X-14885A, A NOVEL DIVALENT CATION IONOPHORE PRODUCED BY A STREPTOMYCES CULTURE: DISCOVERY, FERMENTATION, BIOLOGICAL AS WELL AS IONOPHORE PROPERTIES AND TAXONOMY OF THE PRODUCING CULTURE

CHAO-MIN LIU*, M. CHIN, B. LA T. PROSSER, N. J. PALLERONI, J. W. WESTLEY and P. A. MILLER

Department of Microbiology, Roche Research Center, Hoffmann-La Roche Inc. Nutley, New Jersey 07110, U.S.A.

(Received for publication May 30, 1983)

Antibiotic X-14885A is a novel divalent cation ionophore produced by a Streptomyces culture isolated from soil sample collected in Wyoming. Its cation binding sequence has been found to be: $Mg^{2+} > Ca^{2+}$, $Sr^{2+} > Ba^{2+} \ggg Li^+$, Na^+, Rb^+, K^+, Cs^+.

Of the more than 70 naturally occurring polyether antibiotics described in the literature, the majority are capable of complexing and transporting monovalent cations[1]. A few, such as lasalocid, lysocellin, X-14547A[2], antibiotic 6016[3] and antibiotic X-14868A and B[4], are efficient carriers for both monovalent and divalent cations; just two, A23187[5] and ionomycin[6], are specific for divalent cations. In the course of our search for new ionophore antibiotics, we have encountered a soil isolate capable of producing a new divalent cation ionophore, X-14885A (Fig. 1). Antibiotic X-14885A is a novel pyrrole ether antibiotic structurally related to A23187. The isolation and structure determination of the antibiotic has been reported elsewhere[7]. In this paper, we describe the taxonomy of the producing culture, fermentative production, and the biological and ionophore properties of the antibiotic.

Taxonomy of the Producing Organisms

Strain X-14885 (NRRL 12350) was isolated from a soil sample collected at the bank of Dead Indian Creek, Wyoming. It produces a substrate mycelium which does not fragment into spores, and aerial mycelium forming *retinaculum-apertum* spore chains with approximately 25 spores per

Fig. 1.

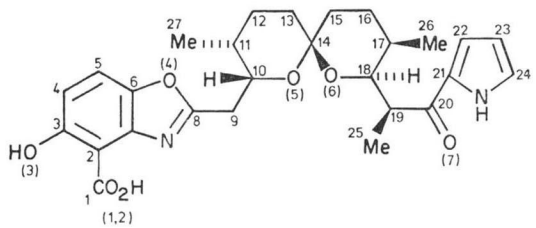


Fig. 2. Smooth-walled aerial mycelium of Streptomyces strain X-14885.

The mycelium does not appear differentiated into spores. Culture was grown on ISP 2 agar at 28°C for two weeks. ×10,000.

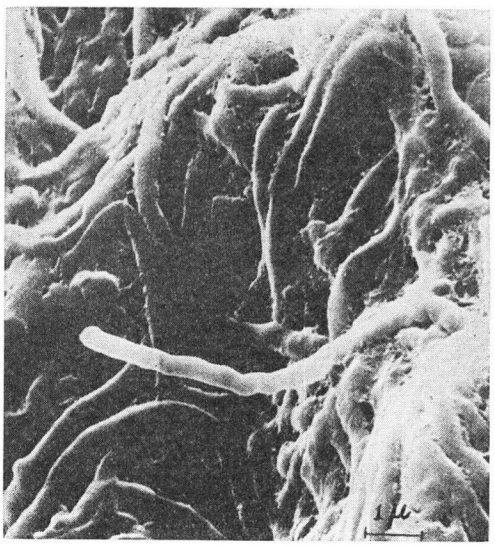


Table 1. Cultural characteristics of strain X-14885.

