NOVEL POLYETHER ANTIBIOTICS X-14667A AND X-14667B FROM *STREPTOMYCES CINNAMONENSIS* SUBSP. *URETHANOFACIENS*

DISCOVERY, FERMENTATION, BIOLOGICAL AS WELL AS IONOPHORE PROPERTIES AND TAXONOMY OF THE PRODUCING CULTURE

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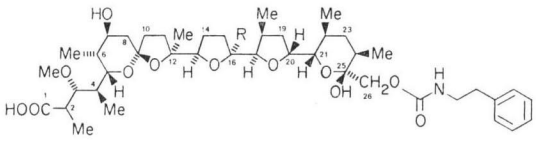
X-14667A and X-14667B are two novel polyether antibiotics produced by *Streptomyces cinnamonensis* subsp. *urethanofaciens*. The antibiotics are active *in vitro* against Gram-positive bacteria and exhibit high affinity for monovalent cations.

In the course of our search for new polyether antibiotics, several strains of *Streptomyces* soil isolates have been found to produce two novel antibiotics which we designated X-14667A and X-14667B (Fig. 1). In this report we describe the taxonomy of the antibiotic producing cultures, fermentation conditions for antibiotic production, and biological and ionophore properties of these antibiotics. The isolation and structure elucidation of the antibiotics are described in the following paper[1].

Fig. 1. Structure of X-14667A and X-14667B.



R = Me , X-14667 A
R = Et , X-14667 B

Taxonomy

Among the strains of *Streptomyces* isolates found to produce antibiotic X-14667A and X-14667B, strain X-14667 was isolated from a soil collected at Aesculapius Temple, Epidaurus, Greece. Strain X-14573 originated from a cactus garden soil from the University of Arizona, Tempe, Arizona. Strain X-14575 was isolated from a cornfield soil sample obtained in the Catskills, New York. These cultures have all been deposited at both the Northern Regional Research Laboratory, Peoria, Illinois and the American Type Culture Collection, Rockville, Maryland, where they have been assigned the numbers: NRRL 11336 and ATCC 31551 (for strain X-14667); NRRL 11337 and ATCC 31552 (for X-14573) and NRRL 11338 and ATCC 31553 (for X-14575).

All three strains produce a substrate mycelium which does not fragment into spores, and an aerial mycelium which later forms spore chains. After 14 days of incubation at 28°C, the spore chains appear *rectus-flexibilis* in form with greater than 50 spores per chain. The spores of strain X-14667 are smooth and range in size from 0.70 by 0.50 μ to 1.35 by 0.38 μ (Fig. 2). The cell wall of these strains contains the LL-isomer of diaminopimelic acid, which, together with the above characteristics, places these organi-

sms in the genus *Streptomyces*[2]. The growth characteristics of strain X-14667 on various solid media are summarized in Table 1. The two other strains (X-14573 and X-14575) have similar characteristics with slight variations in amount of growth, sporulation, or intensity of pigment. The carbon utilization and other metabolic characteristics of strain X-14667, X-14573 and X-14575, as well as *Streptomyces cinnamonensis* ATCC 15413 and several other cultures having similar physiological properties, are shown in Tables 2 and 3. A comparison of these strains, with the *Streptomyces* species described in BERGEY's manual[6], NONOMURA's key for classification[7], PRIDHAM and LYONS' classification[8] and the patent literature[9], shows that *S. cinnamonensis* is the closest relative to our isolates based on the following combination of characters: gray spore mass color, *rectus-flexibilis* spore chain form, smooth spore surface, chromogenic reaction on ISP media 1 and 6, and carbon utilization characteristics. *S. cinnamonensis* ATCC 15413 is similar to X-14667, X-14573, and X-14575 because they all produce monensin A and monensin B. However, *S. cinnamonensis* ATCC 15413 differs from our isolates in that it could not be shown to produce antibiotics X-14667A and X-14667B under the same fermentation conditions which allow for the production of these antibiotics by our strains. Strains X-14667, X-14573 and X-14575, can therefore be considered to be a subspecies of *S. cinnamonensis* for which we propose the name *S. cinnamonensis* subsp. *urethanofaciens*.

Fig. 2. *Streptomyces* sp. X-14667. Smooth spores on ISP-1 after 14 days of incubation at 28°C. Treatment included glutaraldehyde fixation and critical point drying.

