COUMAMIDINES, NEW BROAD SPECTRUM ANTIBIOTICS OF THE CINODINE TYPE

I. DISCOVERY, TAXONOMY OF THE PRODUCING ORGANISM AND FERMENTATION

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Coumamidines are water-soluble basic antibiotics related to the glycocinnamoylspermidines. They are produced by a soil isolate designated *Saccharopolyspora* sp. AB 1167L-65. The coumamidines have broad spectrum activity and were selected in a screen for substances which inhibit *Pseudomonas aeruginosa*.

Coumamidines, a complex of water-soluble basic antibiotics, have been found in the fermentation broth of a soil isolate, designated *Saccharopolyspora* sp. AB 1167L-65. They are related to the glycocinnamoylspermidines[1] (cinodines). This paper describes the discovery of the coumamidines, their production by fermentation and the taxonomy of the producing organism. The isolation and structure elucidation of the coumamidines and their biological properties are given in companion papers[2,3].

Materials and Methods

Microorganisms

Strain AB 1167L-65 was isolated from a soil collected in a wooded area near Russell, Kentucky. The soil was air-dried overnight, then heated for 1 hour at 120°C. Dilutions of the heated soil were plated on a medium consisting of soluble starch 0.1%, KNO_3 0.1%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.1%, yeast extract (Difco) 0.01%, 1 ml of trace element solution[4] per liter and agar 2%. Cycloheximide (50 μg/ml), nystatin (50 μg/ml) and gentamicin (2.5 μg/ml) were added to the medium. The plates were incubated for 30 days at 30°C. *Pseudomonas aeruginosa* K799/61 was obtained from ZIMMERMANN[5]. Other strains used in the screen were from the American Type Culture Collection (ATCC) or from the stock culture collection in our laboratory.

Discovery Screen

A two stage screen was used to discover substances with activity against *P. aeruginosa*. Agar plugs were cut from cultures growing on the surface of a medium containing glucose monohydrate 2%, Lexein F-152 liquid peptone (Inolex) 1%, yeast extract (Difco) 0.1%, molasses (Del Monte) 0.5%, $CaCO_3$ 0.2% and agar 2%. The plugs were placed on the surface of streptomycin assay agar with yeast extract (BBL) seeded with *P. aeruginosa* K799/61. This strain is a mutant with increased susceptibility to various antibiotics (novobiocin, tetracycline, erythromycin and others), apparently due to the failure of the outer layers of its cell envelope to act as an adequate permeability barrier[5]. Cultures which inhibited this ultra-sensitive strain of *Pseudomonas* were fermented in liquid shaken culture. Concentrates of the fermentation broths were tested for inhibition of clinical isolates of *P. aeruginosa* and other bacteria.

Taxonomic Studies

Methods and media described by the International Streptomyces Project (ISP)[6], WAKSMAN[7], and GORDON *et al.*[8] 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 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 was determined by the method of BECKER *et al.*[9]. Whole-cell sugars were identified by the procedure of LECHEVALIER[10]. Lipid LCN-A was determined by the method of MORDARSKA *et al.*[11]. Menaquinones were extracted as described by ATHALYE *et al.*[12] and phospholipids were prepared and chromatographed according to LECHEVALIER and LECHEVALIER[13].

Fermentation

Vegetative mycelium, stored at −75°C, was used at 0.7% to inoculate 500-ml Erlenmeyer seed flasks containing 100 ml of media. The seed medium consisted of glucose monohydrate 2%, Lexein F-152 liquid peptone (Inolex) 1%, yeast extract (Difco) 0.1%, molasses (Del Monte) 0.5% and $CaCO_3$ 0.2%. The seed flasks were incubated at 28°C for 96 hours on a rotary shaker (5.6 cm stroke) at 225 rpm.

The fermentation medium consisted of glucose monohydrate 3%, Lexein F-152 liquid peptone 2%, yeast extract 0.1% and $CaCO_3$ 0.2%. The medium was dispensed at 100 ml per 500-ml Erlenmeyer flask, sterilized at 121°C for 35 minutes and inoculated at 5% with the seed growth. Incubation was at 28°C on a rotary shaker at 225 rpm. Two ml samples were removed daily from each of six replicate flasks for analysis.

