CHARACTERIZATION OF STRAINS OF *ACTINOPLANES* SPP. THAT ARE POTENTIAL BIOLOGICAL CONTROL AGENTS

By

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LIST OF ABBREVIATIONS

Ara	Arabinose
ATCC	American Type Culture Collection
bp	base pair
CsCl	Cesium Chloride
DL-DAP	D-/L-diaminopimelic Acid
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EtOH	Ethanol
Gal	Galactose
GC rich	Guanine and Cytidine nucleotide rich
Glc	Glucose
GLC	Gas Liquid Chromatography
HCl	Hydroxyl Chloric Acid
kb	kilo-base pair
Man	Mannose
PAGE	Poly-Acrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
Rha	Rhamnose
SDS	Sodium Dodecyl Sulfate
TEMED	N, N. N', N'-Tetramethylethylenediamine
TLC	Thin Layer Chromatography
Xyl	Xylose

CHAPTER I

INTRODUCTION

Actinoplanes spp. are members of the aggregate group actinoplanetes in the family Micromonosporaceae of the order Actinomycetales. *Actinoplanes* spp. produce well-developed, nonfragmenting, branched and septate hyphae that are 0.2-1.6 μm in diameter (Couch, 1974; Vobis, 1989). Aerial mycelium is generally not formed. The mycelium is typically Gram-positive, non-acidfast and produces diffusable pigments of many colors. Colonies are generally brightly colored. Various shades of orange are most frequent, although some strains have black, brown, red, purple, green or blue colored mycelia. *Actinoplanes* spp. typically produce spherical to subspherical shaped sporangia (3-20 x 6-30 μm) borne on sporangiophores (Goodfellow and Cross, 1984; Palleroni, 1989). Spherical, subspherical or rod-shaped spores are formed inside sporangia and become motile when hydrated. Zoospores (motile spores) have a few to many polar flagella (Vobis, 1989).

Other genera of the actinoplanetes include Ampullariella, Pilimelia, and Dactylosporangium (Goodfellow *et al.*, 1990). Species of the actinoplanetes are economically important for their abilities to produce antibiotics, enzymes, immuno-suppressive drugs, steroids, and other industrial and pharmaceutical compounds (Goodfellow *et al.*, 1984; Piret, 1988). Other members of Actinomycetales, e.g. *Streptomyces* spp. have provided greater commercial benefit and have been studied to a greater extent than the actinoplanetes. However, *Actinoplanes* spp. have been reported to be hyperparasitic of oospores of

Phytophthora megasperma glycinea (Filonow and Lockwood, 1985) and similar fungi (Sneh *et al.*, 1977; Sutherland and Lockwood, 1984; Khan *et al.*, 1993).

Identification of microbial species is based on their morphology, physiology, biochemical and nucleic acid composition. Studies of the latter two characteristics have evolved into the fields of chemical and genetic taxonomy. Chemical taxonomy typically involves the determination of biochemical constituents of the cell such as fatty acids profiles, or amino acids in cell walls and sugars in whole cell hydrolysates (Goodfellow and Cross, 1984; Lechevalier, 1970 and Pulleroni, 1989).

Chemical taxonomy often leads to a reclassification of genus and species. Recently, Goodfellow *et al.* (1990) used chemical and numerical taxonomic procedures to clarify the taxonomy of *Actinoplanes* and related taxa. Based on their study, five novel species of *Actinoplanes* were identified and the genus *Ampullariella* was suggested to be a synonym of *Actinoplanes* based on their chemical relatedness.

The characterization of nucleic acid has greatly improved taxonomic study of bacteria and other microbes, especially in non-filamentaneous bacteria. Numerous techniques are available for the characterization of chromosomal or plasmid DNA and of ribosomal RNA (Farina and Bradley, 1970; Hopwood *et al.*, 1983 and Wilson *et al.*, 1990). The use of restriction endonuclease digestion of genomic DNA followed by band separation by agarose or SDS-PAGE gel electrophoresis (DNA fingerprinting) has evolved into a powerful yet relatively simple tool for characterizing bacterial strains and other micro-organisms (Owen, 1989). DNA fingerprinting has been used for characterizing strains of *Streptomyces* (Crameri *et al.*, 1983; Hintermann, 1981), *Nocardia* (Crameri *et al.*, 1983) and *Streptococcus* (Skjold *et al.*, 1987). Moreover, DNA fingerprinting has been applied for identification of closely related species in the genera Bacteroids, Brucella, Mycobacterium, and Lactobacillus (Stahl *et al.*, 1990). Thus, DNA finger-printing is a useful tool for taxonomy.