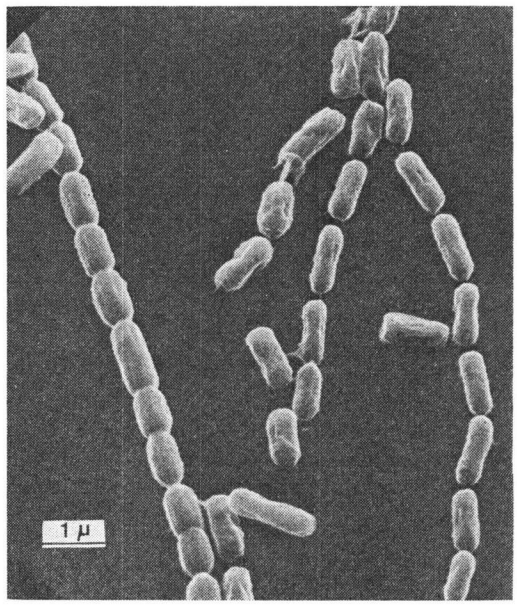


Table 1. Cultural characteristics of strain X-14667.

Agar medium	Amount of growth; Degree of sporulation	Spore mass color[a]	Color of reverse substrate mycelium[a]
Yeast malt extract (ISP-2)[b]	moderate growth; moderate sporulation	*b* (oyster white)	3*lg* (adobe brown)
Oatmeal (ISP-3)[b]	moderate growth; moderate sporulation; slightly pink reverse which turned blue with NaOH	*b* (oyster white)	5*ge* (rose wood)
Inorganic salts starch (ISP-4)[b]	abundant growth; abundant sporulation; hydrolyzes starch	3*cb* (sand)	5*ie* (copper tan)
Glycerol asparagine (ISP-5)[b]	moderate growth; abundant sporulation	*b* (oyster white)	3*lg* (adobe brown); edge of 2*ec* (biscuit)
CZAPEK-DOX[c]	abundant growth; abundant sporulation	3*dc* (natural)	2*gc* (bamboo); edge of 2*ec* (biscuit)

[a] The color scheme used was that taken from the Color Harmony Manual, 4th *ed.*, 1958 (Container Corporation of America, Chicago).

[b] Media recommended by the International Streptomyces Project[3].

[c] CZAPEK-DOX broth (BBL) to which 1.5% agar was added.

Table 3. Comparison of metabolic characteristics of strain X-14667, X-14573, X-14575 and related strains.

Test[a]	X-14667	X-14573	X-14575	*S. cinnamonensis* ATCC 15413	*S. alboniger* ATCC 12461	*S. aureocirculatus* ATCC 19823	*S. ramulosus* ATCC 19802	*S. nitrosporeus* ATCC 19792
ISP-6 darkening[b]	−	−	−	−	−	−(±, 2 weeks)	−, reddish	−
Melanin, ISP-7	−	−	−	−	−	−	−	−
Casein hydrolysis[b]	+	+	+	+	+	++	+, pink	+
Gelatin hydrolysis[c]	+	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+	++
NaCl (%) tolerance[b]	<10, probably=7%	7	7	<10	≤7	<7	≤7	<5
Growth range temperature (°C)	10~37	N.D.	N.D.	10~37	N.D.	N.D.	N.D.	N.D.
ISP-1 darkening	−	+, redwood	± slightly brownish	+ slight	−	−	+	−
Reverse-side pigment	slightly red-brown	brown	brown, occasionally	brownish occasionally	some brown	none	some brown	some brown
Soluble pigment	pink; reversible to blue with NaOH	red-brown	brown	brownish	brown	−	red-brown	−
Antibiotic production	X-14667A X-14667B monensin	X-14667A X-14667B monensin	X-14667A X-14667B monensin	monensin	puromycin	2 antibacterial components	actinomycin and antifungal antibiotic	nitrosporin
Nitrate reduction	+	±	+	± in 2 weeks	−	−	−	+
Hygroscopic property	+	slight on ISP-5	−	−	+	−	−	−

N.D.=not determined

[a] All tests were run at 28°C.

[b] Test was done by the method recommended in reference[4].

[c] See reference 5) for the test.

Table 2. Comparison of carbon utilization by strain X-14667, X-14573, X-14575 and related strains.

Carbon source	Growth response* of:							
	X-14667	X-14573	X-14575	*S. cinnamonensis* ATCC 15413	*S. alboniger* ATCC 12461	*S. aureocirculatus* ATCC 19823	*S. ramulosus* ATCC 19802	*S. nitrosporeus* ATCC 19792
D-Glucose	++	++	++	++	++	++	++	++
D-Xylose	+	++	++	++	±~−	±	−	++
L-Arabinose	++	++	++	+ (+)	++	±	−	++
L-Rhamnose	−	++	−	++	−	−	−	+
D-Fructose	+ (+)	++	++	++	−	++	±	±
D-Galactose	++	++	++	++	++	++	++	++
Raffinose	++	+ (+)	++	+ (+)	−	−	+	−
D-Mannitol	++	++	++	++	++	++	++	±
i-Inositol	+	++	++	++	++	+	−	−
Salicin	±~+	++	++	+ (+)	−	−	−	±
Sucrose	−	−	−	−	−	−	−	±
Cellulose	−	−	−	−	−	−	−	−

* −, Negative response; ±, doubtful response; +, more growth than on carbon control but less than on glucose; ++, positive response equal to the amount of growth on glucose. All tests were done at 28°C.

