A10255, A COMPLEX OF NOVEL GROWTH-PROMOTING THIOPEPTIDE ANTIBIOTICS PRODUCED BY A STRAIN OF *Streptomyces gardneri*

TAXONOMY AND FERMENTATION STUDIES

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A10255 is a complex of new thiopeptide antibiotics characterized structurally by a cyclic peptide core to which is attached a side chain composed of dehydroalanine moieties. The complex contained 80~85% factor B, 15~20% factor G, and trace amounts of factors C, D, E, F, H, and J. Taxonomic studies indicated the producing microorganism to be a strain of *Streptomyces gardneri*. The major portion of the antibiotic produced remained associated with the mycelial biomass, from which it was extracted with polar solvents such as aqueous methanol or aqueous acetone. Initial A10255 yields of $<2\,\mu$g/ml were increased to over $300\,\mu$g/ml in stirred reactors through strain selection, nutritional studies, and conversion of the batch fermentation to a fed-batch mode.

During the process of screening actinomycetes for novel antimicrobial compounds, a new culture was isolated from a soil collected in Huerfand County, Colorado. This isolate produced a previously unreported complex of thiopeptide antibiotics that contained a cyclic peptide core with a side chain composed of dehydroalanine moieties[1]. The complex, identified as A10255, contained 80~85% factor B, 15~20% factor G, and trace amounts of factors C, D, E, F, H, and J. Factor B has a MW of 1,244, an empirical formula of $C_{53}H_{48}N_{16}O_{15}S_3$ and contains six dehydroalanine residues[2]. The antimicrobial spectrum of A10255 includes activity vs. Gram-positive aerobes and anaerobes, with the MIC for most factors being in the range of 0.125~2 μg/ml. Acute LD_{50} values in mice are $>600\,$mg/kg$\times 1$ for factors B, C, G, H, and J[3]. A10255 is active as a growth promotant in poultry, swine and ruminants with all factors being effective; and additionally alleviates acidosis in ruminant animals[4,5]. This paper includes taxonomy and fermentation studies on strain NRRL 15537, and strain NRRL 15922, which was derived from NRRL 15537 by nitrosoguanidine mutagenesis.

Materials and Methods

Cell Wall Analyses

Diaminopimelic acid (DAP) isomers were determined by the method of BECKER *et al.*[6]. Cell wall sugars were identified by the procedure of LECHEVALIER[7].

Taxonomic Methods

Recommendations of the International Streptomyces Project (ISP)[8] and WAKSMAN[9] for the identification of *Streptomyces* species were followed. Identification keys from BERGEY'S Manual[10], KUSTER[11], NONOMURA[12], PRIDHAM *et al.*[13] and SZABO *et al.*[14] 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[15]. Spore mass color names were taken from the system of color wheels recommended by TRESNER and BACKUS[16].

Fatty Acid Analyses

The strain was grown in wide-mouth 250-ml Erlenmeyer flasks containing 50 ml of Trypticase Soy Broth. After incubation at 28°C for 72 hours with orbital shaking at 250 rpm, the cells were harvested by centrifugation at 28°C and washed in sterile deionized water at the same temperature. Approximately 40 mg of cells were transferred to a 13 mm × 100 mm screw-top glass culture tube closed with a PTFE-lined cap. Cellular fatty acids were saponified by heating for 30 minutes at 100°C after addition of 1 ml of 15% NaOH in 50% aqueous MeOH. Methylation of the fatty acids was accomplished by addition of 2 ml of 3.25 N HCl in 45.8% aqueous MeOH and heating for 10 minutes at 80°C. The methylated fatty acids were extracted from this mixture with 1.25 ml of *n*-hexane-methyl-*tert*-butyl ether (1 : 1, v/v) by slow end-over-end rotation of the capped tube for 10 minutes. After withdrawal of the aqueous phase, the residual solvent was washed with 3 ml of 1.2% NaOH by additional end-over-end rotation of the capped tube for 5 minutes prior to transfer of the solvent phase into a capped vial for analysis. Fatty acid methyl esters were analyzed with a Hewlett-Packard Model 5890 Series II gas chromatograph. This unit operated under the control of a Hewlett-Packard 9153 microcomputer driven by version 3.2 of the Hewlett-Packard MIS aerobe library software[17].