However, there is no report of DNA fingerprinting of any member of the Actinoplanaceae, although DNA base composition (Palleroni, 1989) and DNA-DNA reassociation (Farina and Bradley, 1970) studies have been done. Moreover, plasmids have been identified in *Streptomyces* spp. and other actinomycetes (Hopwood and Brodsky, 1986; Hutter and Eckhandt, 1988; Kieser, 1984), yet little is known about the occurrence of plasmids in *Actinoplanes* spp. or the role they may play in the life cycle of *Actinoplanes*. Development of nucleic acid methods for the characterization of *Actinoplanes* spp. is needed to fill this void of knowledge.

The objectives of my research are:

(1) to determine the frequency of occurrence of plasmids in several strains of known and unknown species of *Actinoplanes* that are potential biological agents;

(2) to develop a protocol for the DNA fingerprinting of genomic DNA from these species and strains; and

(3) to compare DNA fingerprinting to the analysis of cell wall amino acids and whole cell sugars as methods for the characterization of strains.

CHAPTER II

MATERIALS AND METHODS

Materials

All restriction endonucleases were purchased from United State Biochemical (USB), Bethesda Research Laboratory (GIBCO BRL), or Promega; all chemicals were purchased from Fisher Scientific, Sigma, or Bio-Rad Laboratories; DNA markers were bought from Promega; most related general supplies were ordered from VWR.

TE buffer is 10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0); the 7.5% PAGE separating gel (20 ml) is made by mixing 5 ml of 30% acrylamide, 3.5 ml of 1% Bis-acrylamide, 8.8 ml of distilled water, 2.5 ml of main gel buffer (3 M Tris pH 8.8, and 0.8% SDS), 100 µl of ammonium persulfate, and 10 µl of TEMED; the PAGE stacking gel (8 ml) is made by mixing 1 ml of 30% acrylamide, 1 ml of 1% Bis-acrylamide, 1 ml of stacking gel buffer (1 M Tris, pH 7.0, and 1% SDS), 5 ml of distilled water, 50 µl of 10% ammonium persulfate, and 10 µl of TEMED; the SDS-PAGE running buffer is 9 mM Tris, 90 mM Boric Acid, and 2 mM EDTA in distilled water; the developing solution for silver staining is 30 g of NaOH and 3.75 ml of 37% formaldehyde in 1 L of distilled water.

Methods

DNA Extraction

Cells of *Actinoplanes* were cultured in (2 x 50 ml) soluble starch yeast extract medium (YPSS) (Sutherland and Lockwood, 1984) at room temperature and 75-100 rpm for a week to a month (length of time depended on the strains). The cells were then

collected by centrifugation at 12,000 g for 10 minutes. The supernatant was discarded and the cell pellets were washed in TE buffer and frozen at -20 °C overnight. The frozen cells were mixed with 1 g of glass beads (0.10-0.11 mm) and ground manually in liquid nitrogen. The ground cells were resuspended in 9.5 ml TE buffer, treated with 0.5 ml SDS (10%) (final concentration 5 mg/ml) and 50 µl pronase E (20 mg/ml) (final concentration 100 µg/ml) at 37 °C for 1-2 hours. Following centrifugation at 5,000 rpm for 10 minutes, the supernatant was collected into a Teflon centrifuge tube. 5 M NaCl was used to adjust the final concentration to 1 M, and an equal volume of chloroform was added followed by shaking gently for at least 10 minutes. After centrifugation at 8,000 rpm for 10 minutes, DNA was precipitated from the upper aqueous phase by adding 0.6-1.0 volume of isopropanol and centrifugation at 7,000 rpm for 10 minutes. The DNA pellet was then dissolved in 4 ml TE buffer for 2 hours to overnight. Incubation at 55 °C hastened the process. Cesium chloride was then added (4.3 g per 4 ml TE buffer) and dissolved completely. After adding 200 µl of 10 mg/ml ethidium bromide, the solution was transferred to 4 ml sealable centrifuge tubes. The volumes were adjusted and the tubes balanced by adding CsCl in TE buffer (1.05 g/ml). Following ultracentrifugation at 55,000 rpm, 20 °C overnight, DNA bands were visualized under long wave UV light, and removed with a sterile 16-gauge needle and a 3 ml plastic syringe. The ethidium bromide was removed by extraction with isoamyl alcohol 2-4 times. The DNA was then precipitated from the aqueous phase by adding 1 volume of TE buffer and 3 volumes of cold (-20 °C) 95% ethanol (EtOH). The chromosomal DNA was visible as a thread like precipitate. It was immediately picked out with a yellow tip and washed in 70% EtOH . The DNA was dissolved in 50 µl of TE buffer and stored at 4 °C.

