A54145, A NEW LIPOPEPTIDE ANTIBIOTIC COMPLEX: DISCOVERY, TAXONOMY, FERMENTATION AND HPLC

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A54145 is a complex of new lipopeptide antibiotics that inhibits Gram-positive bacteria and acts as a growth promotant for broiler chicks. Eight factors; A, B, C, D, E, F, A_1 and B_1; have been isolated and characterized. They contain four similar peptide nuclei, each of which is acylated with either an 2-decanoyl, n-decanoyl, or undecanoyl side chain. Taxonomic studies ascertained that the producing microorganism was a strain of *Streptomyces fradiae*. Fermentation studies determined that superior antibiotic yields were obtained in stirred bioreactors in a soybean flour-molasses medium employing a continuous glucose feed. These findings, interwoven with the selection of hyper-productive mutants, increased fermentation yields from $< 50 \mu g/ml$ to more than 1 mg/ml. An analytical HPLC system was developed for the identification and subsequent quantitation of each factor of the A54145 complex.

While screening actinomycetes for novel antimicrobial substances, a new culture was isolated from a soil sample collected in Mexico. This isolate produced a complex of lipopeptide antibiotics from which eight factors; A, B, C, D, E, F, A_1 and B_1; were isolated and characterized[1]. These factors contained four different cyclic peptide nuclei which varied only in valine/isoleucine and/or glutamate/3-CH_3-glutamate substitutions. The N-terminus of each nucleus was acylated with either an 2-decanoyl (iC_{10}), n-decanoyl (nC_{10}), or undecanoyl (aC_{11}) lipid side chain (Fig. 1)[1,2]. Major factors were A and B_1, both of which contained the iC_{10} fatty acyl units. Although four additional factors could presumably be produced by this combination of nuclei and side chains, these additional factors were not isolated. A54145B, B_1, C and E exhibited *in vitro* MIC values of $0.5 \sim 2 \mu g/ml$ vs. various strains of *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Streptococcus*. Factors A and B were also shown to be active vs. these organisms in mice[3]. The factors additionally increased weight gain and feed efficiency in swine and fowl, being especially effective in chicks and turkeys (R. WELLENREITER, personal communication). A natural variant of the original isolated, NRRL 18158, was characterized as a strain of *Streptomyces fradiae*. This paper describes taxonomy and fermentation studies on the A54145-producing cultures and the analytical HPLC system developed to monitor the A54145 factors.

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] for characterization of *Streptomyces* species were followed. Color names were assigned to reverse pigments

Fig. 1.   Structure of the A54145 factor complex.
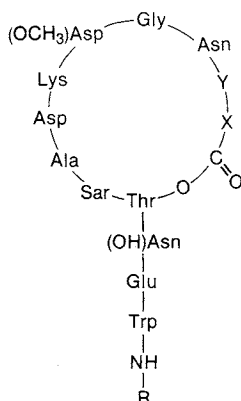
Factor	MW	X	Y	R
A	1,643	Ile	Glu	8-Methylnonanoyl (iC_{10})
A_1	1,643	Ile	Glu	n-Decanoyl (nC_{10})
B	1,657	Ile	3-MethylGlu	n-Decanoyl (nC_{10})
B_1	1,657	Ile	3-MethylGlu	8-Methylnonanoyl (iC_{10})
C	1,657	Val	3-MethylGlu	8-Methyldecanoyl (aC_{11})
D	1,657	Ile	Glu	8-Methyldecanoyl (aC_{11})
E	1,671	Ile	3-MethylGlu	8-Methyldecanoyl (aC_{11})
F	1,629	Val	Glu	8-Methylnonanoyl (iC_{10})

