A41030, A COMPLEX OF NOVEL GLYCOPEPTIDE ANTIBIOTICS PRODUCED BY A STRAIN OF *STREPTOMYCES VIRGINIAE*

TAXONOMY AND FERMENTATION STUDIES

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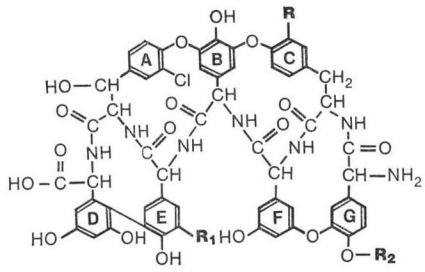
A41030 is a complex of novel glycopeptide antibiotics produced by a culture isolated from a soil. Taxonomic studies have identified the microorganism, NRRL 15156, as a strain of *Streptomyces virginiae*. The major factor, A41030A, and three of the six minor factors are unique among glycopeptides in that they are naturally occurring aglycones, containing no neutral or amino sugars. The A41030 that was not spontaneously released into the fermentation broth could be released from the biomass into aqueous media at pH 10.5. In contrast to the vancomycin and *N*-demethylvancomycin fermentations, A41030 biosynthesis was stimulated by enriching the medium with K_2HPO_4 at a level of 1 mg/ml. Enrichment with putative precursors of the aglycone, however, did not increase the biosynthesis of A41030.

During the process of screening actinomycetes for novel antimicrobial substances, a new strain was isolated from a soil sample collected in Indianapolis, Indiana, USA. This isolate produced a complex of glycopeptide antibiotics composed of seven members, each of which contained a ristocetin-like peptide core. The major component, A41030A, and three of the six minor components were unique among naturally occurring glycopeptides in that they were true aglycones, containing no neutral or amino sugars (Fig. 1). The complex was active *versus* Gram-positive bacteria and demonstrated both growth promotion and feed efficiency improvement in poultry, swine, and ruminants (Eggert *et al.*, in preparation). A natural variant of the original isolate, NRRL 15156, has been characterized as a strain of *Streptomyces virginiae*. This paper presents taxonomy and fermentation studies on NRRL 15156.

Fig. 1. Structure of the A41030 factors.



Factor	MW	R	R_1	R_2
A	1,231	Cl	Cl	H
B	1,197	Cl	H	H
C	1,393	Cl	Cl	Gal
D	1,326	H	Cl	H
E	1,163	H	H	H
F	1,555	Cl	Cl	Gal-Gal
G	1,684	Cl	Cl	Gal-Gal

Factors D and G have two equivalent butyl groups attached to the peptide nucleus at an undetermined location.

Materials and Methods

Cell Wall Analyses

Cell wall sugars were identified by the procedure of Lechevalier[1]. Diaminopimelic acid (DAP) isomers were determined by the method of Becker *et al.*[2].

Taxonomic Studies

The methods and media recommended by the International Streptomyces Project (ISP)[3] for characterization of *Streptomyces* species were followed. Color names were assigned to the reverse pigments and spore mass pigments on the basis of The Inter-Science Color Council-National Bureau of Standards (ISCC-NBS) Centroid Color Charts, standard sample No. 2106[4].

Fermentor Inoculum

NRRL 15156 was propagated on a medium containing dextrin 1%, yeast extract 0.1%, N-Z Amine A (Sheffield Products) 0.2%, beef extract 0.1%, $CoCl_2 \cdot 6H_2O$ 0.001%, and agar 2.0% in deionized water. 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 vessels contained 50 ml of a medium composed of glucose 1.5%, dextrin 2.0%, soybean grits 1.5%, corn steep liquor 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%, v/v).

Fermentors

Wide-mouth 250-ml Erlenmeyer flasks containing 50 ml of culture medium were incubated for 6~10 days at 32°C on a gyrotatory shaker orbiting at 250 rpm in a circle with a diameter of 5.08 cm. Unless otherwise specified, the medium, designated SG, contained dextrin 3.0%, soybean grits 0.6%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1% and $CaCO_3$ 0.2% in tap water, pH 6.7~7.2. Antibiotic activity was quantitated by a turbidimetric assay employing *Staphylococcus aureus,* NRRL B-314, as the test organism. Qualitative evaluations were initially based on a TLC system using Merck-Darmstadt silica gel 60 plates developed in a solvent system containing CH_3CN, EtOH and H_2O (80: 10: 15). Chromatograms were bioautographed vs. *Bacillus subtilis,* ATCC 6633. The Rf value of A41030A in this system was 0.75. Supplementary analyses were performed with a high performance liquid chromatography (HPLC) system utilizing a 4.6×300 mm stainles steel column; Nucleosil 10 C_{18} resin; a solvent composed of H_2O, CH_3CN, and dibutylamine (80.5: 19: 0.5) adjusted to pH 2.5 with H_3PO_4; a flow rate of 1 ml/minute and UV detection at 225 nm.