DNA Digestion

DNA concentration was determined by measurement with a fluorimeter. Approximately 1 µg of DNA was digested with 8-10 units of the following restriction endonuclease enzymes: *EcoR I*, *BamH I*, *Hind III*, *Pst I*, *Not I*, or *Xba I*, in the buffers which came with the specific enzymes, in a total reaction volume of 20 µl at 37 °C for at least 6 hours (typically overnight). Loading buffer was added to stop the reaction, and the fragments were separated by gel electrophoresis.

Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out in horizontal gels of 0.75% agarose in TBE buffer at about 1v/cm for 16 hours. Ethidium bromide was incorporated into the gel at 0.5 µg/ml. Digested DNA was loaded at 2 µg per lane and the results were visualized by illumination with UV light.

PAGE Electrophoresis

The 30 cm x 18 cm glass plates were carefully washed with detergent, tap water, deionized water, 95% EtOH and isopropanol. The apparatus was set up with 3 spacers (one on each side and one on bottom) between 2 glass plates. The 7.5% separating gel was poured carefully to prevent bubbles and allowed to polymerize for at least 2 hours. The 3% stacking gel was then poured and the comb was carefully inserted so as not to introduce bubbles. The stacking gel was allowed to polymerize for 20 minutes to 2 hours. Running buffer was added to the chamber, and the comb was removed carefully. Samples of DNA (20-30 µl) were loaded in each well. DNA molecular weight markers were also loaded in some of the wells. Electrophoresis was conducted under conditions of constant current (17 mA) for 30 hours (Smart *et al.*, 1988).

Silver Staining of Gels

The PAGE gel was washed in a solution containing 10% EtOH and 0.5% acetic acid for 4 hours with 5 changes of solution. The gel was agitated in staining solution (1.9 g/l silver nitrate) on a shaking platform for 2 hours. After rinsing with water for 20 seconds, the gel was incubated with gentle agitation in the developing solution (see above) until the desired color was evident. The gel was then washed in distilled water (Smart *et al.*, 1988).

Gel Documentation and Analysis

The stained gel was photographed with illumination using Kodak technical pan film, using the following aperture settings and exposure times: f4, 1/4 sec.; f4, 1/8 sec.; f8, 1/4 sec.; f8, 1/8 sec.; f5.6, 1/4 sec.; f5.6, 1/8 sec.

Photographs were scanned by the densitometer and the restriction fingerprinting patterns were analyzed by using RFLPrint program (The Discovery Series™ of *pdi*, Model DNA 35). A neighbor-joining method (Saitou and Nei, 1987) was used to generate a phylogenetic tree.

Cell-Wall Amino Acids Analysis

Cells of *Actinoplanes* were cultured in YPSS broth at 22-24 °C for 4-7 days. The cells were collected by centrifugation at 12,000 g for 10 minutes. The pellet was washed once with distilled water, once with 95% ethanol and dried in a vacuum desiccator. Then 1 ml 6 N HCl was added to 9 mg (dry weight) of cells and incubated in a small ampoule at 100 °C for 18 hours. The hydrolysate was filtered through Whatman No. 1 paper. The filtrate was evaporated to dryness in a 100 °C oven, redissolved in distilled water and evaporated to dryness again. The residue was dissolved in 0.3 ml of distilled water, and applied to a cellulose TLC (thin-layer chromatography) sheet. Ascending TLC was performed in methanol-distilled water-6 N HCl-pyridine (80:26:4:10 vol./vol.) (Staneck

