A47934, A NOVEL GLYCOPEPTIDE-AGLYCONE ANTIBIOTIC PRODUCED BY A STRAIN OF *STREPTOMYCES TOYOCAENSIS* TAXONOMY AND FERMENTATION STUDIES

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A47934, a novel glycopeptide-aglycone antibiotic, is produced by a strain of *Streptomyces toyocaensis*, NRRL 15009. A47934 is unique among reported glycopeptides in that it contains a sulfate ester. Like several other glycopeptides, the majority of the A47934 produced remained associated with the producing biomass, from which it could be released into aqueous media by alkalization. Antibiotic biosynthesis was depressed when initial levels of phosphate phosphorus in the medium exceeded the normal level of 35 μg/ml. Enrichment of the fermentation medium with tyrosine depressed A47934 yields while enrichment with *p*-hydroxyphenylglycine or *p*-hydroxyphenylglyoxylic acid stimulated antibiotic biosynthesis.

During the process of screening actinomycetes for novel antimicrobial substances, a new strain was isolated from a sample of sandy soil collected at a low-tide area in the state of Washington, U.S.A. This isolate produced a single antibiotic containing a ristocetin-like peptide core. Like A41030A[1], it contained no neutral or amino sugars. However, unlike A41030A and all previously reported glycopeptide antibiotics, the peptide core contained a sulfate ester (Fig. 1)[2]. The biological spectrum of A47934, typical of glycopeptides, included activity *versus* Gram-positive aerobic and anaerobic bacteria. In addition to protecting mice against infections of *Staphylococcus aureus*, *Streptococcus pyogenes* and *Streptococcus pneumoniae*, it also enhanced both growth and feed efficiency in poultry, swine and ruminants[3]. This paper presents taxonomy and fermentation studies on NRRL 15009.

Materials and Methods

Cell Wall Analyses

Diaminopimelic acid (DAP) isomers were determined by the method of Becker *et al.*[4]. Cell wall sugars were identified by the procedure of Lechevalier[5].

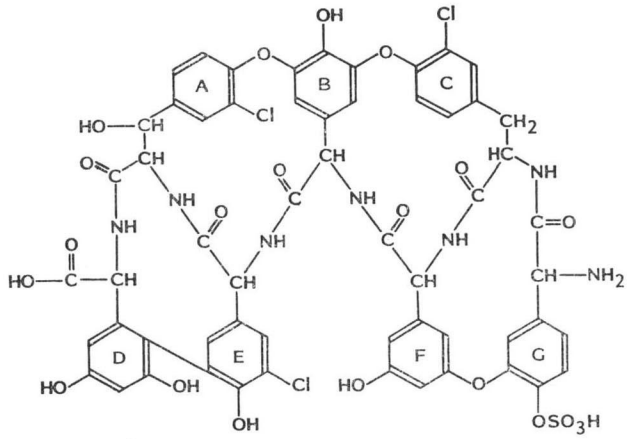
Taxonomic Methods

The methods and media recommended by the International Streptomyces Project (ISP)[6] and Waksman[7] for the identification of *Streptomyces* species were followed. Identification keys from Bergey's Manual[8], Kuster[9], Nonomura[10], Pridham[11] and Szabo[12] were used. Color names were assigned to reverse pigments on the basis of the Inter-Science Color Council-National Bureau of Standards (ISCC-NBS) Centroid Color Charts Standard Sample No. 2106[13]. Spore mass color names were taken from the system of color wheels recommended by Tresner and Backus[14].

Fermentor Inoculum

NRRL 15009 was propagated on a medium containing tomato paste 2%, pre-cooked oatmeal 2% and agar 2% in deionized water (pH 6.7 prior to sterilization). Agar slope cultures were incubated for 6~7 days at 30°C, then suspended in calf serum and lyophilized. Fermentor inoculum was prepared by introducing either lyophilized pellets, or submerged cultures stored in liquid nitrogen, into wide-mouth Erlenmeyer flasks. These 250-ml vessels contained 50 ml of a medium composed of

Fig. 1. Structure of A47934.



glucose 1.5%, potato dextrin 2.0%, soybean grits 1.5%, corn steep liquor 1.0%, yeast extract 1.0% and $CaCO_3$ 0.2% in tap water, adjusted to pH 6.5 with aqueous sodium hydroxide prior to autoclaving. After incubation at 30°C for about 48 hours on a rotary shaker, the resulting mycelial suspension was used to inoculate fermentors (1%).