Fermentation

Vegetative inoculum of X-14667A and B producing culture was obtained by transferring spores of culture *S. cinnamonensis* subsp. *urethanofaciens* to a 500-ml Erlenmeyer flask containing 100 ml of an inoculum medium with the following composition (g/liter): Tomato pomace 5.0, distillers soluble 5.0, O.M. peptone (Oscar Meyer & Co., Madison, Wis.) 5.0, debittered dried yeast 5.0, cornstarch 20.0, $CaCO_3$ 1.0, and K_2HPO_4 1.0. The pH of the medium was adjusted to 7.0 before autoclaving. Inoculated medium was incubated at 28°C for 72 hours on a rotary shaker operating at 250 rpm. A three ml portion of the resulting growth was used to start the fermentation in a 500-ml Erlenmeyer flask containing 100 ml of production medium having the same composition as that described above. The fermentation was carried out for 5 days at 28°C on a rotary shaker with 250 rpm rotation. An example of a shake flask fermentation is shown in Table 4.

For larger scale fermentations, 6 liters of inoculum growth prepared as described above (except that the inoculum was developed in a 6-liter Erlenmeyer flask containing 2 liters of medium) was employed to start the fermentation in a 100-gallon fermentor containing 227 liters of the same production medium used in the shake flask fermentation. The fermentor was stirred at an impeller speed of 280 rpm and aerated at 85 liters per minute. The fermentation was terminated after 5 days of incubation at 28°C. As indicated in the accompanying paper[1], one of the fermentations yielded a mixture of antibiotic components which consisted of about 40% X-14667A, 18% X-14667B, 40% monensin A and 2% monensin B.

Table 4. Time course of X-14667 fermentation in shake flask.

Time (Days)	pH	Potency* (μg/ml)
0	7.1	0
1	6.1	0
2	7.5	0
3	7.5	13.5
4	7.7	18.5
5	7.6	20.0

* Potency was estimated by agar diffusion bioassay against *Staphylococcus aureus* ATCC 6538P using antibiotic X-14667A as standard.

Biological and Ionophore Properties

The *in vitro* antimicrobial activity of X-14667A and X-14667B is shown in Table 5. Like other polyether antibiotics, they are mainly active against Gram-positive bacteria and only weakly active against a certain fungi and yeasts. Both antibiotics were found to be active against *Treponema hyodysenteriae*, *in vitro* (MIC: 0.4~2.0 μg/ml). In addition, the antibiotics are effective in stimulating the production of propionate in an *in vitro* rumen fermentation[10].

Table 5. *In vitro* antimicrobial activity of antibiotic X-14667A and X-14667B.

	MIC (μg/ml)*			MIC (μg/ml)*	
	X-14667A	X-14667B		X-14667A	X-14667B
Pseudomonas aeruginosa ATCC 8705	>1000	>1000	*Bacillus megaterium* ATCC 8011	1.6	0.8
Proteus vulgaris ATCC 6380	>1000	>1000	*Bacillus* sp. E ATCC 27359	0.8	0.4
Escherichia coli ATCC 27856	>1000	>1000	*Bacillus subtilis* NRRL 558	3.1	1.6
Klebsiella pneumoniae ATCC 27858	>1000	>1000	*Bacillus* sp. TA ATCC 27860	3.1	1.6
Serratia marcescens ATCC 27857	>1000	>1000	*Mycobacterium phlei* ATCC 355	15.7	6.3
Serratia sp. ATCC 93	>1000	>1000	*Streptomyces cellulosae* ATCC 3313	12.5	12.5
Acinetobacter calcoaceticus ATCC 10153	>1000	>1000	*Paecilomyces varioti* ATCC 25820	62.5	25
Staphylococcus aureus ATCC 6538P	6.3	6.3	*Penicillium digitatum* ATCC 26821	>1000	250
Micrococcus luteus PCI ATCC 9341	3.1	1.6	*Candida albicans* NRRL 477	62.5	25
			Saccharomyces cerevisiae ATCC 4226	>1000	>1000

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

Table 6. Displacement of $^{86}Rb^{+}$ and $^{45}Ca^{2+}$ from X-14667A, X-14667B and monensin-cation complexes by other cations.