Results and Discussion

Taxonomy

Cultural Characteristics

NRRL 15156 grew abundantly on most complex media. Growth on synthetic media was less abundant. Copious aerial mycelia and sporophores were produced on ISP taxonomy media. The spore mass color was in the gray (GY) color series: 3fe light brownish gray to 5fe light grayish reddish brown in the TRESNER and BACKUS system[5], and 63 light brownish gray in the ISCC-NBS system[4]. The reverse side was a pale yellow, often with a grayish hue. No distinctive pigments were associated with the reverse color. Soluble pigments were not produced. This cultural information is detailed in Table 1.

Morphological Characteristics

NRRL 15156 produced well-developed aerial mycelia with coiled sporophores that also exhibited hooks and loops. It was placed in the *Spirales* (S) section of PRIDHAM *et al.*[6] as a primary morphology type, and in the *Retinaculum-Apertum* (RA) section as a secondary morphology type. The spirals were moderately loose coils of 2~3 turns. The spiral morphology was clearly observed on ISP No. 4 (inorganic salts - starch agar). The RA morphology, represented by large open loops and hooks, was most clearly demonstrated on ISP No. 5 (glycerol - asparagine agar). Neither spirals nor hooks and

Table 1. Cultural characteristics of NRRL 15156 and *S. virginiae*.

Medium		NRRL 15156	*S. virginiae*
ISP No. 2	G:	Abundant	Abundant
	R:	72. d. OY	75. deep yBr
	Am:	Abundant 63.1. brGy	Abundant 63.1. brGY
	Sp:	None	None
ISP No. 3	G:	Good	Fair
	R:	93. yGray	90. gy. Y
	Am:	Good 63.1. brGY	Fair 63.1. brGY
	Sp:	None	None
ISP No. 4	G:	Abundant	Good
	R:	89. p. Y.	91. d. gy. Y
	Am:	Abundant 63. 1. brGY	Good 63. 1. brGY
	Sp:	None	None
ISP No. 5	G:	Abundant	Good
	R:	89. p. Y	89. p. Y
	Am:	Abundant 22. rGY	Good 63. 1. brGY
	Sp:	None	None
CZAPEC'S agar	G:	Fair	Poor
	R:	264. 1. Gray	264. 1. Gray
	Am:	Poor 10. pKGY	Poor 10. pKGY
	Sp:	None	None
TPO	G:	Abundant	Abundant
	R:	72. d. OY	72. deep yBr
	Am:	Abundant 63.1. brGY	Abundant 63.1. brGY
	Sp:	None	None

G=Growth, R=reverse, Am=aerial mycelia, Sp=soluble pigment. Color designations are from the ISCC-NBS system[4].

Fig. 2. Sporophore morphology of strain NRRL 15156 from a 14-day old culture grown on ISP No. 4 agar, ×640.

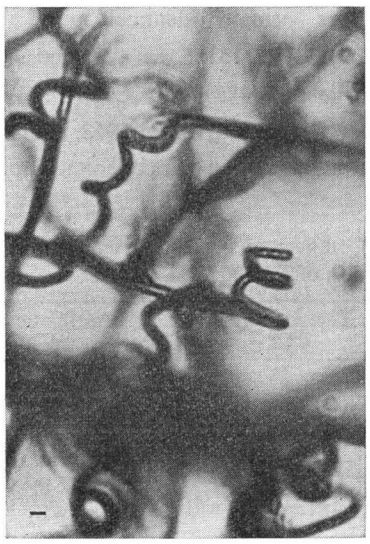
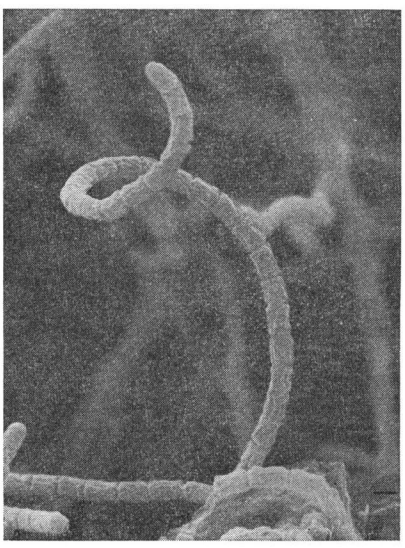


Fig. 3. Electron micrograph of strain NRRL 15156 from a 14-day old culture grown on ISP No. 4 agar. Bar=1.0 μm.



loops were observed on ISP No. 2 (yeast - malt extract agar). The sporophores of NRRL 15156 bore chains of about 50 spores. Spores were oblong, ranging from 0.85~1.43 μm in length and 0.66~0.95 μm in width. The mean size was 0.77×1.19 μm. The spore surface ornamentation was smooth. Neither sclerotia, sporangia, nor motile zoospores were observed.