Fermentors

Wide-mouth 250-ml Erlenmeyer flasks containing 50 ml of culture medium were incubated for 5~7 days at 30°C on a gyratory shaker orbiting at 250 rpm in a circle with a diameter of 5.08 cm. Unless otherwise specified, the medium, designated SGC, contained glucose 2.5%, potato dextrin 3.0%, soybean grits 1.5%, casein 0.5%, blackstrap molasses 0.3% and $CaCO_3$ 0.25% in tap water, pH 6.8~7.0. Antibiotic activity was quantitated microbiologically by an agar-well plate test employing *Bacillus subtilis* ATCC 6633, as the test organism.

Chromatography/HPLC

A47934 was initially monitored with a TLC system using Merck-Darmstadt silica gel 5763 plates developed in a solvent system containing chloroform - methanol - ammonia - butanol - water (25: 50: 25: 25: 10). Chromatograms were bioautographed vs. *B. subtilis* grown in minimal nutrient agar. The Rf value of A47934 in this system was 0.4.

Putative precursors of the antibiotic were monitored with an isocratic HPLC system utilizing a reversed-phase Rad-PAK column (8×100 mm, C_{18}, 5 μM) from Waters Associates; a mobile phase composed of methanol, tetrahydrofuran and triethylamine phosphate buffer (0.5% triethylamine adjusted to pH 3.0 with concentrated H_3PO_4) (7.5: 1: 91.5); a flow rate of 1.5 ml/minute and UV detection at 254 nm.

Results and Discussion

Taxonomy

Cultural Characteristics

NRRL 15009 grew abundantly on both complex organic and chemically defined media. Aerial mycelia were produced. The spore mass color was in the gray series, nearest color tab 2ih, light olive gray. The reverse color ranged from an olive brown to various shades of yellow, depending on the growth medium. Soluble pigments were not produced. These cultural characteristics are summarized in Table 1.

Morphological Characteristics

NRRL 15009 produced well-developed, non-fragmenting, monopodially branched aerial mycelia.

Fig. 2. Photomicrograph illustrating the spiral morphology of NRRL 15009 when grown on CZAPEK's solution agar for 14 days at 30°C.

(a) is brightfield illumination at 640× magnification, (b) is incident light illumination with dark ground field at 900×magnification.

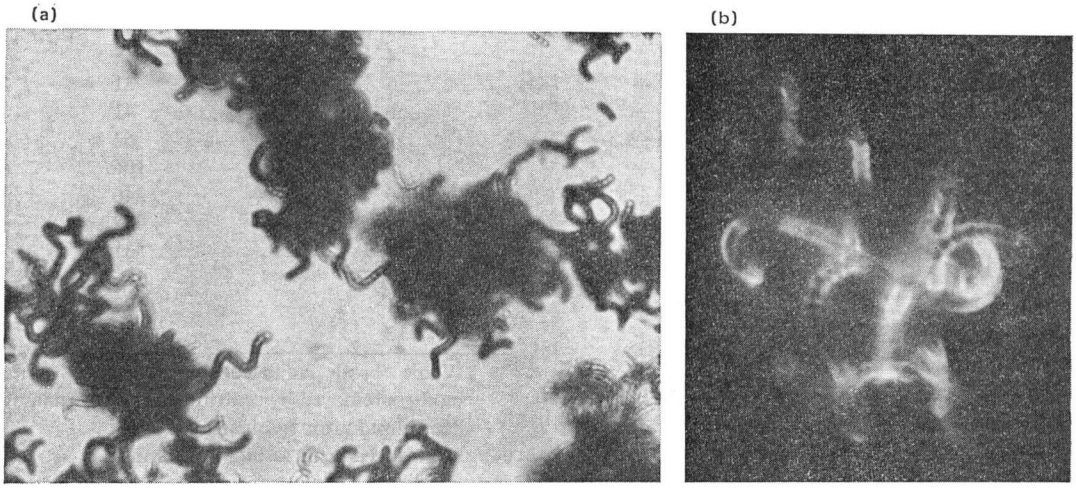


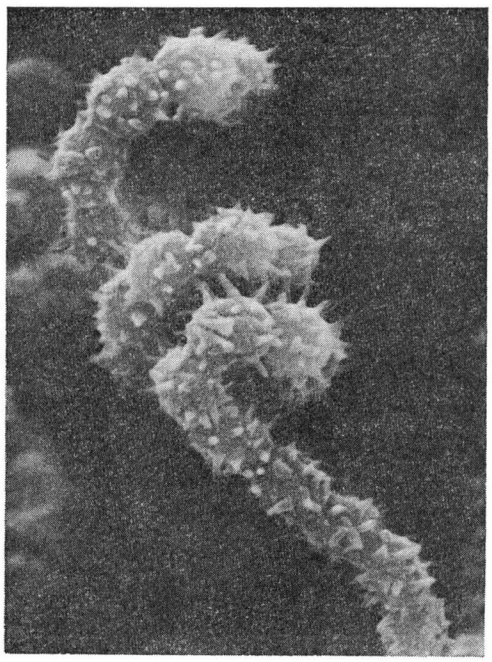
Table 1. Cultural characteristics of NRRL 15009.