Agar medium	Amount of growth; degree of sporulation	Spore mass color[a]	Color of reverse substrate mycelium[a]
Yeast plus malt extract (ISP 2)[b]	Moderate growth; sparse sporulation	b (oyster white) in isolated tufts; 2ge (covert tan) on the rest where not sporulated	2ge (covert tan)
Oatmeal (ISP 3)[b]	Poor growth; no sporulation	2cb (ivory tint) not sporulated	2dc (natural, string)
Inorganic salts - starch (ISP 4)[b]	Poor growth; no sporulation; does not appear to hydrolyze starch in medium	2cb (ivory tint)	2dc (natural, string)
Glycerol - asparagine (ISP 5)[b]	Poor growth; very scant sporulation	2cb (ivory tint)	2dc (natural, string)
Czapek-Dox[c]	Poor growth; almost no sporulation	translucent 2cb (ivory tint)	2dc (natural, string)

[a] The color scheme used was that taken from the Color Harmony Manual, 4th ed., 1958 (Container Corp. of America, Chicago) after incubation for 2 weeks at 28°C.
[b] Media recommended by SHIRLING and GOTTLIEB[10].
[c] Czapek-Dox broth (BBL) to which 1.5% agar was added.

Table 2. Comparison of carbon utilization by strain X-14885 and related strains.

Carbon source	Results[a] in this laboratory			Results reported in BERGEY's Manual[13]
	X-14885	*S. humidus*	*S. lusitanus*	*S. lusitanus*
No carbon control	−	−	−	−
D-Glucose	++	++	++	+
D-Xylose	±	+	−	−
L-Arabinose	±	++	−	±
L-Rhamnose	++	++	−	−
D-Fructose	±	++	−	+
D-Galactose	+	++	+	
Raffinose	−	−	−	−
D-Mannitol	−	++	−	−
i-Inositol	+	+ (+)	−	±
Salicin	+	+	±	−
Sucrose	−	−	−	+
Cellulose	−	−	−	

[a] −: Negative response; ±: doubtful response; +: more growth than carbon control but less than on glucose; + (+): growth nearly equal to amount on glucose; and ++: positive response equal to growth on glucose.
All the tests were performed by the method of SHIRLING and GOTTLIEB[10].

chain. Spores could not be differentiated by scanning electron microscopy (Fig. 2). The surface of what appears to be the spore chain is smooth. The cell wall contains the LL-isomer of diaminopimelic acid which together with the above characteristics places this organism in the genus Streptomyces[8].

Table 1 describes the amount of growth, degree of sporulation, spore mass color and color of the reverse substrate mycelium on various agar media.

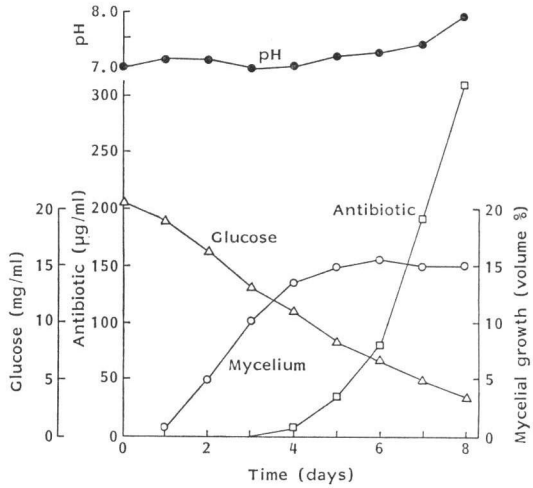
Streptomyces sp. X-14885 hydrolyzes casein, starch and gelatin, but not urea. It decomposes adenine, hypoxanthine and tyrosine, but not xanthine[9]. Table 2 compares the carbon utilization characteristics of *Streptomyces* sp. X-14885 with those of *S. humidus* and *S. lusitanus*, the latter two species chosen for this comparison because they appeared to be the closest relatives based on gray spore mass

Table 3. Metabolic and morphological characteristics of X-14885.