Fig. 2 is a light microscope photomicrograph showing the spiral morphology of NRRL 15156. Fig. 3 is an electron microscope photomicrograph demonstrating the oblong spore shape and smooth spore surface ornamentation.

Physiological Characteristics

Hydrolyzed whole cells contained LL-2,6-diaminopimelic acid. The *meso* isomer was not present. This represents a Type I cell wall, which is indicative of the genus *Streptomyces*[7]. Melanoid pigments were produced in ISP No. 1 broth medium (Tryptone - yeast extract), and on ISP No. 6 (peptone - yeast extract iron agar), but not on ISP No. 7 (tyrosine agar). NRRL 15156 hydrolyzed skim milk, liquefied gelatin, tolerated up to 4% NaCl and grew at temperatures between 10 and 37°C. It utilized cellobiose, fructose, galactose, glucose, maltose, ribose, salicin and succinate. Arabinose, xylose, inositol, mannitol, melibiose, rhamnose, raffinose, lactose, sucrose and acetate were not utilized. Nitrate was not reduced. NRRL 15156 was sensitive to lysozyme, chloramphenicol, erythromycin, novobiocin, rifampicin, streptomycin, tetracycline and vancomycin but resistant to cephalothin and benzylpenicillin.

Species Determination

The characteristics of NRRL 15156 placed it in the genus *Streptomyces*. An examination of the published descriptions of comparable taxa suggested seven similar species; *S. avidinii, S. colombiensis, S. goshikiensis, S. griseolavendus, S. lavendulae, S. toxytricini* and *S. virginiae*. Simultaneous laboratory comparisons showed only insignificant differences between NRRL 15156 and *S. virginiae*. In their description of *S. virginiae*, SHIRLING and GOTTLIEB refer to the presence of both S and RA types of morphology[8]. The carbon utilization pattern of the two cultures was identical with the exception of galactose, which was utilized by NRRL 15156 but not by *S. virginiae*. Minor differences also existed in NaCl tolerance and growth temperature range. Character comparisons of the two cultures are shown in Table 2.

Table 2. Comparison of NRRL 15156 and *S. virginiae*.

Character	NRRL 15156	*S. virginiae*
Aerial spore color	Gray	Gray
Reverse color	Pale yellow	Pale yellow
Soluble pigment	−	−
Melanin production	+	+
Morphology	RA, S	RA, S
Spore shape	Oblong	Oblong
Spore surface	Smooth	Smooth
Carbon utilization	Identical except for galactose	
Galactose utilization	+	−
Gelatin liquefaction	+	+
Skim milk hydrolysis	+	+
NaCl tolerance (percent)	4	5
Nitrate reduction	−	−
Temperature range (°C)	10~37	15~37

These data indicated that culture NRRL 15156 was virtually identical to *S. virginiae*. Therefore NRRL 15156 was classified as a strain of *Streptomyces virginiae* Grundy, Whitman, Rdzok, Hanes and Sylvester 1952. This classification was based on a comparison with published

Table 3. Effect of incubation temperature on biosynthesis of A41030.

Temperature (°C)	Antibiotic level (μg/ml)
25	27
30	103
32	116
33	108
34	61
35	<10

descriptions[8,9] and on direct laboratory comparisons. *S. virginiae* is recognized in the Approved List of Bacterial Names[10] and consequently is a validly published species.

Fermentation

Because the level of antibiotic produced by NRRL 15156 in the original medium was only 8~10 μg/ml, the fermentation was examined for modifications that would increase antibiotic levels. Antibiotic production in fermentation media employed for the biosynthesis of other glycopeptides was inferior to that obtained with the initial medium. Modification of the original medium resulted in the SG medium, in which antibiotic yields were 110~120 μg/ml. The SG medium was used as the basis for all subsequent fermentation studies.

