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Genetic relationships among actinomycetes that produce the immunosuppressant macrolides FK506, FK520/FK523 and rapamycin

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Immunosuppressant; FK506, FK520; FK523; Rapamycin; Ascomycin; Immunomycin; *Streptomyces*; Taxonomy; Gene probes

SUMMARY

A polyphasic taxonomic study was undertaken to establish the genetic and phenotypic relationships among six actinomycetes that produce the immunosuppressant macrolides FK506, FK520/FK523 and rapamycin. Chemotaxonomic studies reveal that all have Type I cell walls. Gas chromatography (GC) of fatty acid methyl esters revealed patterns consistent for strains of *Streptomyces* with 16 : 0 and 15 : 0 *anteiso* predominating. Principal component analysis of GC data revealed distinct profiles for each culture. Reciprocal DNA homology studies at T_m-25 showed the rapamycin-producing strain and one FK506-producing strain to have 38–50% homology with the type strain of *Streptomyces hygroscopicus* (ATCC 27438). The remaining strains exhibited 6–17% homology. To further explore the relationships among these strains all were probed for the presence of an *O*-methyltransferase gene specific to this biosynthetic pathway. Among the strains of interest, only *Streptomyces hygroscopicus* subsp. *yakushimaensis*, the patent strain for FK520/FK523, failed to hybridize with the probes.

INTRODUCTION

In 1987, Kino et al. [8, 10] reported on the discovery of FK506, the first of a new family of immunosuppressant macrolides that was significantly more potent than cyclosporin A (CsA). These compounds are produced by two novel strains of streptomycetes: *Streptomyces tsukubaensis* (FERM BP-927) and *Streptomyces hygroscopicus* subsp. *yakushimaensis* (FERM BP-928). Like CsA, these compounds interact with a cytoplasmic receptor that exhibits a rotamase activity. However, binding studies reveal that these macrolides bind to different immunophilins [17]. Structural studies led to the discovery that the antifungal macrolide rapamycin is an analogue of the FK506 family [20]. Rapamycin has also been shown to have immunosuppressant properties and binds to the same cytoplasmic receptor as FK506. However, rapamycin inhibits T-cell activation by a different mechanism than either FK506 or CsA. Subsequently, three other actinomycetes were reported to either produce FK506 or FK520/FK523 [3,6,7]. One of these cultures, *Streptomyces hygroscopicus* subsp. *ascomyceticus*, was originally reported to produce the antifungal ascomycin [2], which is now known to be structurally identical to FK520 [4]. The other two (actinoplanacete species and *Streptomyces* sp.) were discovered during the screening for immunosuppressant compounds and both produce FK506. Although significant gains have been made in elucidating the mechanism by which these new immunosuppressant macrolides act, little is known about the producing strains or the distribution of such strains in nature. To begin addressing this issue, we have undertaken a comparative study of all the available strains known to produce these compounds. We report here on our findings.

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MATERIALS AND METHODS

Strains, cultivation and maintenance

The strains used in our study were obtained either from public culture collections or were isolated from naturally occurring substrata as part of an ongoing natural products screening programme (Table 1). Cultures were maintained in lyophilized form in 10% skim milk at 5°C. Working stocks were maintained on slants of yeast extract–malt extract agar (ISP no. 2, Difco, Detroit, MI, USA) and/or trypticase soy broth agar (BBL, Baltimore, MD, USA).

Morphological characterization and carbon source utilization

Observations of growth, general culture characteristics and carbon source utilization were made in accordance with the methods of Shirling and Gottlieb [19].

Determination of diaminopimelic acid isomers

The isomeric form of diaminopimelic acid (DAP) was determined by a modification of the method of Lechevalier and Lechevalier [13]. Samples were analysed by ascending TLC on LK2D cellulose plates (Whatman, Clifton, NJ, USA) in a solvent system consisting of methanol/pyridine/4N HCl/H_2O (80:10:4:26).