Fermenter Inoculum

The A10255-producing culture was propagated on a medium containing pre-cooked oatmeal 6%, yeast 0.25%, KCl 0.05%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.01% and agar 2.5% in deionized water (pH adjusted to 7.3 with aqueous NaOH prior to sterilization). Agar slope cultures were incubated for 7~10 days at 30°C, then suspended in calf serum and lyophilized. Fermenter inoculum was grown in wide-mouth 250-ml Erlenmeyer flasks containing 50 ml of a medium composed of glucose 1.5%, dextrin 2.0%, soybean grits 1.0%, corn steep liquor 1.0%, yeast extract 0.1% and $CaCO_3$ 0.5% in tap water, adjusted to pH 6.7 prior to autoclaving. Flasks, inoculated either from lyophilized pellets or liquid nitrogen stocks, were incubated 48~72 hours at 30°C on a gyratory shaker orbiting at 250 rpm in a 5.1-cm diameter circle. The resulting culture was either used directly to provide a 1% (v/v) level of inoculum to flask fermenters or was transferred serially into 400 ml of the same medium in wide-mouth 2-liter flasks and incubated an additional 24 hours to produce large volumes of inoculum for stirred fermenters.

Fermenters

Flask fermenters and incubation conditions were as described above except that the incubation period ranged from 3~7 days and the various media employed are listed in appropriate tables.

Stirred reactors were fully baffled vessels of conventional design with two 6-bladed turbine impellors, a total capacity of 165 liters and a height-diameter ratio of approximately 1 : 1 for the 115 liters of medium. Dissolved oxygen levels were monitored with a galvanic sensor and computer-controlled at 45% of air saturation with an internal head pressure of 0.34 atmospheres. The pH was controlled at 6.9~7.1 with aqueous H_2SO_4 and NH_4OH. Exhaust gases were monitored with a Perkin-Elmer MGA-1200 mass spectrometer interfaced with Hewlett-Packard 2113E/A900 computers.

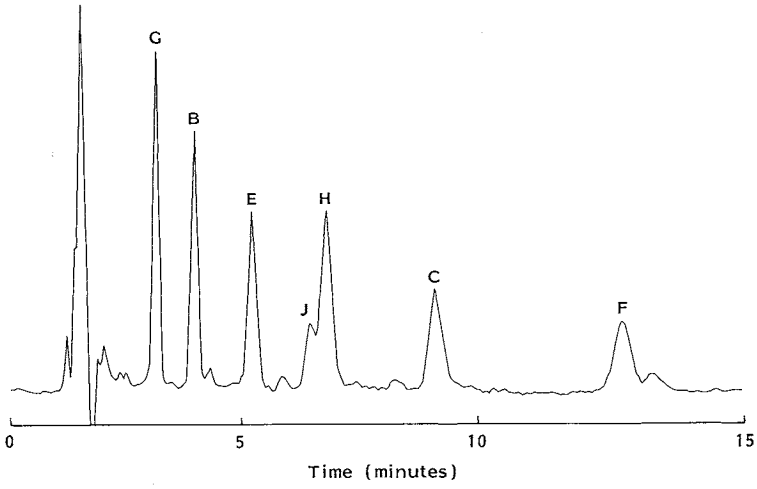
Measurement of Glucose, Glycerol and Inorganic Phosphorus

Glucose was estimated by the glucose oxidase method (Biodynamics/Boehringer Mannheim Corporation, Indianapolis, IN). Automated assays were also employed for the determination of glycerol (modification of Technicon Industrial Method No. 634-81P, Technicon Industrial Systems, Tarrytown, NY) and residual inorganic phosphorus (Technicon Industrial Method No. 93-70w).