and Roberts, 1974) for 2 hours. The TLC plates were removed from the ascending solution air dried, sprayed with 0.2% ninhydrin in acetone and heated at 100 °C for 1-2 minutes. As standards, 1 µl of 0.01 M DL-DAP (diaminopimelic acid) and 1 µl of 0.1 M glycine were used. The DAP spots were seen as gray-green fading to yellow, with the L-isomer moving ahead of the meso-isomer.

Whole Cell Sugar Pattern Analysis

The dried sample (described above in "cell-wall amino acid analysis") was weighed carefully and 200-300 µg of the sample was added to 0.2 ml of 1.5 M methanol in HCl and 50 µl of methyl acetate, and incubated in a small vial at 80 °C overnight. After cooling to room temperature, a few drops of t-butanol were added, and the sample was evaporated under a stream of nitrogen at room temperature to make Trimethylsilyl derivatives. Each sample was then analyzed by GLC (gas liquid chromatography) (Bhatti *et al.*, 1970). Inositol was used as the internal standard.

CHAPTER III

RESULTS AND DISCUSSION

The Frequency of Occurrence of Plasmids in *Actinoplanes*

Total DNAs were prepared from 28 strains (Table I). Ultracentrifugation was then performed to separate genomic and plasmid DNA (if there were any) in a CsCl gradient with ethidium bromide. There was a single chromosomal DNA band in the CsCl gradient of all DNA preparations from all strains, indicating no plasmids in all 28 strains. The experiments were repeated three or more times for each strain. The result suggests that the frequency of occurrence of plasmids in *Actinoplanes* is very low, at least with these 28 strains, which included 16 known species. This is surprising because *Actinoplanes* are famous for their abilities to produce various kinds of antibiotics, enzymes and steroids. (Goodfellow *et al.*, 1985, Piret, 1988). Genes encoding these products are located in plasmids in *Streptomyces* (Hopwood *et al.*, 1983). Plasmid may be lost during culturing of *Actinoplanes* or the copy number of the plasmids maybe too low to be detected. In the later case, plasmid enriching methods such as adding chloramphenicol during culturing may help to alleviate this problem (Maniatis *et al.*, 1982). Also, different densities of CsCl gradients may aid in detecting the presence of plasmids.

Genomic DNA Fingerprinting of *Actinoplanes*

Genomic DNAs were digested by different enzymes in order to get resolvable restriction endonuclease fragment patterns. Since the genome of *Actinoplanes* spp. contains more than 70% of G+C (Goodfellow and Cross, 1984), enzymes cutting GC rich

TABLE I

Strains of *Actinoplanes* spp.*

OSU# Code	Other Code	Identification
1	A 31157	*A. azureus*
2	A 25844	*A. brasiliensis*
4	A 21884	*A. ianthinoqenes*
5	A 27366	*A. italicus*
6	A 14538	*A. missouriensis*
7	A E3-15A	*A. missouriensis*
8	7 - 10	*A. rectilineatus*
9	A 31121	*A. teichomyceticus*
11	B 16253	*A. violaceus*
12	B 16254	*A. yunnahensis*
14	K 30	unknown
15	P 3	unknown
19	R 141Y	unknown
21	W 13	unknown
23	W 178	unknown
24	W 211	unknown
25	W 257	unknown
27	Wi 11	unknown
29	--	unknown
31	(MSU)	unknown
32	(MSU)	unknown
33	--	unknown
	A 12427	*A. philippinensis*
	A 21983	*A. deccanensis*
	A 23056	*A. globisporus*
	B 16714	*B. brasiliensis*
	B 16727	*B. utahensis*

* Most strains of *Actinoplanes* spp. were kindly provided by Dr. M. Lechevalier of Waksman Institute of Rutgers University.