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[7].
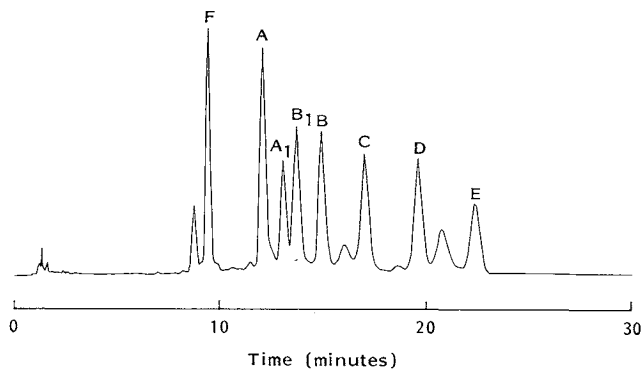
Fermenter Inoculum

Fermenter inoculum was prepared by introducing liquid nitrogen stocks of submerged cultures into wide-mouth 250-ml Erlenmeyer flasks containing 50 ml of medium. For NRRL 18158, the medium was composed of glucose 1.5%, dextrin 2%, soybean grits 1.5%, corn steep liquor 1%, yeast extract 0.1% and $CaCO_3$ 0.5% in tap water, adjusted to pH 6.7 with aqueous sodium hydroxide prior to autoclaving. For NRRL 18160, the medium contained glucose 1%, dextrin 3%, soybean flour 2%, cotton-seed flour 2% and $CaCO_3$ 0.2%. After incubation at 25°C for 48 hours on a gyratory shaker orbiting at 250 rpm in a 5-cm diameter circle, the resulting mycelial suspension was either used directly to provide a 1%-level of inoculum to flask fermenters or was transferred serially into 400 ml of the same medium in wide-mouth 2-liter flasks to produce larger volumes of inoculum for stirred bioreactors.

Fermenters

Flask fermenters and incubation conditions were as described above except that the incubation period was 5~7 days. The flask medium (GSB); containing glucose 5%, soybean grits 3.5%, blackstrap molasses 0.3% and $CaCO_3$ 0.25%; was adjusted to pH 6.9 prior to sterilization.

Stirred bioreactors were fully baffled vessels of conventional design with two 6-bladed turbine impellors, a total capacity of 165 liters and an approximate 1:1 height-diameter ratio for the 115 liters of medium. The medium was sterilized through the application of 22~25 heating units by the F_0 method[8]. Dissolved oxygen levels were monitored with a galvanic sensor and computer-controlled a 40% of air saturation with an internal head pressure 0.34 atmospheres above ambient pressure. The pH was controlled at 6.8~7.0 with aqueous H_2SO_4 and NH_4OH. Exhaust gases were monitored with a Perkin-Elmer MGA-1200 mass spectrometer interfaced with a Hewlett-Packard computer. The stirred bioreactor medium (SBF) contained glucose 4%, soybean flour 2%, blackstrap molasses 0.25%, and ferrous ammonium sulfate 0.06% in tap water, adjusted to pH 7.0 prior to sterilization. Medium SBF-A was identical to SBF except that the initial

Fig. 2.   Analytical HPLC profile of the A54145 factor complex.

glucose level was reduced to 0.3% and additional glucose was supplied as a continuous feed by computer controlled peristalic pumps. The glucose feed was commenced approximately 20 hours after inoculation of the fermenter.

HPLC

The A54145 factors were individually identified and quantitated by HPLC analysis of fermentation broth supernatants. The HPLC system consisted of two Model 510 solvent delivery pumps; a Model 720 gradient controller; a Model 710B WISP autosampler (all from Millipore/Waters, Milford, MA); a DuPont Zorbax 150-C_8 column, 4.6 mm × 25 cm; and a mobile phase of 35% CH_3CN-H_2O, 0.2% triethylamine phosphate at pH 3. The flow rate was 2 ml/minute. A54145 was detected with an HP1040A photodiode array detector (Hewlett-Packard, Palo Alto, CA) at 223 nm. Data plots were recorded with an HP3390A reporting integrator. The analytical HPLC profile of the A54145 factor complex is shown in Fig. 2.

Results and Discussion

Taxonomy

NRRL 18158 was a spontaneous variant of a culture initially isolated from a soil sample obtained in Mexico. Hydrolyzed whole cells contained LL-diaminopimelic acid but did not contain diagnostic sugars or mycolic acids. These traits are characteristic of the genus *Streptomyces*.

