SELECTIVE ISOLATION, CHARACTERISATION AND IDENTIFICATION OF STREPTOSPORANGIA

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PUBLICATIONS

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SUMMARY

Large numbers of actinomycetes were isolated from composite soil samples using procedures considered to be selective for the isolation of streptosporangia and related sporoactinomycetes from environmental samples. The highest streptosporangial counts were obtained when suspensions of air-dried soil were heated in the presence of yeast extract for 20 minutes at 40°C then plated onto humic acid vitamins agar supplemented with actidione (50mg/l) and nalidixic acid (30mg/l) and incubated for 4 weeks at 30°C. The highest count, $7.94 \pm 1.19 \times 10^4$ colony forming units per gram dry soil, were obtained from samples of Ginseng field soil. Representative strains had morphological and chemical properties consistent with their classification in the genus *Streptosporangium*.

Representative isolates and marker strains of the genus *Streptosporangium* were examined for diagnostic features recommended for computer-assisted identification of unknown streptosporangia. Stringent criteria were adopted for positive identifications of both known and unknown strains following a critical evaluation of identification scores obtained for the marker cultures. Sixty-five of the seventy marker strains and twelve of the hundred and thirty six unknown streptosporangia were identified to known streptosporangial taxa. A further nineteen of the isolates were assigned to known taxa using less stringent cut-off points for positive identifications.

5S ribosomal RNA sequences were determined for nine representatives of the genus *Streptosporangium* including centrotype strains of two taxa, clusters 1 and 2, circumscribed in a recent numerical phenetic survey and two Ginseng field soil isolates. The primary and secondary structure of the resultant sequences were of the type characteristic of Gram-positive bacteria with DNA rich in guanine and cytosine. It was evident from the phylogenetic tree that the genus

Streptosporangium is heterogeneous as the type strains of *Streptosporangium albidum* and *Streptosporangium viridogriseum* subspecies *viridogriseum* were sharply separated from the remaining test strains; a result in good agreement with current trends in streptosporangial systematics.

Pilot experiments were designed to determine the potential of Curie point pyrolysis mass spectrometry and rapid fluorogenic enzyme tests in the classification and identification of streptosporangia. The pyrolysis mass spectral data supported the taxonomic integrity of clusters 1 and 2 and showed that *Streptosporangium viridogriseum* subspecies *viridogriseum* had little in common with *bona fide* members of the genus *Streptosporangium*. Pyrolysis data also supported the results of the computer-assisted identification exercise as ten isolates assigned to cluster 1 using stringent cut-off criteria were found to be closely related to representatives of cluster 1. There was evidence that some of the conjugated substrates based on the fluorophores 7-amino-4-methylcoumarin and 4-methylumbelliferone have potential as taxonomic markers for the classification of streptosporangia and related actinomycetes.

INTRODUCTION

A. AIMS

Actinomycetes are an unique source of high value bioactive products, notably antibiotics, enzymes, enzyme inhibitors and vitamins. In particular, they account for sixty percent, that is, more than seven thousand of the naturally occurring antibiotics that have been discovered (Table 1, page 2). Approaches to the search for, and discovery of, new bioactive compounds are generally based on screening both naturally occurring actinomycetes and genetically manipulated strains. Current efforts to find the next generation of new antibiotics of therapeutic value are compromised as the probability of discovering new compounds is declining as the number of known antibiotics is increasing (Okami and Hotta, 1988). It is, therefore, important in search and discovery programmes to screen novel and rare actinomycetes in order to raise the probability of finding novel antibiotics (Nolan and Cross, 1988; Bull *et al.*, 1992).

Given recent developments in microbial systematics it is now possible to recognise and characterise rare and novel actinomycetes derived from the application of selective isolation procedures by detecting key phenetic taxonomic markers (O'Donnell, 1986, 1988; Goodfellow and O'Donnell, 1989; Bull *et al.*, 1992). In addition, molecular taxonomic methods, such as nucleic acid hybridisation, sequencing and fingerprinting techniques, are available for the accurate description of patent strains (Stackebrandt and Goodfellow, 1991). In addition, information in numerical taxonomic databases can be used to design media formulations for the selective isolation of specific fractions of the actinomycete community from soil and other natural habitats (Vickers *et al.*, 1984; Williams *et al.*, 1984a; Williams and Vickers, 1988; Goodfellow and O'Donnell, 1989; Bull *et al.*, 1992). Screening methods, which are increasingly target

Table 1 Number of antibiotics produced by members of selected actinomycete genera*

Genus	1974	1980	1984	1988	1993
Streptomyces	1934	2784	3477	4876	5645
Micromonospora	41	129	269	398	535
Nocardia	45	74	107	262	287
Actinomadura	-	16	51	164	248
Actinoplanes	6	40	95	146	169
Streptoverticillium	19	41	64	138	169
Streptosporangium **	7	20	26	39	57
Saccharopolyspora	-	4	33	44	55
Dactylosporangium	-	4	19	31	40
Amycolatopsis	-	-	-	-	23
Kibdelosporangium	-	-	-	7	18
Actinosynnema	-	-	5	14	17
Microbispora **	4	6	6	10	15
Streptoalloteichus	-	3	4	12	14
Kitasatosporia	-	-	-	11	14
Planobispora **	-	-	-	-	10
Microtetraspora **	-	-	-	-	4
Planomonospora **	-	-	-	-	2

* Data from Bérdy (1974, 1984), Nisbet (1982) and Bérdy database (August, 1993; Data from Medha Athalye, Smithkline Beechams, Brockham Park, Betchworth, Surrey, U.K.).

** Members of the family *Streptosporangiaceae* (Goodfellow *et al.*, 1990a).

directed, are also important in the search for new products (Okami and Hotta, 1988; Bull *et al.*, 1992).

There is evidence that the genus *Streptosporangium* may become an increasingly rich source of commercially significant products, notably antibiotics (Table 2, page 4). In addition, cystathionine γ-lyase has been detected in a strain of *Streptosporangium* (Nagasawa *et al.*, 1984). This enzyme catalyses the α, γ-elimination reaction of L-cystathionine and also the γ-replacement of L-homoserine in the presence of various thiol compounds (Kanzaki *et al.*, 1986a). An efficient method based on the reaction of γ-replacement has been developed for the preparation of L-cystathionine (Kanzaki *et al.*, 1986b), a product with potential value as it has been shown to be deficient in the brains of homocystinuric patients (Gerritsen and Waisman, 1964). The procedure described by Kanzaki and his colleagues allows the total conversion of L-cysteine into L-cystathionine and *O*-succinyl-L-homoserine.

The discovery of additional commercially significant natural products from *Streptosporangium* strains is hindered by the lack of effective procedures for the selective isolation, classification and identification of streptosporangia from environmental samples (Goodfellow, 1991; Hayakawa *et al.*, 1991). Current isolation methods are empirical and depend upon drastic, heat pretreatment of air dried soil samples and the plating out of serial dilutions onto basal media supplemented with selective agents (Nonomura and Ohara 1969a, b; Hayakawa and Nonomura, 1987a; Nonomura and Hayakawa, 1988). Little attempt has been made to evaluate the effectiveness of these selective isolation procedures partly because of the poor taxonomy of the genus *Streptosporangium*.

In a comprehensive chemical and numerical phenetic survey of the genus *Streptosporangium* marker and fresh isolates were assigned to five major, seven minor and eighteen single membered clusters (Whitham, 1988; Whitham *et al.*,

Table 2 Members of the genus *Streptosporangium* known to produce novel bioactive compounds

Name	Product	Reference
Streptosporangium albidum	Aculescimycin	Murata *et al.* (1989)
"*Streptosporangium brasiliense*"	Selenomycin	U.S. patent 3,683,074
Streptosporangium fragile	Anthracycline	Shearer *et al.* (1983)
	fragilomycin complex	U.S. patent 4,293,546
"*Streptosporangium indica*"	Antimicrobial agent	Rao *et al.* (1987)
"*Streptosporangium karnatakensis*"	Antimicrobial agent	
Streptosporangium pseudovulgare	Antitumour antibiotics	Umezawa *et al.* (1976)
	Sporamycin	Komiyama *et al.* (1977)
"*Streptosporangium sibiricum*"	Sibiromycin	Brazhnikova *et al.*(1972)
Streptosporangium violaceochromogenes	Platomycins A and B	Takasawa *et al.* (1975)
	Victomycin	Kawamoto *et al.* (1975)
Streptosporangium viridogriseum subsp. *kofuense*	Chloramphenicol	Tamura *et al.* (1971)
Streptosporangium viridogriseum subsp. *viridogriseum*	Sporaviridin	Okuda *et al.* (1966a, b)
"*Streptosporangium* sp."	Cystathionine γ-lyase	Nagasawa *et al.* (1984)

" ", Species not on the Approved Lists of Bacterial Names (November, 1993).

1993). The results underpinned the taxonomic integrity of the genus *Streptosporangium* and most of its constituent species but indicated that the taxon was heterogeneous. Information from the numerical taxonomic database was used to generate a theoretically sound frequency matrix for the computer-assisted identification of unknown environmental isolates belonging to the genus.

The initial aim of the present project was to evaluate the selectivity of procedures currently recommended for the selective isolation of streptosporangia from environmental samples (Nonomura and Ohara, 1969a; Nonomura, 1989). Representative isolates and marker strains of numerically circumscribed clusters containing streptosporangia (Whitham *et al.*, 1993) were then examined for properties considered to be diagnostic for the identification of streptosporangia in order to evaluate the computer-assisted procedure (Whitham, 1988). The taxonomic status of authentic and putatively novel species of *Streptosporangium* were then evaluated using a rapid automated enzymatic procedure (Hamid *et al.*, 1993) and Curie-point pyrolysis mass spectrometry (Magee, 1993a, b). Finally, the phylogenetic relationships of representative streptosporangia were the subject of 5S ribosomal RNA sequencing studies carried out in collaboration with Dr. Y-H. Park of the Genetic Engineering Research Institute in Daejon, Korea.

B. AN HISTORICAL SURVEY OF THE GENUS *STREPTOSPORANGIUM*

Couch (1955a) proposed the genus *Streptosporangium* for sporangiate actinomycetes that formed nonmotile sporangiospores on abundant aerial hyphae. Initially, only one species, *Streptosporangium roseum,* was recognised. Additional taxa were added to the genus which now includes fourteen validly described species (Table 3, page 6; Nonomura, 1989; Mertz and Yao, 1990). Members of these taxa characteristically form aerial hyphae that carry, on either

Table 3 Validly described species and subspecies of the genus *Streptosporangium*

Taxon	Authors	Habitat	Type Strain
Streptosporangium albidum	Furumai *et al.* (1968)	Soil, Mount Tonigawa, Japan	ATCC 25243
Streptosporangium album	Nonomura and Ohara (1960)	Soil, Japan	DSM 43023
Streptosporangium amethystogenes	Nonomura and Ohara (1960)	Soil, Japan	ATCC 33327
Streptosporangium carneum	Mertz and Yao (1990)	Soil, River Tana Nairobi, Kenya	NRRL 18437
Streptosporangium corrugatum	Williams and Sharples (1976)	Beach sand, Freshfield, Lancashire, U.K.	ATCC 29331
Streptosporangium fragile	Shearer *et al.* (1983)	Soil, Anaikota, Sri Lanka	ATCC 31519
Streptosporangium longisporum	Schäfer (1969)	Steppe soil, Turkey	ATCC 25212
Streptosporangium nondiastaticum	Nonomura and Ohara (1969b)	Soil, Japan	ATCC 27101
Streptosporangium pseudovulgare	Nonomura and Ohara (1969b)	Soil, Japan	ATCC 27100
Streptosporangium roseum	Couch (1955a)	Vegetable garden soil	ATCC 12428
Streptosporangium violaceochromogenes	Kawamoto *et al.* (1975)	Swamp soil, Japan	ATCC 21807
Streptosporangium viridialbum	Nonomura and Ohara (1960)	Soil, Yotei, Hokkaido, Japan	ATCC 33328
Streptosporangium viridogriseum subsp. *kofuense*	Nonomura and Ohara (1969b)	Soil, Japan	ATCC 27102
Streptosporangium viridogriseum subsp. *viridogriseum*	Okuda *et al.* (1966a)	Soil, Japan	ATCC 25242
Streptosporangium vulgare	Nonomura and Ohara (1960)	Soil, paddy field, Anjo, Aichi Prefecture, Japan	ATCC 33329

ATCC, American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, U.S.A.; **DSM**, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Mascheroder Weg 1B, D-38124 Braunschweig, Federal Republic of Germany; **NRRL**, Northern Research and Development Division, United States Department of Agriculture, Peoria, Illinois, U.S.A.

short or long sporangiophores, single or clustered spore vesicles which are commonly 7 to 20μm, but may be up to 40μm, in diameter.

Spore vesicles contain coiled chains of arthrospores that are formed by septation of an unbranched, spiral hypha within each expanded sporangiophore sheath (Vobis and Kothe, 1985). Since spore formation is not endogenous, the term "spore vesicle" has greater precision than the original term "sporangium" (Cross 1970; Sharples *et al.*, 1974; Goodfellow, 1991). Studies on spore maturation have shown that the spores in both spore vesicles and spore chains are formed in essentially the same way. In each case, spores are differentiated by fragmentation of a hypha within a sheath, the latter either expands to form the envelope of the spore vesicle or remains around the spore chain (Lechevalier *et al.*, 1966; Sharples *et al.*, 1974; Vobis and Kothe, 1985; Goodfellow, 1991).

Streptosporangia have a wall chemotype III (M.P. Lechevalier and Lechevalier, 1970a), that is, they have *meso*-diaminopimelic acid in the wall peptidoglycan but do not contain characteristic sugars other than madurose (3-*O*-methyl-D-galactose; Lechevalier and Gerber, 1970) which can be detected in whole-organism hydrolysates. The peptidoglycan is of the A1γ type (Schleifer and Kandler, 1972). The organisms are rich in *iso*-, *anteiso*-, saturated, unsaturated, and methyl-branched fatty acids (pattern 3C; Kroppenstedt, 1985; Kudo *et al.*, 1993; Stackebrandt *et al.*, 1993; Whitham *et al.*, 1993), contain dihydrogenated and tetrahydrogenated menaquinones with nine isoprene units as predominant isoprenologues (Kroppenstedt, 1985; Kudo *et al.*, 1993; Stackebrandt *et al.*, 1993; Whitham *et al.*, 1993), and have phospholipid patterns characterised by glucosamine-containing lipids together with phosphatidylethanolamine, diphosphatidylglycerol and phosphatidylinositol (phospholipid pattern IV; Lechevalier *et al.*, 1977, 1981; Kudo *et al.*, 1993; Stackebrandt *et al.*, 1993; Whitham *et al.*, 1993). The DNA base composition is between 69 and 71

mol % guanine (G) plus cytosine (C) (Jones and Bradley, 1964; Tsyganov *et al.*, 1966; Yamaguchi, 1967; Farina and Bradley, 1970; Stackebrandt *et al.*, 1993).

Streptosporangium species can be distinguished on the basis of spore vesicle size, sporangiophore length, spore shape, and aerial spore mass and substrate mycelium pigmentation (Table 4, page 9). They may also be subdivided according to the nature of their vesicular walls. At one extreme, the spore vesicular membrane of *Streptosporangium fragile* is so thin that is cannot be detected by light microscopy (Shearer *et al.*, 1983); this may lead to difficulty in differentiating such organisms from *Actinomadura* and *Microtetraspora*, as some strains may produce "pseudosporangia" covered by a slimy substance (Nonomura and Ohara, 1971b). In contrast, the spore vesicles of *Streptosporangium albidum* and *Streptosporangium viridogriseum* have thick and strong walls that enclose a "sheathed" chain of spores (Nonomura and Ohara, 1969b). The genus *Kibdelosporangium* (Shearer *et al.*, 1986) bears a close morphological resemblance to these streptosporangia but has a wall chemotype IV, that is, strains contained *meso*-diaminopimelic acid and the sugars arabinose and galactose (M.P. Lechevalier and Lechevalier, 1970a). The wall components of *Streptosporangium albidus* and *Streptosporangium viridogriseum* strains need to be re-examined to clarify the relationship of these organisms to the genus *Kibdelosporangium* and other taxa assigned to the family *Pseudonocardiaceae* (Embley *et al.*, 1988). *Streptosporangium corrugatum* produces characteristically small, club-shaped spore vesicles and those of the remaining species thin vesicular membranes that are readily disrupted in water (Lechevalier *et al.*, 1966a; Williams *et al.*, 1973; Sharples *et al.*, 1974).

Streptosporangium strains usually grow well between 25°C and 30°C though *Streptosporangium nondiastaticum* and *Streptosporangium pseudovulgare* grow better at 42°C and 55°C, respectively (Nonomura and Ohara, 1969b).

Table 4 Characteristics differentiating validly described species of the genus *Streptosporangium**

	S album	*S albidum*	*S amethystogenes*	*S carneum*	*S corrugatum*	*S fragile*	*S longisporum*	*S nondiastaticum*	*S pseudovulgare*	*S roseum*	*S violaceochromogenes*	*S viridialbum*	*S viridogriseum* subsp. *viridogriseum*	*S viridogriseum* subsp. *kofuense*	*S vulgare*
Colour of substrate mycelium															
Brown-black	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Red or orange	-	-	-	-	-	-	+	+	+	+	-	-	-	-	+
Yellowish brown to brown	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+
Colour of spore mass															
Greenish gray	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-
Pink	-	-	+	+	-	+	+	+	+	+	+	-	-	-	+
White	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-
Spore vesicle size															
1-5 μm	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
6-10 μm	+	-	+	-	-	+	+	-	+	+	+	+	-	-	+
11-20 μm	-	(+)	-	-	-	+	+	+	-	(+)	-	-	-	+	-
21-30 μm	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-
31-50 μm	-	-	-	(+)	-	-	-	-	-	-	-	-	+	-	-
Sporangiophore size															
Short (10 μm)	+	-	+	-	+	+	+	+	+	+	+	+	-	+	+
Long (50 μm)	-	+	-	+	-	-	-	-	-	-	-	-	+	+	-
Spore shape															
Spherical-oval	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+
Rod	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-
Soluble pigments[a]	-	-	-	-	-	+	-	-	-	+	+	-	-	-	-
B vitamin required	+	-	+	-	-	-	-	+	+	+	-	+	-	-	+
Growth at:															
42°C	-	-	-	-	-	+	-	+	+	-	-	-	+	+	-
50°C	-	-	-	-	-	-	-	-	-	-	-	-	+	(+)	-
Gelatin liquefaction	+	-	-	-	ND	-	ND	+	+	+	(+)	d	+	+	d
Iodinin production	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	-	+	+	-	-	+	(+)	+	+	+	+	d	+	-	-
Starch hydrolysis	-	-	+	-	-	+	+	-	+	+	+	+	+	+	+
Utilisation of:															
Adonitol	+	ND	+	-	+	-	+	+	+	+	ND	-	-	-	+
Arabinose	+	ND	ND	-	+	+	+	+	+	+	ND	-	-	-	+
Galactose	+	ND	-	+	-	+	-	+	-	+	ND	-	+	+	+
Glycerol	-	ND	ND	-	-	-	-	-	+	+	ND	-	+	+	+
Inositol	-	ND	+	-	-	-	-	-	-	+	(+)	+	+	+	+
Mannitol	+	ND	ND	-	-	+	-	+	+	-	ND	+	+	+	-
Rhamnose	-	ND	+	-	-	+	-	-	-	+	(+)	+	+	(+)	+
Turanose	+	ND	ND	ND	-	+	-	+	+	+	ND	+	-	+	+

* Data taken from Nonomura (1989), Mertz and Yao (1990), Goodfellow (1991) and Whitham *et al.*(1993).

Symbols: +, positive reaction; (+), weak positive reaction; -, negative reaction; d, doubtful; ND, not determined.

[a]Other than pale yellow-brown.

"*Streptosporangium album* subsp. *thermophilus*" (Manachini *et al.*, 1965) is a thermophilic organism that was wrongly classified as it belongs to the genus *Thermoactinomyces* (Goodfellow and Cross, 1984). Most *Streptosporangium* strains grow well at pH 6.8 to 7.0.

Differences in gelatin liquefaction, nitrate reduction, starch hydrolysis and the production of iodinin crystals have also been recommended for the identification of streptosporangia. However, little credence can be placed in the predictiveness of such properties given the small sample of strains and tests examined. Nevertheless the status of most validly described species of *Streptosporangium* was supported in the numerical phenetic survey of Whitham *et al.* (1993) though it was evident that the *Streptosporangium viridogriseum* strains clustered apart from the other streptosporangia.

Little is known about the metabolism and genetics of streptosporangia. Protoplasting and regeneration protocols have been developed for *Streptosporangium viridogriseum* (Oh *et al.*, 1980); plasmids have also been isolated from this organism (Fare *et al.*, 1983) though attempts to isolate phage have been unsuccessful (Prauser, 1984). Plasmid pSg V-1 from *Streptosporangium viridogriseum* had an estimated *M*r of 54 x 10^6 whereas the pSg B-1 plasmid was found to be phenotypically cryptic. An unusual expressed trait resembling phage plaques has been associated with the *Streptosporangium viridogriseum* plasmid pSg V-1.

Nonomura and Ohara (1969a) demonstrated that streptosporangia were components of the actinomycete community in soil. Previous workers had only isolated these organisms infrequently from soil, dung (Couch, 1955a) and leaf litter (Van Brummelen and Went, 1957; Potekhina, 1965), but streptosporangial populations of 10^4 to 10^6 colony-forming units (cfu) per gram dry weight of sample were reported for various Japanese soils (Nonomura and Ohara, 1969a;

Nonomura, 1984). Recently, Hayakawa *et al.* (1991) recovered 10^5 streptosporangial colony forming units/g dry weight samples from vegetable field soil in Japan by plating suspensions of heat pretreated air dried soil onto HV agar supplemented with leucomycin and nalidixic acid. The organisms were abundant in Japanese soils rich in humus and with an acidic reaction (Nonomura and Hayakawa, 1988).

Streptosporangia have also been isolated from lake sediments (Willoughby, 1969a; Johnston and Cross, 1976), beach sand (Williams and Sharples, 1976), pasture and woodland soils (Whitham *et al.*, 1993), and one strain, "*Streptosporangium bovinum*",was reported from infected bovine hooves (Chaves Batista *et al.*, 1963). "*Streptosporangium indianensis*" Gupta, 1965, isolated from an Indian soil, was transferred to the genus *Streptomyces* as *Streptomyces indiaensis* (Kudo and Seino, 1987) as it does not form true spore vesicles (Schäfer, 1969) and has morphological and chemical properties characteristic of streptomycetes (Kudo and Seino, 1987; Whitham *et al.*, 1993). It seems likely that the original author mistook spore aggregates, resulting from autolysis of sporulating aerial hyphae, for spore vesicles. Similarly, strains labelled *Streptosporangium* type 1 from stream water (Willoughby, 1969b) probably belong to the genus *Actinoplanes* given their morphological properties and capacity to form motile spores (Goodfellow and Cross, 1984).

Couch (1955a) classified *Streptosporangium* in the family "*Actinosporangiaceae*" together with sporangiate actinomycetes belonging to the genus *Actinoplanes*. The family "*Actinosporangiaceae*" was subsequently renamed *Actinoplanaceae* (Couch, 1955b). In addition to *Actinoplanes*, the type genus, this taxon encompassed the genera *Amorphosporangium*, *Ampullariella*, *Dactylosporangium*, *Kitasatoa*, *Pilimelia*, *Planobispora*, *Planomonospora*, *Spirillospora* and *Streptosporangium* (Couch and Bland, 1974). Members of all of

these genera were considered to form spore vesicles (sporangia). It was subsequently shown that *Planobispora*, *Planomonospora*, *Spirillospora* and *Streptosporangium* formed a DNA homology group that was readily separated from a second aggregate group that encompassed the genera *Actinoplanes*, *Ampullariella* and *Dactylosporangium* (Farina and Bradley, 1970). The two groups were also separated by chemotaxonomic markers. Organisms assigned to the first group contained madurose and had a wall chemotype III whereas those in the second group had a wall chemotype II, that is, they contained *meso*- and/or hydroxy diaminopimelic acid and glycine (Lechevalier *et al.*, 1971). The genera *Actinoplanes*, *Dactylosporangium*, *Micromonospora* and *Pilimelia* are now known to have many properties in common and are classified in the family *Micromonosporaceae* (Krassilnikov, 1938; Goodfellow *et al.*, 1990a). In the meantime the genus *Kitasatoa* has become a synonym of the genus *Streptomyces* (Goodfellow *et al.*, 1986) and the genera *Amorphosporangium* and *Ampullariella* have been reduced to synonyms of the genus *Actinoplanes* (Stackebrandt and Kroppenstedt, 1987).

Goodfellow and Cross (1984) assigned the oligosporic genera *Actinomadura* (H.A. Lechevalier and Lechevalier, 1970), *Microbispora* (Nonomura and Ohara, 1957) and *Microtetraspora* (Thiemann *et al.*, 1968) and the sporangiate genera *Planobispora* (Thiemann and Beretta, 1968), *Planomonospora* (Thiemann *et al.*, 1967), *Spirillospora* (Couch, 1963) and *Streptosporangium* (Couch, 1955a) to an aggregate group, the maduromycetes. Apart from *Spirillospora*, these taxa form a recognisable suprageneric group based on 16S ribosomal RNA cataloguing and sequencing data (Stackebrandt, 1986). The genus *Spirillospora* is currently considered to be a genus in search of a family (Stackebrandt *et al.*, 1981; Goodfellow, 1986, 1989a).