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. Glucose concentration was determined enzymatically with glucose oxidase (Sigma Diagnostics kit No. 510-A). 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.

Results

Discovery

The coumamidine producing culture was selected from other soil isolates in the first stage of the screen by its inhibition of K799/61, a highly sensitive strain of *P. aeruginosa*. In the second stage, using concentrates of fermentation broths, strain AB 1167L-65 was selected because its activity against clinical strains of *P. aeruginosa* was comparable to its activity against other microorganisms such as *S. aureus*.

Fig. 1. Scanning electron micrograph of strain AB 1167L-65 grown on GAUSE No. 1 modified agar for 10 days at 28°C.

Bar represents 1 μm.

Taxonomy

The vegetative mycelium of strain AB 1167L-65 is branched and does not fragment. Spores are borne in straight to flexuous chains on aerial

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

Table 1. Cultural characteristics of strain AB 1167L-65.

Medium		Cultural characteristics
Yeast extract - malt extract agar (ISP 2)	G:	Abundant
	AM:	White (263) and brownish pink (33)[a]
	R:	Light yellowish brown (76)
	SP:	Absent
Oatmeal agar (ISP 3)	G:	Moderate
	AM:	White (263)
	R:	Yellowish white (92)
	SP:	Absent
Inorganic salts - starch agar (ISP 4)	G:	Poor
	AM:	White (263)
	SP:	Absent
Glycerol - asparagine agar (ISP 5)	G:	Moderate
	AM:	White (263)
	R:	Pale yellow (89)
	SP:	Absent
Peptone - yeast extract - iron agar (ISP 6)	G:	Abundant
	AM:	Yellowish white (92)
	R:	Light yellowish brown (76)
	SP:	Absent
Tyrosine agar (ISP 7)	G:	Moderate
	AM:	White (263) and brownish pink (33)
	R:	Light yellowish brown (76)
	SP:	Absent
Nutrient agar	G:	Abundant
	AM:	White (263)
	R:	Light yellow (86)
	SP:	Absent
CZAPEK's agar	G:	Poor
	AM:	Sparse; white (263)
	R:	Yellowish white (92)
	SP:	Absent
Calcium malate agar	G:	Moderate
	AM:	White (263)
	R:	Grayish yellow (90)
	SP:	Absent Calcium is partially solubilized
ATCC No. 172	G:	Moderate
	AM:	White (263)
	R:	Pale yellow (89)
	SP:	Absent
GAUSE No. 1 modified (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%, agar 1.5%)	G:	Moderate
	AM:	Pinkish white (9)
	R:	Pinkish white (9) and white (263)
	SP:	Absent

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

Observations after incubation for 27 days at 28°C.

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

Table 2. Utilization of various compounds as the sole source of carbon[6] by strain AB 1167L-65.

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

Incubation was at 28°C for 43 days.
++: Good utilization, +: poor utilization, −: did not utilize.

Table 3. Physiological characteristics of strain AB 1167L-65.

Test	Reaction[a]
Starch hydrolysis	−
H_2S production	+
Melanin formation	
Peptone - yeast extract - iron agar	−
Tyrosine agar	−
Litmus milk	Alkaline digestion
Decomposition of:	
Adenine	−
Casein	+
L-Tyrosine	+
NaCl tolerance	Growth at 7% but not 10%
Temperature range[b]	Growth at 21 to 37°C
	No growth at or above 42°C
	Optimum at 21°C
Resistance to lysozyme	Sensitive
Resistance to antibiotics[c]	
Erythromycin	−
Gentamicin	−
Kanamycin	−
Novobiocin	−
Oxytetracycline	−
Rifampicin	+
Streptomycin	−
Vancomycin	−

[a] Observations after incubation for 27 days. Incubation at 28°C except for the temperature range study.
[b] Medium was ATCC No. 172.
[c] Medium ISP No. 2 with antibiotics added at 50 μg/ml.

Table 4. Chemical composition of strain AB 1167L-65.

