THREE NOVEL POLYETHER ANTIBIOTICS X-14889A, C, AND D FROM A STREPTOMYCETE

TAXONOMY OF THE PRODUCING OGRANISM, FERMENTATION PRODUCTION AND BIOLOGICAL PROPERTIES OF THE ANTIBIOTICS

CHAO-MIN LIU, JOHN W. WESTLEY, JENNIFER CHU, T. E. HERMANN, MARK LIU and N. PALLERONI

Roche Research Center, Hoffmann-La Roche Inc., Nutley, N.J. 07110, U.S.A.

(Received for publication September 30, 1992)

Antibiotic X-14889A, C, and D are novel polyether antibiotics related to lysocellin and antibiotic X-14873A. They are produced by a streptomycete isolated from a soil of Wisconsin. The antibiotic X-14889C is active against Gram-positive bacteria and exhibits ionophore property.

Our investigation into the ionophorous antibiotic-producing microorganisms[1,2] has resulted in the isolation of three novel polyether antibiotics related to lysocellin[3] and antibiotic X-14873A[4]. In this report, we present taxonomic description of the polyether-producing organism *Streptomyces* sp. X-14889, fermentation condition for the antibiotic production, as well as the biological activity and ionophore property of antibiotic X-14889C. The isolation and structure determination of antibiotic X-14889A, B, C, D and the other metabolite are described in the accompanying paper[5].

Materials and Methods

Microorganisms

Streptomyces sp. X-14889 was isolated from a creek mud soil sample collected near LaFarge, Wisconsin. The culture was designated strain X-14889 and has been deposited at Northern Regional Research Laboratory (NRRL), USDA, Peoria, IL. where it was assigned the number NRRL 15517. Other organisms used (Table 2) were either from American Type Culture Collection (ATCC) or from NRRL.

Taxonomic Studies

Methods and media for characterization of *Streptomyces* sp. X-14889 were those described by the International Streptomyces Project[6], SKERMAN[7] and BECKER *et al.*[8]. The color scheme used was that of Color Harmony Manual, 4th Ed., 1958, Container Corporation of America, Chicago, IL.

Fermentation

The X-14889 culture was grown and maintained on a starch-casein agar slant consisting of soluble starch 1%, casein 0.1%, K_2HPO_4 0.05%, $MgSO_4$ 0.05%, agar 2% in distilled water, pH 7.4. A piece of agar containing the sporulating culture was used to prepare inoculum in a medium containing Soylose 105 (Archer Daniels Midland Co.) 1%, Cerelose (technical grade glucose) 2%, $CaCO_3$ 0.2%, $CoCl_2 \cdot 6H_2O$ 0.00001% in tap water, pH 6.0. A 72-hour old culture grown in a 500-ml Erlenmeyer flask containing 70 ml of the medium, was incubated at 28°C on a rotary shaker operating at 250 rpm with a 5.1-cm gyration. This was used as inoculum (1.5% v/v) to start fermentation. The composition of the fermentation medium was the same as that of the inoculum medium. The fermentation was carried out for 140 hours either in a shake flask in the medium and fermentation conditions as described for inoculum preparation, or in a 380-liter fermentor containing 240-liter medium agitated at 280 rpm. The aeration in the fermentor was

0.75 v/v/m. Antibiotic titer in the broth was estimated by agar cup assay using *Staphyococcus aureus* ATCC 6538P as a test organism. Cell growth during fermentation was estimated by measuring the packed cell volume in broths. This was carried out by centrifugation of whole broths at 1,600 rpm for 10 minutes in graduated glass centrifuge tubes (13 × 130 mm) with a Sorval GLC-2B bench-top centrifuge.

Determination of Cation Selectivity Sequence and Cation Transport

The cation selectivity sequence of antibiotic X-14889C was determined by the method of LIU and HERMANN[9]. Two μmoles of antibiotic in 2 ml of 70% toluene - 30% *n*-butanol were equilibrated by shaking for 2 hours at 20°C with 2 μmoles of $^{86}RbCl$ or 1 μmole of $^{45}CaCl_2$ (in 1 ml of dimethyl glycine - tetramethyl ammonium hydroxide buffer, 25 mM, pH 10). Complex formation was measured by liquid scintillation counting of both phases. Displacement of labeled cations from the ionophore complexes was determined by the addition of unlabeled cations (2 μmoles of monovalent or 1 μmole of divalent metal chloride).