Purification and labelling of chromosomal DNA

Biomass for DNA extraction was prepared by growing cultures in trypticase soy broth (50 ml 250 ml flask^{-1}, 220 rpm, 1 in throw). Chromosomal DNA was prepared using a modification of the method of Marmur [14] following breakage of the cells in a French Pressure Cell (SLM Instruments, Urbana, IL, USA) at 10,000 lbs/in^2. DNA purity was assessed spectrophotometrically and extraction continued until a hyperchromic shift of ≥20% was obtained at 260 nm, following thermal denaturation in 0.41-M NaCl/20% dimethylsulphoxide (DMSO). Probe DNA was labelled with [α-^{32}P]dCTP (800 Ci mmol^{-1}, Amersham) prepared by the nick translation method of Rigby et al. [16] using a commercial kit (Gibco/BRL, Gaithersburg, MD, USA). Unincorporated label was separated from probe DNA on Sephadex G-50 fine (Pharmacia, Piscataway, NJ, USA) by spun-column chromatography [1].

Preparation of FKMT gene probe

A 0.7-kb *Bam*HI-*Bgl*II fragment containing most of the 31-*O*-desmethyl-FK506 *O*: methyltransferase (FKMT) coding region (H. Motamedi, unpublished and [18]) from *Streptomyces* sp. MA6858 was subcloned into the *Bam*HI site of the plasmic vector pGEM3Z (Promega, Madison, WI, USA), followed by transformation into *E. coli* strain DH5α (BRL/Gibco). Plasmid DNA was prepared from the transformant, grown in LB broth in the presence of ampicillin at 100 μg ml^{-1}, using Qiagen columns (Qiagen, Studio City, CA, USA) following the supplier's protocol. Labelling of plasmid DNA was done by the nick translation method described above.

Preparation of IMT gene probe

A 7-kb *Bam*HI fragment containing the entire 31-*O*-desmethylimmunomycin *O*: methyltransferase (IMT) coding region and surrounding area (H. Motamedi, unpublished) from *Streptomyces hygroscopicus* subsp. *ascomyceticus* was cloned into the *Bam*HI site of pGEM3Z. IMT probe was prepared as described for FKMT.

Determination of DNA relatedness

DNA homology studies were carried out using a modification of the dot-blot method of Kafatos et al. [9]. Unlabelled DNA (5 μg 500 μl^{-1}) was denatured with alkali (0.4 M NaOH, 10 mM EDTA) for 10 min at 100°C and immobilized on nylon membranes (Zeta Probe, BioRad, Hercules, CA, USA) held in place in a 96-place microtiter format filtration manifold. Following immobilization, filters were air dried, then baked at 80°C in vacuo for 30 min. Filters were prehybridized in 100 μl cm^{-2} of hybridization buffer (0.205 M Na_2HPO_4, 1 mM EDTA, 7% SDS, 20% formamide, pH 7.2), at 65°C for 5 min. Following prehybridization, the buffer was drained and replaced with fresh hybridization buffer to which 500 μl of denatured probe DNA (ca. 5 × 10^5 cpm ml^{-1}) was added. Bags were resealed and incubation continued for 24 h at 65°C (T_m-25°C) in an oscillating water bath (Belly-Dancer, Stovall Instruments, Greensboro, NC, USA). Filters were washed once with each of the following buffers in succession for 15 min at room temperature with vigorous shaking: 2×SSC/0.1% SDS, 0.5×SSC/0.1% SDS and 0.1×SSC/0.1% SDS. DNA relatedness was calculated as a percentage of hybridization observed in heterologous reactions as compared to homologous reactions. A minimum of three replicate measurements were made for each possible pairing.

Detection of FKMT and IMT homologues

Hybridization conditions used for the detection of the FKMT and IMT genes were modified as follows. Hybridization buffer consisted of 0.25 M Na_2HPO_4, 7% SDS, 1 mM EDTA, the quantity of unlabelled DNA loaded onto filters was 1 μg 500 μl^{-1} and filters were washed once with each of the following buffers in succession for 15 min at room temperature with vigorous shaking: 2×SSC/0.1% SDS at room temperature, 1.5×SSC/0.1% SDS at room temperature and 1.0× SSC/0.1% SDS at 65°C.