Medium	Cultural characteristics
ISP No. 2	G: Abundant
	R: 68.S.OY
	Am: Good, (2ih), light olive gray
	Sp: None
ISP No. 3	G: Good
	R: 91.d.gy.Y
	Am: Good, (2ih), light olive gray
	Sp: None
ISP No. 4	G: Abundant
	R: 94.1.0lBr
	Am: Abundant, (2ih), light olive gray
	Sp: None
ISP No. 5	G: Abundant
	R: 67.brill.OY
	Am: Good, (2ih), light olive gray
	Sp: None
CZAPEK's agar	G: Good
	R: 91.d.gy.Y
	Am: Good, (2ih), light olive
	Sp: None
TPO	G: Abundant
	R: 94.1.0lBr
	Am: Abundant, (2ih), light olive gray
	Sp: None

G=Growth, R=reverse, Am=aerial mycelia, Sp=soluble pigment.

Color designations are from the ISCC-NBS system[13].

Fig. 3. Electron micrograph illustrating the spiral morphology and spiny spore surface ornamentation of NRRL 15009 when grown on CZAPEK's solution agar for 14 days at 30°C.



Sporophores were formed in short, open spirals of 2~3 coils (Fig. 2). These characteristics placed it in the spirales section of PRIDHAM

Table 2. Comparison of NRRL 15009 and *S. toyocaensis*.

Characteristic	NRRL 15009	*S. toyocaensis*
Aerial spore mass color	Gray	Gray
Carbon utilization:		
L-Arabinose	+	+
L-Rhamnose	−	−
D-Xylose	+	±
D-Glucose	+	+
D-Fructose	+	+
Inositol	+	+
D-Mannitol	−	+
Sucrose	−	−
Raffinose	−	−
Gelatin liquefaction	+	+
Melanoid pigment	−	−
Morphology	spiral	spiral
Nitrate reduction	+	+
Skim milk	+	+
Starch hydrolysis	+	+
Soluble pigments	−	−
Reverse color	Y-Br	Y-Br
Spore shape	Oblong	Oblong
Spore surface	Spiny	Spiny

Table 3. Effect of pH on release of A47934 from the producing biomass.

pH[a]	Antibiotic level in broth (μg/ml)
3	<12
4	18
5	31
6	41
7	62
8	106
9	246
10	448
10.5	531
10.5[b]	119
11	523
12	365

[a] Whole broth adjusted to indicated value, biomass removed by centrifugation, supernatant neutralized prior to bioassay.
[b] Neutralized prior to biomass removal.

et al.[11].

Mature spore chains contained from 10 to 50 spores. The spore shape was oval to oblong with spiny surface ornamentation. Spores ranged from 0.5 to 0.7 μM in width and 0.7 to 0.8 μM in length. Fig. 3 is an electron micrograph demonstrating the spore surface ornamentation.

Physiological Characteristics

Hydrolyzed whole cells contained LL-2,6-diaminopimelic acid, glucose and ribose. These components represent a Type I cell wall[15] and a Type C sugar pattern[5].

NRRL 15009 utilized arabinose, ribose, xylose, glucose, galactose, fructose, inositol and cellobiose. Rhamnose, sucrose, melibiose, raffinose and mannitol were not utilized. Melanoid pigments were not produced. Skim milk and starch were hydrolyzed, gelatin was liquefied and nitrate was reduced to nitrite. NRRL 15009 tolerated up to 9% NaCl and grew at temperatures of 15~40°C.

