N. gonorrhoeae CMX 557	4
N. gonorrhoeae CMX 558	2
N. gonorrhoeae CMX 591	2
N. gonorrhoeae CMX 664	4
N. gonorrhoeae 35F AMP-intermediate	1
N. gonorrhoeae 389 AMP-resistant	2
N. gonorrhoeae F 28	1

AMP: Ampicillin.

Table 8. Antibacterial activity of coloradocin against *Haemophilus influenzae*.

Organism	MIC (μg/ml)
Haemophilus influenzae 519 A	2
H. influenzae 588 A	16
H. influenzae 632 A	2
H. influenzae 667 A	2
H. influenzae 747 C	8
H. influenzae DILL AMP-resistant	8
H. influenzae SPK AMP-resistant	8
H. influenzae SOL AMP-resistant	2
H. influenzae 1435	1
H. influenzae ATCC 9795	32
H. influenzae ATCC 19418	8
H. influenzae ATCC 10211	2

AMP: Ampicillin.

known *Actinoplanes* species showed that it most closely resembled *A. deccanensis* and *A. missouriensis*. Our isolate, however, could be differentiated from these two species in a number of physiological, biochemical and morphological features as indicated in Table 4. In addition the appearance of AB 921J-26 was different from *A. deccanensis* on ISP 6 and ATCC 172 agars and from *A. missouriensis* on ISP 4, ISP 5 and ATCC 172 agars.

Fermentation

The time course study of the coloradocin fermentation is shown in Fig. 2. As the carbohydrate was depleted, the cells began to lyse with an increase in pH. Coloradocin yields reached a peak of 69 mg per liter at 138 hours and did not decrease appreciably over the following 24 hours.

Biological Evaluation

In Vitro Activity

The activity of coloradocin against 28 anaerobic strains, including 7 species of *Bacteroides* is shown in Table 5. The MICs ranged from 0.5 to 64 μg/ml. By comparison, the MICs against eight Gram-positive and nine Gram-negative aerobic bacteria were $\geq$100 μg/ml (Table 6). The MICs of coloradocin against eight strains of *N. gonorrhoeae*, including one with ampicillin-resistance, ranged from 1 to 4 μg/ml (Table 7). The MICs of coloradocin against twelve strains of *H. influenzae*, including three strains resistant to ampicillin, ranged from 1 to 32 μg/ml (Table 8).

Acute Toxicity of Coloradocin

The LD_{50} in mice injected intraperitoneally was >500 mg/kg.

Discussion

The whole-cell sugar pattern of strain AB 921J-26 is unusual. Members of the genus *Actinoplanes* characteristically contain arabinose and xylose[9], but AB 921J-26 has no arabinose and only a trace of xylose. The presence of a major amount of galactose is of doubtful taxonomic significance as this sugar is not considered to have diagnostic value for actinoplanetes[10]. Another unusual feature of AB 921J-26 is the small size of the sporangia. RUAN *et al.*[11] described small sporangia (2~5 μm) for *A. minutisporangius* but this culture is differentiated from AB 921J-26 by its brownish-black color and other characteristics. Although strain AB 921J-26 has some similarities with many of the orange-colored *Actinoplanes* species, it is sufficiently different to prevent assignment to any previously described species[12~15]. Therefore, we are designating this isolate a new species of the genus *Actinoplanes*

and propose the name *Actinoplanes coloradoensis* sp. nov. (M.L. adj. pertaining to Colorado).

Coloradocin has good to moderate activity against a broad range of anaerobic and microaerophilic bacteria. It is inactive against most aerobic bacteria. Structurally related antibiotics, nargenicin[16] and nodusmicin[17], are reported to inhibit *Staphylococcus aureus* whereas coloradocin is not active against *S. aureus* at 100 μg/ml. Of the pathogenic bacteria investigated, coloradocin is most effective against strains of *N. gonorrhoeae* and *H. influenzae*, including ampicillin-resistant strains. The selective potency of coloradocin against *N. gonorrhoeae* and *H. influenzae* is possibly related to its larger molecular size and the greater permeability of these organisms. Coloradocin has low acute toxicity.

Although coloradocin was identified as luminamicin[1], it is produced by a strain of *Actinoplanes* while luminamicin is produced by another genus, *Nocardiodes*[18]. A similar antibiotic lustromycin is produced by *Streptomyces*[19].

Acknowledgments

The authors wish to thank M. F. MILLER for scanning electron microscopy and L. S. OHEIM for reducing sugar analyses.

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