Test[a]	Result
Spore mass color	Gray-white
Spore chain form	*retinaculum-apertum*
ISP 6, darkening	—
Melanin, ISP 7	—
ISP 1, darkening	—
Gelatin hydrolysis[b]	+
Casein hydrolysis[c]	+
Starch hydrolysis[d]	+
NaCl (%) tolerance[b]	3
Growth range temperature (°C)	10~28
Reverse-side pigment	None
Soluble pigment	None
Streptomycin sensitivity (10 μg disc)	+ 9 mm
Nitrate reduction[c]	± slight
Hygroscopic property	—
DAP isomer	LL

[a] All tests were done at 28°C.
[b] See reference 11 for the test.
[c] Determined by the method recommended in reference 12.
[d] Determination was made after growth on agar of Actinomyces broth (Difco) with 0.25% soluble starch by flooding the plates with iodine-KI solution.

Fig. 3. Time course of X-14885A fermentation in shake flask.
Experimental details are described in the text.



color, smooth spores in a spiral to *retinaculum-apertum* spore chain, lack of melanin and a similar but not identical carbon utilization pattern[13]. Table 3 sets forth the morphological and metabolic characteristics of *Streptomyces* sp. X-14885.

Since antibiotic X-14885A is a pyrrole ether antibiotic similar in structure to antibiotic A23187 produced by *S. chartreusis*[14], a comparison was also made between strain X-14885 and *S. chartreusis*. These two cultures differ in the following respects: color of spore mass is blue in *S. chartreusis* and gray for strain X-14885; melanin production is positive for the former and negative for the latter; and *S. chartreusis* produces tunicamycin[15] while piericidine A is produced by strain X-14885.

Based on the various taxonomic criteria examined, it has not been possible to assign *Streptomyces* sp. X-14885 to any of the previously described species of Streptomyces. However at this time we defer assigning the species until further studies are performed.

Fermentation

Production of antibiotic X-14885A by fermentation was carried out with an inoculum medium composed of (in g/liter distilled water): Tomato pomace 5.0, distillers soluble 5.0, OM peptone (Oscar Meyer and Co., Madison, Wis.) 5.0, debittered dried yeast 5.0, corn starch 20.0, Cerelose (C.P.C. International Co., Englewood Cliffs, N.J.) 5.0, $CaCO_3$ 1.0, K_2HPO_4 1.0, and $CoCl_2 \cdot 6H_2O$ 0.00024; the pH was adjusted to 7.0 before sterilization. The inoculated flask was grown on a rotary shaker (250 rpm, 5-cm gyration) at 28°C for 3 days. Three ml of the resulting vegetative growth were used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of medium with the following ingredients (in g/liter distilled water): Cerelose 25.0, corn steep liquor (wet) 15.0, distillers soluble 10.0, Pharmamedia (Traders Protein, Division of Traders Oil Mill Co., Fort Worth, Tx.) 5.0, $CoCl_2 \cdot 6H_2O$ 0.01, and $CaCO_3$ 3.0; the pH was adjusted to 7.3 before sterilization. Fermentation was on a rotary shaker (250 rpm, 5-cm gyration) at

Table 4. *In vitro* antimicrobial activity of antibiotic X-14885A.

Organism	MIC (μg/ml*)	Organism	MIC (μg/ml*)
Pseudomonas aeruginosa ATCC 8705	>1,000	*Micrococcus luteus* ATCC 9341	3.13
Proteus vulgaris ATCC 6380	>1,000	*Bacillus megaterium* ATCC 8011	0.39
Escherichia coli ATCC 27856	>1,000	*Bacillus* sp. E ATCC 27859	0.19
Klebsiella pneumoniae ATCC 27858	>1,000	*B. subtilis* NRRL 558	0.19
Serratia marcescens ATCC 27857	>1,000	*Bacillus* sp. ATCC 27860	0.39
Serratia sp. ATCC 93	>1,000	*Mycobacterium phlei* ATCC 12290	3.9
Acinetobacter calcoaceticus ATCC 10153	>1,000	*Streptomyces cellulosae* ATCC 3313	1.57
Staphylococcus aureus ATCC 6538P	0.79	*Paecilomyces varioti* ATCC 26820	>1,000
Streptococcus faecium ATCC 8043	0.79	*Penicillium digitatum* ATCC 26821	>1,000
		Candida albicans NRRL 477	>1,000
		Saccharomyces cerevisiae ATCC 4226	>1,000