The taxonomic status of the genera assigned to the maduromycetes was formalised with the proposal that *Streptosporangium* be recognised as the type genus of a new suprageneric taxon, the family *Streptosporangiaceae* (Goodfellow *et al.*, 1990a). In addition to the type genus, this family was introduced to accommodate the genera *Microbispora*, *Microtetraspora* (including the *Actinomadura pusilla* group; Kroppenstedt *et al.*, 1990), *Planobispora*, *Planomonospora* and tentatively *Spirillospora*. Recently, a seventh member, *Herbidospora*, has been added (Kudo *et al.*, 1993). Members of the family *Streptosporangiaceae*, which may also include the genus *Planotetraspora* (Runmao *et al.*, 1993), have a pattern of chemical and molecular taxonomic properties that distinguishes them from all other actinomycete families (Goodfellow, 1989a; Kudo *et al.*, 1993; Stackebrandt *et al.*, 1993).

Streptosporangiaceae strains have many chemical features in common but form a morphologically diverse group (Table 5, pages 14 to 15). Nevertheless, strains that bear spore vesicles (*Planobispora*, *Planomonospora* and *Streptosporangium*) are closely related to organisms that form paired (*Microbispora*) or longer spore chains (*Herbidospora* and *Microtetraspora*) but have little in common with sporangiate actinomycetes (*Actinoplanes*, *Dactylosporangium* and *Pilimelia*) classified in the family *Micromonosporaceae* (Goodfellow *et al.*, 1988, 1990a; Stackebrandt *et al.*, 1981, 1983).

It is now apparent that the genus *Streptosporangium* is heterogeneous given scanning electron microscopy studies on the morphology of spores and spore vesicles (Nonomura, 1989), analysis of the electrophoretic mobility of ribosomal protein AT-L30 (Ochi and Miyadoh, 1992) and analyses of 16S rDNA (Kemmering *et al.*, 1993) and 5S rRNA (Kudo *et al.*, 1993). Stackebrandt *et al.* (1993) found that while representative streptosporangia had many chemical properties in common they fell into two groups on the basis of chemical

Table 5 Characteristics of genera classified in the family *Streptosporangiaceae*[*]

	Herbidospora	*Microbispora*	*Microtetraspora*	*Planobispora*	*Planomonospora*	*Spirillospora*	*Streptosporangium*
Morphological characters							
Substrate mycelium	STABLE	STABLE	STABLE	STABLE	STABLE	STABLE	STABLE
Aerial mycelium-spores:							
Absent or in chains	-[a]	+	+	-	-	-	-
Sporangiophores	-	-	-	+	+	+	+
Spore per chain-spore vesicle	MANY	TWO	TWO TO MANY	TWO	ONE	MANY	MANY
Spore motility	-	-	-	+	+	+	-
Temperature range	MESOPHILIC	MESOPHILIC & THERMOPHILIC	MESOPHILIC & THERMOPHILIC	MESOPHILIC	MESOPHILIC	MESOPHILIC	MESOPHILIC
Chemical characters							
Wall chemotype [b]	III	III	III	III	III	III	III
Peptidoglycan type [c]	A1γ	A1γ	A1γ	A1γ	A1γ	A1γ	A1γ
Characteristic sugar	MADUROSE	MADUROSE	MADUROSE	MADUROSE	MADUROSE	MADUROSE	MADUROSE
Fatty acid profile [d]	3c	3c	3c	3c	3c	3a	3c
Predominant menaquinone (MK-) [e]	-10 (H_4)	-9 (H_4)	-9 (H_4)	-9 (H_2, H_4)	-9 (H_4)	-9 (H_4, H_6)	-9 (H_2, H_4)
Phospholipid pattern [f]	IV	IV	IV	IV	IV	I / II	IV
Mole% G+C of DNA	66-71	67-74	64-69	70-71	72	71-73	69-71

Table 5 continued

* Data from Goodfellow (1989a, b, 1991), Goodfellow *et al.*(1990a) Kroppenstedt *et al.*(1990) and Kudo *et al.*(1993).

[a] Symbols : +, present; -, absent.

[b] Major constituents : alanine, glutamic acid, glucosamine, *meso*-diaminopimelic acid and muramic acid (M.P. Lechevalier and Lechevalier, 1970b).

[c] A, cross-linkage between positions 3 and 4 of adjacent peptide subunits; 1, peptide bridge absent; γ, *meso*-diaminopimelic acid at position 3 of tetrapeptide subunits (Schleifer and Kandler, 1972).

[d] Saturated fatty acids, unsaturated fatty acids, *iso*-fatty acids, *anteiso*-fatty acids (variable) and methyl-branched fatty acids (Kroppenstedt, 1985).

[e] *Herbidospora* strains contain tetrahydrogenated menaquinones with ten isoprene units saturated at sites III and IX (Kudo *et al.*, 1993); organisms in the remaining taxa contain tetrahydrogenated menaquinones with nine isoprene units saturated at sites III and VIII (Kroppenstedt, 1982).

[f] Diagnostic phospholipid patterns: I, phosphatidylglycerol (variable); II, only phosphatidylethanolamine; VI, phospholipids containing glucosamine (with phosphatidylmethylethanolamine variable) (Lechevalier *et al.*, 1977).

differences. Most species, including *Streptosporangium corrugatum* and *Streptosporangium roseum*, had a phospholipid pattern type IV and predominant menaquinones of the MK-9 (H_2) and MK-9 (II, VIII-H_4), MK-9 and/or MK-9(H_6) type. The second group, which contained *Streptosporangium albidum* and the subspecies of *Streptosporangium viridogriseum*, were characterised by principal isoprenoid quinones of the MK-9 (II, III-H_4) type and phospholipids typical of pattern II. These results are in excellent agreement with corresponding 16S rRNA sequencing data (Kemmering *et al.*, 1993). Stackebrandt and his co-workers proposed that *Streptosporangium albidum*, *Streptosporangium viridogriseum* subspecies *kofuense* and *Streptosporangium viridogriseum* subspecies *viridogriseum* be assigned to a new genus, *Kutzneria*, as *Kutzneria albida*, *Kutzneria viridogrisea* and *Kutzneria kofuensis*. There is a wealth of evidence to support the classification of the genus *Kutzneria* in the family *Pseudonocardiaceae* (Ochi and Miyadoh, 1992; Kemmering *et al.*, 1993; Kudo *et al.*, 1993; Stackebrandt *et al.*, 1993) though additional work is needed to determine the relationship of this taxon with established members of the family.

The proposal for the genus *Kutzneria* leaves *Streptosporangium* as a homogeneous genus encompassing twelve validly described species. Improved phenotypic tests are needed both for the circumscription of new and established species of *Streptosporangium* and for the rapid identification of streptosporangia if the full potential of these organisms as sources of commercially significant natural products is to be realised.

C. NUMERICAL SYSTEMATICS

1. CLASSIFICATION

It has been repeatedly shown in actinomycete systematics that overreliance on morphological properties can lead to the circumscription of markedly heterogeneous taxa (Bousfield and Goodfellow, 1976; Williams *et al.*, 1983a; Goodfellow and Cross, 1984; Kroppenstedt *et al.*, 1990). The family *Actinoplanaceae* (Couch, 1955b; Couch and Bland, 1974) is a case in point as organisms subsequently shown to be unrelated were assigned to this taxon solely on the basis of a capacity to form spore vesicles. Similarly, strains shown to be only distantly related were classified in the genus *Streptosporangium* only on the basis of a few chemical and morphological properties (Ochi and Miyadoh, 1992; Kemmerling *et al.*, 1993; Stackebrandt *et al.*, 1993). Taxonomies based on single characteristics, or a series of single characteristics, are termed monothetic classifications (Sneath, 1962). These artificial classifications are notoriously unreliable as they have a low information content and cannot readily accommodate strain variation due to mutation or test error (Goodfellow and O'Donnell, 1993). It can, for instance, be difficult to distinguish spore vesicles from aggregates of spores in some actinomycetes (Nonomura and Ohara, 1971b; Nonomura, 1989).

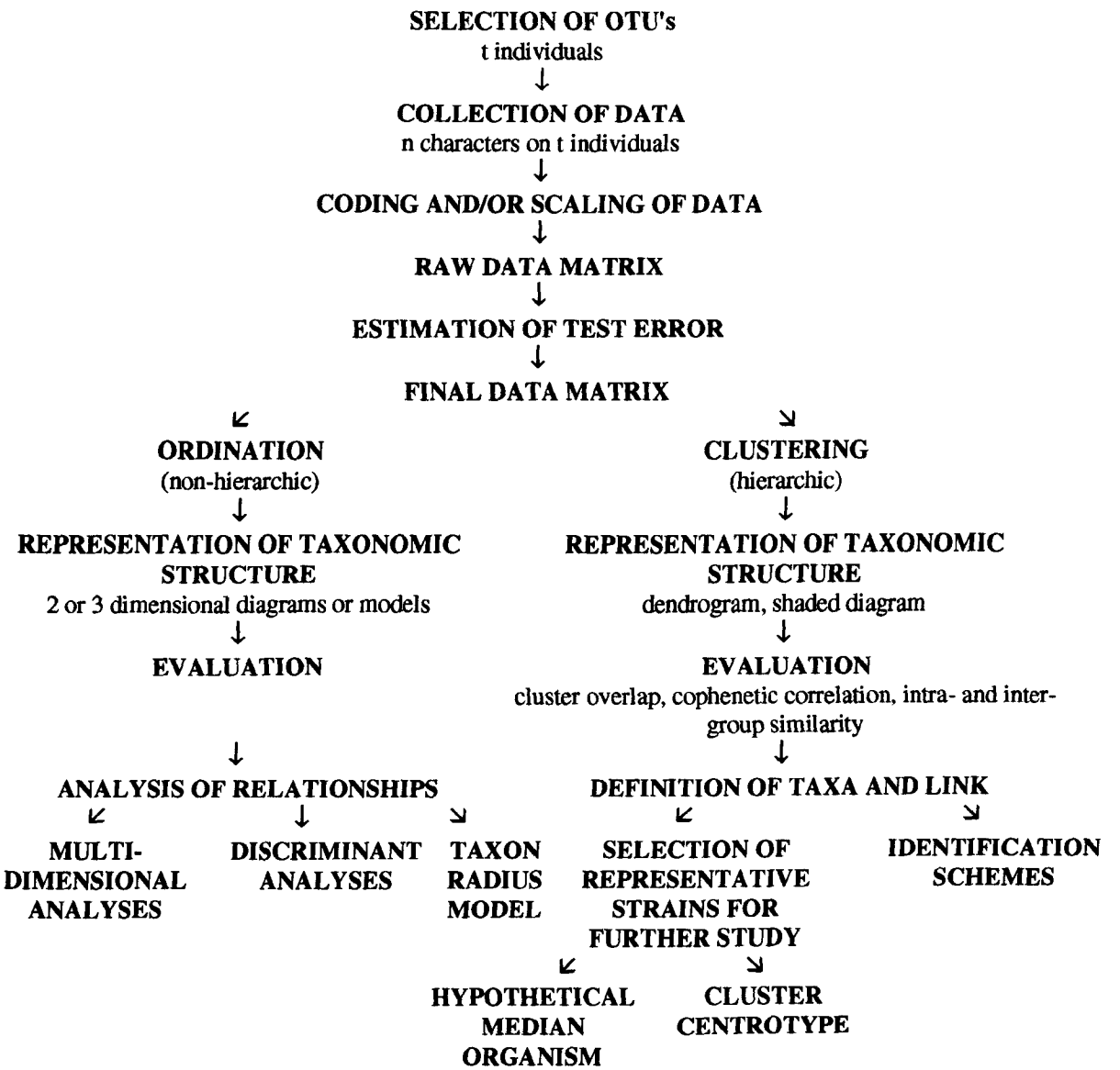
The structural weaknesses of monothetic classifications led some bacterial systematists to believe that stable taxonomies could only be achieved when many bacteria were examined for a large balanced set of properties. Such numerical taxonomies have a high information content and are often described as general purpose classifications *sensu* Gilmour (1937) since they can be of value to many different microbiologists (Goodfellow and O'Donnell, 1993). Sneath (1957a) noted that scientific classification was based upon the assumption that there is a natural order to the microbial world that can be discovered by careful investigation.

A reliable and relatively quick way of establishing centres of variation amongst bacteria is to examine many strains for a large number of equally weighted characters. This is the basis of the numerical taxonomic method introduced to bacteriology by P.H.A. Sneath (1957a, b) and subsequently widely applied (Sneath and Sokal, 1973; Goodfellow and Dickinson, 1985; Macdonell and Colwell, 1985; Sackin and Jones, 1993). The theoretical basis of numerical taxonomy is well documented (Sneath, 1971, 1972; Sneath and Sokal, 1973; Goodfellow, 1977; Sneath, 1978a, b; Sackin and Jones, 1993) and will only be briefly described here. An outline of the operational procedure involved is given in Figure 1, page 19.

Classifications derived from an examination of a large number of organisms and many characters are polythetic as they have high information contents and are based on a complete set of recorded characters not on the presence or absence of series of single characters. Polythetic classifications can accommodate a degree of strain variation and are objective in the sense that they are not sensitive to the addition of more strains or characters.

The entities to be classified, such as strains, species or genera, are referred to collectively as operational taxonomic units (OTU's). The latter should include type strains, well studied reference strains and duplicated cultures to provide a check on test error. In practice, numerical phenetic studies should be based on at least sixty but preferably more strains (Sneath and Sokal, 1973; Sackin and Jones, 1993). It is important to select tests that yield characters that are genetically stable and not sensitive to experimental or observational uncertainties (O'Brien and Colwell, 1987). The usual practice is to choose a set of biochemical, cultural, morphological and physiological characters to represent the entire phenome, that is, the genome and phenotype (Sneath, 1978a, b). It has been recommended that

Figure 1 Operational numerical taxonomic procedure



between 100 and 200 characters should be studied with a lower limit of about 60 (Sackin and Jones, 1993).

The similarities or dissimilarities between test strains can be estimated once the final data matrix has been obtained. Many different resemblance coefficients have been used but only a few have found favour in microbial taxonomy (Sneath, 1972, 1978a; Austin and Colwell, 1977; Sackin and Jones, 1993). The two most commonly used resemblance coefficients are the simple matching (S_{sm}; Sokal and Michener, 1958) and the Jaccard (S_J; Jaccard, 1908) coefficients which measure similarity between OTU's based on binary data. The S_{sm} is used to calculate similarities based on both positive and negative matches whereas with the S_J coefficient negative matches are ignored. Thus, taking two OTU's, x and y, data for all the character states can be summarised as shown below:

		OTU Y	
		+	-
OTU X	+	**a** (++)	**b** (+-)
	-	**c** (-+)	**d** (--)

$$n = a + b + c + d$$

Thus, the S_{sm} coefficient can be defined as Sxy = (a + d) / n, that is, the ratio of the total number of matches to the total number of characters included in the data matrix. Similarly, the S_J coefficient can be defined as Sxy = a / (n - d), that is, the ratio of the total number of positive matches to the total number of characters.

A third and particularly useful coefficient used to estimate relationships is the pattern difference coefficient (D_p; Sneath, 1968). This measures dissimilarity

between strains and omits components of dissimilarity due to differences in vigour. Sneath (1968) divided the total difference between two OTU's into two components termed "vigour" and "pattern" differences. The pattern difference coefficient corresponds to the proportion of the total number of differences between OTU's (D_T) minus the number of differences due to vigour (D_V). These values are defined by the following equations:

$$D_T = \frac{c+b}{n} \qquad D_V = \frac{c-b}{n}$$

The D_p coefficient is expressed as $D_p^2 = D_T^2 - D_V^2 = 4bc / n^2$ in order that the terms are additive and D_p is a positive value.

D_p values can be converted to a measure of similarity, S_p, by using the formula:

$$S_p = (1 - D_p) \times 100$$

Conversion of D_p to similarity values (S_p) facilitates comparisons with classifications based upon the S_{SM} and S_J coefficients. The pattern coefficient has been effectively used in studies on *Actinomadura* (Athalye *et al.*, 1985), *Actinoplanes* (Goodfellow *et al.*, 1990a), *Mycobacterium* (Runyon *et al.*, 1974) and *Streptosporangium* (Whitham *et al.*, 1993) where test strains have included both slow and fast growing organisms.

Operational taxonomic units are assigned to groups (clusters) on the basis of shared similarities. Several techniques are available for clustering organisms into groups (Austin and Colwell, 1977; Sackin and Jones, 1993). The single linkage method is the simplest clustering algorithm as the similarity between two clusters is defined as that of the most similar pair, only one pair per cluster being considered (Sneath, 1957b; Sackin and Jones, 1993). Thus, two clusters may join merely because two constituent OTU's share a higher overall similarity with one another than with any of the remaining test strains. With the single linkage

algorithm, groups of OTU's tend to be linked at relatively low similarities by "chains" of OTU's lying between them. Consequently, this method fails to resolve relatively distinct groups should "intermediate" OTU's be present.

The most commonly used algorithm is the average linkage method, the most popular variant of which is the unweighted pair group method with arithmetic averages (UPGMA; Sokal and Michener, 1958). This algorithm gives equal weight to all of the clusters formed regardless of the numbers of OTU's they contain (Sneath, 1978a). The similarity between two clusters is defined as the average of the similarities between all pairs of OTU's from each cluster. In general, clusters formed using average linkage algorithms are more compact than those based on the single linkage technique.

Hierarchical clustering techniques impose structures on data that may or may not be true representations of the original relationships between OTU's as implied by their similarity values. The suitability of test data for hierarchical clustering can be assessed by determining the cophenetic correlation coefficient (r; Sokal and Rohlf, 1962; Sneath, 1978a; Sackin and Jones, 1993). The cophenetic correlation value between OTU's is the difference between their actual similarities, calculated using any of the various similarity or distance coefficients, and their observed similarities as seen on a hierarchical dendrogram. In practice, complete agreement between dendrograms and resemblance matrices cannot be achieved given the taxonomic distortion introduced when representing multidimensional data in two dimensional form. Typical cophenetic correlation values vary from 0.6 to 0.95 (Jones and Sackin, 1980; Sackin and Jones, 1993). Scores above 0.85 are considered good whereas those below 0.7 imply that only limited confidence can be given to relationships depicted in dendrograms. Farris (1969) demonstrated that of all possible clustering algorithms the UPGMA technique gave the highest

cophenetic correlation values. This observation helps to explain why the UPGMA algorithm is widely employed in numerical phenetic studies.

Once OTU's have been assigned to individual clusters several calculations can be performed to yield taxonomically useful information. The compactness of clusters and the degree of separation between them can be determined from intra- and inter- cluster similarities, respectively. In addition the 95% taxonomic radius of each cluster can be calculated. The latter represents the distance from the centroid of each cluster within which 95% of the members of the cluster would be expected to fall assuming a Gaussian distribution of strains. A high intra-cluster similarity and low 95% taxonomic radius is indicative of a tight, homogeneous cluster and high inter-cluster similarity denotes poor separation of clusters.

Numerical taxonomies, irrespective of the statistics used, are only as good as the data upon which they are based. Test error in polythetic taxonomies, although less serious than in monothetic classifications, serves to lower observed similarities between OTU's and if high erodes the taxonomic structure of the classification (Sneath and Johnson, 1972; Jones and Sackin, 1980; Sackin and Jones, 1993). It is now common practice in numerical taxonomic studies to determine test error by examining duplicated cultures under code and using the average probability of error in an analysis of test variance (Sneath and Johnson, 1972). The assessment of test error is especially important if data from more than one operator is used to generate a numerical classification. In general, inter-operator generation tends to be higher, with values of 8% to 15% (Sneath and Johnson, 1972; Sneath, 1974), than intra-operator test error where values are usually below 4% (Sneath, 1974; Whitham *et al.*, 1993).

Taxonomic structure can also be determined using ordination techniques. Principal components and principal coordinates analyses are two of several ordination techniques available for this purpose (Alderson, 1985). Principal

component analysis (PCA) was first proposed for use with continuous data but the technique can also be applied to binary data (Gower, 1966). Each OTU is represented as a point in multidimensional space where the number of dimensions is equal to the number of variables examined. The points are then projected onto a line through space the direction of which is calculated to represent the maximum variation between OTU's. This line is referred to as the first principal component. A second line is then plotted to account for as much of the remaining variation as possible. Additional principal components are determined in the same way. Scores for the first two or three principal components are often plotted on orthogonal axes and the distribution of OTU's in the 2 or 3 dimensions may reveal valuable information on the taxonomic structure of the OTU's (Dunn and Everitt, 1982). Principal component analysis is only suitable when the distances between OTU's in the original multidimensional data are Euclidean.

An alternative ordination technique known as multidimensional scaling is concerned with the distribution of points in Euclidean space. This method reflects the relationships between OTU's whether Euclidean or not and is achieved using principal coordinates analysis. The results obtained are the same as for PCA when the observed distances are Euclidean. The products of principal coordinates analysis can also be represented in the form of two or three-dimensional plots. The method is considered satisfactory if the variation in the plotted principal coordinates is at least 40% of the original total variation (Sneath and Sokal, 1973; Alderson, 1985).

Ordination techniques have been used successfully to represent relationships between large groups but such analyses can distort affinities between close neighbours (Alderson, 1985). In contrast, hierarchical clustering methods are reliable when depicting relationships between closely related organisms but do

not always satisfactorily represent affinities between large heterogeneous groups (Sneath and Sokal, 1973; Sneath, 1978a).

Numerical classfications need to be interpreted with care as similarity values between strains can be distorted by factors such as test and sampling error, the statistics used, and failure to allow for differences in growth rates and metabolic activity (Sneath and Johnson, 1972; Goodfellow *et al.*, 1979, 1990a). Most confidence can usually be placed in the major centres of variation defined in numerical analyses, it is the relationships of strains lying towards the periphery of clusters that are not always clear (Goodfellow and O'Donnell, 1993). It is, therefore, important to evaluate numerical taxonomies in the context of other taxonomic methods such as chemotaxonomic and molecular systematics. It can also be important to identify OTU's that are most typical of each cluster and therefore suitable for representing clusters in additional studies. The OTU which is the most typical of a phenon and lies closest to the centroid of the cluster is the centrotype, this organism shows the highest average similarity of all the OTU's in the cluster. Centrotype, type and additional representative strains should be included where appropriate in analyses designed to evaluate numerical taxonomies.

In most numerical taxonomic surveys the majority of test strains have been assigned to a small number of major clusters that are often equated with taxospecies (Goodfellow and Dickinson, 1985; Goodfellow *et al.*, 1990a; Goodfellow and O'Donnell, 1993). Single membered clusters that include only a few strains tend to be overlooked. These minor or single membered clusters, however, may represent nuclei of novel groups, genetically unstable strains or organisms of established taxa lacking plasmids (Goodfellow *et al.*, 1987a), and need to be given more consideration when interpreting numerical taxonomies.

2. IDENTIFICATION

Numerical taxonomic surveys provide data on the test reactions of strains within each taxon circumscribed in the classification. Results are usually expressed as the percentage of the strains in each cluster that give a positive result for the characters used to generate the taxonomy. Diagnostic characters can then be selected from the percentage positive frequency table, that is, by *a posteriori* weighting, and used to generate dichotomous keys, diagnostic tables and computer identification matrices. Computer-assisted identification is preferred to conventional keys and tables as it is relatively quick and simple (Lapage *et al.*, 1970; Hill, 1974; Priest and Williams, 1993) with chances of misidentification due to erroneous results greatly reduced (Sneath, 1974a).

Surprisingly few numerical classifications have been supported by probabilistic identification schemes. Theoretically sound, workable schemes are available for the identification of slow-growing mycobacteria (Wayne *et al.*, 1980), neutrophilic streptomycetes (Williams *et al.*, 1983b; Langham *et al.*, 1989), streptosporangia (Whitham, 1988), streptoverticillia (Locci *et al.*, 1986), vibrios (Dawson and Sneath, 1985; Bryant *et al.*, 1986), aerobic, endospore-forming bacilli (Priest and Alexander, 1988; Alexander and Priest, 1990) and for bacteria isolated from Alaskan outer continental shelf regions (Davis *et al.*, 1983). Probabilistic identification schemes using less comprehensive data than is provided by numerical phenetic studies are also available for the identification of Gram-negative aerobic rods (Bascomb *et al.*, 1973), anaerobic bacteria (Kelley and Kellog, 1978) and aerobic endospore-forming bacilli (Willemse-Collinet *et al.*, 1980; Priest, 1989).

The first stage in the generation of a frequency matrix is the selection of a small number of diagnostic tests that are sufficient to differentiate all of the taxa in the numerical taxonomic database. Programs available for this purpose include

CHARSEP (Sneath, 1979b) and DIACHAR (Sneath, 1980a). CHARSEP is used to calculate values for five separation indices, the most useful of which, the VSP index, gives high scores for presumptive diagnostic tests. The DIACHAR program is used to calculate diagnostic scores for each of the characters included in the database with tests then being ranked in order of descending score. The DIACHAR program has been included in the TAXON program which was written in order to facilitate analysis of numerical taxonomic data (Ward, unpublished data, Appendix A). A sound frequency matrix contains sufficient information to define each taxon by several diagnostic properties.