The U-tube system described by ASHTON and STEINRAUF[10] was employed for the assay. Five ml of a chloroform solution of antibiotic (1×10^{-4} M) were added to a glass U-tube. Two ml of an aqueous tris-HCl buffer (20 mM, pH 9.5) containing 1 mM ^{45}Ca-calcium chloride or ^{86}Rb-rubidium chloride were placed in one arm of the U-tube and an equal volume of the same buffer solution with unlabeled calcium or rubidium chloride was placed in the other arm. The chloroform phase separating the two aqueous phases was then gently stirred. The rate of appearance of radioactive calcium or rubidium in the label-free side was determined by counting samples (50 μl) taken from both aqueous phases with 10 ml aquasol (New England Nuclear, Boston, MA.) in an Intertechnique liquid scientillation spectrometer.

Results and Discussion

Taxonomy

Microscopic and Chemical Characteristics

The polyether antibiotics-producing strain X-14889 grows in agar media of various compositions giving a type of growth characteristic of many aerobic actinomycetes. A substrate mycelium penetrates into the agar and remains unfragmented, while part of the aerial mass of growth differentiates into chains of spores. The spore chains are open and tight spirals, and include more than ten spores per chain. The spores are spiny and their average dimensions are 0.65 × 0.91 μm.

Chromatographic analysis of whole cell hydrolysates[8] revealed the presence of LL-diaminopimelic acid which, in addition to the properties listed above, suggest the assignment of strain X-14889 to the genus *Streptomyces*.

Macroscopic Characteristics

The characteristics of growth in different agar media are summarized in Table 1. The data were

Table 1. Cultural characteristics of X-14889.

Medium	Amount of growth and degree of sporulation	Color of the aerial mass	Color of the reverse mycelium
Yeast - malt extract (ISP-2)	Abundant growth; waxy appearance; no sporulation	Luggage tan (4 pg)	Luggage tan (4 pg)
Oatmeal agar (ISP-3)	Abundant growth; good sporulation	Ashes (5 fe)	Gray (g), mixed with tan (3 gc)
Inorganic salts - starch agar (ISP-4)	Abundant growth; no sporulation	Oak brown (4 pi)	Light brown (4 ng)
Glycerol - asparagine agar (ISP-5)	Poor growth; no sporulation	Light gray (c)	Light gray (c)

recorded after fourteen days of incubation at 28 °C.

Physiological Characteristics

Culture X-14889 can grow at the expense of glucose, fructose, arabinose, rhamnose, mannose, mannitol, sucrose and raffinose. There is no growth on xylose, inositol or cellulose, and slight of doubtful growth on salicin. Growth is inhibited by streptomycin. Melanin production on ISP-7 medium, H_2S production in ISP-6 medium, and nitrate reduction in ISP-8 mdium are negative. Gelatin and casein are hydrolyzed, but starch is not. NaCl tolerance is low (less than 2%).

According to phenotypic data reported[11~16], the phenotypic characteristics listed above for culture X-14889 allow the assignment of this culture to a group of streptomycete species which have marked resemblence to the strain under study. These species, *S. albaduncus*, *S. canus*, *S. cuspidosporus*, *S. olivoviridis*, *S. craterifer*, *S. saraceticus* and *S. sparsogenes*. *S. albaduncus* differ from X-14889 in the utilization of xylose, inositol and sucrose; *S. canus* utilizes xylose and inositol; *S. cuspidosporus* grows on xylose, inositol and salicin; *S. olivoviridis* is negative on raffinose and sucrose and is positive on xylose; *S. craterifer* utilizes xylose but the utilization of sucrose and raffinose is doubtful; *S. saraceticus* utilizes xylose, but the utilization of rhamnose is doubtful. *S. sparsogenes* is perhaps the species that more closely resembles X-14889. Neither organism sporulates well on selected media, and the utilization of sugars is similar with the exception of xylose, which is utilized by *S. sparsogenes*, and of galactose, for which this species is negative. Other differences include indistinct individual spores in the spore chains of *S. sparsogenes*, which suggest the presence of a sheath and the hygroscopic character of the spore mass. Thus, even through strain X-14889 resembles several other species of *Streptomyces*, the production of unique antibiotics and several physiological and morphological characteristics set it apart from all other species with which it has been compared. We feel that X-14889 may represent a new species of the genus *Streptomyces*, but we prefer to postpone a formal proposal until more strains are collected and an appropriate intraspecies comparative study can be preformed.

Fig. 1. Time course of fermentation in shake flasks by *Streptomyces* sp. X-14889.

○ pH, ● cell volume, △ antimicrobial titer.