Analysis of whole cell fatty acids by gas–liquid chromatography

Cultures were grown as confluent patches on 60-mm plates of trypticase soy broth agar at 28°C for 4 days. Vegetative growth (approximately 60–80 μg) was gently scraped from the surface with sterile, stainless steel spatulas and placed into Teflon-capped test tubes. Fatty acids methyl esters (FAMEs) were prepared and extracted by the procedure of Miller and Berger [15]. Analysis of the FAMEs was accomplished by capillary gas chromatography (GC) using a Hewlett-Packard Model 5890 gas chromatog-

TABLE 1

Strains used in this study

Name	Strain designation	Reported product
Streptomyces tsukubaensis	FERM BP-927, MA6492	FK506
Streptomyces hygroscopicus subsp. *yakushimaensis*	FERM BP-928, MA6531	FK520/FK523
Streptomyces hygroscopicus subsp. *ascomyceticus*	ATCC 14891, MA6572	Immunomycin (Ascomyin, FK520/FK523)
Streptomyces sp.	ATCC 55098, MA6858	FK506
Streptomyces sp. (actinoplanacete sp.)	ATCC 53770, MA6548	FK506
Streptomyces hygroscopicus subsp. *hygroscopicus*	NRRL 5491, MA6434	Rapamycin
Streptomyces hygroscopicus subsp. *hygroscopicus*	ATCC 27438, MA5993	Type strain

raph/MIDI system (Microbial ID, Inc., Newark, DE, USA) equipped with a 5% phenylmethyl silicone column (0.2 mm×25 m). Chromatography conditions were as recommended by the manufacturer. Peak areas were calculated using a Hewlett-Packard Model 3396 series II integrator. Individual FAMEs were identified on the basis of retention time which is a function of equivalent chain length, using the Microbial Identification System software. Quantities of each fatty acid are expressed as percentages of the total named FAME peak area. Each sample was analysed a minimum of five times and composite profiles were created as library entries using the library generation component of the MIDI software.

Statistical analysis

Fatty acid methyl ester profiles were analysed by principal component analysis using Systat for the Macintosh, Version 5.1 (SYSTAT Inc., Evanston, IL, USA) on an Apple Macintosh IIfx. Plots of the data were created from the component loadings for the first three principal components, which accounted for >98.5% of the variability among the strains.

RESULTS AND DISCUSSION

A list of the strains under study with their reported products and phenotypic characteristics are presented in Tables 1 and 2 respectively. Cell wall analysis reveals that all strains contain L-DAP (Type I cell wall). Although three of the strains (*S. hygroscopicus yakushimaensis* MA6531, *S. hygroscopicus* subsp. *ascomyceticus* MA6572 and *S. hygroscopicus* subsp. *hygroscopicus* MA6434) exhibit a characteristic grey spore mass that tends to blacken and coalesce during maturation, only one strain, *S. hygroscopicus* subsp. *ascomyceticus* (MA6572), conforms to the current species description with regard to utilization of sucrose and raffinose as sole carbon sources [12]. The remaining strains (*S. tsukubaensis* MA6492, *Streptomyces* sp. MA6548 and *Streptomyces* sp. MA6858) are distinct from one another with regard to sporophore arrangement and utilization of key carbohydrates. Two of these strains exhibit either straight (MA6858) or flexous (MA6492) sporophores that are non-coalescent, while the third strain (MA6548) exhibits spiral sporophores in pseudosporangia that coalesce into amorphous masses as the culture ages.

Gas chromatographic analysis of FAMEs revealed all of the strains produce both saturated and unsaturated fatty acids with branched chain fatty acids predominating (Table 3). For the *Streptomyces*, key diagnostic fatty acids are $C_{15:0iso}$, $C_{15:0anteiso}$, $C_{16:0iso}$, $C_{17:0iso}$ and $C_{17:0anteiso}$. [11] These forms comprise 69.86 ± 6.48% of the total fatty acid content of *Streptomyces* species in the current version of the MIDI actinomycete data base (G. Garrity, unpublished). All of the strains in this study exhibit such a pattern and fall within the expected range (64.73 ± 6.73%). *Streptomyces*

TABLE 2

Key cultural characteristics of FK506, FK520/FK523 and rapamycin producing strains and selected reference strains