The importance of evaluating identification matrices has been stressed (Sneath and Sokal, 1973; Sneath, 1978b; Williams *et al.*, 1985b; Priest and Williams, 1993). The computer program OVERMAT (Sneath, 1980c) can be used to determine the degree of overlap between taxa represented in identification matrices. Unknown strains cannot be unambiguously identified when there is considerable overlap between clusters. OVERMAT determines both the disjunction index (W) for each pair of taxa (Vg) from the percentage positive data. Any pair of taxa which overlap by more than a chosen cut-off value (Vo), usually 1%, will have a value for W which is less than that for the cut-off point. Further tests selected using the DIACHAR program are then added to the matrix in order to distinguish between taxa that overlap by more than the chosen cut-off value.

The computer program MOSTTYP (Sneath, 1980b) is used to calculate identification scores for the most typical organisms, that is, the hypothetical median organism (HMO), in each constituent cluster. When identification matrices are sound the HMO of each cluster will be assigned to its taxon with very high identification scores. The calculation of identification scores for HMO's can be achieved using the procedure COMPARE in the TAXON program (Ward, unpublished data; Appendix A). Probabilistic identification matrices can be

further assessed by treating strains included in the original numerical taxonomic study as known organisms then calculating identification scores using the original classification data obtained for the diagnostic tests.

The MATIDEN program (Sneath, 1979a) is often used to obtain the best identification scores for known or unknown strains against defined groups in frequency matrices (Williams *et al.*, 1983b; Dawson and Sneath, 1985; Locci *et al.*, 1986; Priest and Alexander, 1988; Alexander and Priest, 1990). Three of the five identification coefficients included in this program are commonly used:

(i) Willcox probability (Willcox *et al.*, 1973). This is the likelihood of an unknown (u) against taxon J divided by the sum of the likelihood's of u against all q taxa. Scores approaching 1.0 denote a good fit between the unknown to a group in the frequency matrix.

$$\text{Willcox probability} = L_{Uj} / \sum_{j=1}^{q} L_{Uj}$$

$$\text{where } L_{UJ} = \prod_{i=1}^{m} [U_i + PiJ - 1]$$

for the m characters considered, where U_i is the character i and P_{iJ} is the proportion of positives for character i of taxon J (Sneath, 1974).

(ii) Taxonomic distance (d): This expresses the distance of an unknown (u) from the centroid of the group with which it is being compared hence low scores indicate relatedness.

$$d = \sqrt{[\sum_{i=1}^{m} (U_i - P_{iJ})^2 / m]}$$

The taxonomic distance should not be significantly more than the 95% taxonomic radius of the cluster (Williams *et al.*, 1983b).

(iii) The standard error of taxonomic distance (S.E. [d]). This coefficient is based on the assumption that the organisms are in hyperspherical normal clusters. An acceptable score is less than about 2.0 to 3.0, and about half of the members of a taxon will have negative scores, that is, they are closer to the centroid than average.

The criteria chosen for a successful identification are somewhat arbitrary (Williams *et al.*, 1985a; Priest and Williams, 1993). Those adopted by Williams *et al.* (1983b) for the identification of streptomycetes were:

(i) A Willcox probability greater than 0.850 with low scores for taxonomic distance and its standard error.
(ii) All first scores significantly better than those for the next best two alternative clusters.
(iii) A small number of characters of the unknown listed as being atypical of those of the cluster in which it was placed.

All of these criteria were derived from the application of the MATIDEN program (Sneath, 1979a). More stringent Willcox probabilities have been recommended for the identification of unknown Gram-negative bacteria (Lapage *et al.*, 1973), corynebacteria (Hill *et al.*, 1978) and slow growing mycobacteria (Wayne *et al.*, 1980).

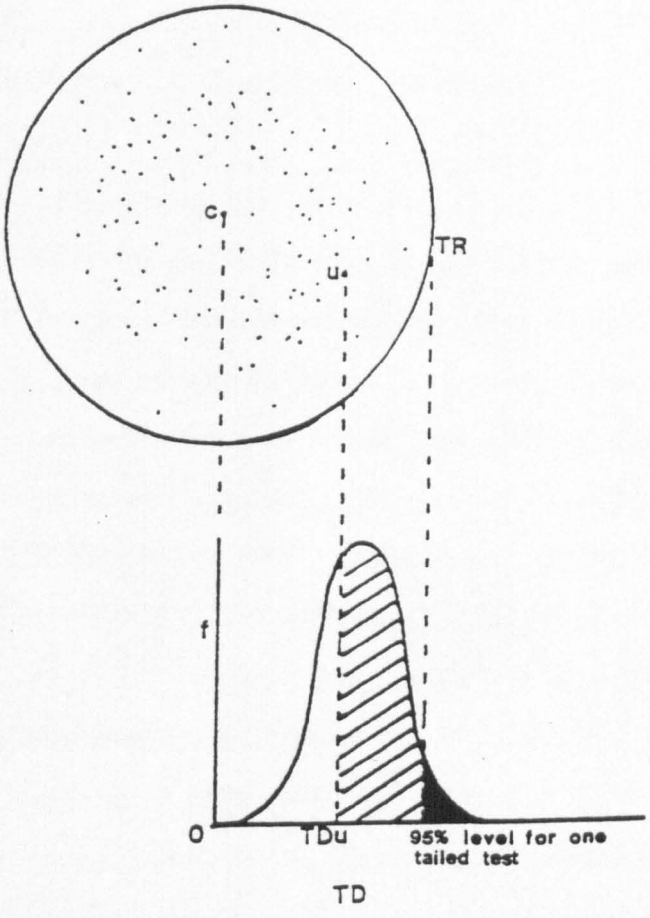
The algorithms used to calculate Willcox probability and taxonomic distance values are part of the IDENTIFY procedure in the TAXON program (Ward, unpublished data; Appendix A). Two additional values, the 95%

taxonomic radius of clusters and the Gaussian distance probability coefficient, can also be used for the identification of unknown strains to taxa included in probabilistic identification matrices. The Gaussian distance probability is a measure of the percentage probability of members of the cluster to which the unknown strain is being compared lying further away from the centroid of that cluster than the unknown strain. The derivation of these two additional coefficients is given in Figure 2, page 31.

The two additional identification values offer some advantages over the existing coefficients as the homogeneity and compactness of each cluster within a frequency matrix is expressed as a numerical value. A high taxonomic distance may be acceptable if the cluster to which an unknown strain is identified is heterogeneous or contains a relatively small number of strains and therefore has a large 95% taxonomic radius. In contrast, compact clusters, that is, those containing a large number of strains with a very high overall similarity will require a low taxonomic distance for good identification. The definition of an acceptable identification should therefore include a comparison between the taxonomic distance of an unknown strain from the cluster centroid and the 95% taxonomic radius of the cluster.

Probabilistic identification procedures allow a probability to be fixed to an identification so that a measure of confidence can be placed in the identification of unknown organisms. In general, such identifications are not normally affected by an occasional erroneous result. Numerical identification procedures can also cope with missing information, that is, adequate identifications can be obtained when only a proportion of tests have been performed. Automated procedures for microbial identification and commercial identification kits are based on the concepts underpinning numerical identification as they require an unknown

Figure 2 Derivation of the 95% cluster radius and Gaussian distance probability



C, cluster centroid; f, number of strains; TR, 95% taxonomic radius; TD, taxonomic distance from cluster centroid; TDu, taxonomic distance of u from cluster centroid and u, unknown strain.

The figure shows a single cluster represented as a number of strains (.) whose phenetic position in multidimensional space is represented in two dimensions. The lower part of the figure shows how the 95% taxonomic radius of the cluster was found using a one-tailed test; a Gaussian distribution of strains is assumed.

The position of the unknown strain (u) is shown. The area under the Gaussian distribution curve having a greater taxonomic distance away from the cluster centroid is hatched. This value, the Gaussian distance probability, is expressed as a percentage of the total area under the curve to give the percent probability of a strain lying further away from the cluster centroid than u.

organism to be compared against information in databases (Priest and Williams, 1993).

3. COMPUTER-ASSISTED CLASSIFICATION AND IDENTIFICATION OF *STREPTOSPORANGIUM* AND RELATED TAXA

a. Classification

Numerical taxonomic procedures have been successfully used in the reclassification of several actinomycete taxa, notably *Actinomadura* (Athalye *et al.*, 1981), *Actinomyces* (Schofield and Schaal, 1981), *Actinoplanes* (Goodfellow *et al.*, 1990a), *Corynebacterium* (Jones, 1975), *Gordona* (Goodfellow *et al.*, 1991), *Mycobacterium* (Goodfellow and Wayne, 1982), *Nocardia* (Goodfellow, 1971; Orchard and Goodfellow, 1980), *Rhodococcus* (Goodfellow *et al.*, 1990b), *Streptomyces* (Williams *et al.*, 1983a; Kämpfer *et al.*, 1991), *Thermomonospora* (McCarthy and Cross, 1984) and *Tsukamurella* (Goodfellow *et al.*, 1991). In contrast, relatively few numerical taxonomic surveys have included, let alone focused on streptosporangia and related strains.

Members of the family *Streptosporangiaceae* have featured, albeit peripherally, in several broadly based numerical phenetic studies (Silvestri *et al.*, 1962; Jones and Bradley, 1964; Goodfellow and Pirouz, 1982) but little credence can be given to numerical taxonomic relationships based on single representatives of specific taxa (Wilkinson and Jones, 1977; Whitham *et al.*, 1993). This is borne out by the fact that *Streptosporangium* strains included in broadly based studies have been reported to have relatively high overall similarities with organisms as taxonomically diverse as *Nocardia* (Silvestri *et al.*, 1962), *Actinoplanes* and *Micromonospora* (Jones and Bradley, 1964) and *Thermomonospora* and *Spirillospora* (Goodfellow and Pirouz, 1982).

It has already been pointed out that streptosporangia and related actinomycetes were the subject of an extensive numerical phenetic survey (Whitham, 1988; Whitham *et al.*, 1993). One hundred and twenty-two streptosporangia, including isolates from natural habitats, and 37 marker strains of *Microbispora*, *Microtetraspora*, *Planobispora*, *Planomonospora* and *Streptosporangium* were examined for 199 unit characters. The data were examined using the pattern, simple matching and Jaccard coefficients and clustering achieved with average (UPGMA), complete and single linkage clustering algorithms. Two reduced data sets were also examined using the same proximity coefficients and the UPGMA clustering algorithm. Good agreement was obtained between the classifications based on the combined data set and with the latter minus the antibiotic sensitivity data. Cluster composition was not markedly affected by the statistics used or by test error, which was low at 2.4%. The product of the Dp, UPGMA analysis on the whole data set was considered in detail and the five aggregate groups defined were equated with the genera *Microbispora*, *Microtetraspora*, *Planobispora* and *Planomonospora*, and *Streptosporangium*. The streptosporangia were recovered in five major, seven minor and twenty single membered clusters. Ten of the multimembered clusters contained putatively novel environmental isolates.

b. Identification

Identification of actinomycetes can be considered as a two-fold process (Goodfellow, 1986, 1989a). Reliable criteria are needed to assign unknown organisms to family and generic rank prior to the use of diagnostic tests for identification to species level and below. Identification to the genus level and above can usually be achieved by using a combination of morphological and chemical properties (Lechevalier, 1989), but few reliable and well tested schemes

are available for the separation of species and biotypes. Most of the recommended procedures tend to be labour intensive and hence are of little value for ecological studies that involve many strains.

Members of the family *Streptosporangiaceae* can be distinguished from all other actinomycetes using a combination of chemical and morphological features. Unidimensional thin-layer chromatographic analysis of whole-organism hydrolysates is used to determine whether an organism contains diaminopimelic acid and, if so, whether this component is in the LL- or *meso*- form (Lechevalier and Lechevalier, 1980; Kroppenstedt, 1985). The presence of *meso*-diaminopimelic acid and madurose with the absence of other characteristic sugars serves to separate *Streptosporangiaceae* strains from those of *Actinoplanes* and related genera, *Nocardia* and related genera, *Pseudonocardia* and related genera, *Nocardiopsis* and *Thermomonospora*, but not from the genera *Dermatophilus* and *Frankia*. The latter can readily be distinguished from *Streptosporangium* and allied taxa on morphological grounds.

In an extension of the survey outlined above a frequency matrix was generated for the identification of unknown *Streptosporangium* strains. Twenty-six characters, selected using the DIACHAR program (Sneath, 1980a), served to distinguish between twelve multimembered numerically circumscribed clusters encompassing streptosporangia. Application of the OVERMAT (Sneath, 1980c) and MOSTTYP (Sneath, 1980b) programs showed the frequency matrix to be theoretically sound.

D. MOLECULAR SYSTEMATICS

The bacterial genome is a rich source of taxonomic data (Stackebrandt and Goodfellow, 1991; Stackebrandt and Liesack, 1993; Palleroni, 1993). DNA sequencing of large sections of the genome should eventually provide data of great value for bacterial systematics but large-scale sequencing studies are currently time-consuming and expensive. However, encouraging results have been obtained from comparative sequence analyses of genes encoding elongation factors EF-Tu and β-subunits of F_1 F_0 type ATP synthases (Amann *et al.*, 1988; Schleifer and Ludwig, 1989; Ludwig *et al.*, 1993).

One of the major limitations of taxonomic methods such as chemotaxonomy is that they are sensitive to changes in the growth regime of the test organisms (O'Donnell, 1988a, b; Suzuki *et al.*, 1993). Thus, when comparing bacteria for chemical markers, such as the discontinuous contribution of fatty acids, it is important that any variation observed is an expression of genetic differences and not a result of the differential effects of the growth conditions. This problem can be contained by growing cultures under identical conditions and, in some cases, to the same stage of the growth cycle (Saddler *et al.*, 1986), but this approach is difficult, indeed sometimes impossible, when physiologically diverse organisms are being compared. In sharp contrast, the chemical composition of chromosomal DNA and RNA is not affected by growth conditions though the amounts of these macromolecules may fluctuate with growth rate. Nucleic acids are, therefore, the only macromolecules that can be used to compare and contrast very diverse microorganisms (Stackebrandt and Goodfellow, 1991).

The bacterial chromosome can be analysed at two levels:

(i) The gross composition of the four nucleotide bases in DNA can be estimated. The guanine (G) plus cytosine (C) content in bacterial DNA varies from 24 to 76 mol% (Stackebrandt and Liesack, 1993).

(ii) Sequence relatedness between DNA macromolecules from two bacteria can be estimated by determining the extent of renaturation of DNA molecules from the two strains in DNA "reassociation" or relatedness experiments (Johnson, 1985b, 1991; Stackebrandt and Liesack, 1993).

The % G+C content of DNA has been determined for members of many bacterial taxa using well described methods (Johnson, 1985a; Owen and Pitcher, 1985; Tamaoka, 1993). Indeed, the analysis of the mean nucleotide composition of DNA (De Ley, 1970; Johnson, 1985 a, b; Tamaoka, 1993) can be considered to be an integral part of the minimal description of bacterial taxa (Lévy-Frébault and Portaels, 1992). It is, however, important that the results of % G+C determinations be interpreted in the light of other taxonomic data as unrelated organisms can have identical base compositions. Generally, strains with DNA base compositions that differ by more than 5% mol G+C should not be classified in the same species and those that differ by more than 10% should not be assigned to the same genus (De Ley, 1970; Owen and Pitcher, 1985; Goodfellow and O'Donnell, 1993). Conversely, two organisms that have DNA with widely different base compositions will only be distantly related. In the first instance, DNA base compositions of representatives of numerically circumscribed clusters provide a way of assessing their homogeneity.

Assessment of base sequence similarity between DNA from two organisms may be readily achieved by DNA reassociation experiments where DNA is rendered into single strands by alkaline or thermal denaturation and subsequently allowed to reanneal in the presence of a second denaturated DNA molecule

(Johnson, 1985b, 1991). If the nucleotide sequences of the two DNA samples are homologous, hybrid duplexes will be formed by base pairing. In contrast, duplex formation will be negligible if there are few sequences in common. The amount of molecular hybrid formed and its thermal stability provides a measure of homology. Heterologous duplexes are less stable than their homologous counterparts due to imperfect base pair matching. It has been estimated that if 1% of the bases are unpaired within a heteroduplex then its thermal melting temperature (T_m value) is lowered by 1 to 1.5% (Britten and Kohne, 1966).

It has been recommended that genomic species should encompass strains with approximately 70% or more DNA:DNA relatedness with a difference of 5°C or less in thermal stability (ΔT_m; Wayne *et al.*, 1987). Values from 30 to 70% reflect a moderate degree of relationship while values become increasingly unreliable once they fall below the 30% level. DNA relatedness experiments, therefore, provide a quantitative estimate of DNA sequence relatedness between organisms and have become the gold standard for the recognition of bacterial species (Goodfellow and O'Donnell, 1993).

Several detailed protocols are recommended for the determination of DNA relatedness (Owen and Pitcher, 1985; Johnson, 1985b, 1991; Stackebrandt and Liesack, 1993). In all cases it is important that DNA reassociation assays be carefully standardised if reproducible results are to be obtained since the extent and specificity of reassociation is influenced by external conditions and the physical state of the DNA. Given careful attention to these parameters DNA reassociation studies are usually accurate and repeatable with congruent results being obtained.

DNA reassociation techniques have limitations. In particular, they do not lend themselves to rapid, automated procedures. Further, due to the labour intensive nature of the work, full similarity (reassociation) matrices with estimates

of DNA relatedness between each and every strain are rare (Grimont *et al.*, 1982; Goodfellow and O'Donnell, 1993). Most DNA:DNA relatedness studies tend to be restricted to comparisons between judiciously chosen reference strains and a variety of test organisms. Generally, this approach is reliable but it is important that reference strains are representative of the taxa under study (Hartford and Sneath, 1988).

DNA relatedness studies have contributed to improved classification of several actinomycete taxa, notably rhodococci (Zakrzewska-Czerwínska *et al.*, 1988), "sporangiate" actinomycetes (Farina and Bradley, 1970; Stackebrandt *et al.*, 1981) and streptomycetes (Mordarski *et al.*, 1986; Labeda and Lyons, 1991a, b; Labeda, 1992). DNA:DNA pairing studies, however, are mainly of value in establishing relationships between closely related species as DNA relatedness values fall to low levels, below 20%, for species that are only moderately different phenotypically (Goodfellow and O'Donnell, 1993).

Ribosomal (r) RNA sequencing analyses and DNA:rRNA pairing studies have been widely used to establish suprageneric relationships between bacteria (Woese *et al.*, 1985; Woese, 1987; Stackebrandt and Liesack, 1993) as base sequences of rRNA cistrons are more highly conserved than most genes in the bacterial genome (Doi and Igarashi, 1965; Dubnau *et al.*, 1965). The methods used to determine DNA:RNA similarities have been extensively reviewed (Kilpper-Bälz, 1991). DNA:rRNA hybridisation techniques have not been as widely used as DNA:DNA relatedness procedures despite their undoubted significance in unravelling relationships between taxa within the class *Proteobacteria* (De Vos *et al.*, 1989).

A major breakthrough in determining relationships between distantly related bacteria was achieved by sequence analysis of linear rRNA. Initially, partial catalogues of oligonucleotide sequences, derived from T1 ribonuclease

digestion of purified 16S rRNA, were generated for test strains (Fox *et al.*, 1977a, b, 1980; Stackebrandt *et al.*, 1980, 1985). Oligonucleotides produced by the digestion procedure were sequenced in their entirety to give nucleotide catalogues. An average catalogue consisted of about 80 fragments (7-20 nucleotides in length) that were evenly distributed over the primary structure accounting for between 35 and 45% of a complete sequence. The relationship between any two given strains was expressed as a similarity coefficient (S_{AB}) calculated on the basis of the proportion of identical nucleotides in the respective catalogues. The resultant matrices of S_{AB} values were examined using appropriate algorithms to generate dendrograms. As new catalogues became available they were compared to all previous catalogues regardless of the laboratory of origin. This continually growing database represented a significant advantage over hybridisation methods as the latter rely on comparisons against a few judiciously chosen reference strains usually at one point in time.

The need to derive more information from 16S rRNA, and to extend sequencing studies to the larger 23S rRNA macromolecules, led to the development of the reverse transcriptase sequencing technique (Qu *et al.*, 1983; Lane *et al.*, 1985). This method was derived from the deoxynucleotide chain-terminating, copying method of Sanger *et al.* (1977). In this method an oligonucleotide is annealed to a template nucleic acid under conditions such that it anneals at only one location. The oligonucleotide serves as a primer for the synthesis of a DNA copy of the template extending from the 3' terminus of the primer that is incorporated into the reverse transcript. The template strand is copied from 3' to 5' and the synthesised DNA strand from 5' to 3'. Inclusion of low levels of one dideoxynucleotide triphosphate in the reaction together with all four deoxynucleotide triphosphates results in the random termination of reverse transcriptase at positions corresponding to the nucleotide component of that

dideoxynucleotide in the template strand. The sequences obtained can be used to calculate homology values for the construction of phylogenetic trees, and to choose primers for amplification of 16S DNA genes using the polymerase chain reaction (Saiki *et al.*, 1988; Ludwig, 1991).

5S rRNA has also been sequenced for taxonomic purposes (Fox and Stackebrandt, 1987; Hori and Osawa, 1986; Stackebrandt and Liesack, 1993) but the small size of this molecule has tended to detract from its value in measuring distant phylogenetic relationships. Nevertheless, comparative 5S rRNA sequencing studies have been used to clarify relationships between closely related bacteria, including actinomycetes (Park *et al.*, 1987a, b, 1991, 1993). Two techniques are applied to achieve the necessary base-specific cleaves of RNA, namely the chemical (Peattie, 1979; Waldmann *et al.*, 1987; Zhang *et al.*, 1987) and enzymatic methods (Donis-Keller *et al.*, 1977; Krupp and Gross, 1979, 1983).

The genus *Streptosporangium* has been the subject of few molecular systematic studies. Stackebrandt *et al.* (1993) undertook 16S rDNA / RNA analyses of five members of the genus *Streptosporangium.* The type species *Streptosporangium roseum, Streptosporangium nondiastaticum* and *Streptosporangium pseudovulgare* formed an highly related cluster with *Streptosporangium corrugatum* peripherally associated. In contrast, *Streptosporangium viridogriseum* subspecies *viridogriseum* fell within the radiation of the family *Pseudonocardiaceae* showing a close similarity to *Saccharothrix australiensis.* The results were in good agreement with those of an earlier study on the electrophoretic mobility of ribosomal proteins which also showed that streptosporangia could be assigned to three groups (Ochi and Miyadoh, 1992). The first group contained *Streptosporangium albidum, Streptosporangium viridogriseum* subspecies *kofuense* and *Streptosporangium viridogriseum* subspecies *viridogriseum*, the second *Streptosporangium album,*

Streptosporangium amethystogenes, *Streptosporangium fragile*, *Streptosporangium nondiastaticum*, *Streptosporangium roseum*, *Streptosporangium violaceochromogenes* and *Streptosporangium vulgare*, and the third *Streptosporangium corrugatum*. Kudo *et al.* (1993) carried out 5S rRNA sequencing studies on *Herbidospora* strains and found that one of them had very similar sequences to those of *Streptosporangium roseum* and *Planobispora longispora* strains.

E. CHARACTERISATION OF STREPTOSPORANGIA

1. RAPID ENZYME TESTS

a. Background

The discontinuous distribution of enzymes between members of microbial species can provide information of value for classification and identification (Manafi *et al.*, 1991; Goodfellow and James, 1993; James, 1993). Indeed, for the best part of a century, diagnostic tests have been used which are dependent on the presence or absence of particular enzymes. Most early enzymatic tests were applied empirically, their underlying biochemical basis only becoming clear at a later date. Early biochemical tests included examination for enzymes of the hydrolase, lyase and oxidoreductase groups, as well as for the end-products of metabolic pathways.

The use of enzymes as taxonomic markers offers advantages over some other taxonomic methods. These include ease of performance, flexibility in a variety of situations such as in agar and liquid media, ability to test diverse organisms in the same study, for example, fast and slow growing organisms in the same microtitre plate, and the capacity to acquire data quickly. In addition, tests designed to detect individual enzymes may be rapidly performed and are simple in operation often without the need for reagent additions.

One of the outstanding properties of enzymes is their specificity. Some enzymes have almost absolute specificity for a given substrate and do not attack even closely related molecules; others are less so and act on a class of compounds. The specificity of enzymes combined with their ability to catalyse reactions of substrates at low concentrations is significant in biochemical evaluation. As with other bacterial proteins, the type of enzyme produced by members of a particular taxon is an expression of their genetic potential though microorganisms can regulate the amount and activity of the enzymes that they produce. Thus, for example, enzyme tests carried out on an organism grown on different culture media can give differing results due to the induction of catabolic enzymes or the repression of biosynthetic pathways hence standardisation of enzyme test procedures is essential.

b. Enzymes as Taxonomic Markers

Enzymes are not distributed uniformly amongst prokaryotes hence their discontinuous distribution can be weighted for classification and identification. Among the oxidoreductases, catalase, cytochrome oxidase and nitrate reductase have long been useful for classification and identification. Catalase is present in most cytochrome containing aerobic and facultatively anaerobic bacteria, with the notable exception of *Streptococcus* species, and is responsible for the decomposition of hydrogen peroxide to water and oxygen. The usual test procedure is simply to add hydrogen peroxide to a colony and observe the emission of oxygen bubbles (McFaddin, 1980). However, aromatic amines and phenols may also be used as oxygen acceptors. Hanker and Rabin (1975) developed a test whereby dopamine and phenylenediamine were oxidised to a coloured derivative by hydrogen peroxide in the presence of catalase.