* Lowest two-fold dilution given a zone of inhibition in an agar-diffusion assay.

Table 5. Displacement of $^{45}Ca^{2+}$ from X-14885-cation complex by other cations.

Displacing cation	Cation radius (Å)	$^{45}Ca^{2+}$ remaining in complex (%)
None	—	100
Mg^{2+}	0.82	56
Ca^{2+}	1.18	65.5
Sr^{2+}	1.12	66.5
Ba^{2+}	1.34	74.5
Li^{+}	0.68	89
Na^{+}	0.97	93.3
K^{+}	1.33	98
Rb^{+}	1.47	93.3
Cs^{+}	1.67	98

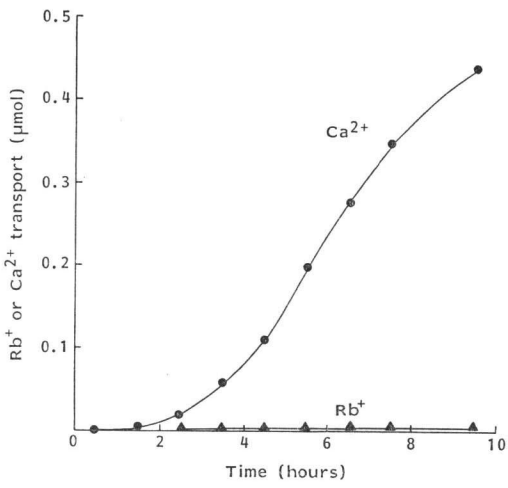
The cation selectivity sequence of X-14885 was determined by the method described previously[6]. Two μmol of antibiotic (in 2 ml of 70% toluene - 30% 1-butanol) were equilibrated by shaking for 2 hours at 20°C with 2 μmol of $^{86}RbCl$ or 1 μmol of $^{45}CaCl_2$ (in 1 ml of dimethyl glycine - tetramethylammonium hydroxide buffer, 25 mM pH 10.0). Complex formation was measured by liquid scintillation counting on both phases (see Fig. 3).

Displacement of labelled cation from the ionophore complexes was determined by the addition of unlabelled cations (2 μmol of monovalent or 1 μmol of divalent metal chloride).

28°C for 8 days. Broth samples were taken daily and analyzed for glucose content (by a Beckman glucose analyzer II), packed cell volume (by centrifugation of broth at 1,500 rpm for 10 minutes

Fig. 4. Time course of Ca^{2+} and Rb^{+} transport by antibiotic X-14885A in a U-tube.

The U-tube system described by ASHTON and STEINRAUF[16] was employed for the assay. A glass U-tube was filled with 5 ml of a chloroform solution of antibiotic (1×10^{-4} M). 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 was placed in one arm of the U-tube and an equal volume of the same buffer solution with unlabelled calcium or rubidium chloride in the other arm. The reaction was started by the addition of the respective labelled metal chloride and the chloroform phase separating the two aqueous phases was then gently stirred with a magnetic stirrer. 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, Mass.) in an intertechnique liquid scintillation spectrometer.



in an International centrifuge), and antibiotic potency in broth (by an agar well diffusion assay using *Staphylococcus aureus* ATCC 6538 P as test organism). The time course of an X-14885A fermentation is shown in Fig. 3. Under the conditions described, a potency of more than 300 μg/ml was obtained after 8 days of fermentation.