Species Determination

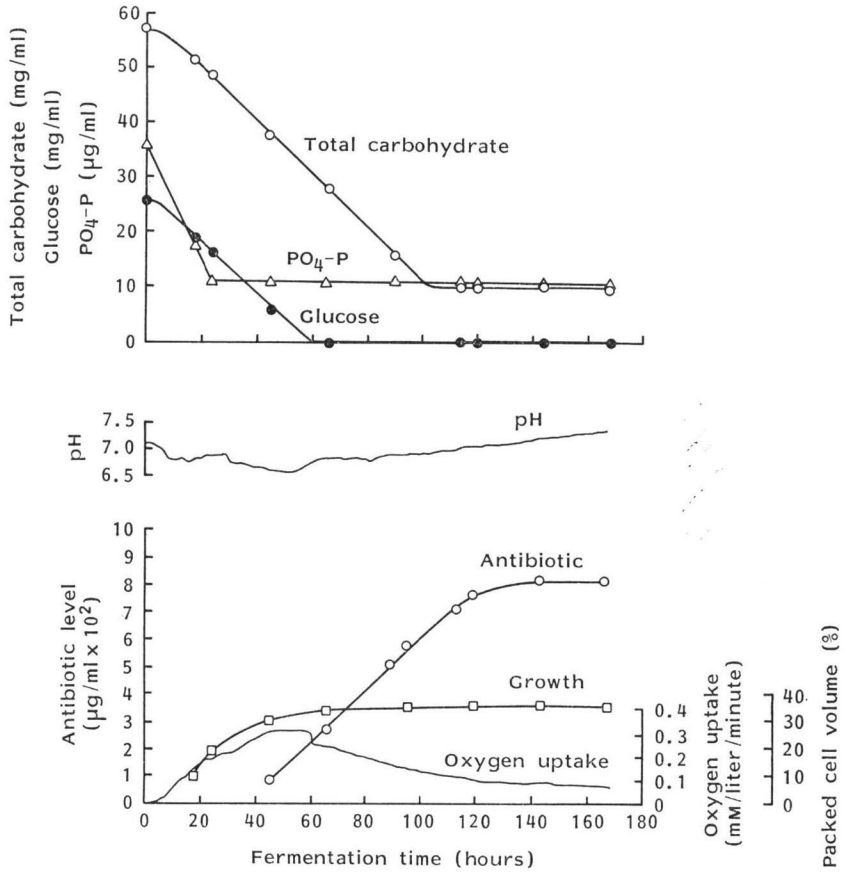
The characteristics of NRRL 15009 placed it in the genus *Streptomyces*. An examination of the published descriptions of similar taxa suggested a close resemblance to *Streptomyces griseoflavus* and *S. toyocaensis*. Simultaneous laboratory comparisons subsequently determined that NRRL 15009 was virtually identical to *S. toyocaensis* (Table 2).

Based on these data, NRRL 15009 is classified as a strain of *S. toyocaensis* Nishimura, Katagiri, Sato, Mayama and Shimaoka ATCC 19814. *S. toyocaensis* was first described in the Japanese patents in 1954[16]. Because it is not included in the Approved List of Bacterial Names[17], however, it is not a validly published species.

Fermentation

The fermentation was initially examined for modifications that would increase A47934 production. The antibiotic level produced in fermentation media employed for the biosynthesis of other glycopeptides was inferior to that obtained in the initial medium. Modification of the original medi-

Fig. 4. Typical time course of the A47934 fermentation.



um resulted in the SGC medium, in which antibiotic yields were approximately 800 μg/ml. The SGC medium was used as the basis for all subsequent fermentation studies.

A47934 was present in both the fermented broth and the mycelial mass of *S. toyocaensis*. The percentage of antibiotic in each location varied with the fermentation conditions employed and the age of the fermentation. In the SGC medium, increasingly larger percentages of A47934 were released into the broth as the fermentation progressed. Even so, the majority of the antibiotic produced consistently remained associated with the producing biomass. Early levels in the biomass were about 90% of the total, while harvest levels were 60~70% of the total. The antibiotic associated with the biomass could be extracted with acetone or, like actaplanin[18] and A41030[1], other glycopeptide antibiotics, released into aqueous systems by increasing their alkalinity (Table 3). Maximum amounts of A47934 were released at pH 10.5~11. This procedure, followed by immediate neutralization after removal of the biomass, was routinely employed to quantitate the total antibiotic present.

A typical profile of the fermentation time course in the SGC medium is shown in Fig. 4. The stationary portion of the trophophase was achieved by 65 hours. The idiophase was initiated at approximately 35 hours, before the logarithmic phase of growth was completed, and continued linearly until maximum antibiotic levels were attained on the 5th or 6th day. The pH descended from slightly above neutrality at inoculation to 6.6 at about 55 hours, then trended gradually upward to a terminal

Table 4. Effect of incubation temperature on biosynthesis of A47934.

Temperature (°C)	Antibiotic level (μg/ml)
25	318
28	786
30	793
32	791
34	547
37	163

Table 5. Effect of carbohydrates and lipids on biosynthesis of A47934.