The cytochrome oxidase enzyme mediates the oxidation of reduced cytochrome by molecular oxygen which in turn acts as an electron acceptor in the terminal stage of the electron transfer system. In this test, tetramethylphenylenediamine is oxidised by molecular oxygen in the presence of cytochrome C to give a coloured compound, Wurster's blue (Kovacs, 1956). Nitrate reductase catalyses the reduction of nitrate to nitrite. The nitrite reductase test relies on the formation of an azo dye from nitrite, sulphanilic acid and naphthylamine (Bachmann and Weaver, 1951).

Enzymes of the lyase group that are commonly used in systematics include decarboxylases, tryptophanase and tryptophan deaminase. The decarboxylase enzymes are numerous and each is totally specific for a given substrate. In bacterial classification and identification arginine, lysine and ornithine are generally used since they are decarboxylated to produce diamines which may be readily detected using a pH indicator. Tryptophanase and tryptophan deaminase form products which may be readily detected. Indole produced by the former reacts with 4-dimethylaminobenzaldehyde to give a red-violet compound (McFaddin, 1980) and phenylpyruvic acid produced by the latter complexes with ferric chloride to produce a green colouration (Blazevic and Ederer, 1975).

One of the first tests to be used in bacterial classification which involved the detection of a hydrolase enzyme was that for β-glucosidase. The naturally occurring compound esculin (6-*O*-β-D-glucosyloxy-7-hydroxycoumarin) was used as the test substrate (Meyer and Schönfeld, 1926). This compound is cleaved in the presence of β-glucosidase and the 6, 7-dihydroxycoumarin (esculetin) released forms a dark-brown chelate with ferric ions in the growth medium.

In the search for new diagnostic tests chromogenic hydrolase substrates developed for biochemical applications were adapted for bacterial identification. Phenolphthalein diphosphate has been used to detect alkaline phosphatase activity

(Lewis, 1961) and *O*-nitrophenyl-β-D-glucoside has been employed as a rapid test for lactose fermenting bacteria (Lowe, 1962). Muftic (1967) demonstrated the value of aminopeptidases in the classification of mycobacteria; naphthylamide substrates were employed and free naphthylamine released in the reaction was visualised with a diazonium salt.

Fluorescent techniques provide a much more sensitive way of detecting enzyme activity than colorimetric based procedures. The latter may be adapted for fluorometry by adding a fluorophore with a spectrum that is quenched by a product of the enzymatic reaction or by using fluorescent indicators, notably 7-amino-4-methylcoumarin (7-AMC) and 4-methylumbelliferone (4-MU). When conjugated derivatives of these molecules are cleaved by the relevant hydrolytic enzyme the parent molecules are released. The latter are intensely fluorescent in the visible region of the electromagnetic spectrum whereas the corresponding derivatives are only weakly fluorescent in this region (Figure 3, page 45).

Maddocks and Greenan (1975) introduced a simple test procedure that involved the use of 4-methylumbelliferyl glucosides to differentiate between *Escherichia coli* and *Pseudomonas aeruginosa* strains. A restricted number of fluorogenic probes have been used to classify and identify actinomycetes, notably gordonae (Goodfellow *et al.*, 1991), mycobacteria (Grange, 1978; Grange and Clark, 1977; Slosarek, 1980), streptomycetes (Goodfellow *et al.*, 1987b) and a range of carboxydotrophic and mycolic acid containing actinomycetes (Goodfellow *et al.*, 1988, 1990b, 1991; O'Donnell *et al.*, 1993). The ability of actinomycetes to produce endo- and exo-peptidases from 7-amino-4-methylcoumarin conjugated substrates has also been demonstrated (Goodfellow *et al.*, 1987c, 1990b, 1991). These preliminary studies indicate that fluorogenic probes prepared from 7-AMC and 4-MU provide a simple, rapid and inexpensive way of detecting specific enzymes in small amounts of whole actinomycetes.

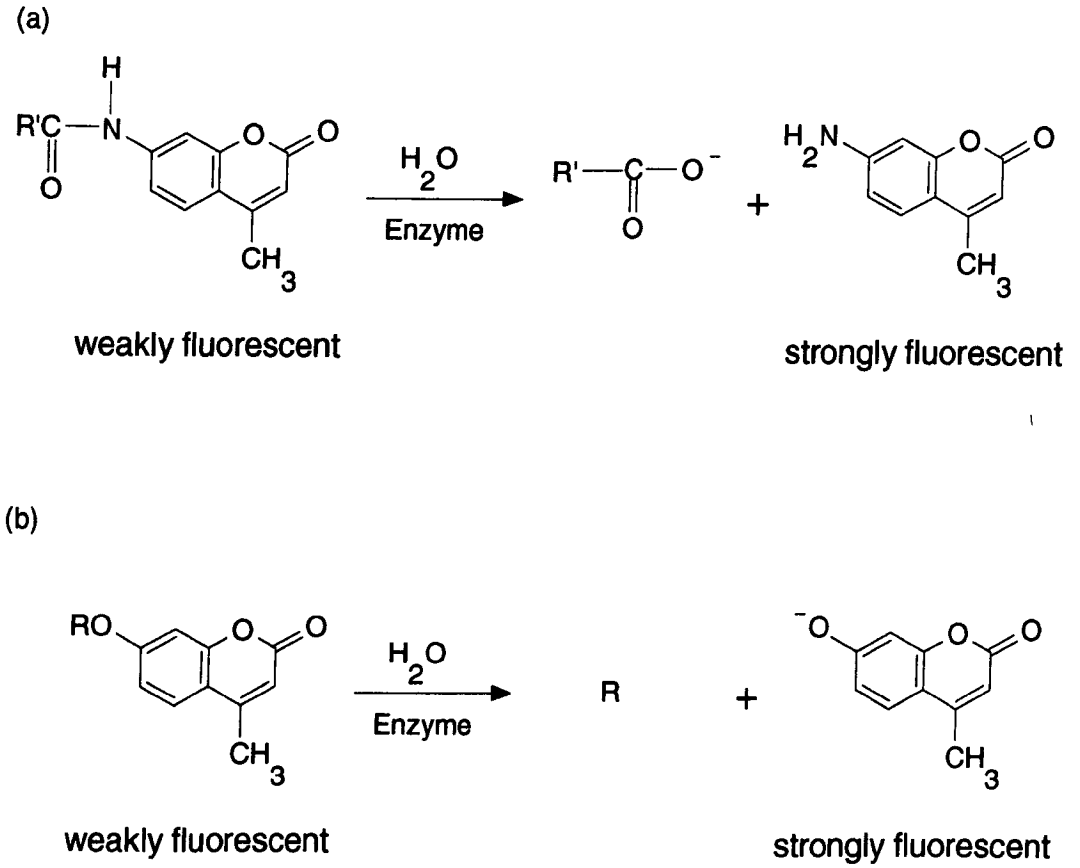


Figure 3 Cleavage of conjugated substrates to release (a) 7-amino-4-methylcoumarin and (b) 4-methylumbelliferone.

Abbreviation: R, fatty acid or sugar; R', amino acid or peptide chain.

c. Types of Biochemical Tests

1) Colorimetric Liquid Media

Originally most biochemical tests were carried out using bottles or tubes containing broth test media. Preparation time and costs made it impossible to carry out effective test schemes routinely on large numbers of isolates and in an effort to solve the problem diagnostic companies developed test kits. The latter are usually based on a series of microcupules containing dehydrated sterile media. A suspension of test organism is added to these and the strip or plate incubated. After reagent addition, if required, colour formation is estimated visually or measured using a colorimeter. Most tests in kits used to be of the classical biochemical type but these have been replaced by chromogenic hydrolase substrates. Esterase and glycosidase substrates are usually based on *para*-nitrophenol although absorption of the free chromophore is fairly weak making it difficult to see low levels of hydrolysis with the naked eye.

In the Zyme kit marketed by API (API System S.A., La Balme Les Grottes, France) the esterase and glycosidase substrates are based on naphthylamine. The free amine or phenol released in the reactions are coupled with a diazonium salt to produce a strongly absorbing chromophore. Zyme kits have found widespread use in the classification and identification of various groups of actinomycetes, including *Actinomyces* (Kilian, 1978), *Brevibacterium* (Freney *et al.*, 1991), *Gordona* (Goodfellow *et al.*, 1991), *Nocardia* (Boiron and Provost, 1990), *Rhodococcus* (Goodfellow *et al.*, 1991), *Streptomyces* (Kämpfer *et al.*, 1991a, b) and carboxydotrophic actinomycetes (O'Donnell *et al.*, 1993). This system provides information on nineteen enzyme activities in four hours. Encouraging results have also been obtained with the API Coryne strip developed for the identification of Gram-positive rods, notably clinically important brevibacteria and corynebacteria (Freney *et al.*, 1991).

2) Multipoint Inoculated Agar Plates

Many biochemical tests are carried out using agar plates. An indicator incorporated in the medium or a reagent added after incubation leads to the production of coloured rings around positive test colonies. With multipoint inoculation devices up to forty different microbial strains may be inoculated onto a single agar plate leading to inexpensive data acquisition.

Mast (Mast International Ltd., Bootle, U.K.) market an identification system based on agar plates. Results may be interpreted by eye, more objectively, using an image analyser. Unfortunately, some reaction products readily diffuse through the media and confusion can occur if a coloured zone spreads over adjacent colonies. Tests that generate highly insoluble products are, therefore, preferred. Esculin hydrolysis is useful in this respect as the ferric-esculetin complex produced is poorly soluble and forms an easily discernible ring around positive colonies. Another chelating compound, 8-hydroxyquinoline, has been used for enzyme testing (Fishman and Green, 1955). It produces a highly insoluble black chelate with a large molar extinction coefficient. James and Yeoman (1987, 1988) examined members of the family *Enterobacteriaceae* with 8-hydroxyquinoline-β-D-glucoside and 8-hydroxyquinoline-β-D-glucuronide in agar plates and achieved much more compact zones around positive colonies than were obtained with esculin.

3) Fluorometric Testing

Fluorescence of molecules is caused by absorption of electromagnetic radiation, i.e. ultraviolet, visible and infra-red, leading to promotion of electrons from the ground to an excited state. The latter has a finite lifetime during which some loss of vibrational energy occurs. The residual energy is either lost by collisional deactivation or by re-emission of radiant energy (fluorescence). The

loss of vibrational energy means that fluorescence energy is less than the energy of absorption and the wavelength maximum correspondingly longer than the absorption maximum. Absorption of energy leads to electronic excitation.

In most cases substrate specificity is wide enough to permit a variety of groups on one side of the bond to be broken. Artificial substrates may thus be used with a chromophore or fluorophore attached to the group of interest. A chromophore should have strong absorption capacity to maximise sensitivity and it is beneficial if this absorption is in the visible spectrum. Generally, wavelength and intensity of absorption are increased if conjugation in the molecule is increased. Electron donating groups such as the halogens also increase the wavelength of absorption. If, however, another molecule is attached to the amino or hydroxy groups the electron donating effect is reduced and absorption is at a lower wavelength. A substrate formed by such a linkage will have a lower wavelength of absorption than the free chromophore so that enzyme activity may be readily followed.

Fluorescence is enhanced by electronic conjugation and by the planarity (flat, stable nature) of the molecule. It is for this reason that most highly fluorescent substances have rigid aromatic ring structures. Fluorescent emission is shifted to longer wavelength by electron donating groups and fluorogenic substrates may be created in a similar manner to their chromogenic counterparts. The coumarinic compounds 7-AMC and 4-MU have structural features which predispose towards fluorescence. These include planarity, rigidity, electron delocalisation *via* an efficient conjugation system and the presence of at least one electron releasing group.

A restricted range of 4-MU linked substrates have been used in the classification and identification of actinomycetes including mycobacteria (Grange, 1978; Grange and Clark, 1977; Hamid *et al.*, 1993), renibacteria (Goodfellow *et*

al., 1985) and streptomycetes (Goodfellow *et al.*, 1987b). Kenneth *et al.* (1992) have developed a rapid and sensitive assay for chitinolytic activity based on the use of fluorogenic 4-methylumbelliferone glycosides of N-acetylglucosamine oligosaccharides in order to characterise the enzymes associated with chitinolytic microorganisms, notably exo- and endo-chitinases and N-acetylglucosamine. Similarly, enzyme activity profiles of mycobacteria and related actinomycetes have been obtained using peptide hydrolase substrates based on 7-amino-4-methylcoumarin (Goodfellow *et al.*, 1987c; Hamid *et al.*, 1993).

Fully automated systems are needed to determine the full potential of rapid enzyme tests in bacterial systematics. Microscan (Sacramento, California, U.S.A.) and Sensititre Ltd. (East Grinstead, U.K.) market automated systems. With the Sensititre procedure conjugated enzyme substrates are held in microtitre plates and after inoculation results are read quantitatively using a fluorometric plate reader attached to a computer for instant data acquisition. The raw data are interpreted using appropriate software. A variant of the Sensititre system has been developed and applied to the classification and identification of rapidly growing mycobacteria and nocardiae (Hamid *et al.*, 1993). This latter procedure was used in the present study to assign unidentified streptosporangia from soil to artificial groups.

d. Enzymes as Taxonomic Markers in Streptosporangial Systematics

Little attempt has been made to determine the enzymatic profile of representatives of the genus *Streptosporangium*. The restricted number of enzyme tests currently used in streptosporangial systematics (Nonomura, 1989; Goodfellow, 1991) can be attributed to the traditional emphasis placed on morphological and physiological criteria and to the problem of detecting small amounts of end product in large volumes of test media.

The most comprehensive study to date was carried out by Whitham *et al.* (1993) who examined one hundred and twenty-two streptosporangia, including isolates from soil, and 37 marker strains of *Microbispora*, *Microtetraspora*, *Planobispora*, *Planomonospora* and *Streptosporangium*, for their ability to cleave twenty-six 4-MU conjugated derivatives. Some of the tests were of presumptive diagnostic value for the delineation of the taxa classified in the family *Streptosporangiaceae*. Most of the microbisporae were characterised by their ability to cleave 4MU-β-D-glucuronide; the planobisporae and planomonosporae were unusual in cleaving 4MU-pyrophosphate. All of the streptosporangia were active against 4MU-2-acetamido-2-deoxy-β-D-galactopyranoside, 4MU-β-D-fucopyranoside, 4MU-β-D-galactopyranoside, 4MU-α-D-glucopyranoside and 4MU-β-D-glucopyranoside, but gave different patterns of reaction with 4MU-2-acetamido-2-deoxy-β-D-glucopyranoside, 4MU-N-acetyl-β-D-glucosamide, 4MU-α-L-arabinofuranoside, 4MU-α-L-arabinopyranoside, 4MU-β-D-cellobiopyranoside, 4MU-α-D-galactopyranoside, 4MU-β-D-glucuronide, 4MU-α-D-mannopyranoside, 4MU-β-D-mannopyranoside, 4MU-bisphosphate, 4MU-pyrophosphate, 4MU-heptanoate, 4MU-nonanoate and 4MU-palmitate indicating that these enzymes were of potential value for the classification and identification of streptosporangia.

2. CHEMOSYSTEMATICS

Simple rapid methods have been developed to detect the distribution of taxonomically useful components of the bacterial wall peptidoglycan. These qualitative techniques are widely used for primary classification and identification of actinomycetes as they can be applied to large numbers of strains (Schaal, 1985; Williams *et al.*, 1989; Suzuki *et al.*, 1993). More analytical techniques have been used to elucidate the primary structure of actinomycete cell walls but they involve

procedures that are not readily applicable to more than a few strains (Schleifer and Kandler, 1972; Schleifer and Seidl, 1985; Kodama *et al.*, 1992; Hancock, 1993). However, quantitative data on wall amino acid composition can be obtained relatively quickly by gas chromatography (O'Donnell *et al.*, 1985).

Qualitative analyses of components of cell walls and whole-organisms led to the classification of actinomycetes into eight aggregate groups or wall chemotypes based on the discontinuous distribution of major wall amino acids and sugars (Table 6, page 52; Becker *et al.*, 1965; Lechevalier *et al.*,1966b; Lechevalier and Gerber, 1970; M.P. Lechevalier and Lechevalier, 1970a, b; Minnikin and O'Donnell, 1984; Suzuki *et al.*, 1993). Members of the family *Streptosporangiaceae* contain *meso*-DAP as the major wall diamino acid but lack any characteristic sugars and hence belong to wall chemotype III (M.P. Lechevalier and Lechevalier, 1970a, b). The isomers of diaminopimelic acid are key chemical markers particular since the detection of the LL-isomer distinguishes streptomycetes from all other sporoactinomycetes. Current methods for the detection of DAP by thin layer chromatography (TLC) are not satisfactory as it can be difficult to distinguish the LL-, DD- and *meso*-DAP isomers from one another on TLC plates.

Peptidoglycans have been classified according to their chemical composition by Ghuysen (1968) and Schleifer and Kandler (1972). Both classifications stress the importance of the mode of cross linkage, but the tri-digital system proposed by Schleifer and Kandler (1972) is the one that is commonly used (Suzuki *et al.*, 1993). The variation in peptidoglycan composition has provided invaluable information for the reclassification of actinomycetes (Goodfellow and Cross, 1984), notably for the so called coryneform actinomycetes (Goodfellow, 1989a). Members of the family *Streptosporangiaceae*

Table 6 Cell wall chemotypes of some actinomycete taxa*

Wall Chemotype	Major Constituents**	Families / Genera
I	LL-diaminopimelic acid, glycine	*Streptomycetaceae*
II	*meso*-diaminopimelic acid, glycine	*Micromonosporaceae*
III A	*meso*- diaminopimelic acid, madurose	*Dermatophilaceae* *Frankiaceae* *Streptosporangiaceae*
III B	*meso*- diaminopimelic acid	*Brevibacteriaceae* *Thermomonosporaceae*
IV A	*meso*- diaminopimelic acid, arabinose, galactose, mycolic acids	*Corynebacteriaceae* *Mycobacteriaceae* *Nocardiaceae*
IV B	*meso*- diaminopimelic acid, arabinose, galactose	*Pseudonocardiaceae*
V	lysine, ornithine	*Actinomyces israelii*
VI	lysine (aspartic acid, galactose)***	*Microbacterium* *Oeskovia*
VII	diaminobutyric acid, glycine (lysine)	*Agromyces* *Clavibacter*
VIII	ornithine	*Curtobacterium* *Cellulomonas*

*, Data modified from M.P. Lechevalier and Lechevalier (1970b) and Goodfellow and O'Donnell (1989).

, All wall preparations contain major amounts of alanine and glutamic acid; *, variable constituents.

have a peptidoglycan type A1γ (Schleifer and Kandler, 1972; Nonomura, 1989; Goodfellow, 1991).

Lipid analyses have yielded valuable data for bacterial classification and identification (Mayer *et al.*, 1985; Tornabene, 1985; Jantzen and Bryn, 1993), especially for actinomycete systematics (Minnikin, 1982; Minnikin and O'Donnell, 1984; O'Donnell, 1985, 1988a, b; Saddler *et al.*, 1987; O'Donnell *et al.*, 1993; Suzuki *et al.*, 1993). Most attention has focused on the use of fatty acids, isoprenoid quinones and polar lipids as chemical markers.

A functional plasma membrane requires the presence of a suitable mixture of both relatively fluid and solid fatty acids esterified to polar head groups. Several different types of fatty acid mixtures are found in actinomycetes. At one extreme, straight chain fatty acids occur with fluid monounsaturated components and acids biosynthetically derived from unsaturated fatty acids such as tuberculostearic and cyclopropane acids (e.g. *Actinomyces*, *Corynebacterium* and *Mycobacterium*). In contrast, other actinomycetes have *iso*- fatty acids as their main relatively solid base although smaller amounts of straight chain components are usually present. The fluid elements in patterns of this type are composed solely of *anteiso*-fatty acids (e.g. *Actinopolyspora*, *Cellulomonas*, *Oerskovia*, *Thermomonospora*, *Saccharomonospora* and *Streptomyces*).

Members of the genus *Streptosporangium* have complex fatty acid patterns consisting of major amounts of saturated straight chain, *iso*- and methyl- branched fatty acids and minor and variable amounts of unsaturated and *anteiso*- acids, respectively (Kroppenstedt, 1985; Poschner *et al.*, 1985; Kudo *et al.*, 1993; Whitham *et al.*, 1993). There is also evidence that quantitative analyses of fatty acids extracted from representative strains of the family *Streptosporangiaceae* grown under carefully standard conditions will yield valuable data for the

classification and identification of the constituent taxa (Mertz and Yao, 1990; Kudo *et al.*, 1993; Stackebrandt *et al.*, 1993).

Information of value for actinomycete systematics has also been derived from two-dimensional thin-layer chromatographic analyses of polar lipids (Minnikin and O'Donnell, 1984; Suzuki *et al.*, 1993). These lipids form the structural basis of bacterial plasma membranes, phospholipids are the most common type found in actinomycetes. Phospholipids commonly detected in actinomycete wall envelopes include phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE) and phosphatidylinositol (PI). Methylated derivatives of PE, such as phosphatidylmethylethanolamine (PME) and phosphatidylcholine (PC) are less commonly encountered. Other polar lipids of diagnostic potential which lack phosphorus groups include the amphipatic glycolipids and acylated long-chain ornithine and lysine amides (Minnikin and O'Donnell, 1984). Actinomycetes have been assigned to five groups on the basis of the discontinuous distribution of certain nitrogenous phospholipids (Lechevalier *et al.*, 1977, 1981; Goodfellow, 1989b).

Polar lipid data provide further evidence of a close relationship between the genera *Microbispora*, *Microtetraspora*, *Streptosporangium*, *Planobispora* and *Planomonospora*; strains in these taxa have a type IV phospholipid pattern *sensu* Lechevalier *et al.* (1977), that is, they contain glucosamine. Whitham *et al.* (1993) found that nearly all of their streptosporangia contained major amounts of diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol with most also characterised by the presence of phosphatidylinositol, phosphatidylinositol mannosides and phosphatidylmethylethanolamine. Similar findings were reported from earlier studies (Kroppenstedt, 1985; Mertz and Yao, 1990) though the failure of some investigators (Lechevalier *et al.*, 1977;

Hasegawa *et al.*, 1979) to detect phosphatidylglycerol can be attributed to the use of different media and cultivation regimes.

Actinomycetes can also be assigned to several aggregate groups on the basis of the types of menaquinones found in their plasma membranes. Menaquinones vary in the length and degree of hydrogenation of double bonds of their isoprene units (Collins and Jones, 1981; Collins, 1993; Suzuki *et al.*, 1993). Initial menaquinone analyses provided qualitative or semi-quantitative information (Minnikin *et al.*, 1978; Minnikin and O'Donnell, 1984) but it is now commonplace to derive quantitative profiles by using high performance liquid chromatography (Collins *et al.*, 1984; Kroppenstedt, 1982, 1985; Suzuki *et al.*, 1993). Quantitative menaquinone data, however, have to be interpreted with care as Saddler *et al.* (1986) found that the menaquinone composition of *Streptomyces cyaneus* NCIB 9616 varied at different stages of the growth cycle. Most *Streptosporangiaceae* strains have profiles rich in MK-9 (H_0), MK-9 (H_2) and MK-9 (H_4) (Poschner *et al.*, 1985; Stackebrandt *et al.*, 1993; Whitham *et al.*, 1993). Members of the genus *Streptosporangium* are characterised by the presence of major amounts of di- and tetra-hydrogenated menaquinones with nine isoprene units (Collins *et al.*, 1984; Kroppenstedt, 1985; Mertz and Yao, 1990; Kudo *et al.*, 1993; Stackebrandt *et al.*, 1993; Whitham *et al.*, 1993).

The sites of hydrogenation of the isoprenyl side chains of menaquinones also provide useful taxonomic information. Prior to the application of mass spectrometry/mass spectrometry (MS/MS; Tamaoka, 1987) the hydrogenated positions of bacterial menaquinones were determined by NMR analysis. Di-, tetra-, hexa- and octahydrogenated menaquinones tend to be hydrogenated at positions 'II', 'II and III', 'II, III and VIII', and 'II, III, VIII and IX', respectively (Suzuki *et al.*, 1993). The order of hydrogenation would appear to be 'II >III >VIII >IX'. Streptosporangia contain major amounts of tetrahydrogenated

menaquinones with nine isoprene units hydrogenated at positions II and VIII (Stackebrandt *et al.*, 1993).