The effect of incubation temperature on antibiotic biosynthesis is shown in Table 3. Maximum yields were obtained at 32°C. A typical profile of the fermentation time course in the SG medium is shown in Fig. 4. The stationary portion of the trophophase was achieved by 48 hours. The idiophase was initiated at approximately 24 hours, well before the logarithmic phase of growth was completed. The pH remained constant at 7.2~7.3 for 120 hours, then began to trend slowly upward. Phosphate was assimilated rapidly during the first 30 hours. The residual phosphate remaining thereafter may represent bound phosphate that was not biologically available, as well as small amounts that may have been released by mycelial lysis late in the fermentation. The total carbohydrate level declined steadily,

Fig. 4. Typical time course of the A41030 fermentation.

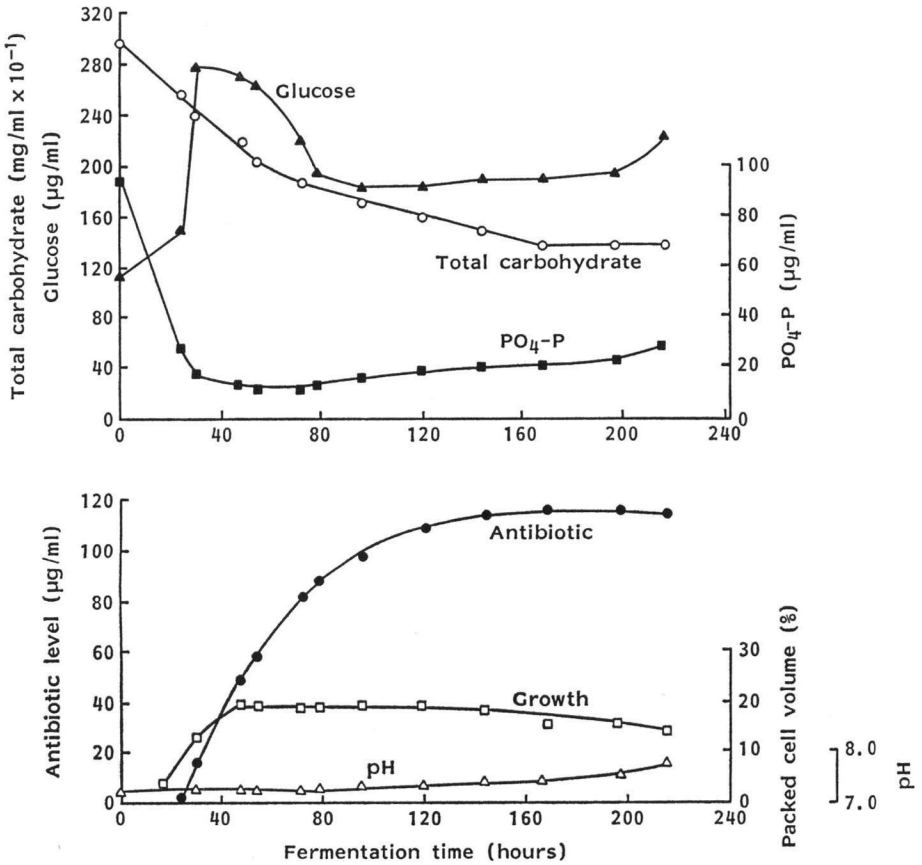


Table 4. Effect of pH on release of A41030 from the producing biomass.

pH[a]	Antibiotic level in broth (μg/ml)
1	76
7	78
8	86
9	95
10	112
10.5	114
11	116
12	108

[a] Whole broth adjusted to indicated value, biomass removed by centrifugation, and the supernatant neutralized prior to bioassay.

Table 5. Effect of carbohydrates and lipids on biosynthesis of A41030.

Carbon source[a] (3% level)	Terminal		Antibiotic potency (μg/ml)
	pH	Growth (Vol%)	
None[a]	7.9	2	<3
Glucose	7.7	10	9
Galactose	6.7	21	11
Mannose	8.0	16	94
Dextrin	7.0	11	116
Corn starch	7.4	16	104
Soluble starch	6.9	16	103
Glycerol	8.1	20	34
Peanut oil	7.0	38	20
Cotton-seed oil	7.2	19	103
Methyl oleate	7.0	15	23

[a] SG medium minus dextrin.

though biphasically, until a stationary level was reached at 168 hours. The sharp early increase in the low level of glucose, as well as the subsequent decline, may reflect minor differences in the level of amylase activity and the rate of glucose utilization.