A10255 Assay and Chromatography

Although small amounts of A10255 were excreted into the aqueous fermentation medium, the major portion of the antibiotic synthesized remained associated with the biomass. Extraction of this cell-associated material was accomplished more efficiently with 80% aqueous methanol or acetone than with anhydrous solvents. The extraction of whole broth with four volumes of anhydrous solvent per volume of broth provided a convenient method for measuring total antibiotic with a single sample. Antibiotic activity was initially quantitated microbiologically by employing *Bacillus subtilis* (ATCC 6633), in an agar-well plate

Fig. 1. Analytical HPLC profile of the A10255 factor complex.



test with methanol-pH 6.0 phosphate buffer (1:2) as the sample diluent.

Composition of the A10255 factor complex was initially monitored with a TLC system using Merck-Darmstadt silica gel 5763 plates developed in a solvent system containing chloroform-methanol (4:1). Chromatograms were bioautographed *vs. Micrococcus luteus* (ATCC 9341) grown in nutrient agar at pH 6.0.

In addition to the microbiological assay and the TLC procedure, an isocratic analytical HPLC system can be used to quantitatively measure individual factors of the complex. This analytical technique utilizes a Model 710B WISP autosampler and 6000A delivery pump (both from Millipore/Waters, Milford, MA), a Nova C_{18} Rad-PAK column, a mobile phase composed of acetonitrile-water (38:62) with 0.5% ammonium acetate (w/v), a flow rate of 2.0 ml/minute and detection at 247 nm with a Spectroflow SF770 variable wavelength UV detector (Schoeffel Instrument Corporation). The profile of A10255 factor standards in this system (except factor D, which was unavailable) is shown in Fig. 1.

Results and Discussion

Taxonomy

Cultural Characteristics

Strain NRRL 15537 was characterized by limited vegetative growth and such poorly developed aerial mycelia that color determination of the latter was difficult. The spore mass color was in the white to gray series. The nearest color tab in the white series was b, oyster white, while the nearest color tab in the gray series was d, light gray. The reverse color, which was not affected by pH, ranged from orange-yellow to yellow-brown. Soluble pigments were limited to a light brown on tyrosine and tomato paste-oatmeal agars, and a light orange on glycerol-glycine agar. These cultural characteristics are summarized in Table 1.

Morphological Characteristics

Strain NRRL 15537 produced sparse, non-fragmenting, monopodially branched aerial mycelia (Fig. 2). Sporophores were arranged as straight and flexuous branches containing 10~50 spores per chain. Spores were cylindrical with an average width of 0.6 μM and an average length of 1.6 μM. Surface ornamentation was smooth. Neither spirals, sclerotia, sporangia nor motile spores were formed.

Table 1. Cultural characteristics of strain NRRL 15537.

Medium	Cultural characteristics	Medium	Cultural characteristics
ISP No. 2	G: Fair	Calcium malate	G: Fair
	R: 72 d OY		R: 79 I gy yBr
	Am: Poor: b White (edges only), d light gray		Am: None to trace
			Sp: None
	Sp: None	CZAPEK's agar	G: Fair
ISP No. 3	G: Trace to fair		R: 93 y Gray
	R: 70 I OY		Am: Poor: b White
	Am: Trace: b White (edges only)		Sp: None
	Sp: None	Glucose-asparagine	G: Fair
ISP No. 4	G: Fair		R: 72 d OY
	R: 71 m OY		Am: Poor: b White
	Am: Poor: b White		Sp: None
	Sp: None	Glycerol-glycine	G: Fair
ISP No. 5	G: Fair		R: 53 m O (no pH change)
	R: 70 I OY		Am: None
	Am: Fair: b White to d I. Gray		Sp: Light orange
	Sp: None		
ISP No. 7	G: Good		
	R: 54 br O		
	Am: Poor: b White		
	Sp: Light-brown		

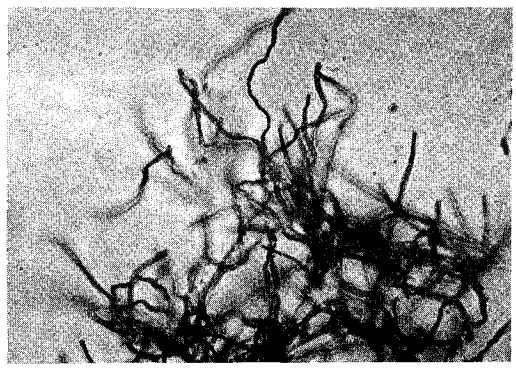
G: Growth, R: reverse, Am: aerial mycelia, Sp: soluble pigment.