3. PYROLYSIS MASS SPECTROMETRY

The need to classify, identify and type microorganisms is a central theme in microbiology but many of the methods currently used for these purposes are complex, expensive in labour and materials and quite often give poor reproducibility. There is a real need to develop rapid, reproducible and cost-effective methods for the classification, identification and typing of microorganisms. It has already been demonstrated that chemical and molecular techniques have helped to generate a lot of new information for microbial classification and identification (Goodfellow and O'Donnell, 1993) and have provided valuable data for the revision of many bacterial taxa, including those assigned to the order *Actinomycetales* (Williams *et al.*, 1989; Kroppenstedt *et al.*, 1990; Manchester *et al.*, 1990; Ochi and Miyadoh, 1992; Stackebrandt *et al.*, 1993; Whitham *et al.*, 1993). However, many of these techniques are relatively expensive in both cost per sample and in time. This is not the case with analytical chemical techniques, notably Curie-point pyrolysis mass spectrometry (Magee, 1993a, b).

Curie-point pyrolysis mass spectrometry (PyMS) of whole-organisms was introduced by Meuzelaar (1974) to facilitate rapid characterisation and identification of microorganisms. Pyrolysis, the thermal degradation of material in an inert atmosphere or vacuum, leads to the production of volatile fragments from non-volatile materials such as microorganisms. Under controlled conditions the breakdown is reproducible and the fragments are characteristic of the original material hence the pyrolysate is a "fingerprint" of the original sample. Following pyrolysis, the fragments are ionised then separated by mass spectrometry. The

method involves organisms taken directly from growth media. Sample preparation and total running time are very fast and inexpensive. The analytical system for pyrolysis and data acquisition can also be automated thereby allowing the pyrolysis of multiple samples.

Pyrolysis mass spectrometry was first used to investigate complex biopolymers such as albumin and pepsin (Zemany, 1952). Interest soon waned in PyMS as workers turned to pyrolysis gas-liquid chromatography (PyGS) as this system was less expensive (Davison *et al.*, 1954). Pyrolysis gas-liquid chromatography was used to separate molecules on the basis of their relative polarity, that is, the constituent components of the pyrolysate were separated by the difference in retention time of each fragment in the chromatographic column. This system was used in the late 1960's and early 70's (Gutteridge and Norris, 1979) but it's application to microbial systematics was limited given problems of long-term reproducibility, lack of speed and inadequate data handling facilities (Gutteridge, 1987). An automated PyGC system was subsequently developed, but not used, for the detection of possible extra-terrestrial life in the Surveyor series of lunar landings (Wilson *et al.*, 1962).

The National Aeronautics and Space Administration (NASA) later developed a pyrolysis mass spectrometer for the detection of microorganisms in dust samples on Mars. The technique involved heating lunar dust samples and analysing the resultant degradative products but convincing traces of organic material were not found (Gutteridge and Norris, 1979). An improved pyrolysis mass spectrometer was introduced by Meuzelaar and Kistemaker (1973) for the characterisation of complex biological samples including microorganisms. The procedure was seen as rapid, universally applicable, and relevant for the characterisation of organisms at generic, specific and subspecific levels but the two commercially available machines gave data that were poorly reproducible. In

addition, the cost of running the instruments was high and the analysis of samples could not be achieved in less than 5 minutes.

A major breakthrough in pyrolysis mass spectrometry came with the introduction of the Horizon PyMS-200X, an instrument based on the PyMS quadrupole mass spectrometric system of Prutee Limited (Aries *et al.*, 1986). The superior performance of this machine over the earlier models can be attributed to an improved electron multiplier, which led to faster analysis times (2 minutes per sample), enhanced reproducibility and an upgraded statistical package for data analysis. The operational procedure has been described in detail by Magee (1993a, b) and hence will not be considered here.

Curie-point pyrolysis mass spectrometry is increasingly being applied in microbial systematics (Magee, 1993a, b). The procedure has been used to confirm the homogeneity within and discrimination between taxa circumscribed using conventional taxonomic methods (French *et al.*, 1989). Good correlation was found when PyMS data were compared with those obtained using conventional clustering techniques, for example, in comparative taxonomic studies of *Fusobacterium ulcerans* (Adriaans and Shah, 1988; Magee *et al.*, 1989b). In some instances, however, as Gutteridge and Norris (1979) anticipated, pyroclassifications differ from more conventionally based taxonomies thereby providing another perspective on the classification of test organisms (Hindmarch *et al.*, 1990).

Excellent results have been obtained in applications of PyMS to microbial identification. Pyrolysis mass spectrometric identification of clinically significant mycobacteria (Wieten *et al.*, 1981a, b; 1983) was shown to be reliable given > 90% agreement with conventional procedures in discriminating between organisms in the *Mycobacterium tuberculosis* complex. In these studies challenge sets were compared with marker organisms included in each batch of analyses, a technique

termed operational fingerprinting. Identification of staphylococci to species using the PyMS procedure also showed around 90% agreement with conventional identification systems (Magee *et al.*, 1983; Hindmarch and Magee, 1987). Saddler *et al.* (1989) used PyMS in the selection of unusual actinomycetes for pharmacological screening programmes.

The most important application of Curie-point PyMS, to date, has been in the area of microbial epidemiology (Magee, 1993a). A broad range of species have been studied including *Bacteroides ureolyticus* (Duerden *et al.*, 1989), *Candida albicans* (Magee *et al.*, 1988), *Mycobacterium xenopi* (Sisson *et al.*, 1992), *Pseudomonas aeruginosa* (Sisson *et al.*, 1991), *Salmonella enteritidis* (Freeman *et al.*, 1990a) and *Staphylococcus epidermidis* (Freeman *et al.*, 1991). Studies of three *Streptococcus pyogenes* outbreaks (Magee *et al.*, 1989a; Freeman *et al.*, 1990b) showed almost total agreement with conventional typing; interest in this species reflects the dire consequences of misjudgement in potential hospital outbreaks. Many authors cite the speed and lack of necessity for species-specific modification of methods with PyMS as clear advantages over traditional techniques.

The application of PyMS to actinomycete systematics has mainly been restricted to examination of a few clinically significant isolates. Meuzelaar *et al.*(1976) were able to distinguish between several mycobacterial species but were unable to show differences between *Mycobacterium avium*, *Mycobacterium bovis* and *Mycobacterium xenopii* strains. Wieten *et al.*(1981a, b) was able to separate *Mycobacterium bovis*, *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis* strains using a PyMS generated database derived from a small set of molecular masses. Curie-point pyrolysis mass spectrometry has also been used to characterise *Mycobacterium kansasii* (Wieten *et al.*, 1979), *Mycobacterium leprae*

(Wieten *et al.*, 1982) and *Mycobacterium* strains (Sisson *et al.*, 1992), and to distinguish between *Corynebacterium* species (Hindmarch *et al.*, 1990).

Pyrolysis mass spectrometry has also been used in the classification, identification and typing of industrially significant actinomycetes (Saddler *et al.*, 1988; Sanglier *et al.*, 1992). Sanglier *et al.* (1992) pyrolysed members of representative actinomycete genera in order to study the effects of media design, incubation time, sample preparation and the effects of sporulating versus non-sporulating actinomycete strains on experimental data. It was concluded that reproducible data could be obtained given vigorous standardisation of growth and pyrolysis conditions. These workers were also able to distinguish between actinomycete strains at and below the species level. In particular, representatives of three closely related *Streptomyces* species, namely *Streptomyces albidoflavus*, *Streptomyces anulatus* and *Streptomyces halstedii* were distinguished. The separation of these numerically circumscribed streptomycete species (Williams *et al.*, 1983a; Kämpfer *et al.*, 1991a; Goodfellow *et al.*, 1992) suggests that PyMS may be a useful way of evaluating clusters defined in numerical taxonomic surveys.

It is essential to perform multivariate statistical analyses to interpret pyrolysis mass spectral data. The mathematical procedure involves several stages; pre-processing to remove the effects of variation in the amount of sample analysed; univariate analysis to remove masses with poor reproducibility and some form of multivariate analysis to resolve the complex statistical structure of the remaining data to yield results that can be used for either classification or identification. It can be difficult to represent the large amounts of information derived from PyMS studies and this may necessitate further processing to obtain three dimensional scatter plots and dendrograms. Much of this data handling can now be rapidly performed on micro-computers using commercial software.

Statistical packages used to analyse PyMS data include ARTHUR (Kowalski, 1975), BMDP (Dixon, 1975), GENSTAT (Nelder, 1979) SIMCA (Wold, 1976) and SPSS (Nie *et al.*, 1975).

The multivariate technique, principle components analysis, is commonly used to analyse PyMS data. Principle components analysis involves data reduction to obtain a two or three dimensional graphical representation of multidimensional data in order to display relationships within datasets and to detect outliers (Gutteridge *et al.*, 1979; McFie and Gutteridge, 1982; Shute *et al.*, 1985; Magee, 1993a, b). Canonical variate analysis is used in PyMS to determine relationships between the groups defined by principle components analysis. In PyMS analysis replicate samples, usually three, of a single strain are considered as a group. Canonical variate analysis minimises intra-replicate variation and maximises differences between strains.

The use of artificial neural networks (ANNs) for the analysis of pyrolysis mass spectral data provides an even more robust and effective approach towards pattern recognition (Goodacre *et al.*, 1992; Chun *et al.*, 1993a, b; Freeman *et al.*, 1993). Artificial neural networks are composed of processing elements which are analogous to neurons in the brain. Each of the processing elements (PE) has a number of weighted inputs that are summed to give the internal activation level of the PEs. Artificial neural networks use many processing elements which are usually arranged in three layers: an input layer, one or more hidden layers and an output layer. Such networks learn by modifying the weights or strengths of the connections between the PEs though finding ways of teaching/training networks were a major stumbling block during early research (Morris and Boddy, 1992). The artificial neural network system offers considerable advantages over current practice in the analysis of PyMS data as it is not necessary to examine duplicate or triplicate samples or to analyse reference and unknown strains in a single run.

Artificial neural networks are increasingly being used to detect complex, non-linear relationships in multivariate data. They have been used to analyse data generated by PyMS (Goodacre *et al.*, 1992) in order to discriminate between bacterial species (Bungay and Bungay, 1991), in fermentation control (Lant *et al.*, 1990), and in gene (Wu *et al.*, 1990) and protein classification (Qian and Sejnowski, 1988; Wu *et al.*, 1991). In the case of bacterial identification the underlying rationale is that it is possible to acquire sets of multivariate data from representatives of taxa and train ANNs using the known identities as the derived outputs. Once neural networks have been trained they can be used to discriminate between the taxa under study (Chun *et al.*, 1993a, b; Freeman *et al.*, 1993). Artificial neural networks have been shown to provide a rapid, reliable and cost-effective method of identifying *Streptomyces* species (Chun *et al.*, 1993a, b).

F. SELECTIVE ISOLATION OF STREPTOSPORANGIA

1. BACKGROUND

Actinomycetes are a successful group of bacteria that live in a variety of natural and man-made environments. Most are strict saprophytes but some form parasitic or mutualistic associations with animals and plants (Goodfellow and Williams, 1983; Williams *et al.*, 1984b; Schaal and Lee, 1992). The primary natural reservoir of actinomycetes is soil where strains are believed to have a role in the recycling of nutrients (Williams *et al.*, 1984b; McCarthy and Williams, 1992). Soil particles carrying spores are widely dispersed which means that actinomycetes can be isolated from most natural habitats.

Actinomycetes are usually isolated from environmental samples by applying appropriate selective pressures at various stages of the dilution plate procedure (Williams and Wellington, 1982; Williams *et al.*, 1984a; Goodfellow and Williams, 1986; Goodfellow and O'Donnell, 1989; Bull *et al.*, 1992).

Samples may be taken at random or from habitats where the microbial community is adapted to relatively extreme external factors. It is usually necessary to eliminate or reduce fungal and unwanted bacterial growth on isolation media in order to selectively isolate actinomycetes from natural habitats. Fungal contaminants are usually controlled by supplementing isolation media with antifungal antibiotics such as cycloheximide (actidione; Phillips and Hanel, 1950; Cork and Chase, 1956; Porter *et al.*, 1960), nystatin or pimaricin (Porter *et al.*, 1960; Tsao and Thieleke, 1966).

2. CHOICE OF ENVIRONMENTAL SAMPLES

Many thousands of actinomycetes have been isolated from the environment but relatively little is known about the ecological or geographical distribution of even the better known neutrophilic streptomycetes (Goodfellow and Simpson, 1987; Bull *et al.*, 1992). Ecological studies have been few in number and have tended to concentrate on the detailed taxonomic analysis of a limited number of strains isolated from a few environmental samples (Orchard and Goodfellow, 1980; Goodfellow *et al.*, 1990a; Whitham *et al.*, 1993). The lack of convincing evidence for the production of antibiotics and other useful metabolites in nature compounds this problem (Williams, 1982). Given this situation, it is difficult to predict the sites where particular actinomycete taxa or strains occur. Notable exceptions include the association of thermotolerant and thermophilic actinomycetes involved in the turnover of self-heating composts and other vegetable material (Lacey, 1988) and the coprophilous life cycle of *Rhodococcus coprophilus* (Rowbotham and Cross, 1977). Nevertheless the selection of macro- or micro- habitats as a source of commercially useful actinomycetes remains something of a lottery.

3. PRETREATMENT OF SAMPLES

A wide range of pretreatment regimes have been used to enhance the isolation of actinomycetes from environmental samples (Cross, 1982; Williams *et al.*, 1984a; Goodfellow and O'Donnell, 1989). These include physical and chemical treatments or enrichments of samples. Many pretreatment regimes exert a clear selectivity for the isolation of members of particular actinomycete taxa, but the scientific basis for their action is rarely evident. A novel pretreatment method involves the addition of a mixture of polyvalent streptomycete phage to soil suspensions to selectively reduce the growth of streptomycete colonies on isolation plates and thereby raise the proportion of other genera isolated (Williams and Vickers, 1988).

Membrane filtration and centrifugation can be used to concentrate actinomycete propagules in soil, water and sediment samples (Trolldenier, 1966; Goodfellow and Haynes, 1984). Similarly, nutrient enrichment of environmental samples and soil suspensions have been used to increase the number of streptomycetes and streptosporangia prior to isolation (Williams and Mayfield, 1971; Nonomura and Hayakawa, 1988). A useful departure from the dilution plate procedure involves the isolation of thermophilic actinomycetes from dry, self-heating plant material. The latter is shaken in a wind tunnel (Gregory and Lacey, 1963) or sedimentation chamber (Lacey and Dutkiewicz, 1976) and the spore clouds obtained impacted onto surface-dried isolation plates held in an Andersen (1958) sampler. This method has been successfully used to isolate mycolateless wall chemotype IV actinomycetes which are a source of several important antibiotics (Whitehead, 1989).

Hirsch and Christenson (1983) used membrane filters to reduce the number of contaminating bacteria on isolation plates. Soil dilutions were inoculated onto the surface of membrane filters placed onto the surface of isolation media. After

incubation for three days, the filters were removed and the plates re-incubated for a further seven days. Actinomycete spores can germinate and grow through pores in membrane filters onto the media below whereas non-mycelial bacteria are restricted to the upper surface of filters and are therefore removed with them.

A variety of baiting procedures have been developed for the selective isolation of specific actinomycetes from environmental samples. The "baits", once colonised, are removed and placed onto plates of nutrient media for subsequent culturing and examination. A variety of baits have been employed for the isolation of members of the genera *Planobispora, Planomonospora*, *Spirillospora* and *Streptosporangium* (Couch, 1954; Schäfer, 1973; Goodfellow, 1991a). A method based on the chemotactic response of motile spores was successfully used to isolate *Spirillospora* strains from soil (Palleroni, 1980).

Actinomycete propagules are more resistant to desiccation than most vegetative bacteria so that the simple practice of air-drying soil samples, prior to plating suspensions onto suitable selective media, can significantly increase the chances of isolating spore-forming actinomycetes, especially when isolation media are not highly selective (Meiklejohn, 1957; Williams *et al.*, 1972; Hunter, 1978; Nonomura and Hayakawa, 1988; Hayakawa *et al.*, 1991). Resistance to desiccation is usually accompanied by a measure of heat resistance; dry soils can be heated to over 100°C to reduce the number of unwanted bacteria in order to enhance the recovery of rare actinomycetes (Nonomura and Ohara, 1969a; Athalye *et al.*, 1981). Less extreme heat pretreatment regimes have been recommended for the isolation of micromonosporae (Goodfellow and Haynes, 1984), nocardiae (Orchard, 1975), rhodococci (Rowbotham and Cross, 1977) and streptomycetes (Williams *et al.*, 1972; Goodfellow and Haynes, 1984). It is not known why actinomycete propagules such as spores (e.g. streptomycetes), spore vesicles (e.g. streptosporangia) and hyphal fragments (e.g. rhodococci) are more resistant to

heat and desiccation than vegetative cells of Gram negative bacteria. Some of the physical pretreatments used to isolate members of the family *Streptosporangiaceae* are shown in Table 7, page 67.

The effectiveness of chemical pretreatment regimes depends on the higher resistance of actinomycete propagules to antimicrobial compounds such as chlorine (Burman *et al.*, 1969), phenol (Speer and Lynch, 1969; Pantier *et al.*, 1979; Nonomura and Hayakawa, 1988) and quaternary ammonium compounds (Phillips and Kaplan, 1976; Du Moulin and Stottmeier, 1978). Although toxic such compounds have been found useful in the reduction of unwanted bacteria on isolation plates. Sodium dodecyl sulphate (0.05%) and yeast extract (6%) have also been used inhibit the growth of bacteria, they enhance germination of actinomycete spores (Nonomura and Hayakawa, 1988).

4. CHOICE OF ISOLATION MEDIA

The selectivity of an isolation medium is influenced by its nutrient composition, pH, the addition of selective inhibitors and by incubation conditions (Williams *et al.*,1984a; Hayakawa and Nonomura, 1987a). These factors determine which fractions of natural populations from environmental samples will compete successfully and thereby be recovered on selective isolation media. Innumerable media formulations have been recommended for the isolation of actinomycetes in general and for selected genera in particular.

Surprisingly, many "general" or "non-selective" media were designed without regard either to the nutrient properties or tolerances of the target actinomycetes. Thus, media such as colloidal chitin (Lingappa and Lockwood, 1961; Hsu and Lockwood, 1975), half-strength nutrient (Gregory and Lacey, 1963), glucose-asparagine (Crook *et al.*, 1950), glycerol-arginine (Benedict *et al.*, 1955), M3 (Rowbotham and Cross, 1977) and starch-casein-nitrate agars (Küster

Table 7 Pretreatment regimes used for the selective isolation of members of the family *Streptosporangiaceae*

Treatment	Substrate	Isolation Medium	Isolates	References
Baiting				
Paspalum grass	Soil	3%, w/v agar	*Streptosporangium* (also *Actinoplanes*)	Couch (1954, 1955a, 1963)
Pollen	Soil and water	3%, w/v agar	*Spirillospora* (also *Actinoplanes*)	
Pollen and hair	Soil	3%, w/v agar	*Planobispora, Planomonospora*	Bland and Couch (1981)
Chloride ions	Soil	Starch-casein agar	*Spirillospora* (also *Actinoplanes*)	Palleroni (1980)
Physical				
Air-dry and heat at 100°C or 120°C for 1 hour	Soil	Arginine-vitamins agar supplemented with actidione and nystatin (50 µg/ml)	*Microbispora, Streptosporangium*	Nonomura and Ohara (1969a, b)
Air-dry and heat at 100°C or 120°C for 1 hour	Soil	Glucose-asparagine with soil extract agar supplemented with actidione (50 µg/ml) and polymixin B (4 µg/ml)	*Microtetraspora*	Nonomura and Ohara (1971a, b)
Air-dry and heat at 120°C for 1 hour and treated 10^{-1} dilution with 1.5% phenol	Soil	Humic acid-vitamins agar supplemented with actidione (50 µg/ml) and nalidixic acid (30 µg/ml)	*Microbispora*	Hayakawa and Nonomura (1988)
Air-dry and heat at 120°C for 1 hour	Soil	Humic acid-vitamins agar supplemented with actidione (50 µg/ml) and nalidixic acid (30 µg/ml)	*Streptosporangium* (also *Dactylosporangium*)	Nonomura (1984), Hayakawa and Nonomura (1988)
Air-dry and heat at 120°C for 1 hour and treated 10^{-1} dilution with 0.01% benzethonium chloride	Soil	Humic acid-vitamins agar supplemented with leucomycin (1.0 µg/ml) and nalidixic acid (30 µg/ml)	*Streptosporangium*	Hayakawa *et al.* (1991)
Desiccate at 28°C for 1 week	Soil	Yeast extract agar supplemented with actidione (50 µg/ml) and nystatin (50 µg/ml)	*Herbidospora*	Kudo *et al.* (1993)

and Williams, 1964) are widely used though little attempt has been made to evaluate their effectiveness (Williams *et al.*, 1984a). This point was clearly demonstrated when the use of various objectively formulated selective media resulted in the isolation of different streptomycete species from the same sand dune sample (Vickers *et al.*, 1984). The media recommended by Nonomura and Ohara (1969a, b) for the isolation of *Microbispora* and *Streptosporangium* were empirical.

It is now apparent that most "non-selective" media recommended for the isolation of actinomycetes are selective for streptomycetes, notably for those belonging to the *Streptomyces albidoflavus* (*griseus*) group (Vickers *et al.*, 1984). This observation helps to explain why actinomycetes such as streptosporangia have only occasionally been isolated on conventional isolation media such as cellulose, chitin, starch-ammonium or starch-casein agars (Van Brummelen and Went, 1957; Potekhina, 1965; Willoughby, 1969b; Williams and Sharples, 1976).

5. TAXONOMIC APPROACH TO SELECTIVE ISOLATION

Selective media can be designed on objective criteria given recent improvements in actinomycete systematics (Vickers *et al.*, 1984; Williams *et al.*, 1984a; Goodfellow and O'Donnell, 1989; Bull *et al.*, 1992). Indeed, numerical taxonomic databases, which contain extensive information on the biochemical, nutritional, physiological and antibiotic sensitivity profiles of constituent actinomycete taxa are ideal resources for the formulation of isolation media deemed selective for industrially significant actinomycete taxa. Further, improved diagnostic methods allow selective isolation media to be evaluated as representative colonies can be identified with confidence.

a. Formulation and Evaluation of New Selective Media

The discovery that Diagnostic Sensitivity Test agar supplemented with tetracyclines was selective for *Nocardia asteroides* (Orchard and Goodfellow, 1974) was based on antibiotic sensitivity data (Orchard, 1975; Orchard and Goodfellow, 1974) and the product of an earlier numerical phenetic survey (Orchard and Goodfellow, 1980). A logical extension of this work was to visually scan percentage positive frequency tables for antibiotics that could form the basis of selective isolation media. In one such numerical taxonomic survey streptoverticillia were found to have a higher resistance to neomycin and oxytetracycline than neutrophilic streptomycetes (Williams *et al.*, 1985a). This observation raised the possibility of reducing the number of streptomycetes, and other unwanted bacteria on isolation plates, with a view to isolating streptoverticillia, a commercially significant group of actinomycetes rarely recovered on "non-selective" isolation media. Hanka *et al.* (1985) were able to raise the proportion of *Streptoverticillium* colonies on isolation plates using an agar medium supplemented with oxytetracycline and the membrane-filter stripping method of Hirsch and Christenson (1983). Hanka and Schaadt (1988) further enhanced the recovery of streptoverticillia by the addition of lysozyme, as well as oxytetracycline, to the agar medium.

Another approach to selective isolation involves tailoring media to the nutritional requirements of target organisms. This has also been achieved by taking the relevant information from numerical taxonomic databases. The most extensive studies have been carried out by Williams and his colleagues (Vickers *et al.*, 1984; Williams *et al.*, 1984a; Williams and Vickers, 1988) who used particular combinations of carbohydrate and amino acid, with and without antibacterial antibiotics, to favour the growth of uncommon streptomycetes previously shown to be a promising source of antibiotically active metabolites, or

to discourage the growth of the ubiquitous *Streptomyces albidoflavus*. The selective agents were chosen objectively by examining the neutrophilic streptomycete database (Williams *et al.*, 1983b) using the DIACHAR program (Sneath, 1980a). The highest diagnostic scores were given by characters which were all positive or negative for strains in one cluster when compared with all of the other numerically defined taxa (Vickers *et al.*, 1984).

Other workers have also used the growth responses of actinomycetes to sole nitrogen and/or carbon sources to selectively isolate other antibiotic-producing taxa. To this end, selective media have been designed to isolate specific fractions of the acidophilic streptomycete microflora (Goodfellow and Simpson, 1987) and mycolateless wall chemotype IV actinomycetes (Whitehead, 1989) found in natural habitats (Table 8, page 71).