Moderate vegetative growth and limited aerial hyphae were produced on both complex and defined media. Vegetative growth was yellow-brown. The aerial spore mass color was red. No distinctive or soluble pigments were observed. Mycelia were non-fragmenting and monopodially branched. Sporophores consisted of short chains containing 10 or more spores arranged in hooks and loops typical of the *Retinaculum-Apertum* morphology of PRIDHAM et al.[9]. *Rectus-Flexibilis* morphology was also observed. Scanning electron micrography revealed oblong spores with smooth surfaces and an average size of 1.0 × 1.5 μm.

L-Arabinose, ribose, xylose, glucose, galactose, mannose, fructose, lactose, cellobiose, and trehalose were utilized. Adonitol, cellulose, dextran, inositol, inulin, mannitol, melibiose, raffinose, rhamnose, salicin, sucrose and xylitol were not utilized. Adenine, hypoxanthine, DNA, casein, elastin, esculin and starch were degraded. Calcium malate, chitin, hippurate, keratin, xanthine and tyrosine were not degraded. Catalase, phosphatase, urease and hydrogen sulfide were produced. Gelatin was liquefied, nitrate was reduced and 6% NaCl was tolerated. The culture did not produce melanoid pigment, was not resistant to lysozyme, and did not hydrolyze or peptonize skim milk. It grew at temperatures up to 45°C and survived exposure to 50°C for 8 hours.

NRRL 18158 was compared with published descriptions of other *Streptomyces* species that likewise

belong in the red color series, possess *Retinaculum-Apertum* and *Rectus-Flexibilis* sporophore morphology, have smooth spore surface ornamentation, lack melanoid and distinctive pigments, and exhibit carbon utilization patterns and other cultural characteristics similar to those of NRRL 18158. Direct laboratory comparison of six similar cultures showed good agreement between NRRL 18158 and *S. fradiae*. Therefore, NRRL 18158 was classified as a strain of *S. fradiae* (WAKSMAN and CURTIS, 1916), WAKSMAN and HENRICI, 1948. NRRL 18160 was derived from NRRL 18158 by nitrosoguanidine mutation.

Fermentation

Initial fermentation yields of A54145 were < 50 μg/ml. Preliminary studies with the wild-type culture and optimization of the screening medium resulted in the isolation of NRRL 18158, which, in the GSB medium, produced A54145 at a level of ~100 μg/ml. Examination of alternate carbon sources in this medium determined that a number of carbohydrates and lipids supported excellent growth and the biosynthesis of fair amounts of A54145 (Table 1). Glycerol was the only compound tested that was not metabolized, and glucose was one of the most productive carbon sources. Comparison of alternate complex nitrogen sources then determined that all soybean products; grits, flour and hydrolysate; produced substantially better quantities of A54145 than any other substrate. Most of the latter, in fact, produced very low levels of the antibiotic (Table 2).

Time-course profiles of the residual medium glucose level and A54145 biosynthesis by NRRL 18160 in stirred bioreactors with medium SBF (Fig. 3) indicated that when 4% glucose was included in the medium at make-up, the entire amount was consumed within 90 hours. After depletion of the glucose the A54145 level did not increase appreciably, and maximum yields did not exceed 400 μg/ml. Inclusion of higher glucose levels in the medium did not increase the biosynthesis of A54145. In addition, the regulatory

Table 1.   Effect of carbon source on the biosynthesis of A54145 in shaken flasks.

Carbon source[a]	Level (g/liter)[b]	Terminal		A54145 (μg/ml)	Carbon source[a]	Level (g/liter)[b]	Terminal		A54145 (μg/ml)
		pH	Solids (vol. %)				pH	Solids (vol. %)	
—	—	8.7	5	< 12	Potato dextrin	50	7.6	34	47
Glucose	30	7.9	17	71	Glycerol	30	8.6	6	< 12
	50	7.0	24	103	Methyl oleate	30	7.8	27	96
Galactose	30	7.0	23	94	Refined soybean oil	30	7.6	23	59
	50	6.9	22	68	Corn oil	30	7.5	28	61
Maltose	50	6.8	32	84	Peanut oil	30	7.8	18	78
Corn starch	50	7.7	30	42	Cotton-seed oil	30	7.7	27	73

[a]   Basal medium GSB minus glucose.
[b]   Optimum level when only one level is shown.