Physiological and Chemotaxonomic Characteristics

Whole-cell hydrolysates of strain NRRL 15537 contained LL-2,6-diaminopimelic acid; glucose, mannose and ribose. These characteristics represent a type I cell wall and a "no characteristic" sugar pattern, indicative of the genus *Streptomyces*.

L-Arabinose, D-xylose, D-glucose, D-galactose, D-fructose, inositol and raffinose were utilized for growth. D-Mannitol, L-rhamnose, salicin and sucrose did not support growth. Starch and skim milk were hydrolyzed, catalase was produced, gelatin was liquified and nitrates were reduced. Melanoid pigments were produced in Tryptone-yeast extract broth (ISP No. 1) and on peptone-yeast extract iron agar (ISP No. 6), but not on tyrosine agar (ISP No. 7).

Fig. 2. Photomicrograph illustrating *Rectus-Flexibilis* (RF) morphology.



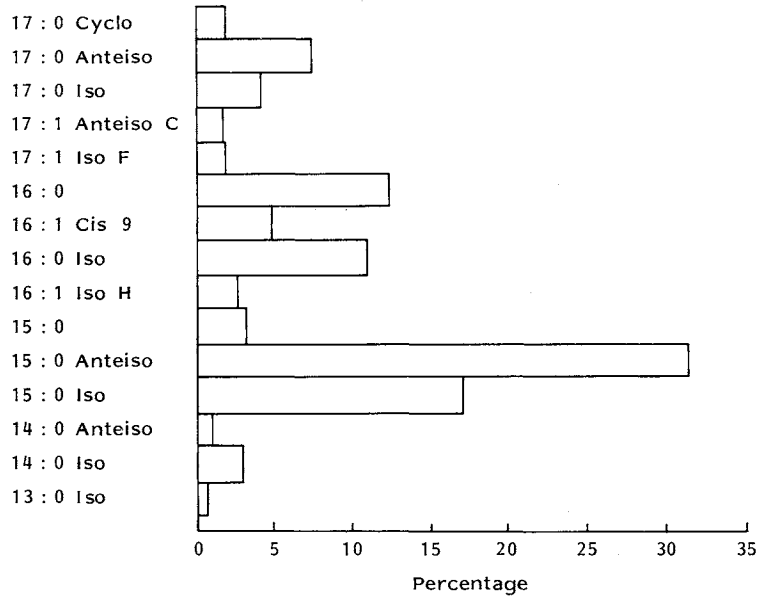
Magnification is 640×. Culture grown on ISP No. 5 agar medium for 14 days at 30°C.

The whole cell fatty acid profile is shown in Fig. 3. With the exception of three unbranched lipids and a small amount of 17:0 cyclopropane, the entire pattern is composed of iso- and anteiso-branched fatty acids, which is characteristic of the genus *Streptomyces*. The most abundant single component is the 15:0 anteiso-branched fatty acid.

Species Determination

The characteristics of strain NRRL 15537 placed it in the genus *Streptomyces*. An examination of the

Fig. 3. Fatty acid profile of *Streptomyces gardneri* (NRRL 15537).



published descriptions of similar taxa suggested a close resemblance to four species: *Streptomyces aureofasciculus*, *S. aureomonopodiales*, *S. flavochromogenes* and *S. gardneri*. Direct laboratory comparisons with these species indicated significant differences from *S. aureofasciculus* and *S. flavochromogenes* but good agreement with *S. aureomonopodiales* and *S. gardneri*. However, the differences between *S. aureomonopodiales* and *S. gardneri* are insufficient to justify their separation into independent species, and the former is not listed in the Approved List of Bacterial Names. Therefore, *S. aureomonopodiales* was removed from consideration.