It is essential to evaluate, and if necessary modify, computer-generated media formulations by altering combinations of selective agents in order to enhance the recovery of target organisms (Goodfellow and O'Donnell, 1989). With neutrophilic streptomycetes, the employment of objectively designed media, and subsequent computer-assisted identification of isolates, showed that it was possible to increase the number of particular species and decrease others but not with all of the soils tested (Vickers *et al.*, 1984). It was noted that some species increased or decreased in a manner that could not be predicted by comparison with information in the data matrix. This is not altogether surprising for the surface of an isolation plate is the site of intense competition between many bacterial colonies and the outcome of the struggle for survival will not only be influenced by the composition of the medium but also by the mix of species in the inoculum that are able to grow. This apparent problem can be turned to good advantage when selective pressures generated by the medium reveal the presence of rare and novel species in soil samples. Thus, the role of computer-assisted

Table 8 Objectively formulated media based on selective agents derived from numerical taxonomic databases using the DIACHAR program

Selective Agents	Target Strains	References
Adenine and streptomycin	'*Saccharopolyspora*'-like organisms	Goodfellow (unpublished data)
Raffinose and histidine	*Streptomyces chromofuscus*, *S. cyaneus* and *S. rochei*	Vickers *et al.* (1984), Williams *et al.* (1984a)
Rifampicin	*Streptomyces atroolivaceus*, *S. diastaticus*	Vickers *et al.* (1984), Williams *et al.* (1984a)
Sodium chloride	*Streptomyces albidoflavus*, *S. atroolivaceus*	Williams and Vickers (1988)
Rifampicin	Acidophilic streptomycetes-cluster 25	Simpson (1987)
Butylene glycol, chlorotetracycline HCl and sodium chloride	*Rhodococcus rhodochrous*-cluster 8	Thomas (1991)
Arginine, glycerol and starch	*Streptomyces lividans*	Herron and Wellington (1990)
Neomycin, streptomycin, thiostrepton, viomycin	*Streptomyces lividans*	Wellington *et al.* (1990)
Aminobutyric acid and rhamnose	*Streptomyces albidoflavus*, *S. violaceusniger*	Williams and Vickers (1988)

methods in the development of targeted selective media must not be underestimated. Indeed, such methods are a vital element for the isolation of novel and target isolates from natural habitats.

6. INCUBATION REGIMES

The temperature and length of incubation also contribute to selectivity. Incubation at 25°C to 30°C favours mesophilic bacteria and incubation at 55°C enhances the chances of isolating thermotolerant and thermophilic actinomycetes. Novel and unusual isolates may be overlooked unless incubation periods are extended. Nonomura and Ohara (1971a) succeeded in isolating several new species of less common actinomycete genera when they incubated isolation plates at 30°C and 40°C for up to one month. Little attention has been paid to the selective isolation of psychrophilic, anaerobic or autotrophic actinomycetes which represent potential sources of commercially important novel compounds. However, large numbers of carboxydotrophic actinomycetes have been isolated using carbon monoxide as a sole carbon source and shown to form a distinct and taxonomically diverse group (Falconer *et al.*, 1993; O'Donnell *et al.*, 1993).

7. SELECTION OF COLONIES

The ability to recognise members of actinomycete taxa on primary isolation plates requires the use of a microscope fitted with a high powered, long working distance objective. Continued developments in image analysis and the application of sophisticated software may pave the way for automated systems capable of recognising certain colony types directly on isolation plates. However, in general, it is not possible to distinguish between members of species of the same genus on isolation plates, a fact which may result in serious duplication and wasted effort in industrial screening programmes. Much subsequent duplication of effort can be

avoided by a preliminary grouping of isolates. With some genera this can be achieved very easily. Thus, most streptomycete soil isolates, grouped together on the basis of their easily determined pigmentation characteristics, were identified to same cluster (taxospecies) in a frequency matrix designed for the identification of unknown streptomycetes (Williams and Vickers, 1988).

8. SELECTIVE ISOLATION OF STREPTOSPORANGIA

Isolation procedures may combine one or more selective regimes (Goodfellow and Williams, 1986; Goodfellow and O'Donnell, 1989). Thus, enrichment or pretreatment of environmental samples is often followed by incubation on an appropriate selective isolation medium. Nonomura and Ohara (1969a) introduced a combined technique for the selective isolation of streptosporangia from soil. Air-dried soil samples were passed through a 2mm sieve, ground using a pestle and mortar, spread on filter paper and heated in a hot air oven at 120°C for one hour prior to the preparation of soil dilutions and surface incubation of arginine-vitamins (AV) agar plates (Nonomura and Ohara, 1969a). Inoculated plates were incubated at 30°C for four to six weeks when colonies bearing spore vesicles on an abundant aerial mycelium, that is, streptosporangia, were observed on isolation plates.

Nonomura and Ohara (1969a) isolated streptosporangia from several Japanese soils using arginine-vitamins (AV) medium in numbers up to 2.0×10^4 colony forming units per gram dry weight of soil. They also recovered large numbers of *Microbispora* strains, 2.8×10^4 colony forming units per gram dry weight, from the same soils. Hayakawa and Nonomura (1987a, b) successfully used humic acid-vitamins agar for the isolation of large numbers of actinomycetes belonging to the genera *Dactylosporangium*, *Microbispora*, *Micromonospora*, *Microtetraspora*, *Nocardia*, *Streptomyces*, *Streptosporangium* and

Thermomonospora. Humic acid-vitamins (HV) agar supplemented with nalidixic acid (30mg/l) and cycloheximide (50mg/l) was subsequently used to selectively isolate *Microbispora* and *Streptosporangium* strains (Nonomura and Hayakawa, 1988). Recently, Hayakawa *et al.* (1991) introduced a new procedure for the selective isolation of streptosporangia which involved heat pretreatment (120°C/1hour) of air-dried soil with benzethonium chloride (0.01%) prior to plating out onto HV agar supplemented with leucomycin (1mg/l) and nalidixic acid 30mg/l. Little attempt has been made to evaluate the effectiveness of these procedures for the selective isolation of streptosporangia.

MATERIALS AND METHODS

A. SELECTIVE ISOLATION, ENUMERATION AND CHARACTERISATION OF STREPTOSPORANGIA

1. ENVIRONMENTAL SAMPLES

Soil samples collected from diverse habitats were used to prepare composite samples (Table 9, page 76) that were used for the isolation of streptosporangia.

2. SOIL REACTION

The pH of all of the soil samples was determined using the method of Reed and Cummings (1945). Each sample (*ca.* 20-25g) of fresh material was placed into a 100 ml beaker and deionized water added slowly while agitating until the sample was thoroughly wetted, that is, when a thin layer of water had appeared on the surface of the sample. Samples prepared in this way were left to equilibrate for one hour when the pH was determined using a glass electrode pH meter (Model 292, Pye Unicam Ltd., Cambridge, England, U.K.). The electrode was pushed well down into the sample and the reading allowed to stabilise before being taken. The final pH was taken as an average of several readings since small local variations may exist within samples.

3. PRETREATMENT AND DILUTION PLATE PROCEDURES

The composite soil samples were dried at room temperature for four weeks. Some of the samples were then heated at 120°C for an hour in a hot air oven (Nonomura and Ohara, 1969a). A series of dilutions were made as shown in Table 10, page 77 using both heat pretreated and non-heat pretreated preparations.

Composite soil samples (*ca* 1g) were accurately weighed and aseptically added to sterile Universal bottles of known weight. Both treated and untreated

Table 9 Source of soil samples used for selective isolation of streptosporangia

Number of Laboratory Soil	Source	Time of Sampling
433-434	Garden soil, Hibuya Park, Tokyo, Japan	May, 1989
435-436	Garden soil, Tsukuba University, Tsukuba, Japan	May, 1989
443-444	Garden soil, IMTECH, Chandigarh, India	April, 1990
482-489	Tropical rainforest soil, Meru Betini, Indonesia	July, 1991
512-513	Rim of crater, Mount Bromo, Bromo, Indonesia	July, 1991
515-516	Garden soil, Yogyakarta, Indonesia	July, 1991
576-577	Soil rich in humus, Keswick, England, U.K.	September, 1991
579-581	Ginseng field (post harvest), Kumsan, Republic of Korea	September, 1991
583-584	Ginseng field (post harvest), Kumsan, Republic of Korea	September, 1991
585-587	Ginseng field (young plant), Kumsan, Republic of Korea	September, 1991
604-605	Woodland soil, Mount Sorak, Republic of Korea	September, 1991
A2	Subtropical rainforest soil, Brazil	

Table 10 Pretreatment and dilution plate regimes* used for the selective isolation of members of the genera *Microbispora* and *Streptosporangium* from diverse composite soil samples

Pretreatment of Soil	10^{-1} Dilution
Heat 120°C/1hour	Saline solution (0.9ml, 0.85%, w/v)
Heat 120°C/1hour	Phenol (0.9ml, 1.5%, w/v, 30°C/30 minutes)
Sodium dodecyl sulphate (0.05%, w/v, 40°C/20minutes)	Saline solution (0.9ml, 0.85%, w/v)
Yeast extract (6%, w/v, 40°C/20minutes)	Saline solution (0.9ml, 0.85%, w/v)

*, Procedure recommended by Nonomura and Hayakawa (1988).

10^{-1} suspensions were shaken on a Griffin flask shaker (Griffin and George Ltd., Manchester, England, U.K.) for 30 minutes at speed setting 8 to disperse bacteria. Tenfold dilutions of the suspensions were made by transferring 1ml samples aseptically, using automatic pipettes (P1000; Gilson, Anachem Ltd., Luton, Bedfordshire, England, U.K.) fitted with sterile tips, to sterile test tubes containing 9.0 ml of sterile saline solution (0.85%, w/v) and mixing on a Vortex mixer (Fisons Scientific Apparatus Ltd., Loughborough, Leicestershire, England, U.K.).

Aliquots (0.1ml) of each dilution were aseptically pipetted, using an automatic pipette (P200; Gilson, Anachem Ltd.) fitted with sterile tips, onto the surface of humic acid and vitamins (HV) agar (Nonomura, 1984; Hayakawa and Nonomura, 1987a) supplemented with nalidixic acid (30mg/l) and actidione (50mg/l) (Nonomura and Hayakawa, 1988). Four plates were prepared for each dilution, aliquots (0.1 ml) of the 10^{-1} to10^{-4} dilutions were dispersed over room dried agar surfaces of the isolation medium (Vickers and Williams, 1987), using sterile glass spreaders. Inoculated plates were incubated at 30°C for up to four weeks. The number of target organisms, and the total number of actinomycetes, growing on the isolation plates were recorded and expressed as the mean number of colony forming units (cfu) per gram dry weight soil.

4. SELECTION, PURIFICATION AND MAINTENANCE OF ISOLATES

After incubation, isolation plates were examined both by eye and using a binocular microscope fitted with long distance objectives (X400, magnification; Nikon Kogaku K.K., Tokyo, Japan). Organisms were tentatively assigned to the genus *Microbispora* if they produced aerial hyphae with sporophores bearing two spores, to the genus *Microtetraspora* if aerial hyphae carried four spores, to the genus *Streptosporangium* if spore vesicles were detected on aerial hyphae, and to a catch all group labelled "unknown actinomycetes" if they did not fall into any of

the previous catagories. It was observed that many of the organisms growing on the selective isolation plates had long spore chains like those characteristic for some *Microtetraspora*, *Saccharopolyspora* and *Streptomyces* species; these organisms were categorised as "unknown actinomycetes". Photographs were taken of thirty-six putative streptosporangia growing on isolation plates using a Nikon camera (35mm, Nikon Kokaku K.K., Tokyo, Japan) fitted to the binocular microscope.

One hundred and fifty-three putative streptosporangia, that is, all that were detected, were taken from the isolation plates (Table 20, pages 133 to 136) using sterile tooth-picks and inoculated onto HV agar plates (Nonomura, 1984; Hayakawa and Nonomura, 1987a), which were incubated at 30°C for two weeks. All of the strains were checked for purity both by eye and the using the binocular microscope, single colonies were then picked from the plates using sterile loops and inoculated onto HV plates (Nonomura, 1984; Hayakawa and Nonomura, 1987a) which were incubated for two weeks at 30°C. This procedure was repeated until pure cultures of all of the isolates were obtained. In addition, 46 randomly selected unknown strains growing on isolation plates were purified and maintained as frozen glycerol suspensions (Table 11, page 80).

The pure cultures were maintained on modified Bennett's agar slopes (Agrawal, unpublished data) and stored at room temperature. Each slope was used to inoculate two modified Bennett's agar plates that were incubated at 30°C for 10 days. Glycerol suspensions were prepared by scraping aerial and substrate mycelium from the incubated plates and making heavy suspensions in 1.5ml of glycerol (20%, v/v, BDH) in each of two Cryo vials (2.5ml; Whatman Ltd., Maidstone, England, U.K.) that were stored at -25°C (Wellington and Williams, 1978). One glycerol suspension was used as a working suspension and the other kept as a stock culture. The frozen glycerol suspensions served as both a

Table 11 Source of unknown actinomycetes isolated using HV agar supplemented with actidione (50mg/l) and nalidixic acid (30mg/l) and incubated at 30°C for 4 weeks

Strain Number (A)	Soil Numbers	Selective Isolation Procedure
001, 003-005, 007, 009-011	433-434	Dried soil heated at 120°C/1hour
013-015, 018-021		Dried soil heated at 120°C/1hour and treated with phenol (1.5% w/v; 30°C/30minutes)
022-024, 026, 029-030		Dried soil treated with sodium dodecyl sulphate (0.05% w/v; 40°C/20minutes)
033-036, 039, 041-042		Dried soil treated with yeast extract (6% w/v; 40°C/20minutes)
046-047, 049	443-444	Dried soil heated at 120°C/1hour
050-052, 055		Dried soil heated at 120°C/1hour and treated with phenol (1.5% w/v; 30°C/30minutes)
056-058, 061-062		Dried soil treated with sodium dodecyl sulphate (0.05% w/v; 40°C/20minutes)
064-067, 069-070		Dried soil treated with yeast extract (6% w/v; 40°C/20minutes)

convenient means of long term preservation and as a source of instant inoculum. Working inocula were obtained by thawing glycerol suspensions at room temperature for about 15 minutes when they were treated as conventional broth cultures. After use, the glycerol suspensions were promptly frozen and stored once again at -25°C. Although repeated freezing and thawing decreases cell viability, this method of preservation compares favourably with other techniques for storing cultures, including lyophilisation (Wellington and Williams, 1978).

5. CHARACTERISATION

a. Analysis for Diaminopimelic Acid

1) Growth and Harvesting

One hundred and thirty-six of the 153 putative streptosporangia (Table 12, pages 82 to 83) and the 46 unknown actinomycetes (Table11, page 80) were examined for the presence of isomers of diaminopimelic acid. Thawed glycerol suspensions were used to inoculate sterile cellulose nitrate membrane filters (0.45 μm pore size, 47mm diameter; Whatman Ltd., Maidstone, England, U.K.) which had been placed in the centre of Petri dishes on modified Bennett's agar plates (Agrawal, unpublished data) which were incubated for 2 weeks at 30°C. After incubation, the test strains were scraped from the surfaces of the membrane filters and put into sterile Universal bottles. The samples were kept in a freezer (-20°C) for 1hour then lyophilised (Edward's High Vacuum, Model EF03, Crawley, England, U.K.).

2) Whole-Organism Hydrolysis

Dried biomass (*ca* 20mg) was hydrolysed with 1ml of 6N HCl in a screw-capped tube at 100°C for 18 hours. After cooling, the hydrolysate was filtered (Whatman No.1 Filter Paper, Whatman Ltd., Maidstone, England, U.K.) and

Table 12 Source of *Streptosporangium* isolates examined for the presence of diaminopimelic acid and for diagnostic characters needed for numerical identification

Strain Number (HJ)	Soil Numbers	Selective Isolation Procedure
001-002	585-587	Dried soil heated at 120°C/1hour
005-006		Dried soil heated at 120°C/1hour and treated with phenol (1.5% w/v; 30°C/30minutes)
008		Dried soil treated with sodium dodecyl sulphate (0.05% w/v; 40°C/20minutes)
009-011		Dried soil treated with yeast extract (6% w/v; 40°C/20minutes)
012-017, 019-024	579-581	Dried soil heated at 120°C/1hour
025-033		Dried soil heated at 120°C/1hour and treated with phenol (1.5% w/v; 30°C/30minutes)
034-051		Dried soil treated with sodium dodecyl sulphate (0.05% w/v; 40°C/20minutes)
052-087		Dried soil treated with yeast extract (6% w/v; 40°C/20minutes)
090-092	583-584	Dried soil heated at 120°C/1hour
093-094, 096-097		Dried soil heated at 120°C/1hour and treated with phenol (1.5% w/v; 30°C/30minutes)
098-099		Dried soil treated with sodium dodecyl sulphate (0.05% w/v; 40°C/20minutes)
100-103		Dried soil treated with yeast extract (6% w/v; 40°C/20minutes)
104	443-444	Dried soil heated at 120°C/1hour
105-106		Dried soil treated with yeast extract (6% w/v; 40°C/20minutes)

Table 12 continued

Strain Number (HJ)	Soil Numbers	Selective Isolation Procedure
107-109	433-434	Dried soil heated at 120°C/1hour
111		Dried soil treated with yeast extract (6% w/v; 40°C/20minutes)
112-114	435-436	Dried soil heated at 120°C/1hour
115-116		Dried soil treated with yeast extract (6% w/v; 40°C/20minutes)
117-118	482-489	Dried soil heated at 120°C/1hour
122		Dried soil heated at 120°C/1hour and treated with phenol (1.5% w/v; 30°C/30minutes)
123-124		Dried soil treated with sodium dodecyl sulphate (0.05% w/v; 40°C/20minutes)
125-126, 128-129		Dried soil treated with yeast extract (6% w/v; 40°C/20minutes)
130-132, 133	515-516	Dried soil heated at 120°C/1hour
135		Dried soil heated at 120°C/1hour and treated with phenol (1.5% w/v; 30°C/30minutes)
138-141		Dried soil treated with yeast extract (6% w/v; 40°C/20minutes)
143	604-605	Dried soil heated at 120°C/1hour
144, 146-147		Dried soil treated with yeast extract (6% w/v; 40°C/20minutes)
148-149	576-577	Dried soil treated with sodium dodecyl sulphate (0.05% w/v; 40°C/20minutes)
150-153		Dried soil treated with yeast extract (6% w/v; 40°C/20minutes)

washed twice with 1ml sterile distilled water. The combined filtrates were concentrated to dryness using a vacuum pump. The dried extract was dissolved in 1ml of distilled water and dried again until the smell of HCl was lost. The residue was then redissolved in distilled water (0.3ml) and transferred to an Eppendorf tube.

3) One-Dimensional Thin Layer Chromatography

Eight or nine samples of residue were applied as aliquots (5µl) onto the base line of a cellulose TLC plate (20x20cm, Merck 5716, Merck Ltd., Warwickshire, England, U.K.). In addition, a standard was also applied, namely 5µl mixed isomers of α,ε-diaminopimelic acid (Sigma 1377). The plates were developed in methanol:water:10N HCl:pyridine (80:26.25:3.75:10, v/v) for up to 4 hours, that is, until the solvent front had nearly reached the top of the plate. The plates were dried in a fume cupboard and spots visualised by spraying with ninhydrin in acetone (0.2%, w/v) followed by heating at 100°C for 5 minutes. The diaminopimelic isomers were detected after about 3 minutes as brown coloured spots; the remaining amino acids were visualised as blue-violet coloured spots. The spots corresponding to the diaminopimelic acid isomers became yellow after 24 hours.

b. Morphological Studies

Three of the putative *Streptosporangium* strains, namely HJ47, HJ84 and HJ94, isolated from Ginseng field soil (Kumsan, Republic of Korea), were examined to determine the morphology of spore vesicles using a Scanning Electron Microscope (Cambridge S240, Cambridge Instrument Ltd., Cambridge, England, U.K.). All of the test strains produced abundant aerial mycelium on arginine-vitamins (AV) agar plates (Nonomura and Ohara, 1969a).

Plugs (5 mm diameter) of the agar medium were taken from plates of the test strains grown on AV agar for 2 weeks at 30°C, placed into 2 ml of gluteraldehyde (25%) /Ca codylate (1M) held in bijoux bottles and kept in the fixative solution for 3 hours at 20°C. The fixed samples were dehydrated in a graded alcohol series (10%, 25%, 50%, 75%, 90%, 100%) for 10 minutes consecutively, then dried in a Biorad Critical Point Drier (BIORAD CDP750, VG Microtech Ltd., East Sussex, England, U.K.) using liquid CO_2 as the transition fluid. The plugs were then mounted onto stubs using 'silver dag' adhesive and coated with gold by means of a Polaron Sputter Coater (E 5100, Fisons Instruments Ltd., East Sussex, England, U.K.). The gold-coated specimens were observed by Scanning Electron Microscopy using a accelerating voltage of 12KV and photographed. Photographs were taken with an amplication range of 638X to 11.6KX magnification.

B. NUMERICAL IDENTIFICATION

1. PRACTICAL EVALUATION OF THE *STREPTOSPORANGIUM* FREQUENCY MATRIX

Seventy strains representing the twelve major *Streptosporangium* clusters (Table 13, pages 86 to 90) defined in the numerical taxonomic survey of *Streptosporangium* and related taxa (Whitham, 1988; Whitham *et al.*, 1993) were examined under code for each of the diagnostic tests recommended for the computer-assisted identification of streptosporangia (Whitham, 1988; Table 14, pages 91 to 93). All of the test strains were examined in duplicate. Identification scores were determined using the IDENTIFY procedure in the TAXON program (Ward, unpublished data; Appendix A).

Table 13 Designation and source of strains representing twelve multimember streptosporangial clusters used to evaluate the *Streptosporangium* frequency matrix*

Cluster Number	Name of Cluster and Designation	Strain Identity	Strain History / Source	Time of Sampling
AGGREGATE GROUP A (*Streptosporangium* spp.)				
1 (*Streptosporangium* sp.)				
	166**	*Streptosporangium* sp.	Plot 5, Cockle Park Experimental Farm, Northumberland, England, U.K.	October 1984
	256, 263, 266		Plot 9, Cockle Park Experimental Farm, Northumberland, England, U.K.	October 1984
	320		Plot 10, Cockle Park Experimental Farm, Northumberland, England, U.K.	October 1984
	101, 104, 106, 117, 121, 127, 128, 129, 143, 144, 145a, 153, 159, 165, 179, 218, 220, 222, 224, 235, 245, 253		Oak copse, Corbridge, Northumberland, England, U.K.	March 1986
	353, 354		Adjacent to River Wear, North-east Durham, England, U.K.	May 1986
	369		Burton Bushes, Beverley Westwood, Hull, England, U.K.	May 1986

Table 13 continued

Cluster Number	Name of Cluster and Designation	Strain Identity	Strain History / Source	Time of Sampling
2 (*Streptosporangium* sp.)				
	269, 270, 271, 274	*Streptosporangium sp.*	Plot 3, Cockle Park Experimental Farm, Northumberland, England, U.K.	October 1984
	282, 286, 292**, 303		Plot 10, Cockle Park Experimental Farm, Northumberland, England, U.K.	October 1984
	115		Oak copse, Corbridge, Northumberland, England, U.K.	March 1986
	366		Burton Bushes, Beverley Westwood, Hull, England, U.K.	May 1986
3 (*Streptosporangium* sp.)				
	541, 547	*Streptosporangium sp.*	Plot 9, Cockle Park Experimental Farm, Northumberland, England, U.K.	October 1984
4 (*Streptosporangium* sp.)				
	116**, 161, 163, 254, 375	*Streptosporangium sp.*	Oak copse, Corbridge, Northumberland, England, U.K.	March 1986
5 (*Streptosporangium* sp.)				
	168	*Streptosporangium sp.*	Plot 5, Cockle Park Experimental Farm, Northumberland, England, U.K.	October 1984
6 (*Streptosporangium* sp.)				
	169	*Streptosporangium sp.*	Plot 5, Cockle Park Experimental Farm, Northumberland, England, U.K.	October 1984

Table 13 continued

Cluster Number	Name of Cluster and Designation	Strain Identity	Strain History / Source	Time of Sampling
	136, 141**, 148, 227, 251	*Streptosporangium sp.*	Oak copse, Corbridge, Northumberland, England, U.K.	March 1986
	393		Burton Bushes, Beverley Westwood, Hull, England, U.K.	May 1986
7 (*Streptosporangium* sp.)				
	226**, 232	*Streptosporangium sp.*	Oak copse, Corbridge, Northumberland, England, U.K.	March 1986
8 (*Streptosporangium* sp.)				
	170	*Streptosporangium sp.*	Plot 5, Cockle Park Experimental Farm, Northumberland, England, U.K.	October 1984
	276		Plot 10, Cockle Park Experimental Farm, Northumberland, England, U.K.	October 1984
	209, 213**		Oak copse, Corbridge, Northumberland, England, U.K.	March 1986
	355		Adjacent to river Wear, North-east Durham, England, U.K.	May 1986
9 (*Streptosporangium roseum* / *Streptosporangium vulgare*)				
	005**T	*Streptosporangium roseum* Couch, 1955a	DSM 43021; A. Seino, KCC A-0005; K. Tubaki, NI 9100; J.N. Couch (ATCC 12428; CBS 313.56; IFO 3776; RIA 470)	

Table 13 continued

Cluster Number	Name of Cluster and Designation	Strain Identity	Strain History / Source	Time of Sampling
	007^T	*Streptosporangium vulgare* Nonomura and Ohara, 1960	DSM 43802; G. Vobis, MB-T9; H. Nonomura, FYU S-1. Soil, paddy field, Anjo, Aichi, Prefecture, Japan. (ATCC 33329; CBS 344.61; IFO 13985; KCC A-0028; NRRL B-2633; RIA 765)	
10 (*Streptosporangium amethystogenes* / *Streptosporangium corrugatum* / *Streptosporangium longisporum*)				
	001^T	*Streptosporangium amethystogenes* Nonomura and Ohara, 1960	DSM 43179; A. Seino, KCC A-0026; H. Nonomura, FYU S-5. Soil. (ATCC 33327; CBS 429.61; NRRL B-2639; RIA 764)	
	002**^{T}	*Streptosporangium corrugatum* Williams and Sharples, 1976	DSM 43316; S. T. Williams, E-90. Beach sand. (ATCC 29331; NCIB 11120)	
11 (*Streptosporangium* sp.)				
	126**, 155	*Streptosporangium sp.*	Oak copse, Corbridge, Northumberland, England, U.K.	March 1986
12 (*Streptosporangium* sp.)				
	182**, 194	*Streptosporangium sp.*	Oak copse, Corbridge, Northumberland, England, U.K.	March 1986

*, Clusters and frequency matrix generated by Whitham (1988).