Biological and Ionophore Properties

The *in vitro* antimicrobial activity of antibiotic X-14885A is shown in Table 4. The antibiotic is active against Gram-positive bacteria but exhibits no activity against any of the Gram-negative bacteria and fungi tested.

Based on the structure similarity of A23187 and X-14885A, it was expected that antibiotic X-14885A would be preferentially a divalent cation ionophore. Examination of cation binding and transporting properties of the antibiotic shown in Table 5 and Fig. 4 indicates that this is indeed the case. The cation binding sequence of X-14885A was found to be $Mg^{2+} > Ca^{2+}$, $Sr^{2+} > Ba^{2+} \ggg Li^{+}$, Na^{+}, Rb^{+}, K^{+}, Cs^{+}.

References

1) WESTLEY, J. W.: Polyether Antibiotics: Naturally Occurring Acid Ionophores. Vol. 1. Biology. pp. 1~20, Marcel Dekker, Inc., N. Y., 1982
2) LIU, C.-M.; T. E. HERMANN, B. LA T. PROSSER, N. J. PALLERONI, J. W. WESTLEY & P. A. MILLER: X-14766A, a halogen containing polyether antibiotic produced by *Streptomyces malachitofuscus* subsp. *downeyi* ATCC 31547. Discovery, fermentation, biological properties and taxonomy of the producing culture. J. Antibiotics 34: 133~138, 1981
3) ŌTAKE, N. & M. MITANI: Ionophorous properties of antibiotic 6016, a novel magnesium selective ionophore. Agric. Biol. Chem. 43: 1543~1546, 1979
4) 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
5) REED, P. W. & H. A. LARDY: A-23187: a divalent cation ionophore. J. Biol. Chem. 247: 6970~6977, 1972
6) LIU, C.-M. & T. E. HERMANN: Characterization of ionomycin as a calcium ionophore. J. Biol. Chem. 253: 5892~5894, 1978
7) WESTLEY, J. W.; C.-M. LIU, J. F. BLOUNT, L. H. SELLO, N. TROUPE & P. A. MILLER: Isolation and characterization of a novel polyether antibiotic of the pyrrolether class, antibiotic X-14885A. J. Antibiotics 36 (10): 1983, in press
8) LECHEVALIER, H. A.; M. P. LECHEVALIER & N. N. GERBER: Chemical composition as a criterion in the classification of actinomycetes. Adv. Appl. Microbiol. 14: 47~72, 1971
9) GORDON, R. E.: The taxonomy of soil bacteria. *In* T. R. G. GRAY & D. PARKINSON *Ed.*, Ecology of Soil Bacteria. pp. 293~321, Liverpool University Press, Liverpool, 1967
10) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
11) SKERMAN, V. B. D.: A Guide to Identification of the Genera of Bacteria. The Williams and Wilkins Co., Baltimore, 1967
12) GORDON, R. E. & M. M. SMITH: Rapidly growing and fast bacteria. J. Bacteriol. 66: 41~48, 1953
13) BUCHANAN, R. E. & N. E. GIBBONS (*Eds.*): BERGEY's Manual of Determinative Bacteriology. 8th Edition. Williams and Wilkins Co., pp. 748~829, 1974
14) GALE, R. M.; C. E. HIGGINS & M. M. HOEHN: Antibiotic A-23187 and process for the preparation thereof. U.S. Patent 3,923,823, 1975
15) HAMILL, R. L. & L. W. CRANDALL: Polyether antibiotics. *In* Journal of Chromatography Library. Vol. 15. Antibiotics: Isolation, Separation and Purification. M. J. WEINSTEIN & G. H. WAGMAN *Eds.*, pp. 479~520, Elsevier, N. Y., 1978
16) ASHTON, R. & L. K. STEINRAUF: Thermodynamic consideration of the ion transport antibiotics. J. Mol. Biol. 49: 547~556, 1970