Strain NRRL 15537 was tantamount to *S. gardneri* in cultural, morphological and physiological characteristics. A distinguishing attribute of both strains was their poor aerial hyphae formation on most media. Strain NRRL 15537 was concequently classified as a strain of *S. gardneri* (WAKSMAN, 1942) WAKSMAN 1961.

Fermentation

The low levels of A10255 produced by the original soil isolate in the screening medium, $<2\,\mu g/ml$, severely hampered supply and isolation of the new antibiotic. Therefore, shaken flasks were used to examine the fermentation for modifications that would increase productivity. Fermentation media employed for the biosynthesis of other thiopeptides were found to support even lower levels of antibiotic production than the screening medium. Consequently, the screening medium was selected for further development.

Modification of the screening medium included examination of the effects of molasses, $CaCO_3$, sulfate and alternate carbon sources. Although sucrose did not support growth in the taxonomic or fermentation studies, blackstrap molasses was essential for the biosynthesis of A10255, with a level of 4% being optimum. Low levels of $CaCO_3$ maintained the pH of the medium near neutrality. Omission of carbonate resulted in acidification of the medium and reduced antibiotic yields while extremely high levels exhibited no evidence of detrimental effects. Enrichment of the medium with sulfate had no effect. Only three carbon/energy substrates; two hexoses, glucose and fructose, and glycerol; supported growth and appreciable

Table 2. Effect of various carbon sources on biosynthesis of A10255 in shaken flasks[a].

Carbon source	Level (%)	Terminal pH	Growth (vol %)	Antibiotic (μg/ml)
—		8.7	3	<2
Glucose	3	8.0	12	23
Fructose	3	7.8	11	19
Glycerol	3	7.9	13	52
Glycerol + glucose	3+1	7.9	12	67

[a] Basal medium contained Bacto-Peptone 1.5%, blackstrap molasses 4.0% and $CaCO_3$ 0.5% in tap water. Wild-type culture.

Table 3. Effect of various nitrogen sources on A10255 biosynthesis in shaken flasks[a].

Substrate	Level	Growth (vol %)	Antibiotic (μg/ml)
—		2	<2
Bacto-Peptone	1.5%	11	81
Hy-soy	1.5	10	60
Hy-Case Amino	1.5	18	72
Casamino acids	1.5	18	59
Glycine	0.05 M	12	26
Alanine	0.05	14	28
Serine	0.05	11	28
Asparagine	0.03	10	25
Glutamine	0.03	11	26
NH_4Cl	0.05	12	21
$(NH_4)_2SO_4$	0.03	12	22

[a] Basal medium contained glucose 1.0%, glycerol 3.0%, blackstrap molasses 4.0% and $CaCO_3$ 0.5% in tap water. Culture was NRRL 15537.

Table 4. Effect of polypropylene glycol (P-2000) on A10255 fermentation yields[a] in shaken flasks.

Polypropylene glycol level (%)	Terminal pH	Growth (vol %)	Antibiotic (μg/ml)
0	7.8	14	80
0.1	7.7	13	102
0.2	7.9	15	123
0.3	7.8	14	98

[a] Basal medium contained glucose 1%, glycerol 3%, Bacto-Peptone 1.5%, blackstrap molasses 4.0% and $CaCO_3$ 0.5% in tap water. Culture was NRRL 15537.

antibiotic synthesis. The combination of 1% glucose with 3% glycerol was superior to any level of either substrate singly (Table 2). Lactose, sucrose, dextrin, and various oils were not utilized for growth or antibiotic synthesis when tested as the sole carbon source. In combination with glycerol, they either had no effect or reduced antibiotic yields.