Carbon source[a] (4% level)	Terminal pH	Growth (vol %)	Antibiotic level (μg/ml)
None	8.9	7	<10
Glucose	7.6	24	551
Galactose	7.1	28	184
Dextrin	7.7	27	619
Corn starch	7.4	33	604
Soybean oil	7.3	51	336
Peanut oil	7.3	50	269
Methyl oleate	7.0	47	32

[a] SGC medium minus glucose and dextrin.

value near 7.4. After declining rapidly during the first 24 hours, phosphate remained at a constant level of 10 μg/ml through the 7th day.

The levels of glucose and total carbohydrate declined in parallel until glucose was exhausted at 60 hours. Total carbohydrate reached a stationary level of 10 mg/ml shortly after 100 hours. This residual carbohydrate may reflect unhydrolyzed polysaccharide units remaining beyond the $1\sim6$ glycoside branches of the dextrin molecules.

The effect of incubation temperature on antibiotic synthesis is shown in Table 4. Maximum yields were obtained at $28\sim32$°C. Table 5 summarizes the effect of various carbon sources on the biosynthesis of A47934. Glucose, dextrin and corn starch supported the highest yields. Lipids produced larger quantities of biomass accompanied by lower levels of antibiotic. Glycerol, disaccharides, fructose and mannose supported normal growth but little antibiotic synthesis.

Substantially greater yields of A47934 were produced by soybean products, cotton-seed meal, casein hydrolysates or combinations of these ingredients than by any of numerous other complex nitrogen sources tested. Although the antibiotic contains sulfate, enrichment of the medium with sulfate salts did not enhance antibiotic levels. Nitrate and ammonium salts, when substituted for the soybean grits and casein in the SGC medium at levels of $1\sim2\times10^{-2}$ M, supported some growth and the biosynthesis of about $60\sim80$ μg/ml of A47934.

Omission of $CaCO_3$ from glucose-containing media resulted in acidic fermentations containing little growth and virtually no antibiotic. Although the initial medium level of phosphate phosphorus was normally low, about 35 μg/ml, the addition of 20 μg/ml of supplemental orthophosphate depressed antibiotic synthesis by 26%. This response is unlike that of the *Streptomyces virginiae* strain which produces A41030[1] but is similar to that of the *Nocardia orientalis* strains that produce vancomycin and *N*-demethylvancomycin[19].

There is limited information concerning biogenesis of the unusual aromatic amino acid residues in glycopeptide aglycones. Four of the aromatic rings in vancomycin can be derived from tyrosine (TYR) while *p*-hydroxyphenylglycine (*p*-HPG) is also the precursor of two of the same residues[20]. The *p*-HPG moieties of ristocetin are likewise derived from TYR[21]. Biosynthesis of *N*-demethylvancomycin is increased by enrichment of the medium with TYR, *p*-HPG or *p*-hydroxyphenylglyoxylic acid (*p*-HPGA)[19]. Except for depression by TYR, however, these compounds have little affect on the fermentation yields of A41030[1]. The effect of these putative precursors on broth HPLC profiles and the biosynthesis of A47934 is shown in Table 6.

Shikimic acid (SA) did not affect antibiotic yields and was not converted to metabolites iden-

Table 6. Effect of putative precursors on broth HPLC profiles and biosynthesis of A47934.

Putative precursor (5×10^{-3} M)	Relative level at harvest[a] (HPLC)				Antibiotic level (% of control)	
	SA	TYR	*p*-HPG	*p*-HPGA	0-hour addition	72-hour addition
Control	−	−	±	+	100	100
SA	++++	−	±	+	102	101
TYR	−	+++	+	+	76	83
p-HPG	−	−	±	++++	148	136
p-HPGA	−	−	±	++++	146	143

[a] 0-hour addition.

tifiable by the HPLC system used. TYR, which depressed yields as much as 24%, was decreased slightly while the *p*-HPG peak increased by a corresponding amount. Both *p*-HPG and *p*-HPGA increased A47934 biosynthesis nearly 50% when pulsed into the medium at inoculation. No detectable change in the amount of *p*-HPGA occurred during the fermentation but the additional *p*-HPG appeared to be stoichiometrically converted to *p*-HPGA. Similar, but less complete, deamination of *p*-HPG by *S. virginiae* was also observed in the A41030 fermentation, although A41030 biosynthesis was not stimulated[1]. Conversion of *p*-HPG to *p*-HPGA suggests that the similar stimulatory effect of the two compounds on A47934 biosynthesis may actually be due to *p*-HPGA. This interesting possibility must await further elucidation of the glycopeptide biosynthetic pathway, however, inasmuch as there are no published reports on the possible role of *p*-HPGA in glycopeptide biosynthesis.

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