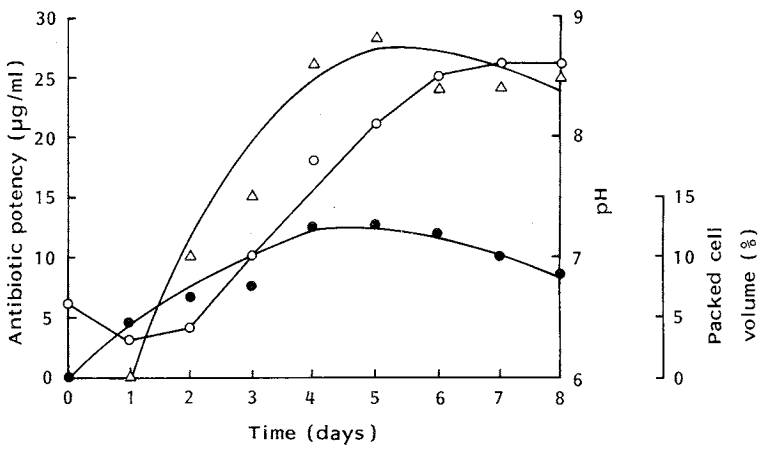


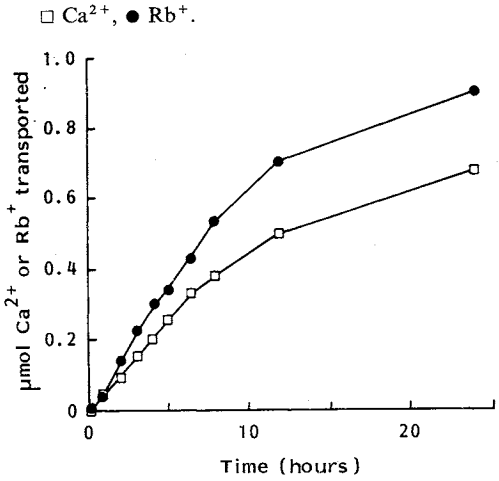
Table 2. *In vitro* antimicrobial activity of antibiotics X-14889A, X-14889C, and X-14889D (μg/ml).

Microorganism (ATCC No.)	X-14889A	X-14889C	X-14889D
Streptococcus faecium 8043	1.57	0.08	125
Staphylococcus aureus 6538P	6.25	5	125
Micrococcus luteus 9341	6.25	5	
Bacillus megaterium 8011	6.25	2.5	1,000
Bacillus sp. E 27359	3.13	0.63	7.9
Bacillus subtilis 558	3.13	1.25	1,000
Bacillus sp. TA 27860	3.13	1.25	1,000
Mycobacterium phlei 355	100	50	1,000
Streptomyces cellulosae 3313	100	10	1,000
Paecilomyces variotii 28820	100	125	1,000
Penicillium digitatum 26821	100	250	1,000
Candida albicans NRRL 477	100	62.5	1,000
Saccharomyces cerevisiae 4226	100	500	1,000

Table 3. Displacement of $^{86}Rb^+$ or $^{45}Ca^{2+}$ from X-14889C-cation complex by other cations.

Displacing cation	Cation radius (Å)	$^{86}Rb^+$ or $^{45}Ca^{2+}$ remaining in complex (%)	
		$^{86}Rb^+$	$^{45}Ca^{2+}$
None	—	63	81
Mg^{2+}	0.82	48	47
Ca^{2+}	1.18	53	48
Sr^{2+}	1.12	58	64
Ba^{2+}	1.34	50	55
Li^+	0.68	60	81
Na^+	0.97	46	46
K^+	1.33	33	11
Rb^+	1.47	38	21
Cs^+	1.67	42	29

Fig. 2. Time course of Ca^{2+} and Rb^+ transport by antibiotic X-14889C in a U-tube.



Fermentation

Time course of antibiotic X-14889A, B, C, and D fermentation in shake flasks is presented in Fig. 1. The potency of antimicrobial activity produced by the strain X-14889 in broth, increased in parallel with the broth pH. Activity reached approximately 25 mg/liter after five days fermentation in shake flasks.

Antimicrobial Activity and other Biological Properties

The *in vitro* antimicrobial activity of antibiotics X-14889A, C and D are shown in Table 2. Antibiotics X-14889A and C are mainly active against Gram-positive bacteria. The minimum inhibitory concentrations (MICs) of antibiotic X-14889C against the Gram-positive bacteria tested range from 0.08 to 6.25 μg/ml. The MICs of antibiotic X-14889A, a descarboxy derivative of X-14889C are higher against these Gram-positive bacteria, ranging from 1.5 to 6.25 μg/ml. Antibiotic X-14889D is virtually inactive against all of the organisms tested.