sites tended to give small fragments. This was true even when *Not I* was used, which recognizes 8-base pair sites and normally gives large fragments. On the other hand, enzymes cutting AT rich sites such as *EcoR I* and *HindIII* gave few and larger fragments. Enzymes whose recognition sites contain about 70% G+C composition usually produced about 200 fragments within the size range of 500 bp to 2.0 kb. These enzymes include *BamH* I, *Pst* I, *Xho* I, and *Kpn* I. Initially, agarose gel electrophoresis was used to resolve the DNA fragments generated by endonuclease digestion. However, this simple method could not separate DNA bands to high resolution (data not shown), so SDS-PAGE was later employed to achieve high resolution separation. When *BamH* I fragments were separated by SDS-PAGE and stained with silver nitrate, each strain displayed a reproducible restriction fragment pattern and different strains showed different patterns (Fig. 1). But two strains of *A. missouriensis* had identical DNA fingerprints (Fig. 2), so they could actually be the same. Different enzymes should be used to confirm this result. Fingerprinting profiles of *BamH* I digestion were analyzed by densitometer, the degree of similarity between organisms was quantitated and a phylogenetic tree was generated by the neighbor-joining method (Fig. 3).

The relationship between restriction patterns and phenotypes were examined. One way to do this is to create mutations. If the mutant can be detected to have band differences, it might mean that these bands contain the region of the gene responsible for this phenotype. So it is possible to clone the gene by this way. I analyzed two spontaneous color mutants, A321121 (*A. teichomyceticus*, usually is orange) and A 321121w (a white color mutant of *A. teichomyceticus*), but no difference was detected between their band patterns (Fig. 4). It is possible that the mutation in the genome is point mutation, and a different enzymes could identify the difference. The other possibility is that this color mutation is just caused by the environment factors.

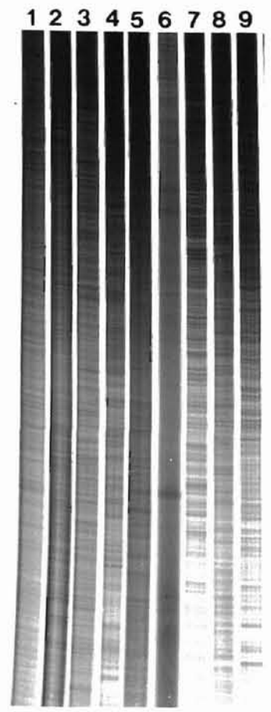

Figure 1. Restriction pattern of *Actinoplanes* by *BamH I*.

Genomic DNA was digested by *BamH I* and separated by SDS-PAGE electrophoresis. Different strains have different restriction patterns. Lane 1: *A. brasiliensis*; Lane 2: OSU #33; Lane 3: OSU #21 W13; Lane 4: OSU #14 K30; Lane 5: *A. ianthinogenes*; Lane 6: A. *utahensis*; Lane 7: OSU #15 P3; Lane 8: *A. italicus*; Lane 9: *A. rectilineatus*.

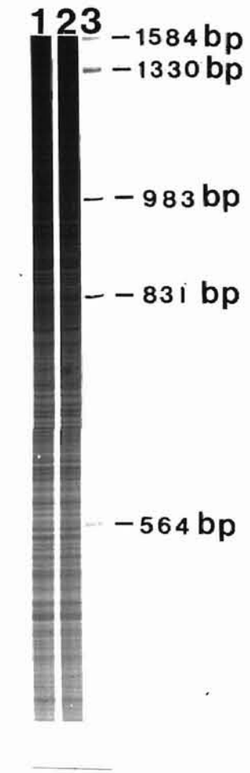

Figure 2. Two strains of *A. missouriensis* have identical DNA fingerprints.

DNA samples from two strains of *A. missouriensis* were digested by *BamH I* and were shown to have identical fingerprints on SDS-PAGE gel. Lane 1: OSU #32 *A. missouriensis*; Lane 2: OSU #6 A. 14538 *A. missouriensis*; Lane 3: λ DNA digested by *Hind III* and *EcoR I* serving as DNA molecular weight marker.