Characteristic	MA6492	MA6531	MA6572	MA6434	MA6548	MA6858	MF5993
Coloration (SP no. 2)							
vegetative	yellow	yellowish-brown	yellowish-brown	yellow-brown	yellow-brown	yellow-brown	yellow
aerial	grey	grey	grey	grey	none	whitish-grey	grey
Chromogenicity	−	−	−	+	−	−	+
Melanoid pigments	−	−	−	−	−	−	−
Sporophore morphology	RF	RA-S	S	S	S with occasional pseudospornagia	RF-straight	S
Hygroscopic spore mass	−	+	+	+	+	−	+
Utilization of							
raffinose	±	−	−	±	+	±	−
sucrose	−	+	−	−	+	−	−
DAP isomer	LL-DAP	LL-DAP	ND	ND	LL-DAP	LL-DAP	LL-DAP
Environmental substrate and geographical locale	soil Tsukuba, Ibaraki, Perfecture, Japan	soil Yaku-shima, Kagoshima Perfecture, Japan	soil Sannomiya, Hyoga Perfecture Japan	soil Easter Island	soil Kaiholena Valley, Koele, Lanai, Hawaii, USA	deer dung Poverty Creek drainage basin, West Virginia, USA	Type strain

MA6492, *S. tsukubaensis*; MA6531, *S. hygroscopicus* subsp. *yakushimaensis*; MA6572, *S. hygroscopicus* subsp. *ascomyceticus*; MA6858, *Streptomyces* sp.; MA6548, *Streptomyces* sp.; MA6434, *S. hygroscopicus* subsp. *hygroscopicus*; MA5993, *S. hygroscopicus* subsp. *hygroscopicus*. RA, retinaculum apertum; RF, retcus flexibilis; S, spirales; +, positive utilization at 28 days; −, no utilization at 28 days; ±, poor utilization at 28 days.

TABLE 3

Fatty acid composition of FK506, FK520/FK523 and rapamycin producing strains and selected reference strains

Fatty acid	% in profile						
	MA6492	MA6531	MA6572	MA6434	MA6548	MA6858	MA5993
$C_{13:0\ iso}$				0.69			0.4
$C_{14:0\ iso}$	7.7	11.7	10.01	5.43	1.74	7.79	9.29
$C_{15:0\ iso}$	5.82	10.96	17.73	25.95	8.5	10.79	14.68
$C_{15:0\ anteiso}$	13.89	9.89	13.55	10.97	28.95	20.09	17.82
$C_{15:1\ B}$	1.14		0.38	0.69			0.67
$C_{15:0}$	5.1	2.15	1.22	2.66	4.22	1.56	1.01
$C_{16:1\ iso\ H}$	6.6	7.92	4.2	2.35	2.1	7.01	8.1
$C_{16:0\ iso}$	29.39	28.96	25.63	17.88	16.28	27.29	25.5
$C_{16:1\ cis\ 9}$	6.46	0.91	2.69	3.77	1.42	1.09	4.25
$C_{15:0\ anteiso\ 2\ OH}$		4.63	0.53			2.09	
$C_{16:0}$	2.64	4.36	2.53	3.17	4.56	3.69	3.09
$C_{16:0\ 9?\ CH3}$	3.17	1.79	5.28	7.77	4.13	2.69	3.56
$C_{17:1\ anteiso\ C}$	2.98	2.47	2.21	1.31	4.32	2.67	2.54
$C_{17:0\ iso}$	1.36	2.53	5.43	10	4.47	3.62	3.58
$C_{17:0\ anteiso}$	4.1	3.47	5.93	3.58	13.63	6.03	4.87
$C_{17:1\ cis\ 9}$	4.24			0.88	0.8		
$C_{16:0\ iso\ 2OH}$		1.76					
$C_{17:0\ cyclo}$	1.99			1.23	1.61	1.53	
$C_{17:0}$	0.67			0.72	0.78	0.34	
$C_{17:0\ 10\ CH3}$	0.81						
$C_{18:1\ iso\ H}$	1.04					0.68	0.43
unk 17.595					1.11		
$C_{17:0\ iso\ 2\ OH}$	0.57				0.54		

MA6492, *S. tsukubaensis*; MA6531, *S. hygroscopicus* subsp. *yakushimaensis*; MA6572, *S. hygroscopicus* subsp. *ascomyceticus*; MA6858, *Streptomyces* sp.; MA6548, *Streptomyces* sp.; MA6434, *S. hygroscopicus* subsp. *hygroscopicus*; MA5993, *S. hygroscopicus* subsp. *hygroscopicus*; unk, unknown.