Antibiotic X-14889C is active against *Plasmodium berghei* at 22 mg/kg in a mouse model[17]. It is also

effective in increasing the molar proportion of propionate of the volatile fatty acids (acetate, propionate, and butyrate) produced by the rumen fluid at 50 ppm (data not shown). This activity suggests that antibiotic X-14889C is a potential candidate as a ruminant performance enhancer[12]. The LD_{50} of the antibiotic in mice is 190 mg/kg (po); 120 mg/kg (ip).

Ionophore Properties of Antibiotic X-14889C

Antibiotic X-14889C complexes and transports both monovalent and divalent metal cations as demonstrated in the experiment shown in Table 3 and Fig. 2. The cation selectivity for the antibiotic as deduced from the ion-competition experiment shown in Table 3 is:

$$K^+ > Rb^+ > Cs^+ > Na^+ > Li^+$$

$$Mg^{2+} = Ca^{2+} > Ba^{2+} > Sr^{2+}$$

References

1) LIU, C.-M.: Microbial aspects of polyether antibiotics: Activity, production, and biosynthesis. *In* Polyether Antibiotics: Naturally Occurring Acid Ionophores. Vol. 1. Biology. *Ed.*, J. W. WESTLEY, pp. 44~102, Marcel Dekker, Inc., New York, 1982
2) LIU, C.-M.; T. E. HERMANN, A. DOWNEY, B. LA, T. PROSSER, E. SCHILDKNECHT, N. J. PALLERONI, J. W. WESTLEY & P. A. MILLER: Novel polyether antibiotics X-14868A, B, C, and D produced by a Nocardia. Discovery, fermentation, biological as well as ionophore properties and taxonomy of the producing culture. J. Antibiotics 36: 343~350, 1983
3) OTAKE, N.; M. KOENUMA, H. KINASHI, S. SATAO & Y. SAITO: The crystal and molecular structure of the silver salt of lysocellin, a new polyether antibiotics. J. Chem. Soc. Chem. Commun. 1975: 92~93, 1975
4) LIU, C.-M.; J. W. WESTLEY, T. H. HERMANN, B. LA, T. PROSSER, N. PALLERONI, R. H. EVANS & P. A. MILLER: Novel polyether antibiotics X-14873A, G and H produced by a *Streptomyces*: Toxonomy of the producing culture, fermentation, biological and ionophorous properties of the antibiotics. J. Antibiotics 39: 1712~1718, 1986
5) WESTLEY, J. W.; C.-M. LIU, J. F. BLOUNT, L. TODARO, L. H. SELLO & N. TROUPE: Isolation and characterization of four polyether antibiotics, X14889A, B, C, and D, closely related to lysocellin and the ferensimycins. J. Antibiotics 46: 280~286, 1993
6) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
7) SKERMAN, V. B. D. (*Ed.*): A guide to Identification of the Genera of Bacteria. Williams & Wilkins Co., 1967
8) 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
9) LIU, C.-M. & T. E. HERMANN: Characterization of ionomycin as a calcium ionophore. J. Biol. Chem. 253: 5892~5894, 1978
10) ASHTON, R. & L. K. STEINRAUF: Thermodynamic consideration of the ion transport antibiotics. J. Mol. Biol. 49: 547~556, 1970
11) RUFF, M. D.: Veterinary application. *In* Polyether Antibiotics: Naturally Occurring Acid Ionophores. Vol. 1. Biology. *Ed.*, J. W. WESTLEY, pp. 303~332, Marcel Dekker, Inc., New York, 1982
12) BUCHANAN, R. E. & N. E. GIBBONS (*Ed.*): BERGEY's Manual of Determinative Bacteriology. 8th Ed. Williams and Wilkins Co., Baltimore, 1974
13) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. II. Species descriptions from first study. Int. J. Syst. Bacteriol. 18: 69~189, 1968
14) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. III. Additional species descriptions from first and second studies. Int. J. Syst. Bacteriol. 18: 279~392, 1968
15) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. IV. Species descriptions from the second, third and fourth studies. Int. J. Syst. Bacteriol. 19: 391~512, 1969
16) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type strains of *Streptomyces*. V. Additional descriptions. Int. J. Syst. Bacteriol. 22: 265~394, 1972
17) SCHILDKNECHT, E. G.; D. SIEGEL & R. W. RICHLE: Antiparasitic activity of natural and semisynthetic monensin urethanes. Chemotherapy (Basel) 29: 145~152, 1983