Displacing cation	Cation radius (Å)	$^{86}Rb^{+}$ or $^{45}Ca^{2+}$ remaining on complex (%)					
		X-14667A		X-14667B		Monensin	
		$^{86}Rb^{+}$	$^{45}Ca^{2+}$	$^{86}Rb^{+}$	$^{45}Ca^{2+}$	$^{86}Rb^{+}$	$^{45}Ca^{2+}$
none	—	67.3	62.5	68.3	58.4	59.1	58.7
Ca^{2+}	0.82	66.0	38.7	67.0	36.0	49.2	35.4
Mg^{2+}	1.18	68.0	41.0	66.0	39.0	39.0	16.3
Sr^{2+}	1.12	67.5	49.4	67.0	41.0	52.5	43.9
Ba^{2+}	1.34	68.5	46.3	66.5	37.7	44.7	25.6
Li^{+}	0.68	45.6	17.5	41.4	11.6	33.5	18.5
Na^{+}	0.97	54.5	26.0	50.0	18.5	1.4	0.4
K^{+}	1.33	28.8	5.7	24.5	2.3	13.2	1.8
Rb^{+}	1.47	39.0	11.7	37.3	8.7	32.6	18.0
Cs^{+}	1.67	53.1	26.7	49.2	25.2	46.7	43.8

The cation selectivity sequence of X-14667A, X-14667B and monensin was determined by the method described previously[11]. Two μmole of antibiotic (in 2 ml of 70% toluene-30% *n*-butanol) were equilibrated by shaking for 2 hours at 20°C with 2 μmole of $^{86}RbCl$ or 1 μmole of $^{45}CaCl_2$ (in 1 ml of dimethyl glycine-tetramethyl-ammonium hydroxide buffer, 25 mM pH 10.0). Complex formation was measured by liquid scintillation counting of both phases (see Fig. 3).

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

Since X-14667A and X-14667B are urethane derivatives of monensins (see Fig. 1), the ionophore property of these antibiotics was compared with that of monensin. The affinity of the antibiotics to some monovalent and divalent cations was examined by a cation competition experiment described in Table 6. In contrast to monensin which complexes most tightly with Na^+, X-14667A and X-14667B bind most tightly with K^+. The affinity of these new antibiotics for monovalent cations is so much stronger than for divalent cations that hardly any displacement of rubidium ion by divalent cations from a Rb^+-antibiotic complex was observed. On the other hand, binding of monensin with Mg^{2+} is comparable to that of Rb^+ or Li^+ and is stronger than Cs^+, The cation selectivity sequence of X-14667A, X-14667B and monensin are:

X-14667A: $K^+ > Rb^+ > Li^+ > Na^+, Cs^+ \gg Ca^{2+} \geq Mg^{2+} \geq Ba^{2+}, Sr^{2+}$

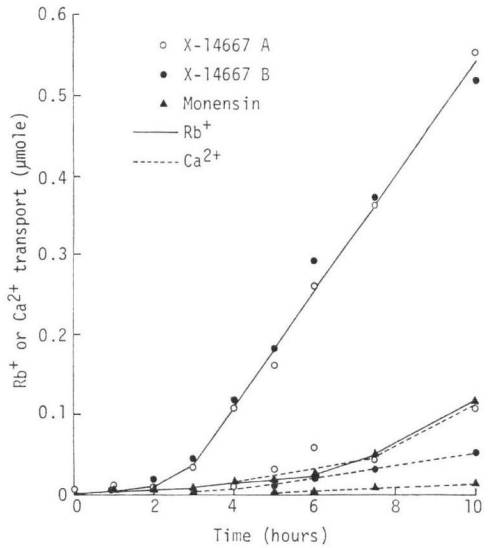
X-14667B: $K^+ > Rb^+ > Li^+ > Na^+, Cs^+ \gg Ca^{2+} \geq Mg^{2+} \geq Ba^{2+}, Sr^{2+}$

Monensin: $Na^+ \gg K^+ \gg Rb^+, Li^+, Mg^{2+} > Ba^{2+} > Ca^{2+} > Sr^{2+}, Cs^+$

The rate of cation transport by these antibiotics in a U-tube system[12] is shown in Fig. 3. Rb^+ is transported across the chloroform barrier from one aqueous phase to another at a much faster rate by X-14667A and X-14667B than by monensin, whereas Ca^{2+} is only slowly transported by either of these antibiotics.

Fig. 3. Time course of Ca^{2+} and Rb^+ transport by antibiotic X-14667A, X-14667B and monensin in a U-tube.

The U-tube system described by ASHTON and STEINRAUF[12] 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.



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