tsukabaensis (MA6492) is unique among these strains studied as it also produces trace amounts of $C_{17:0\ 10\ CH3}$. This fatty acid is not generally produced in detectable amounts by *Streptomyces* species. To further explore the similarity among these cultures based upon fatty acid composition, the data presented in Table 3 were ordinated in three dimensions using principal component analysis (Figure 1). Such an analysis allows one to accurately summarize multivariate data by decomposing a correlation matrix to uncover the minimum number of variables or combination of variables that explain most of the joint variation. In the case of FAME analysis, if most of the variability can be explained by three to five principal components, strains that share a common identity (e.g. strains of the same species) will cluster tightly together. Unrelated strains will be widely separated. Strains *S. hygroscopicus* subsp. *ascomyceticus* MA6572 and *S. hygroscopicus* subsp *hygroscopicus* MA6434 were found to cluster with the type strain of *Streptomyces hygroscopicus* subsp. *hygroscopicus* (MA5993). Two other strains, *Streptomyces* sp. MA6548 and *Streptomyces* sp. MA6858 were also found in close proximity to one another. These strains are quite distant from the first cluster along the axis of the first principal component. In addition, the distance between these strains is greater than that observed in the first cluster. Strains *S. tsukubaensis* (MA6492) and *S. hygroscopicus* subsp. *yakushimaensis* MA6531 are distinct from the other strains as well as from one another.

Reciprocal DNA homology studies reveal that all of the strains are genetically distinct from one another (Table 4). Species-level relationships (≥70% homology) were not observed for any combination, however, two strains (*S. hygroscopicus* subsp. *hygroscopicus* MA6434 and *S. hygroscopicus* subsp. *ascomyceticus* MA6572) show intermediate levels of homology (38–50%) to the type strain of *S. hygroscopicus*. This finding is consistent with the results of fatty acid analysis. The remaining strains exhibit low levels of homology to one another, as is expected for members of the same genus (10–15%). The apparent relationship between *Streptomyces* sp. MA6548 and *Streptomyces* sp. MA6858, observed in fatty acid studies, was not borne out by DNA homology experiments. We believe that the Euclidean distance between these two strains, observed in the principal component plots, exceeds the limits of species relationships, as defined by DNA homology.

Recently, we have isolated two genes (FKMT and IMT) that encode for two methyltransferases in the biosynthesis

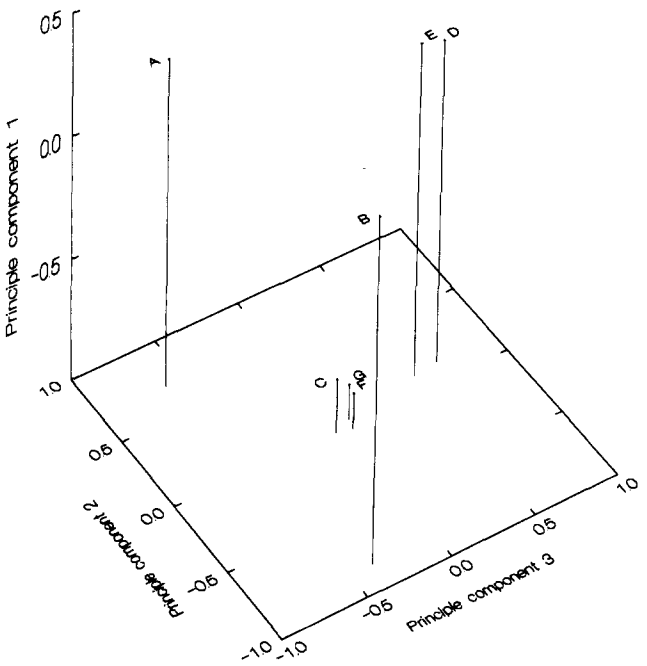

Fig. 1. Principle component plot of fatty acid composition. A, *S. tsukubaensis*; B, *S. hygroscopicus* subsp. *yakushimaensis*; C, *S. hygroscopicus* subsp. *ascomyceticus*; D, *Streptomyces* sp.; E, *Streptomyces* sp.; F, *S. hygroscopicus* subsp. *hygroscopicus*; G, *S. hygroscopicus* subsp. *hygroscopicus*.