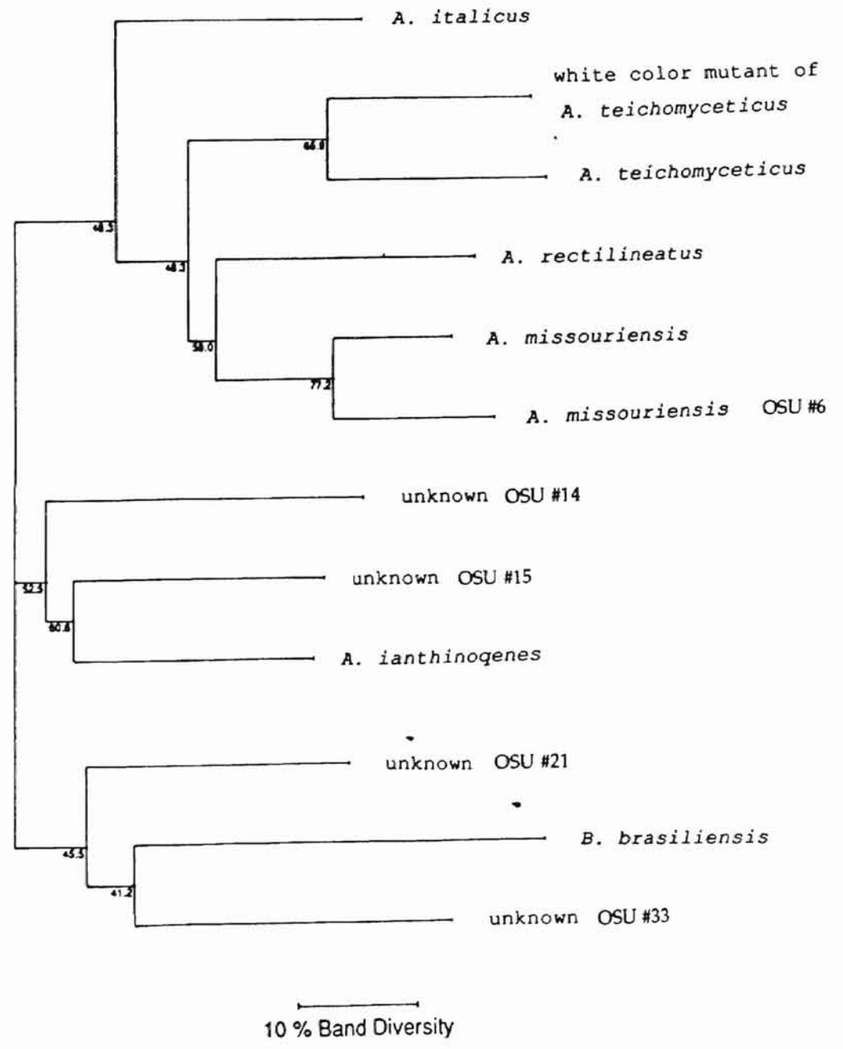

Figure 3. Phylogenetic tree generated by neighbor-joining method.

Restriction endonuclease fingerprinting profiles of *BamH I* digestion were analyzed by densitometer, a phylogenetic tree was generated by the neighbor-joining method based on the degree of similarity between organisms. The numbers on the converging points of the phylogenetic tree stand for degrees of similarity between strains connected by the converging points. 0.5% tolerance was applied to this tree.

Figure 4. A spontaneous color mutant has identical DNA fingerprint with its parent.

A spontaneous color mutant, A 321121w (a white color mutant of *A. teichomyceticus*), had identical fingerprint compared to its parent strain A 321121 (*A. teichomyceticus*, usually orange colored) when DNA samples from both strains were digested by *BamH I*. Lane 1: A 321121w (mutant); Lane 2: A 321121 (parent).

X-ray or ultraviolet (UV) irradiation can be used to create mutation, such as altered parasitic ability in *Actinoplanes* spp.. If a mutant with changed ability of hyperparasitism of the oospore of Pythium (improved or reduced) can be found, it will be interesting to find out the band difference in the genomic restriction patterns, and it will help to clone the gene controlling the ability of the hyperparasitism of the *Actinoplanes*.

I also tried the random primer PCR amplification method and pulse-field fingerprinting by rare-cutting enzymes of genomic DNA of *Actinoplanes* spp.. These two methods improved the resolvability of the band patterns and reduced the background (data not shown), but the disadvantage is that they increase the cost and reduce the number of bands and the information we can get from the band patterns.