Alternate nitrogen sources such as soybean flour, cottonseed meal, peanut meal, fish meal, pork blood meal, yeast, meat peptone and corn steep liquor were utilized for growth but supported the synthesis of $<6\,\mu g/ml$ of A10255. Only Bacto-Peptone and hydrolysates of soybeans and casein produced good antibiotic levels (Table 3). However, ammonium salts and several short-chain aliphatic amino acids also supported acceptable levels of growth and additionally sustained the biosynthesis of $20\sim30\,\mu g/ml$ of antibiotic. These amino acids included glycine, alanine, serine, aspartate and glutamate.

A10255 was known to be insoluble in aqueous liquids such as fermentation media. The antifoam agent P-2000 (polypropylene glycol, MW $\sim$2,000) had previously been shown to enhance the productivity of another hydrophobic metabolite in our laboratories (A32256, a phenazine, unpublished data), and a similar response has been reported with avermectin[18]. Inclusion of P-2000 as a medium adjuvant at the optimum level of 0.2% increased A10255 production by as much as 50% in shaken flasks (Table 4) and 20% in stirred reactors. However, the solubility of P-2000 in organic solvents allowed co-extraction with A10255 during isolation of the antibiotic. The difficulty of separating P-2000 from A10255 during subsequent purification steps eventually resulted in omission of P-2000 from the fermentation medium.

Phosphate has been shown to exert a strong influence on the production of numerous antibiotics, either stimulating or inhibiting biosynthesis[19]. The A10255 fermentation was stimulated by the addition of orthophosphate. Antibiotic synthesis was increased approximately 50% by enriching the medium with $NaH_2PO_4 \cdot H_2O$ at the optimum level of 0.01% (Table 5). In order to investigate the contribution of each medium component to the total phosphate pool of the medium, samples from a stirred fermenter were

Table 5. Effect of phosphate enrichment on A10255 biosynthesis in stirred reactors[a].

Phosphate addition level (%)	A10255 titer (μg/ml)
0	118
0.005	143
0.01	174
0.02	166

[a] Medium contained glucose 1%, glycerol 3%, Bacto-Peptone 1.5%, blackstrap molasses 4.0% and $CaCO_3$ 0.5% in tap water. Culture was NRRL 15922.

Table 6. Phosphate-phosphorus content of A10255 medium components and the complete medium.

Medium component	Component level (%)	Phosphorus[a] contribution (μg/ml)	Total medium phosphorus[a] (μg/ml)
Tap water		<0.05	<0.05
Polypropolyene glycol	0.2	<0.05	<0.05
Glucose	1.0	<0.05	<0.05
Glycerol	4.0	<0.05	<0.05
Bacto-Peptone	0.9	15	15
Hy-Case Amino	0.9	49	64
Blackstrap molasses	4.0	3	67
$NaH_2PO_4 \cdot H_2O$	0.01	24	91
$CaCO_3$	0.5	<0.05	74
Total pre-sterilization phosphorus			74
Total post-sterilization phosphorus			48

[a] Measured as phosphate-phosphorus.

analyzed for phosphate-phosphorus content during medium preparation. Individual ingredients were added in the order listed in Table 6. After a suitable mixing period following each addition, samples were withdrawn for analysis. Bacto-Peptone, Hy-Case Amino and blackstrap molasses respectively contributed 15, 49 and 3 μg/ml of phosphate-

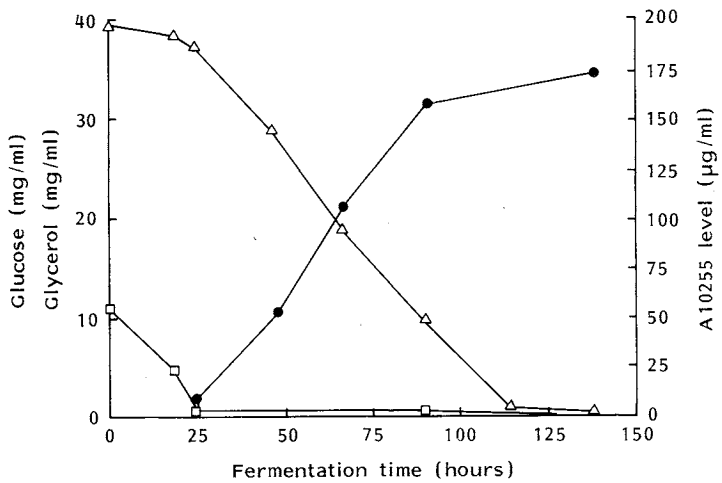
Table 7. A10255 fermentation media and antibiotic yields in 165-liter fermenters.
Media:

Medium component	Medium designation		
	Initial	PAH	HCA
Glucose	1.0%	1.0%	0.1%[a]
Glycerol		4.0	
Bacto-Peptone (Difco)	0.5	0.9	
Hy-Case Amino (Sheffield)		0.9	1.6[b]
Molasses	2.0	4.0	4.0
$NaH_2PO_4 \cdot H_2O$		0.01	0.01
$CaCO_3$	0.2	0.5	0.5
Polypropylene glycol		0.2	0.2

[a] Plus continuous feed of $1.6 \sim 1.8 \times 10^{-1}$ mg/ml/hour.
[b] Plus continuous feed of 1.25×10^{-1} mg/ml/hour initiated after 24 hours.

Strains:	Wild type	NRRL 15922	NRRL 15922
Yields (μg/ml):	<2.0	170.0	330.0

Fig. 4. Time-course profile of the A10255 fermentation in the PAH medium.
□ Glucose, △ glycerol, ● A10255.



phosphorus. Monobasic sodium phosphate contributed 24 μg/ml (theoretical contribution = 22.5 μg/ml). The subsequent addition of $CaCO_3$ then reduced the detectable phosphorus level from 91 to 74 μg/ml and sterilization of the medium by autoclaving further reduced the measurable phosphorus level to 48 μg/ml. These reductions in the measurable phosphorus level presumably resulted from the formation of insoluble phosphate salts that sedimented during clarification of the medium by centrifugation prior to assay.

Integration of these findings resulted in the PAH medium shown in Table 7, in which NRRL 15922 produced 170 μg/ml of A10255 after an incubation period of six days, as shown in the profile of the fermentation time course (Fig. 4). Examination of that profile indicated the entire initial glucose level of 1.0% was consumed within the first 24 hours while only 0.3% glycerol was metabolized during the same period. After exhaustion of the glucose, glycerol consumption increased and continued at a constant rate until the glycerol supply was depleted at approximately 115 hours. Although high levels of glucose repressed antibiotic biosynthesis, glucose was metabolized preferentially when both glucose and glycerol were available to *S. gardneri*. The glycerol was subsequently replaced with a continuous glucose feed, at the rate of $1.6 \sim 1.8 \times 10^{-1}$ mg/ml/hour, to provide a constant supply of the preferred carbon source at a low level. This avoided the repression of antibiotic biosynthesis associated with high glucose levels and increased A10255 yields 50%. Bacto-Peptone was replaced with a higher level of Hy-Case Amino and an additional, continuous feed of Hy-Case Amino was initiated after 24 hours at the rate of 1.25×10^{-1} mg/ml/hour. These modifications resulted in the HCA medium where A10255 levels reached 330 μg/ml in stirred reactors after an incubation period of seven days (Table 7).