TABLE 5

Hybridization of FK506, FK520 and FK523 producing strains and selected reference strains with FKMT and IMT probes

Source of chromosomal DNA	Probe	
	FKMT	IMT
S. tsukubaensis MA6492	+++	+++
S. hygroscopicus subsp. *yakushimaensis* MA6531	–	–
S. hygroscopicus subsp. *ascomyceticus* MA6572	+++	+++
Streptomyces sp. MA6858	+++	++
Streptomyces sp. MA6548	+++	+++
S. hygroscopicus subsp. *hygroscopicus* MA6434	+++	+++
S. hygroscopicus subsp. *hygroscopicus* MA5993	–	–

reported to produce FK506, FK520/FK523 or rapamycin probed positive for both the FKMT and IMT genes. The failure of MA6531 to hybridize with either probe was an unexpected result as this culture is one of the patent strains for FK520/FK523. However, this finding is consistent with the failure of this culture to produce either compound (M. Nallin-Omstead, personal communication). These results

TABLE 4

DNA homology at T_m-25 among FK506, FK520/FK523 and rapamycin producing strains and selected reference strains

Source of chromosomal DNA	Source of probe DNA						
	6492	6531	6572	6858	6548	6434	5993
MA6492	100	11	12	8	11	11	11
MA6531	9	100	10	6	10	12	9
MA6572	13	14	100	9	12	43	44
MA6858	13	15	11	100	17	12	13
MA6548	14	15	11	11	100	13	15
MA6434	NT	NT	NT	NT	NT	100	NT
MA5993	12	9	38	9	9	50	100

MA6492, *S. tsukubaensis*; MA6531, *S. hygroscopicus* subsp. *yakushimaensis*; MA6572, *S. hygroscopicus* subsp. *ascomyceticus*; MA6858, *Streptomyces* sp.; MA6548, *Streptomyces* sp.; MA6534, *S. hygroscopicus* subsp. *hygroscopicus*; MA5993, *S. hygroscopicus* subsp. *hygroscopicus*; NT, not tested.

of FK-506 and FK-520 (methylation of the precursors 31-*O*-desmethyl FK-506 and 31-*O*-desmethyl immunomycin). To further explore the relationships of the strains under study, these genes were used as probes to analyse various macrolide producing strains. The results of these experiments are presented in Table 5. As expected, *S. hygroscopicus* subsp. *hygroscopicus* did not hybridize with either probe. Similar findings were obtained when other non-producing strains of *Streptomyces, Actinoplanes* and *Kibdellosporangium* were probed. With the exception of MA6531, all of the strains confirm the specificity of two probes as well as indicate a strong correlation between the presence of the FKMT and IMT genes and the production of FK506, FK520 and FK523. While it is possible that MA6531 has lost the one or more genes in the FK520/FK523 biosynthetic pathway, including the IMT gene, it is more likely that the culture deposited and received as FERM BP-928 is not the same as the culture described in the patent.

In the initial report of the production of FK506 by *Streptomyces* sp. MA6548, this culture was identified as an

actinoplanacete (*sensu* Couch) on the basis of sporangium-like vesicles found in the aerial mass [5,7]. The results of the present study do not support this identification. This culture is most likely a *Streptomyces* species as it has a type I cell wall (L-DAP) and the fatty acid profile of a streptomycete.

Neither DNA homology nor GC of FAMEs revealed close relationships among the strains that produce FK506 and analogues. This, however, is not entirely unexpected, as members of the genus *Streptomyces* are genetically heterogeneous and the metabolites that are produced by members of this genus may not necessarily be restricted to a single named species. The results of our experiments with the FKMT and IMT probes clearly demonstrate this fact. Those experiments also suggest that FKMT and IMT encode for enzymes that must be an essential part of the biosynthetic pathway of the immunosuppressant macrolides FK506, FK520/FK523 and rapamycin. The fact that these compounds have been found in genetically distant strains from geographically dispersed regions suggests that the pathway may be evolutionarily conserved. What remains unknown is how widespread these genes are in nature and whether these metabolites play a role in signal transduction in prokaryotes.

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