Table 2.   Effect of complex nitrogen sources on A54145 biosynthesis in shaken flasks.

Nitrogen source[a]	A54145 Level (μg/ml)	Nitrogen source[a]	A54145 Level (μg/ml)
—	2	Corn gluten meal	9
Soybean grits	94	Acid hydrolyzed casein	12
Soybean flour	108	Enzyme hydrolyzed casein	33
Soybean hydrolysate	98	Yeast	< 2
Peanut meal	67	Fish meal	23
Cotton-seed meal	23	Meat peptone	11

[a]   Basal medium GSB minus soybean grits. Each substrate tested at 35 g/liter, the optimum level for soybean grits.

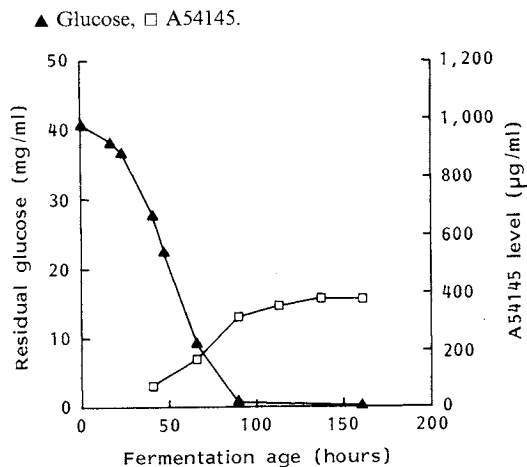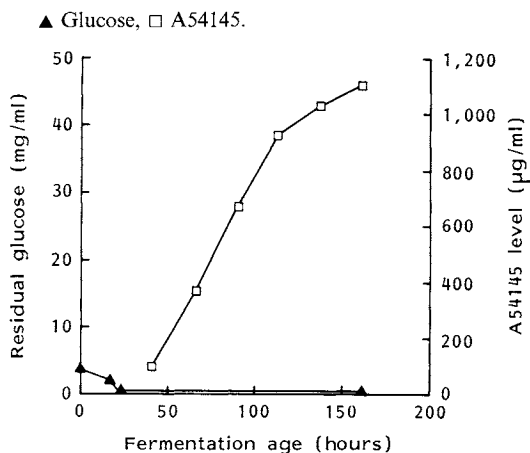Fig. 3.   Time-course profile of residual medium glucose and A54145 biosynthesis in stirred bioreactors.

▲ Glucose, □ A54145.

Fig. 4.   Time-course profile of residual glucose and A54145 biosynthesis with the 100% glucose feed rate in medium SBF-A.

▲ Glucose, □ A54145.

affects of carbon catabolites on secondary metabolism are well known[10,11]. Therefore, the effect of a continuous glucose feed was examined in medium SBF-A. When the glucose feed rate was sufficiently high to maintain a constant extracellular glucose level of $1 \sim 3$ mg/ml throughout the fermentation period, the maximum mean quantity of glucose consumed was calculated to be $\sim 375 \, \mu$g/ml/hour, which was considered the "100% rate". When glucose was fed at this rate, antibiotic yields of about $1,150 \, \mu$g/ml were produced. These yields represented

Table 3.   Effect of glucose feed rate on A54145 biosynthesis.

Feed rate[a] (%)	Antibiotic produced	
	Total (μg/ml)	% of maximum
100[b]	1,145	100
80	1,100	96
60	980	88
40	335	29

[a]   Basal medium was SBF-A.
[b]   Maximum mean quantity metabolized, $\sim 375 \, \mu$g/ml/ hour, when glucose was maintained in slight excess.

an increase of approximately 200% over the yields achieved with the standard SBF medium. A time-course profile of the residual glucose levels and A54145 biosynthesis when glucose was fed at the 100% rate in medium SBF-A is shown in Fig. 4. Reducing the glucose feed to 80% of this rate produced 96% of the maximum antibiotic level while a 60%-feed rate still produced 88% of the maximum A54145 level. However, a glucose feed rate of 40% dramatically reduced antibiotic titers (Table 3), presumably because cell biosynthesis and/or maintenance energy requirements associated with primary metabolism consumed a major portion of this amount and the remainder was inadequate to support high levels of secondary metabolite biosynthesis.