**,Centrotype strains of the cluster; ^{T}, Type strain.

Abbreviations: **ATCC**, American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, U.S.A.; **CBS**, Centraalbureau voor Schimmelcultures, Baarn, Netherlands; **DSM**, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Mascheroder Weg 1B, D-38124, Braunschweig, Federal Republic of Germany; **FYU**, Department of Fermentation Industries, Yamanashi University, Motoganagi-cho, Kofu-shi, Yamanashi-ken, Japan; **IFO**, Institute of Fermentation, 4-54 Juso Nishino-machi, Higashiyodogawa-ku, Osaka, Japan; **KCC**, Kaken Chemical Company Limited, 6-48 Jujodai, 1-Chome, Kita-ku, Tokyo 114, Japan; **NCIB**, National Collection of Industrial Bacteria, St. Machar Drive, Aberdeen, U.K.; **NRRL**, Northern Research and Development Division, United States Department of Agriculture, Peoria, Illinois, U.S.A.; **RIA**, Research Institute for Ampelology, Budapest, Hungary.

Table 14 Percentage positive frequency matrix for the twelve multimember streptosporangial clusters*

CHARACTER / CLUSTER	*Streptosporangium sp.* 1	*Streptosporangium sp.* 2	*Streptosporangium sp.* 3	*Streptosporangium sp.* 4	*Streptosporangium sp.* 5	*Streptosporangium sp.* 6	*Streptosporangium sp.* 7	*Streptosporangium sp.* 8	*S roseum / vulgare* 9	*S amethystogenes/ corrugatum/ longisporum* 10	*Streptosporangium sp.* 11	*Streptosporangium sp.* 12
NUMBER OF STRAINS	57	23	3	5	3	7	2	5	2	3	2	2
BIOCHEMICAL TEST												
Urease production	0	0	0	0	0	0	0	100	0	67	0	100
DEGRADATION TESTS												
DNA	72	95	100	100	100	100	100	100	80	100	100	100
Keratin	0	5	100	0	0	14	0	0	50	0	0	0
Starch	60	62	0	80	0	0	50	0	0	67	100	0

Table 14 continued

CHARACTER CLUSTER	1	2	3	4	5	6	7	8	9	10	11	12
NUMBER OF STRAINS	57	23	3	5	3	7	2	5	2	3	2	2
MORPHOLOGICAL TESTS												
Aerial mycelium colour:												
pink	95	100	100	60	100	0	0	0	50	33	100	0
white	5	0	0	40	0	100	100	100	50	67	0	100
Sole carbon and energy sources:												
Galactose	85	95	100	0	67	86	100	100	100	0	0	100
Mannitol	85	0	100	100	100	100	100	100	0	33	100	100
Turanose	71	71	33	0	67	100	100	60	100	33	100	100
PHYSIOLOGICAL TESTS												
Growth at 37°C	33	95	33	0	33	71	0	100	50	0	0	100
Growth in the absence of:												
B-vitamins	0	5	0	20	33	86	100	40	100	33	0	0
Growth in the presence of:												
Phenyl ethanol (1.0 ml/l)	7	62	67	100	0	14	0	20	100	67	100	100
Sodium chloride (40 g/l)	0	0	0	0	0	0	0	0	0	0	0	100
Thallous acetate (5 mg/l)	2	0	0	20	33	71	50	0	100	100	100	100
Resistance to antibiotics (µg/ml)												
Aminoglycosides:												
Gentamycin sulphate(5)	36	81	0	40	33	0	0	20	100	100	0	0
Neomycin sulphate(25)	0	38	0	0	33	0	0	0	100	0	0	0
Neomycin sulphate(0.5)	98	100	0	60	100	100	100	100	100	33	100	100
Streptomycin sulphate(25)	95	76	100	100	33	0	0	100	100	0	100	0

Table 14 continued

CHARACTER CLUSTER	1	2	3	4	5	6	7	8	9	10	11	12
NUMBER OF STRAINS	57	23	3	5	3	7	2	5	2	3	2	2
β-Lactams												
i)Cephalosporins:												
Cefoxitin (250)	3	76	0	0	100	29	0	100	100	0	0	0
Cephaloridine (50)	26	86	0	100	100	86	0	100	100	0	0	0
Cephradine (500)	0	0	0	0	0	0	0	0	100	0	100	0
ii)Penicillins:												
Amoxicillin (250)	88	91	33	100	100	100	0	100	100	0	100	0
iii)Others:												
Clavulanic acid (250)	95	91	0	80	67	100	100	100	0	0	0	100
Rifampicins:												
Rifampicin (0.5)	100	95	100	100	100	86	0	80	0	33	100	0
Miscellaneous:												
Fusidic acid (5)	2	0	0	0	33	0	100	0	0	0	0	100
Fusidic acid (0.5)	62	81	100	100	100	57	100	0	0	0	100	100

*, Clusters defined by Whitham (1988).

2. IDENTIFICATION OF UNKNOWN STREPTOSPORANGIA

One hundred and thirty-six putative streptosporangia from diverse soil samples (Table 12, pages 82 to 83) were examined in duplicate as described above.

3. CHARACTERISATION OF STRAINS

All of the test strains, that is, the marker strains and fresh isolates, were examined for twenty-six unit characters (Whitham, 1988). Tests were repeated where ambiguous or clearly unexpected results were obtained. Details of media, their preparation and sterilisation are given in Appendix B. Unless otherwise stated, all tests were carried out at pH 7.0.

Where possible, tests were carried out using a multipoint inoculation procedure that involved the use of an automatic multipoint inoculator (Denley-Tech; Denley Instruments Ltd., Daux Road, Billingshurst, Sussex, England, U.K.). This apparatus allows standardised, multiple surface inoculation of 90mm diameter Petri dishes (Sterilin Ltd., Teddington, Middlesex, England, U.K.) with either twenty, twenty-five or thirty different organisms. In this study, the multipoint inoculator was used with an inoculation head that contained twenty pins for culture transfer and a marker pin that provided a reference point to orientate plates. Inocula were pipetted into sterile, inverted Oxoid caps held in an ordered array within a metal template placed inside the base of a 100mm square plastic dish (Sterilin Ltd., Teddington, Middlesex, England, U.K.). For each group of twelve test strains inoculated, control plates of the appropriate basal medium were seeded at the beginning and end of the inoculation procedure. This practice was followed throughout to eliminate false negative results due to loss of inoculum.

a. Biochemical Test

1) Urea

Urea is a product of amino acid metabolism. The hydrolysis of urea was detected using the medium and method of Gordon (1966). Broth cultures were incubated at 30°C and examined after 7, 14, 21, and 28 days. The test medium contained the indicator phenol red. The hydrolysis of urea results in the formation of ammonia and carbon dioxide:

$$\underset{\text{Urea}}{NH_2CONH_2} + \underset{\text{Water}}{H_2O} \xrightarrow{\text{Urease}} \underset{\text{Ammonia}}{2NH_3} + \underset{\text{Carbon Dioxide}}{CO_2}$$

The consequential change in pH from neutral to alkaline is detected by the phenol red indicator.

b. Degradation Test

1) Keratin

Keratin is a highly insoluble protein found in hair, wool, horn and skin. Keratin (5g/l) was incorporated into AV agar (Nonomura and Ohara, 1969a), care being taken to ensure an even distribution of this insoluble compound. Clearing of the compound from under and around the area of test strain growth was taken to denote a positive result. Plates were examined after incubation for 7, 14, 21 and 28 days at 30°C to detect the breakdown of the substrate.

2) Starch

Starch occurs principally as the main reserve polysaccharide in plants and is a composite molecule consisting of α-D-glucopyranose subunits in two different structural configurations; amylose, a linear molecule with α-1,4-linkages and amylopectin, a α-1,4-linked backbone with α-1,6-branches. Both α and β-amylases degrade starch. α-Amylases randomly cleave the α-1,4-glucosidic

linkages so that amylose, for example, is broken down initially to dextrins and then to a mixture of maltose and glucose. β-Amylases act on linear α-1,4-linked glucans and cleave alternate bonds from the non-reducing end of the chain forming maltose.

The production of extracellular amylases was detected in AV agar (Nonomura and Ohara, 1969a) supplemented with potato starch (10g/l). The inoculated plates were incubated for 14 days at 30°C when those supporting good growth were flooded with Lugol's iodine (Cowan and Steel, 1974). Iodine complexes with amylose to form a dark blue starch-iodine complex whereas dextrins, maltose and glucose are unable to do so. A positive result was indicated by a zone of clearing around the area of growth.

3) Deoxyribonucleic Acid

DNA is a linear polymer of deoxyribonucleotides where the adjacent residues are linked by 3', 5' -phosphodiester bridges. Each residue in the chain is linked to one of several nitrogenous bases, namely adenine, thymine, cytosine and guanine and, in some instances, modifications of these bases. All bacteria possess intracellular nucleases for the manipulation of their own nucleic acids but only some produce the extracellular enzymes capable of specific or non-specific hydrolysis of extracellular nucleic acids.

The degradation of DNA was detected using Bacto DNase Test Agar (Difco) which contains DNA at 2 g/l. Inoculated plates were incubated for 14 days at 30°C and checked for good growth before flooding with a molar solution of hydrochloric acid. This test depends on the ability of the deoxyribonucleases to reduce the viscosity of solutions of the appropriate semi-purified nucleic acid extracts. Degradation products from nuclease treated nucleic acids are acid soluble whereas native DNA is precipitated by the addition of 1M HCl. Thus, the

diffusion of any nucleases into the growth medium from a test strain results in a clear zone around and under the colonies after addition of the acid. Zones of clearing were scored as positive results.

c. Morphological Tests

The test strains were grown on oatmeal agar (Küster, 1959) at 30°C for 3 weeks when aerial mycelial pigmentation was recorded using a binocular plate microscope (Nikon Kogaku K.K. Tokyo, Japan) at X40 magnification. The colours of the aerial mycelium were recorded and assigned to two colour groups, namely pink and white.

d. Nutritional Tests

The test strains were examined for their ability to utilise three compounds, D(+)galactose, mannitol and turanose, as the sole source of carbon for energy and growth. These carbon sources (10g/l) were prepared as aqueous solutions and sterilised by steaming at 100°C for 30 minutes on three consecutive days. The sterilised test compounds were added to sterile, molten carbon utilisation agar (ISP 9 medium; Appendix B) recommended by Shirling and Gottlieb (1966).

Inocula were prepared from test strains grown on sterile cellulose nitrate membrane filters (0.45µm pore size, 47mm diameter; Whatman Ltd., Maidstone, England, U.K.) which had been placed in the centre of Petri dishes on AV medium (Nonomura and Ohara, 1969a), inoculation was for two weeks at 30°C. After incubation, aerial mycelia and spores were transferred to 2ml saline solution (0.85%, w/v) in bijoux bottles. The freshly prepared saline suspensions were used to inoculate Petri dishes containing carbon utilisation agar supplemented with a carbon source, plates of basal medium supplemented with glucose (10g/l), the positive control, and plates of basal medium alone, the negative control.

Inoculations were carried out using the multipoint inoculation procedure described earlier with twelve organisms per Petri dish. Inoculated plates were incubated at 30°C and examined for growth after 7, 14 and 21 days. When scoring the plates, growth on the test medium was compared with that on both the positive and negative controls. Growth, when greater than that on the negative control, was scored as positive and growth that was equal to or less than that on the negative control was scored as a negative result.

e. Physiological Tests

1) Growth in The Absence of B-Vitamins

The test strains were examined for their ability to grow on AV agar (Nonomura and Ohara, 1969a) without B-vitamins. Plates were inoculated from saline suspensions (0.85%, w/v) using the multipoint procedure then incubated at 30°C for 14 days. After incubation, plates were scored by comparison with growth on a control plate of AV medium alone. Growth was scored as a positive result.

2) Growth in The Presence of Chemical Inhibitors

The test strains were examined for their ability to grow on AV agar (Nonomura and Ohara, 1969a) supplemented with one of three chemical inhibitors, namely phenyl ethanol (1.0ml/l), sodium chloride (40g/l) and thallous acetate (0.005g/l). Plates were inoculated from saline suspensions (0.85%, w/v) using the multipoint procedure then incubated at 30°C for 14 days. After incubation, plates were scored by comparison with growth on a control plate of AV medium alone. Growth was scored as a positive result.

3) Resistance to Antibiotics

The test strains were examined for their ability to grow in the presence of antibiotics. All but one of the antibiotics were sterilised by Seitz-filtration of aqueous solutions. Rifampicin was dissolved in dimethylformamide (0.2ml; BDH Laboratory Supplies, Warwickshire, England, U.K.) and then added to the appropriate amount of sterile distilled water.

The sterilised antibiotics were added to molten, cooled AV agar, at pH 7.0, to give the appropriate concentrations. Media were dispensed into Petri dishes and inoculated immediately after setting. Plates were inoculated using the automatic multipoint inoculator using suspensions of spores and mycelial fragments in sterile saline solution (0.85%, w/v). Inoculated plates were incubated at 25°C then examined for growth after 4, 7 and 14 days. Growth of cultures in the presence of each antibiotic was compared to that on a control plate consisting of the basal medium alone. Cultures showing resistance were scored positive.

4) Tolerance to Temperature

Organisms were tested for their ability to grow at 37°C. The test strains were inoculated from saline suspensions (0.85%, w/v), using the multipoint inoculation procedure, onto AV agar medium (Nonomura and Ohara, 1969a). Inoculated plates were incubated at 37°C for 14 days and examined for growth against the control plate of AV medium alone. Growth was scored as positive, lack of growth indicated a negative result provided the organism grew on the control plate.

4. CODING AND COMPUTATION

All of the tests were binary and hence were scored "1" for a positive result and "0" for a negative one. The binary test results were typed in a +/- format as input to the TAXON program (Ward, unpublished data; Appendix A) and run on a IBM personal computer; data were stored on hard disc. The results of the duplicated cultures were analysed and the test reproducibility expressed as the test variance (Si^2;formula 15; Sneath and Johnson, 1972). The +/- results in the final data matrix were analysed using the IDENTIFY procedure in the TAXON program (Ward, unpublished data; Appendix A).

5. DETERMINATION OF IDENTIFICATION SCORES

The test strains were identified as far as possible using the frequency matrix (Table14, pages 91 to 93) and algorithms modified from the MATIDEN program (Sneath, 1979a) and incorporated into the IDENTIFY procedure in the TAXON program (Ward, unpublished data; Appendix A). IDENTIFY provides the best identification scores for known and unknown strains against a frequency matrix consisting of q taxa and m unit characters. Percentages in the frequency matrix, with 0 changed to 1 and 100 to 99% (Lapage *et al.*, 1970), are converted into proportions, Pij, for the ith character of taxon j. The character state values of an unknown organism (u) are compared with each taxon in turn and identification coefficients calculated and printed out for all of the clusters. Four identification coefficients were calculated, namely Willcox probability, taxonomic distance (d), the 95% taxonomic radius and Gaussian distance probability.

C. SEQUENCING OF 5S RIBOSOMAL RNA

1. TEST STRAINS

Glycerol suspensions of the test strains (Table 15, page 102) were used to inoculate modified Bennett's agar plates (Agrawal, unpublished) which were incubated at 30°C for 7 days. After incubation, the strains were checked for purity by eye and used to inoculate 200ml of modified Bennett's broths in 500ml long neck flasks which were shaken at 30°C for 5 days on a orbital incubator at 200 rpm. Test strains were harvested using a Beckmann centrifuge (Rotor JA10) at 10,000 rpm for 20 minutes at 4°C.

2. PREPARATION AND SEQUENCING OF 5S rRNA

Wet biomass (*ca* 5g) was homogenised with aluminium oxide (2g), mixed with 7ml of buffer containing 10mM Tris hydrochloride (pH 7.5), 10mM $MgCl_2$, 0.1M KCl and 10µg of DNase 100mg/ml for 10 minutes at 4°C then centrifuged at 5,000 rpm for 15 minutes at 4°C. Ethanol was then added to the aqueous phase which was kept at -20°C. The resultant precipitate was dissolved in 7ml of buffer containing 10mM Tris hydrochloride (pH 7.5), 10mM $MgCl_2$, 0.1M KCl and 10ml of phenol and the preparation centrifuged at 7,000 rpm for 10 minutes at 20°C. The resultant supernatant was kept in ethanol at -20°C. This procedure was repeated to yield more rRNA. The RNA preparation was examined by electrophoresis on a 12% polyacrylamide gel containing 7M urea, 0.1M Tris-borate (pH 8.3) and 1mM EDTA. The electrophoresed preparation was stained with ethidium bromide(20mg/l), and the 5S rRNA band excised and eluted with 0.5M ammonium acetate-0.1mM EDTA-sodium dodecyl sulphate (0.1%, w/v) at 37°C.

The ^{32}P labelling of the 5' terminus of was done with [γ -^{32}P]ATP and polynucleotide kinase after pretreatment of the 5S rRNA with alkaline

Table 15 History of the strains examined in the 5S rRNA sequencing studies

Strain Number	Strain Identity	Strain History / Source
TW 006	*Streptosporangium albidum* (Furumai *et al.*, 1968)	DSM 43870; T. Okuda, MCRL-048; soil, Japan. (ATCC 25243; IFO 13901; KCTC 9237)
TW 001	*Streptosporangium amethystogenes* (Nonomura and Ohara, 1960)	DSM 43179; A. Seino, KCC A-0026; H. Nonomura, FYU S5; soil. (ATCC 33327; CBS 429.61; NRRL B-2639; RIA 764)
TW 004	*Streptosporangium pseudovulgare* (Nonomura and Ohara, 1969b)	DSM 43181; A. Seino, KCC A-0115; H. Nonomura, FYU, S2-32; soil. (ATCC 27100; CBS 881.70; IFO 13991; KCTC 9239)
TW 021	*Streptosporangium viridogriseum* subsp. *viridogriseum* (Okuda *et al.*, 1966a)	DSM 43850; ATCC 25242
TW 007	*Streptosporangium vulgare* (Nonomura and Ohara, 1960)	DSM 43802; G. Vobis, MB-T9; H. Nonomura, FYU S-1; paddy field, Anjo, Aichi Prefecture, Japan. (ATCC 33329; CBS 433.61; KCC A-0028; NRRL B-2633; RIA 765)
TW 166	*Streptosporangium* sp.	Centrotype strain of cluster 1; Plot 5, Cockle Park Experimental Farm, Northumberland, England, U.K.
TW 292	*Streptosporangium* sp.	Centrotype strain of cluster 2; Plot 10, Cockle Park Experimental Farm, Northumberland, England, U.K.
HJ 011	*Streptosporangium* strain	Unidentified strain, Ginseng field (young plant), Kumsan, Republic of Korea
HJ 090	*Streptosporangium* strain	Identified to cluster 1; Ginseng field (post harvest), Kumsan, Republic of Korea

Abbreviations: **ATCC**, American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, U.S.A.; **CBS**, Centraalbureau voor Schimmelcultures, Baarn, Netherlands; **DSM**, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Mascheroder Weg 1B, D-38124, Braunschweig, Federal Republic of Germany; **FYU**, Department of Fermentation Industries, Yamanashi University, Motoganagi-cho, Kofu-shi, Yamanashi-ken, Japan; **IFO**, Institute of Fermentation, 4-54 Juso Nishino-machi, Higashiyodogawa-ku, Osaka, Japan; **KCC**, Kaken Chemical Company Limited, 6-48 Jujodai, 1-Chome, Kita-ku, Tokyo 114, Japan; **KCTC**, Korean Collection of Type Cultures, Genetic Engineering Research Institute, Korean Institute of Science and Technology, Republic of Korea; **NRRL**, Northern Research and Development Division, United States Department of Agriculture, Peoria, Illinois, U.S.A.; **RIA**, Research Institute for Ampelology, Budapest, Hungary.

phosphatase (Donis-Keller, 1980). The ^{32}P labelling of the 3' terminus was performed with [5'-^{32}P] pCp with RNA ligase (Peattie, 1979). The 5'- or 3'-end labelled 5S rRNAs were digested completely by nuclease P_1 or RNase T_2 using thin-layer cellulose plates and autoradiographs (Kuchino et al., 1979).

5S rRNA secondary structure models were constructed using the method of Tinoco *et al.* (1971), as adapted by Hori and Osawa (1986).

3. PHYLOGENETIC ANALYSIS

The evolutionary distance, *Knuc*, and the standard error of *Knuc*, σ_k, between sequences were calculated after Kimura (1980). *Knuc* corresponds to the number of base substitutions per nucleotide site that have occurred in the course of evolution:

$$Knuc = -\frac{1}{2}\log_e[(1-2P-Q)(1-2Q)^{1/2}]$$

where P and Q are the fractions of nucleotide sites showing transition- and transversion-type differences, respectively. A phylogenetic tree was generated by applying the weighted pair group average clustering method with mean averages algorithm (Sneath and Sokal, 1973) to the *Knuc* values to determine the branching order and relative evolution distances.

D. PYROLYSIS MASS SPECTROMETRY

1. TEST STRAINS

Experiments were carried out to evaluate the status of clusters recovered in the numerical phenetic survey of Whitham (1988) and to determine the quality of some of the results obtained in the computer-assisted identification procedure. The strain histories of all of the test strains are shown in Table 16, pages 105 to

108. All of the strains were maintained as frozen glycerol suspensions as described earlier.

(i) Experiment 1: The aim of this experiment was to determine whether representatives of streptosporangial clusters 1 and 2 (Whitham, 1988; Whitham *et al.*, 1993) could be separated by using Curie-point pyrolysis mass spectrometry.

(ii) Experiment 2: This experiment was designed to determine the relationships between representatives of streptosporangial clusters 1 and 2 (Whitham, 1988; Whitham *et al.*, 1993) and type strains of *Streptosporangium* species.

(iii) Experiment 3: The aim of this experiment was to determine whether isolates identified to clusters 1 and 2 grouped with representatives of the respective taxa.

2. GROWTH CONDITIONS

Glycerol stock cultures were used to inoculate sterile polyvinyl membrane filters (0.45 mm, HV type; Millipore) placed over a medium originally designed to inhibit the sporulation of streptomycetes (20 g, Casamino acids; 20 g, starch; 4 g, yeast extract; 18 g, Bacto agar; 1 litre distilled water; pH 6.4-6.6; Sanglier *et al.*, 1992). Duplicated preparations were incubated for 3 days at 30°C and the growth obtained used to inoculate a further set of plates which were incubated under identical conditions.

3. PREPARATION AND ANALYSIS OF SAMPLES

Pyrolysis foils and tubes (Horizon Instruments, Heathfield, East Sussex, England, UK.) were washed in acetone and dried overnight at 27°C. Single foils were inserted, with flamed forceps, into pyrolysis tubes so as to protrude about 6

Table 16 Source of strains examined by Curie point pyrolysis mass spectrometry

Strain Number	Strain History / Source
EXPERIMENT 1	
a) Cluster 1 (*Streptosporangium* sp.)	
166*, 167	Plot 5, Cockle Park Experimental Farm, Northumberland, England, U.K.
256	Plot 9, Cockle Park Experimental Farm, Northumberland, England, U.K.
100, 101, 109, 117, 118, 121, 124, 127, 131, 140, 142, 145a, 159, 179, 225, 235, 245	Oak copse, Corbridge, Northumberland, England, U.K.
369	Burton Bushes, Beverley Westwood, Hull, U.K.
b) Cluster 2 (*Streptosporangium* sp.)	
269, 272, 273, 274, 275, 398, 399	Plot 3, Cockle Park Experimental Farm, Northumberland, England, U.K.
281, 287, 292*, 303, 308	Plot 10, Cockle Park Experimental Farm, Northumberland, England, U.K.
113	Oak copse, Corbridge, Northumberland, England, U.K.
366	Burton Bushes, Beverley Westwood, Hull, U.K.
EXPERIMENT 2	
a) Cluster 1 (*Streptosporangium* sp.)	
166*	Plot 5, Cockle Park Experimental Farm, Northumberland, England, U.K.
101, 109, 118, 124, 142, 145a, 179, 225, 245	Oak copse, Corbridge, Northumberland, England, U.K.