Test	Result
Diaminopimelic acid	*meso*-Isomer
Diagnostic sugars	Arabinose and galactose
Mycolic acids (as LCN-A)	Absent
Major menaquinone	MK-9 (H_4)
Phospholipid pattern	P III

mycelia. Scanning electron microscopy of sporulated aerial mycelia shows a sheath-like covering with spiny protrusions (Fig. 1).

The appearance of strain AB 1167L-65 on eleven media is given in Table 1. White aerial mycelium is formed on most media. Soluble pigments are not produced. The ability of this strain to utilize various carbon compounds in synthetic medium is given in Table 2. Only half of the compounds tested were utilized. Some physiological properties are given in Table 3.

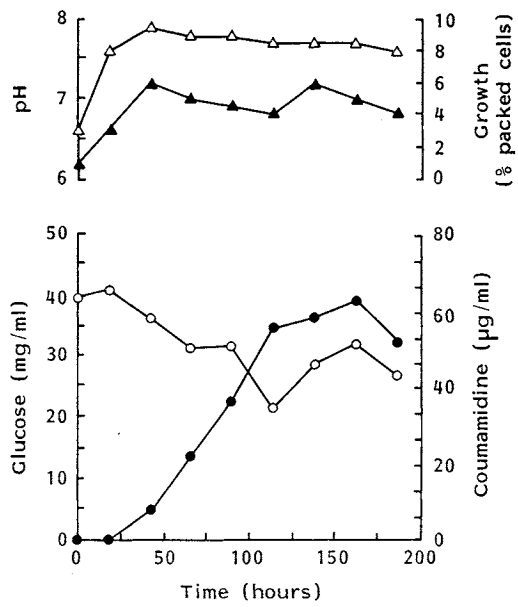
The chemical composition of strain AB 1167L-65 is summarized in Table 4. The presence of *meso*-diaminopimelic acid, arabinose and galactose in whole-cell hydrolysates indicates a cell wall of Type IVA[14]. Mycolic acids (LCN-A)[15] were not observed in lipid extracts. Phosphatidyl choline and phosphatidyl methylethanolamine were found in the polar lipid extracts of strain AB 1167L-65. Mass spectrometry indicated that the major menaquinone had a molecular weight of 788 and was, therefore, tetrahydrogenated with 9 isoprenoid units.

Fermentation

Growth, pH, consumption of glucose and accumulation of coumamidines are plotted in a time course study of a fermentation with strain AB 1167L-65. Growth in terms of cell mass was low, and only half of the glucose was consumed. A maximum potency of 63 μg/ml was achieved at 162 hours.

Fig. 2. Time course of the coumamidine fermentation by *Saccharopolyspora* sp. AB 1167L-65 in shaken flasks.

○ Glucose, ● coumamidine, △ pH, ▲ growth.



Discussion

The coumamidine-producing strain AB 1167L-65 can be assigned to the genus *Saccharopolyspora* on the basis of morphology and chemical composition. Two species have been described for this genus, *Saccharopolyspora hirsuta*[16] and *Saccharopolyspora erythraea*[17]. Strain AB 1167L-65 cannot readily be assigned to either species. It is much less versatile than *S. hirsuta* in the utilization of various compounds as the sole source of carbon and has a lower optimum temperature for growth. AB 1167L-65 has longer spore chains than *S. erythraea* and lacks the characteristic red coloration in its aerial mass as well as the deep reverse color and soluble pigments found in *S. erythraea*. We are designating the coumamidine producer as *Saccharopolyspora* sp. AB 1167L-65. It has been deposited at the Northern Regional Research Center in Peoria, Illinois, U.S.A., where it was assigned the accession number NRRL 18369. The isolation of *Saccharopolyspora* from a temperate forest soil is unusual[16,18] and is undoubtedly due to the selective method used to treat and plate the soil.

When *Saccharopolyspora* sp. AB 1167L-65 was compared with the producer of the glycocinnamoylspermidines, *Nocardia* sp. NRRL 5646[1], it was found to differ notably by the production of abundant aerial mycelium and lack of fragmentation.

Despite poor glucose utilization and sparse vegetative growth, the coumamidine fermentation yielded appreciable quantities of antibiotic. This suggests that media improvement studies would be a route to higher antibiotic yields.