Availability of the analytical HPLC assay permitted quantitative measurement of the individual A54145 factors. A time-course profile of individual factor biosynthesis in the SBF-A medium in stirred bioreactors is shown in Fig. 5. Factors A and B_1 were most abundant throughout the entire fermentation. However, the biosynthesis of A was more rapid initially but ceased by 135 hours while the biosynthesis of B_1, in which 3-CH_3-glutamate replaced the glutamate in the A nucleus, continued linearly. As might be expected, biosynthesis of the two minor factors that also contained the A nucleus, A_1 and D, paralleled the biosynthesis of factor A. Similarly, biosynthesis of factors B and E, which also contain the B nucleus, continued with B_1 even though their rate of accretion was much lower than the rate for B_1. Factor C,

Fig. 5. Time-course profile of A54145 factor biosynthesis.

$\blacksquare$ B_1, $\square$ A, $\bullet$ B, $\circ$ F, $\blacktriangle$ E, $\blacktriangledown$ A_1, $\triangle$ D, $\triangledown$ C.
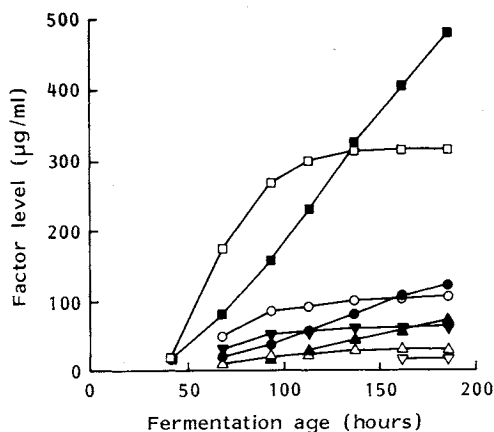
Fig. 6. Time-course profile of biosynthesis of the A54145 nuclei.

$\bullet$ B, $\square$ A, $\blacksquare$ F, $\triangle$ C.
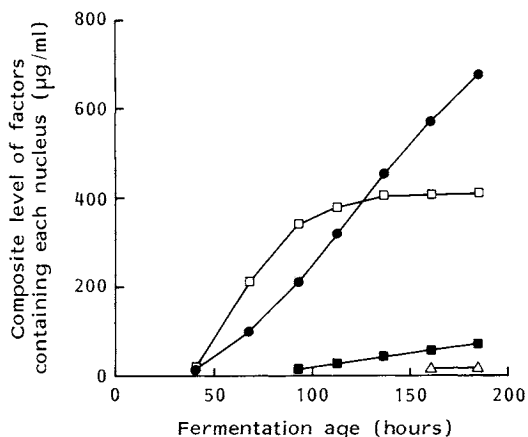
which was extremely minor, could not be detected until the 7th day of fermentation. This time-course factor profile further demonstrates the dynamic nature of the factor complex, in which the relative content of the individual factors continued to change during the entire time-course of the fermentation. The approximate percentage of factors containing each of the three different fatty acyl units attached to the N-terminus of the nuclei after 185 hours of fermentation was as follows: $iC_{10} = 74 \sim 78\%$, $nC_{10} = 13 \sim 15\%$ and $aC_{11} = 9 \sim 11\%$.

Availability of the chemical structure of each factor also permitted evaluation of the time-course biosynthetic profiles of the four A54145 nuclei (Fig. 6). The profiles of the A and B nuclei were virtually identical with the profiles of the A and B_1 factors. Biosynthesis of the two minor nuclei, in which valine replaced isoleucine, was initiated much later. Detectable levels of the F nucleus did not appear until approximately 90 hours and then increased slowly, while the very minor C nucleus was not detectable until 160 hours.

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