Chemical Analysis of *Actinoplanes*

Cell-wall amino acid and whole cell sugar patterns were analyzed for more than 20 strains (Table II). It was reported previously that *Actinoplanes* spp. had cell wall chemotype II containing glycine and meso-DAP or 3-hydroxyl-DAP (Couch, 1974; Goodfellow and Cross, 1984; Palleroni, 1989). Our result confirmed this description (Fig. 5), with the exception of strain ATCC 32937 (*A. caeruleus*) which showed no diaminopimelic acid in the cell wall (data not shown). This result for ATCC 32937 is consistent with the result of Horan and Brodsky (1986). All strains had similar amino acid spot patterns except for the isomers of DAP. Some strains contain only meso-DAP and some have both, but with different quantities (Table II). From sugar patterns, I have identified Arabinose, Xylose, Mannose, Rhamnose, Galactose, Glucose in whole cell hydrolysates of all the strains (Table III). Each strain had characteristic sugar patterns, however two strains (OUS #6 and #32) of the same species (*A. missouriensis*) showed a similar sugar pattern (Fig. 6 and Table III), and they also have the same genomic DNA

fingerprints as described above, which factor suggests the possibility that these two strains are the same. A white colored spontaneous mutant of strain ATCC 21884 (strain ATCC 21884y) seemed to have greater amount of glucose than its parent (Table III).

An attempt to classify these strains of *Actinoplanes* spp. according to their relationship based on the chemical analysis described above, but it was not very successful. There was no obvious correlation between the chemical analysis and the genomic DNA finger printing, except that the two strains of *A. missouriensis* showed consistent results from these two methods. I also could not identify the unknown strains into species solely based on these information. More work should be done in order to achieve this objective.

TABLE II

Cell-Wall Amino Acid Analysis of *Actinoplanes* spp.

Other Code	Identification	Glycine	Meso-DAP	3-hydroxyl-DAP
A25844	*A. brasiliensis*	+++	++	+
A29868	*A. Ferruqineus*	+++	++	++
A21884	*A. ianthinogenes*	+++	++	++
A27366	*A. italicus*	+++	+	+++
A14538	*A. missouriensis*	+++	+	+++
E3-15A	*A. missouriensis*	+++	+++	+
7-10	*A. rectilineatus*	+++	+++	±
A31121	*A. teichomyceticus*	+++	++	+++
A14539	*A. utahensis*	+++	++	+
B16253	*A. violaceus*	+++	+	++
B16254	*A. yunnahensis*	+++	+	+
A-60	*A. minutisporangius*	+++	++	-
P3	unknown	+++	++	++
P114A	unknown	+++	++	-
R141Y	unknown	+++	++	-
W13	unknown	+++	+	-
W57	unknown	+++	+	+
W178	unknown	+++	-	-
W309	unknown	+++	+++	-
Wi11	unknown	+++	+++	-
Z20	unknown	+++	++	++
A32937	*A. caeruleus*	+++	-	-
--	unknown	+++	+	+++

Note: the number of "+" or "-" signs indicates the relative intensities of the spots on the TLC plate resulting from the staining of the amino acids by ninhydrin, with the strongest intensity designated "+++" and the weakest "±" ("-" means "undetectable").

Table III

Whole Cell Sugar Ratio in *Actinoplanes* spp.