A41030 was present in both the fermentation broth and the mycelial mass of *S. virginiae*. The percentage of antibiotic in each location varied with the age of the fermentation and the fermentation conditions employed. In the SG medium, most of the A41030 produced early in the fermentation period remained associated with the biomass. Increasingly larger percentages were released into the broth as the fermentation progressed until, after seven days, approximately 70% of the antibiotic was located in the broth. The antibiotic associated with the biomass could be extracted with lower alcohols or, like actaplanin[11], another glycopeptide antibiotic complex, conveniently released into aqueous systems by increasing the alkalinity (Table 4). Maximum amounts of A41030 were released at pH 10.5~11. This procedure, followed by immediate neutralization of the samples after removal of the biomass, was routinely employed in order to quantitate the total antibiotic present.

The biosynthesis of both vancomycin[12] and *N*-demethylvancomycin[13] by producing strains of *Nocardia orientalis* has been shown to be depressed when the concentration of phosphate in the medium is increased. The A41030 fermentation, however, was stimulated by the addition of orthophosphate. Antibiotic synthesis in the SG medium was increased 60~70% through inclusion of K_2HPO_4 at the optimum level of 1 mg/ml.

The effect of various carbon sources on the biosynthesis of A41030 is shown in Table 5. Dextrin, starch, soluble starch, mannose and cotton-seed oil supported the highest yields. Galactose, glycerol and peanut oil produced larger quantities of biomass but these were accompanied by low levels of antibiotic. Arabinose, sorbose, sugar alcohols, dissacharides and other oils did not support detectable growth or antibiotic synthesis.

Soybean meal and soybean flour produced equivalent yields of A41030, which were substantially greater than the yields produced by any of 17 other complex nitrogen sources tested. Although nitrate was not reduced in the taxonomy tests, both nitrate and ammonium salts, when substituted for soybean meal in the SG medium, supported some growth and the biosynthesis of about 30 μg/ml of A41030. Enrichment of the SG medium with $NaNO_3$ at a level of 0.5 mg/ml increased the yields of A41030 about 80%, to approximately 200 μg/ml. The medium was more alkaline over the entire time course of the

Table 6. Effect of putative precursors on broth HPLC profiles and biosynthesis of A41030.

Putative precursor (5×10^{-3} M)	Relative level at harvest (HPLC)				Antibiotic level (% of control)	
	SA	TYR	*p*-HPG	*p*-HPGA	0 hour addition	72 hours addition
Control	+	+	+	+	100	100
SA	++++	+	+	+	106	104
TYR	+	++++	+	+	88	36
p-HPG	+	+	++	+++	105	107
p-HPGA	+	+	+	++++	91	92
$NaNO_3$					181	162
NH_4Cl					116	112

fermentation when nitrate was added, with the terminal value being pH 8.0~8.3.

Information regarding the biogenesis of the unusual aromatic amino acid residues in glycopeptide aglycones has only recently become available. One study determined that four of the aromatic rings of vancomycin could be precursed with tyrosine (TYR) while two of the same residues could also be precursed with *p*-hydroxyphenylglycine (*p*-HPG)[4]. Another ^{13}C-labeling study on the biosynthesis of ristocetin showed that the *p*-HPG moieties are derived from tyrosine[5]. The synthesis of *N*-demethylvancomycin is known to be increased by the addition of TYR, *p*-HPG or *p*-hydroxyphenylglyoxylic acid (*p*-HPGA) to the medium[13]. The effect of putative aromatic precursors on the broth HPLC profiles and biosynthesis of A41030 is shown in Table 6.

Neither shikimic acid (SA) nor the three aromatic compounds examined produced significant, reproducible increases in A41030 biosynthesis. When pulsed into the medium at the time of inoculation, TYR depressed yields about 12%. When the same pulse was made 72 hours post-inoculation, A41030 biosynthesis was depressed 74% by TYR. Some stimulation was observed with ammonium salts while the addition of $NaNO_3$ increased synthesis by 60~80%.

Examination of the supernatant of the fermentation broth by semi-quantitative analytical HPLC system GL-A (L. D. BOECK and D. M. BERRY, in preparation) indicated that all four of the putative precursors were normally present in the broth at harvest. When the medium was enriched with SA, TYR or *p*-HPGA, either at inoculation or 72 hours post-inoculation, the levels of these compounds in the broth remained essentially unchanged during the remainder of the incubation period. They did not appear to be metabolized or interconverted. The sole exception to this observation was *p*-HPG, which appeared to be extensively deaminated. After the addition of *p*-HPG to the medium, progressive decreases in the level of *p*-HPG were accompanied by similar increases in the level of *p*-HPGA.

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