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References

1) TRESNER, H. D.; J. H. KORSHALLA, A. A. FANTINI, J. D. KORSHALLA, J. P. KIRBY, J. J. GOODMAN, R. A. KELE, A. J. SHAY & D. B. BORDERS: Glycocinnamoylspermidines, a new class of antibiotics. I. Description and fermentation of the organism producing the LL-BM123 antibiotics. J. Antibiotics 31: 394~397, 1978, and references therein
2) CHEN, R. H.; D. N. WHITTERN, A. M. BUKO & J. B. MCALPINE: Coumamidines, new broad spectrum antibiotics of the cinodine type. II. Isolation and structural elucidation. J. Antibiotics 42: 533~537, 1989
3) FERNANDES, P. B.; R. N. SWANSON, D. J. HARDY, C. W. HANSON, D. MCDANIEL, J. BEYER & R. H. CHEN: Coumamidines, new broad spectrum antibiotics of the cinodine type. III. Microbiologic activity of coumamidine γ1. J. Antibiotics 42: 538~541, 1989

4) HUSSEIN, A. M.; A. M. ALLAM & A. M. RAGAB: Taxonomical studies on thermophilic actinomycetes of some soils of Egypt. Ann. Microbiol. (Paris) 25: 19~28, 1975
5) ZIMMERMANN, W.: Penetration through the gram-negative cell wall: a co-determinant of the efficacy of beta-lactam antibiotics. Int. J. Clin. Pharmacol. Biopharm. 17: 131~134, 1979
6) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
7) WAKSMAN, S. A. (*Ed.*): The Actinomycetes. Vol. 2. Classification, Identification and Descriptions of Genera and Species. Williams & Wilkins Co., Baltimore, 1961
8) GORDON, R. E.; D. A. BARNETT, J. E. HANDERHAN & C. H.-N. PANG: *Nocardia coeliaca*, *Nocardia autotrophica*, and the nocardin strain. Int. J. Syst. Bacteriol. 24: 54~63, 1974
9) 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
10) LECHEVALIER, M. P.: Identification of aerobic actinomycetes of clinical importance. J. Lab. Clin. Med. 71: 934~944, 1968
11) MORDARSKA, H.; M. MORDARSKI & M. GOODFELLOW: Chemotaxonomic characters and classification of some nocardioform bacteria. J. Gen. Microbiol. 71: 77~86, 1972
12) ATHALYE, M.; M. GOODFELLOW & D. E. MINNIKIN: Menaquinone composition in the classification of *Actinomadura* and related taxa. J. Gen. Microbiol. 130: 817~823, 1984
13) LECHEVALIER, M. P. & H. A. LECHEVALIER: The chemotaxonomy of actinomycetes. *In* Actinomycete Taxonomy. *Eds.*, A. DIETZ & D. W. THAYER, pp. 227~291, Society for Industrial Microbiology, Arlington, 1980
14) LECHEVALIER, M. P. & H. A. LECHEVALIER: Chemical composition as a criterion in the classification of aerobic actinomycetes. Int. J. Syst. Bacteriol. 20: 435~443, 1970
15) GOODFELLOW, M.; D. E. MINNIKIN, P. V. PATEL & H. MORDARSKA: Free nocardomycolic acids in the classification of nocardias and strains of the "rhodochrous" complex. J. Gen. Microbiol. 74: 185~188, 1973
16) LACEY, J. & M. GOODFELLOW: A novel actinomycete from sugar-cane bagasse: *Saccharopolyspora hirsuta* gen. et sp. nov. J. Gen. Microbiol. 88: 75~85, 1975
17) LABEDA, D. P.: Transfer of the type strain of *Streptomyces erythraeus* (Waksman 1923) Waksman and Henrici 1948 to the genus *Saccharopolyspora* Lacey and Goodfellow 1975 as *Saccharopolyspora erythraea* sp. nov., and designation of a neotype strain for *Streptomyces erythraeus*. Int. J. Syst. Bacteriol. 37: 19~22, 1987
18) SUZUKI, K.; K. NAGAI & T. SAITO: Selective isolation and characterization of *Saccharopolyspora* species. Abstracts of the 7th International Symposium on Biology of Actinomycetes, No. P1-36, p. 102, Tokyo, May 22~26, 1988