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References

1) MICHEL, K. H.; M. M. HOEHN, L. D. BOECK, J. W. MARTIN, M. A. ABBOTT, O. W. GODFREY & F. P. MERTZ: A10255, a new thiopeptide antibiotic complex. 1. Discovery, fermentation, isolation and characterization. Program and Abstracts of the 29th Intersci. Conf. on Antimicrob. Agents Chemother., No. 409, p. 169, Houston, Sept. 17~20, 1989
2) DEBONO, M.; R. M. MOLLOY, J. L. OCCOLOWITZ, J. W. PASCHAL, K. H. MICHEL & J. W. MARTIN: A10255, a new thiopeptide antibiotic complex produced by *Streptomyces gardneri*. 2. Structure determination of A10255B. Program and Abstracts of the 29th Intersci. Conf. on Antimicrob. Agents Chemother., No. 410, p. 169, Houston, Sept. 17~20, 1989
3) COUNTER, F. T.; P. W. ENSMINGER & C. Y. E. WU: A10255, a new thiopeptide antibiotic complex produced by *Streptomyces gardneri*. 3. Microbiological evaluation. Program and Abstracts of the 29th Intersci. Conf. on Antimicrob. Agents Chemother., No. 411, p. 169, Houston, Sept. 17~20, 1989
4) RICHARDSON, L. F.; C. C. SCHEIFINGER & D. A. BECKER: A10255, a new thiopeptide antibiotic complex produced by *Streptomyces gardneri*. 4. Rumen fermentation efficiency enhancement. Program and Abstracts of the 29th Intersci. Conf. on Antimicrob. Agents Chemother., No. 412, p. 169, Houston, Sept. 17~20, 1989
5) RICHARDSON, L. F.; C. C. SCHEIFINGER, D. N. PERRY, D. H. MOWREY, K. H. MICHEL, L. D. BOECK & E. L. POTTER: Effect of compound A10255 on rumen lactic acid accumulation in sheep switched abruptly from roughage to concentrate diets. J. Anim. Sci. 66: 490, Abstract 645, 1988
6) 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
7) LECHEVALIER, M. P.: Identification of aerobic actinomycetes of clinical importance. J. Lab. Clin. Med. 71: 934~944, 1968

8) SHIRLING, E. B. & D. GOTTLIEB: Methods of characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
9) WAKSMAN, S. A. (*Ed.*): The Actinomycetes. Vol. 2. Classification, Identification and Descriptions of Genera and Species. Williams & Wilkins Co., 1961
10) BUCHANAN, R. E. & N. E. GIBBONS (*Ed.*): BERGEY's Manual of Determinative Bacteriology. 8th Ed. Williams & Wilkins Co., 1974
11) KUSTER, E.: Simple working key for the classification and identification of named taxa included in the international *Streptomyces* project. Int. J. Syst. Bacteriol. 22: 139~148, 1972
12) NONOMURA, H.: Key for classification and identification of 458 species of the streptomycetes included in ISP. J. Ferment. Technol. 52: 78~92, 1974
13) PRIDHAM, T. G.; C. W. HESSELTINE & R. G. BENEDICT: A guide for the classification of streptomycetes according to selected groups. Placement of strains in morphological sections. Appl. Microbiol. 6: 52~79, 1958
14) SZABO, I. M.; M. MARTON, I. BUTI & C. FERNANDEZ: A diagnostic key for the identification of "species" of *Streptomyces* and *Streptoverticillium* included in the International *Streptomyces* Project. Acta Botanica Academiae Scientiarium Hungaricae 21: 387~418, 1975
15) KELLY, K. L. & D. B. JUDD: U. S. Department of Commerce. National Bureau of Standards: ISCC-NBS Centroid Color Charts Standard Sample No. 2106. U.S. Government, Washington, D. C., 1958
16) TRESNER, H. D. & E. J. BACKUS: System of color wheels for streptomycete taxonomy. Appl. Microbiol. 11: 335~338, 1963
17) MILLER, L. & T. BERGER: Bacterial identification by gas chromatography of whole cell fatty acids. Hewlett-Packard Application Note 228-41. Hewlett-Packard Co., Palo Alto, CA., 1985
18) BURG, R. W.; B. M. MILLER, E. E. BAKER, J. BIRNBAUM, S. A. CURRIE, R. HARTMAN, Y. KONG, R. L. MONAGHAN, G. OLSON, I. PUTTER, J. B. TUNAC, H. WALLICK, E. O. STAPLEY, R. ŌIWA & S. ŌMURA: Avermectins, new family of potent anthelmintic agents: Producing organism and fermentation. Antimicrob. Agents Chemother. 15: 361~367, 1979
19) BOECK, L. D.; F. P. MERTZ & G. M. CLEM: A41030, a complex of novel glycopeptide antibiotics produced by a strain of *Streptomyces virginiae*. Taxonomy and fermentation studies. J. Antibiotics 38: 1~8, 1985