OSU Code	Strains	Ara	Rha	Xyl	?(1)*	#Man	Gal	Glu
2	A25844	0.0	0.4	0.0	0.6	1.0	2.4	9.9
3	A29868	**0.0	0.0	0.0	0.2	1.0	0.6	3.6
4	A21884	0.0	0.0	0.0	0.2	1.0	0.3	4.1
4***	A21884y	0.0	0.1	0.0	0.2	1.0	0.1	11.6
5	A27366	0.0	0.1	0.0	0.2	1.0	0.8	14.1
6	A14538	0.0	0.2	0.0	0.5	1.0	2.6	2.1
7	E3-15A	0.0	0.5	0.0	0.6	1.0	3.4	10.5
8	7-10	0.0	0.0	0.0	0.2	1.0	0.5	7.8
9	A31121	0.0	0.0	0.0	0.2	1.0	0.1	1.3
10	A14539	0.0	0.2	0.0	0.1	1.0	1.1	3.1
11	B16253	0.0	0.3	0.1	0.8	1.0	0.9	13.0
12	B16254	0.0	0.5	0.0	0.6	1.0	2.2	6.0
13	A-60	0.0	0.1	0.2	0.3	1.0	1.2	5.0
15	P3	0.0	0.3	0.0	1.4	1.0	3.0	4.2
16	P114A	0.0	0.0	0.0	0.1	1.0	0.3	4.5
19	R141Y	0.0	0.1	0.1	0.2	1.0	1.0	32.8
21	W13	0.1	0.2	0.1	0.6	1.0	1.0	32.8
22	W57	0.2	0.1	0.2	0.4	1.0	2.1	8.7
23	W178	0.0	0.3	0.0	1.3	1.0	2.7	25.5
26	W309	0.1	0.2	0.1	0.6	1.0	0.4	10.6
27	Wi11	0.0	0.0	0.0	0.1	1.0	0.5	11.1
30	67-121	0.0	0.3	0.2	1.0	1.0	0.3	5.8
32	--	0.0	0.2	0.0	0.5	1.0	2.7	1.8

: The value of mannose serves as standard to be compared with other sugars.
* : "?(1)" is the unidentified peak in the sugar pattern of the whole-cell hydrolysates existing in all the strains.
** : "0.0" does not mean that there is no sugar of this kind in the whole-cell hydrolysates only that the amount is too small to be detected by the instrument.
***: "A 21884y" is a white color mutant of strain A 21884.

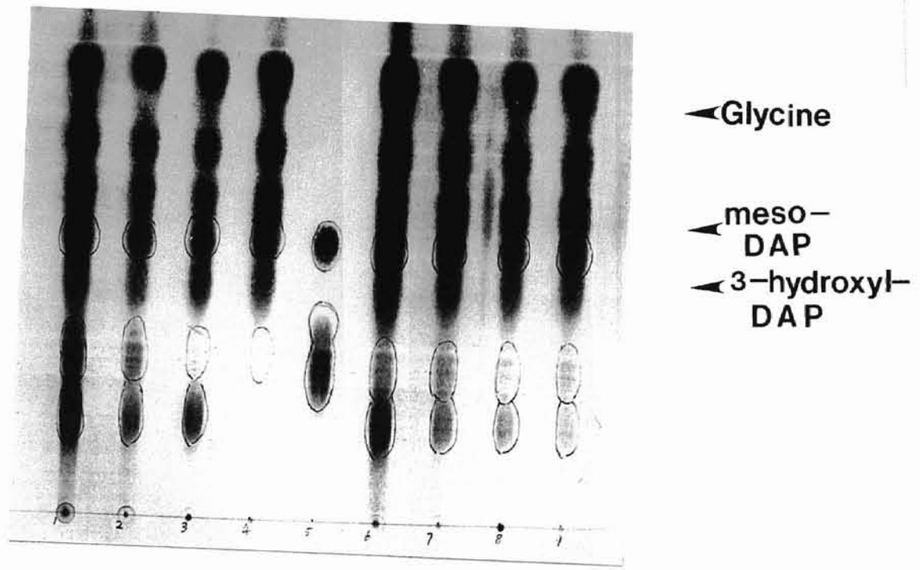

Figure 5. Cell-wall amino acid analysis of *Actinoplanes*.

Cell-wall amino acid analysis for different strains of *Actinoplanes* were carried out using thin-layer chromatography (TLC). The lane assignments are: Lane 1: *A. ianthinogenes*; Lane 2: *A. utahensis*; Lane 3: *A. minutisporamgius*; Lane 4: *A. missouriensis* (B); Lane 5: standard; Lane 6: A. sp.; Lane 7: *A. brasiliensis*; Lane 8: *A. teichomyceticus* (B); Lane 9: *A. missouriensis* (A).

Figure 6. Characteristic whole cell sugar patterns determined by gas-liquid chromatography.

A. ATCC 14538 (*A. missouriensis*);
B. OSU #32 (*A. missouriensis*);
C. ATCC 25844 (*A. brasiliensis*).

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