Table 16 continued

Strain Number	Strain History / Source
b) Cluster 2 (*Streptosporangium* sp.)	
272, 273, 274, 275, 399	Plot 3, Cockle Park Experimental Farm, Northumberland, England, U.K.
287, 292*, 303, 308	Plot 10, Cockle Park Experimental Farm, Northumberland, England, U.K.
113	Oak copse, Corbridge, Northumberland, England, U.K.
c) Type Strains	
Streptosporangium albidum (Furumai *et al.*, 1968)	DSM 43870; T. Okuda, MCRL-048; soil, Japan. (ATCC 25243; IFO 13901)
Streptosporangium amethystogenes (Nonomura and Ohara, 1960)	DSM 43179; A. Seino, KCC A-0026; H. Nonomura, FYU S5; soil. (ATCC 33327; CBS 429.61; NRRL B-2639; RIA 764)
Streptosporangium corrugatum (Williams and Sharples, 1976)	DSM 43316; S.T. Williams, E90; beach sand. (ATCC 29331; NCIB 11120)
Streptosporangium fragile (Shearer *et al.*, 1983)	ATCC 31519; IFO 14311
Streptosporangium nondiastaticum (Nonomura and Ohara, 1969b)	DSM 43848; ATCC 27101
Streptosporangium pseudovulgare (Nonomura and Ohara, 1969b)	DSM 43181; A. Seino, KCC A-0115; H. Nonomura, FYU, S2-32; soil. (ATCC 27100; CBS 881.70)
Streptosporangium roseum (Couch, 1955a)	DSM 43021; A. Seino, KCC A-0005; K. Tubaki, NI 9100; J. N. Couch, UNCC 27B; vegetable garden soil. (ATCC 12428; CBS 313.56; IFO 3776; RIA 470)
Streptosporangium violaceochromogenes (Kawamoto *et al*, 1975)	DSM 43849; Kyowa Fermentation Industry, MK-49: soil swamp, Japan. (ATCC 21807)
Streptosporangium viridialbum (Nonomura and Ohara, 1960)	DSM 43801; G. Vobis, MB-T8; H. Nonomura, FYU S-20; soil, Yotei, Hokkaido, Japan. (ATCC 33328; CBS 432.61; KCC A-0027; NRRL B-2636; RIA 768)

Table 16 continued

Strain Number	Strain History / Source
Streptosporangium viridogriseum subsp. *viridogriseum* (Okuda et al., 1966a)	DSM 43850. (ATCC 25242)
Streptosporangium vulgare (Nonomura and Ohara, 1960)	DSM 43802; G. Vobis, MB-T9; H. Nonomura, FYU S-1; paddy field, Anjo, Aichi Prefecture, Japan. (ATCC 33329; CBS 433.61; KCC A-0028; NRRL B-2633; RIA 765)

EXPERIMENT 3

a) Cluster 1 (*Streptosporangium* sp.)

166*	Plot 5, Cockle Park Experimental Farm, Northumberland, England, U.K.
101, 109, 118, 124, 142, 145a, 179, 225, 245	Oak copse, Corbridge, Northumberland, England, U.K.

b) Cluster 2 (*Streptosporangium* sp.)

272, 273, 274, 275, 399	Plot 3, Cockle Park Experimental Farm, Northumberland, England, U.K.
287, 292*, 303, 308	Plot 10, Cockle Park Experimental Farm, Northumberland, England, U.K.
113	Oak copse, Corbridge, Northumberland, England, U.K.

c) Isolates identified to cluster1 (*Streptosporangium* sp.)

20, 36, 55, 56,	Ginseng field, Kumsan, Republic of Korea
90, 98	
107, 112	Woodland Soil, Tokyo, Japan
123, 125	Rainforest soil, Meru Betini, Indonesia

d) Isolates identified to cluster2 (*Streptosporangium* sp.)

21, 91	Ginseng field, Kumsan, Republic of Korea
126, 129	Rainforest soil, Meru Betini, Indonesia

Table 16 continued

Strain Number	Strain History / Source
e) Unidentified isolates	
9, 14, 64, 93	Ginseng field, Kumsan, Korea
131	Garden soil, Yogyakarta, Indonesia
149	Soil rich in humus, Keswick, England, U.K.

*Centrotype strain.

Abbreviations: **ATCC**, American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, U.S.A.; **CBS**, Centraalbureau voor Schimmelcultures, Baarn, Netherlands; **DSM**, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Mascheroder, Weg 1B, D-38124, Braunschweig, Federal Republic of Germany; **FYU**, Department of Fermentation Industries, Yamanashi University, Motoganagi-cho, Kofu-shi, Yamanashi-ken, Japan; **IFO**, Institute of Fermentation, 4-54 Juso Nishino-machi, Higashiyodogawa-ku, Osaka, Japan; **KCC**, Kaken Chemical Company Limited, 6-48 Jujodai, 1-Chome, Kita-ku, Tokyo 114, Japan; **NCIB**, National Collection of Industrial Bacteria, St. Machar Drive, Aberdeen, U.K.; **NRRL**, Northern Research and Development Division, United States Department of Agriculture, Peoria, Illinois, U.S.A.; **RIA**, Research Institute for Ampelology, Budapest, Hungary.

mm from the mouth. For each strain, small amounts of biomass (*ca.* 50 μg) were taken from different areas of the inoculated plate, using sterile disposable plastic loops, and smeared uniformly onto the surface of the protruding foils. The assembled tubes plus foils were placed in an oven at 80°C for 15 minutes to dry the biomass onto the foils. The dried foils were then inserted into the tubes, using a stainless steel depth gauge, so that the tip of the foils lay 10 mm from the mouth of the tubes. Viton O-ring collars (Horizon Instruments) were positioned 2mm from the edge of the tubes which were loaded onto the PyMS carousel. Each strain was examined in triplicate in order to follow the mass characteristicity discriminate analysis routines.

Pyrolysis was carried out using a Horizon Instruments PyMS 200X mass spectrometer (Aries *et al.*, 1986; Ottley and Maddock, 1986). Prior to the analysis, the inlet heater was set at 160°C and the heated tube loader at 120°C. The assembled tubes were loaded sequentially into the pyrolysis chamber by a robotic arm. Curie-point pyrolysis was carried out at 530°C for 2.4 seconds under vacuum with a temperature rise time of 0.6 of a second. The volatile pyrolysis products were ionised by collision with a crossing beam of low-energy (20eV) electrons and the ions separated in the quadrupole mass spectrometer that scanned the pyrolysate at 0.35 second intervals. Integrated ion counts at unit mass intervals from 51 to 200 were recorded on hard disc together with the pyrolysis sequence number and total ions count for each sample.

4. DATA ANALYSIS

The GENSTAT statistical package (Nelder, 1979) was used to carry out the multivariate statistical analyses. PYSTAT, a program developed for the PyMS 200X by Horizon Instruments, was used to convert the raw PyMS data into a form suitable for analysis, and to provide instructions to GENSTAT as to which

analysis steps were to be carried out. PYSTAT was also used to pre-treat data for analysis on the OPUS V IBM-PC compatible microcomputer. The major steps involved in these procedures are shown in Figure 4, page 111.

The molecular structure of microorganisms shows a high degree of similarity hence pyrolysis mass spectra from different microorganisms are very similar and cannot be differentiated and identified by simple observation. Although the PyMS-200X can scan down to a mass-charge ratio of 11 these low mass ions tend to be derived from gases and water which are not only common derivatives from many organic molecules but may also be derived from leaks and filament oxidation. This problem was overcome in the present study by omitting standard masses below 50 from the analyses (Berkeley *et al.*, 1991).

The quantitative ion count for masses from a particular sample depend upon molecular composition and sample size. It is important to control the sample size as mass ion counts can saturate the detector or at low levels are subject to sensitivity and random fluctuation effects. Consequently, samples with total mass ion counts exceeding 3.000.000 or less than 800.000 were excluded from the analyses. To correct for smaller changes in sample size raw data were normalised such that;

$$M_{IJ}^{C} = (m_{ij} / \sum_{i=51}^{200} m_{ij}) x100$$

where m_{ij}^{c} is the corrected mass ion intensity for sample j mass ion i as a percentage of the total ion intensity, and m_{ij} is the mass ion intensity for sample j mass ion i.

Within any set of spectra some mass ion peaks may show little change between spectra from different organisms while others may show large variations between spectra from duplicated samples. Mass ion peaks that are reproducible

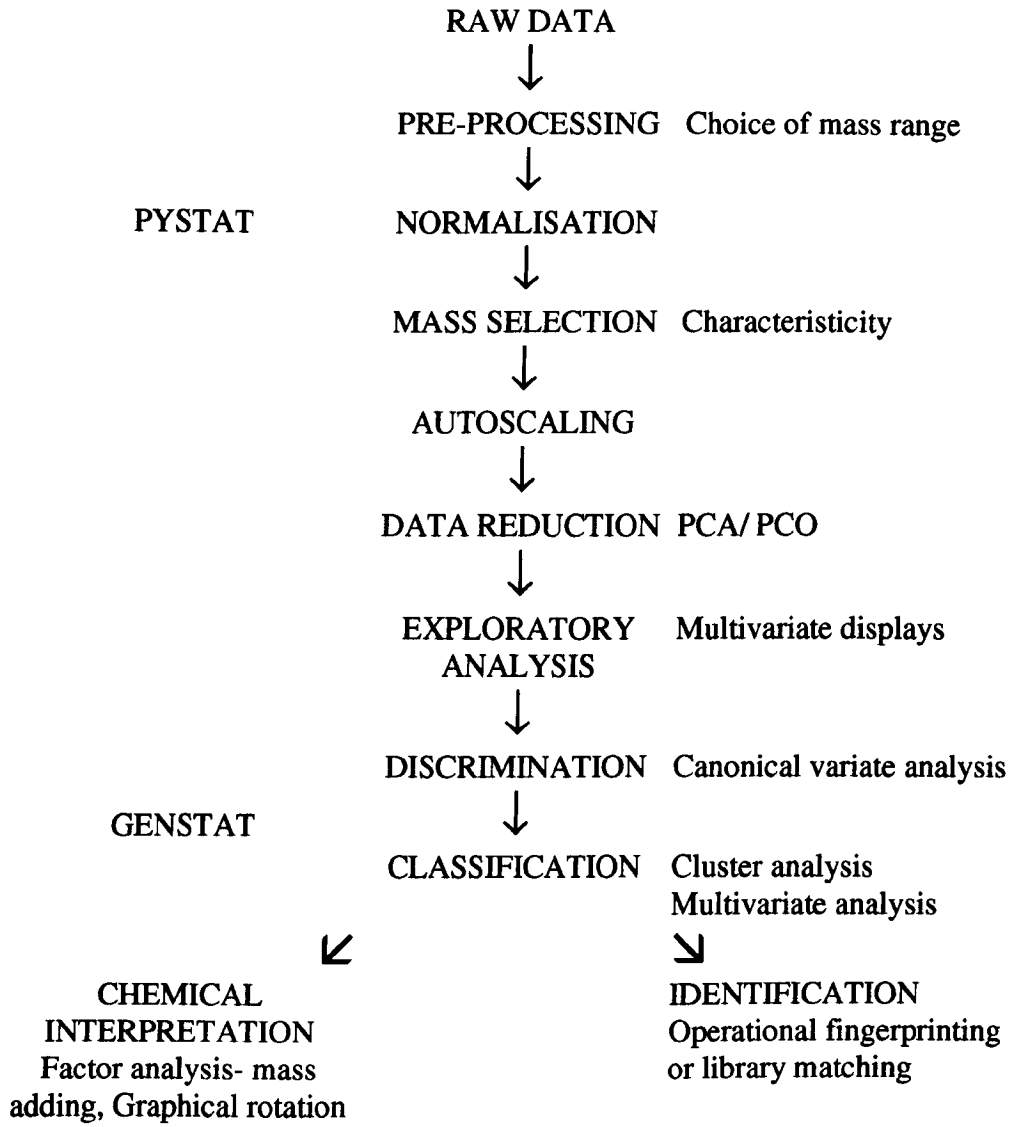


Figure 4 Major steps in handling pyrolysis mass spectrometric data showing the functions performed by the PYSTAT and GENSTAT programs.

within duplicates but vary across spectra from different organisms best distinguish between organisms. Eshuis *et al.* (1977) proposed characteristicity, the ratio of outer variance to inner variance (Kruskal 1964*a*, *b*), as a measure of this discrimination. Consequently, data sets were reduced by selecting the 30 mass ions with the highest characteristicity values. The data needed to calculate the inner variance were derived from the triplicate samples.

After data reduction on the smaller set of masses, re-normalisation changes the normalised mass spectra and re-calculation of characteristicity gives new characteristicity values. In an iterative re-normalisation procedure the whole data set was normalised and the characteristicity values calculated, the five lowest characteristicity masses were removed and the data set re-normalised and the characteristicity recalculated. The average characteristicity of the remaining masses was calculated and the process of removing masses and recalculating repeated until the average characterisiticity did not increase any more. This set of masses is, in some senses, the most characteristic set of masses.

The reduced data set was then analysed by principal components analysis. Plots of the first two or three principal components were produced as plots of the spectral scores, the position of the pyrolysis spectra on the principal component axes. A plot of the mass loadings for the axes gave information about the contribution of masses to the principal component axes. Canonical variate analyses of all of the principal components accounting for more the 0.1% of the total variance was carried out to give a combined principal component-canonical variate analysis (PC-CVA). The data from PC-CVA were plotted as Mahalanobis distances. The Mahalanobis distance matrix was standardised by dividing the maximum intergroup distance and was treated as an ordinary Euclidean distance then converted to a similarity matrix (Gutteridge *et al.*, 1985). The values in the

similarity matrix were examined using the unweighted pair group method with arithmetric averages algorithm (Sneath and Sokal, 1973).

E. RAPID ENZYME TESTS

1. TEST STRAINS AND SUBSTRATES

The 159 test strains included the centrotype strains of clusters 1, 2, 4, 6, 8 (Whitham, 1988; Whitham *et al.*, 1993), 18 type strains representing validly described species of the genera *Microbispora*, *Microtetraspora*, *Planobispora*, *Streptomyces* and *Streptosporangium* and 136 putative streptosporangia from soil; seventeen of the strains were duplicated in order to determine test error. The source and strains histories of the test organisms are given in Table 17, pages 114 to 116. The names and sources of the thirty-six 4-methylumbelliferone (4-MU) and thirty-five 7-amino-4-methylcoumarin (7-AMC) derivatives examined are given in Table 18, pages 117 to 118.

2. ENZYME TESTS

The conjugated substrates were dissolved in 0.5ml of dimethyl sulfoxide (DMSO, Sigma), apart from 4MU-lignocerate, 4MU-palmitate and 4MU-stearate which were solubilised in a few drops of acetic acid and DMSO (0.5ml). The conjugated derivatives were then diluted in absolute alcohol to give a final concentration of 5 x 10^{-4}M. All stock solutions were stored at -25°C. Aliquots of each of the substrates (50 µl) were transferred to the wells of 96 welled microtitre plates (Sensititre Ltd., East Grinstead, Sussex, England, U.K.) using an eight chanelled automatic pipette and the solvent evaporated by leaving the plates in a laminar flow cabinet for 30 minutes. The plates were then sealed with a plastic cover (Sensititre Ltd.) and stored at 4°C until required. Plates were allowed to equilibrate to room temperature prior to inoculation.

Table 17 Test strains examined using the rapid enzyme tests

Strain Number	Strain Identity	Strain History / Source
A. *Streptosporangium* isolates		
HJ 001-002, 005-006, 008, 009*, 010-011	*Streptosporangium* sp.	Ginseng field (young plant), Kumsan, Korea
HJ 012-015, 016*, 017, 019-020, 021*, 022-027, 028*, 029-030, 031*, 032-034, 035*, 036-040, 041*, 042-051, 052*, 053-062, 063*, 064-076, 077*, 078-084, 085*, 086-087, 090-093, 094*, 096-098, 099*, 100-103		Ginseng field (post harvest), Kumsan, Republic of Korea
HJ 104-105, 106*		Garden soil, IMTECH, Chandigarh, India
HJ 107-109, 111		Garden soil, Hibuya Park, Tokyo, Japan
HJ 112-116		Garden soil, Tsukuba University, Tsukuba, Japan
HJ 117-118, 122-126, 128, 129*		Tropical rainforest soil, Meru Betini, Indonesia
HJ 130-132, 133, 135, 138-141		Garden soil, Yogyakarta, Indonesia
HJ 143-144, 146-147		Woodland soil, Moun Sorak, Republic of Korea
HJ 148-153		Soil rich in humus, Keswick, England, U.K.
B. Centrotype strains of numerically circumscribed clusters		
TW 166 (cluster 1)	*Streptosporangium* sp.	Plot 5, Cockle Park Experimental Farm, Northumberland, England, U.K.
TW 292 (cluster 2)	*Streptosporangium* sp.	Plot 3, Cockle Park Experimental Farm, Northumberland, England, U.K.
TW116 (cluster 4)	*Streptosporangium* sp.	Oak copse, Corbridge, England, U.K.
TW141 (cluster 6)	*Streptosporangium* sp.	Oak copse, Corbridge, England, U.K.
TW213 (cluster 8)	*Streptosporangium* sp.	Oak copse, Corbridge, England, U.K.

Table 17 continued

Strain Number	Strain Identity	Strain History / Source
C. Marker strains		
TW 038	*Microbispora chromogenes* (Nonomura and Ohara, 1960)	DSM 43165; A.Seino, KCC A-0022; H.Nonomura, FYU, M22; soil. (CBS 304.61; DSM 43165; KCC 3022; NRRL B-2634)
TW 029	*Microbispora rosea* (Nonomura and Ohara, 1957)	S.T. Williams, Department of Genetics and Microbiology, Liverpool University, Liverpool, U.K.; E6, Japanese soil
TW 041	*Microtetraspora fusca* (Thiemann *et al.*, 1968)	DSM 43841; A.Seino, KCC A-3188; RIA 924; J.E. Thiemann, T457; soil. (ATCC 23058; CBS 623.67; IFO 13915)
TW 030	*Microtetraspora glauca* (Thiemann *et al.*, 1968)	S.T. Williams, E63; ATCC 23057; J.E.Thiemann, T158; italian soil. (CBS 624.27; DSM 43311; KCC A-0300; RIA 925)
TW 023	*Microtetraspora niveoalba* (Nonomura and Ohara, 1971b)	DSM 43174; A. Seino. KCC A-0149; H.Nonomura, FYU Mt3; soil. (ATCC 27301; CBS 834.70; DSM 43174)
TW 032	*Planobispora longispora* (Thiemann and Beretta, 1968)	DSM 43041; A.Seino, KCC A-0092; J.E.Thiemann Pb-1075; soil, shore Uramaco, Venezuela. (ATCC 23867; CBS 115.69; IFO 13879)
TW 008	*Streptomyces indiaensis* (Gupta *et al.*, 1965)	DSM 43803; G. Vobis, MB-T10; T. Frumai, MCRL; K.C. Gupta, RRI. (ATCC 33330; KCC A-0053)
TW 006*	*Streptosporangium albidum* (Furumai *et al.*,1968)	DSM 43870; T. Okuda, MCRL-048; soil, Japan. (ATCC 25243; IFO 13901)
TW 010	*Streptosporangium album* (Nonomura and Ohara 1960)	DSM 43023; A.Seino, KCC A-0025; H. Nonomura, FYU, S2-32; soil, Japan. (ATCC 27100; CBS 881.70)
TW 001*	*Streptosporangium amethystogenes* (Nonomura and Ohara, 1960)	DSM 43179; A. Seino, KCC A-0026; H. Nonomura, FYU S5; soil. (ATCC 33327; CBS 429.61; NRRL B-2639; RIA 764)

Table 17 continued

Strain Number	Strain Identity	Strain History / Source
TW 002	*Streptosporangium corrugatum* (Willams and Sharples, 1976)	DSM 43316; S.T. Williams, E90; beach sand. (ATCC 29331; NCIB 11120)
TW 009	*Streptosporangium fragile* (Shearer *et al.*, 1983)	ATCC 31519; IFO 14311
TW 022	*Streoptosporangium nondiastaticum* (Nonomura and Ohara, 1969b)	DSM 43848; ATCC 27101; H. Nonomura, S 2-31; soil
TW 004	*Streptosporangium pseudovulgare* (Nonomura and Ohara, 1969b)	DSM 43181; A. Seino, KCC A-0115; H. Nonomura, FYU, S2-32; soil. (ATCC 27100; CBS 881.70)
TW 005	*Streptosporangium roseum* (Couch, 1955a)	DSM 43021; A. Seino, KCC A-0005; K. Tubaki, NI 9100; J. N. Couch, UNCC 27B; vegetable garden soil. (ATCC 12428; CBS 313.56; IFO 3776; RIA 470)
TW 020	*Streptosporangium viridogriseum* subsp. *kofuense* (Nonomura and Ohara, 1969b)	DSM 43851; ATCC 27102
TW 021	*Streptosporangium viridogriseum* subsp. *viridogriseum* (Okuda *et al.*, 1966a)	DSM 43850; ATCC 25242
TW 007	*Streptosporangium vulgare* (Nonomura and Ohara, 1960)	DSM 43802; G. Vobis, MB-T9; H. Nonomura, FYU S-1; paddy field, Anjo, Aichi Prefecture, Japan. (ATCC 33329; CBS 433.61; KCC A-0028; NRRL B-2633; RIA 765)

* Duplicated strain

Abbreviations: **ATCC**, American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, U.S.A.; **CBS**, Centraalbureau voor Schimmelcultures, Baarn, Netherlands; **DSM**, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Mascheroder, Weg 1B, D-38124, Braunschweig, Federal Republic of Germany; **FYU**, Department of Fermentation Industries, Yamanashi University, Motoganagi-cho, Kofu-shi, Yamanashi-ken, Japan; **IFO**, Institute of Fermentation, 4-54 Juso Nishino-machi, Higashiyodogawa-ku, Osaka, Japan; **KCC**, Kaken Chemical Company Limited, 6-48 Jujodai, 1-Chome, Kita-ku, Tokyo 114, Japan; **NCIB**, National Collection of Industrial Bacteria, St. Machar Drive, Aberdeen, U.K.; **NRRL**, Northern Research and Development Division, United States Department of Agriculture, Peoria, Illinois, U.S.A.; **RIA**, Research Institute for Ampelology, Budapest, Hungary.

Table 18 The name and source of the 7-amino-4-methylcoumarin and 4-methylumbelliferone enzyme substrates used in the rapid enzyme tests

Substrates	Source	Substrates	Source
7-amino-4-methylcoumarin (7AMC)		**4-Methylumbelliferone (4MU)**	
1) Endopeptidase substrates		**1) Glycosides**	
Boc-L-Leucine-glycine-L-arginine-7AMC	Bachem	4MU-2-Acetamido-4,6-o-benzylidene-2-deoxy-β-D-glucopyranoside	Sigma
Boc-L-Valine-L-leucine-L-lysine-7AMC	Bachem	4MU-2-Acetamido-2-deoxy-β-D-galactopyranoside	NBS
Boc-L-Valine-L-proline-L-arginine-HCl-7AMC	Bachem	4MU-2-Acetamido-2-deoxy-β-D-glucopyranoside	NBS
Boc-*iso*-L-Leucine-L-glutamine-glycine-L-arginine-HCl-7AMC	Nova	4MU-N-Acetyl-β-D-galactosamine	Sigma
Bz-L-Valine-glycine-L-arginine-HCl-7AMC	Bachem	4MU-N-Acetyl-β-D-glucosamine	Sigma
Glutaryl-glycine-glycine-L-phenylalanine-7AMC	Nova	4MU-β-D-Cellobiopyranoside	Sigma
Succinyl-glycine-L-proline-7AMC	Nova	4MU-α-L-Fucopyranoside	NBS
Succinyl-L-leucine-L-tyrosine-7AMC	Nova	4MU-β-D-Fucoside	Sigma
Succinyl-L-alanine-L-alanine-L-phenylalanine-7AMC	Nova	4MU-β-L-Fucoside	Sigma
Succinyl-L-leucine-L-leucine-L-valine-L-tyrosine-7AMC	Nova	4MU-α-D-Galactoside	Sigma
Z-L-Arginine-L-arginine-7AMC	CRB	4MU-β-D-Galactoside	Sigma
Z-Glycine-L-proline-7AMC	CRB	4MU-α-D-Glucoside	Sigma
Z-L-Glycine-glycine-L-leucine-7AMC	Nova	4MU-β-D-Glucoside	Sigma
2) Other peptidase substrate		4MU-α-D-Glucuronide	Sigma
L-Lysine-L-alanine-7AMC	Sigma	4MU-β-D-Maltoside	AJ
L-Alanine-L-phenylalanine-L-lysine-2TFA-7AMC	Bachem	4MU-α-D-Mannopyranoside	Sigma
3) Exopeptidase substrates		4MU-β-D-Mannopyranoside	Sigma
L-Alanine-7AMC	Sigma	4MU-β-D-Ribofuranoside	AJ

Table 18 continued

Substrates	Source	Substrates	Source
β-Alanine-TFA-7AMC	Bachem	4MU-2,3,5-Trio-o-benzyl-α-L-arabinofuranoside	Sigma
D-Alanine-TFA-7AMC	Bachem	4MU-β-D-Xyloside	Sigma
L-Arginine-7AMC	Bachem	4MU-β-D-Xylopyranoside	KL
Asparate-7AMC	Bachem	**2) Inorganic esters**	
L-Asparagine-TFA-7AMC	Sigma	4MU-Phosphate	Sigma
L-Cysteine(Bzl)-7MAC	Bachem	4MU-Pyrophosphate	KL
L-Glutamine-HCl-7AMC	Bachem	4MU-Sulphate	Sigma
L-Glycine-HBr-7AMC	Bachem	*bis*-(4MU)-phosphate	AJ
L-Histidine-7AMC	Bachem	**3) Organic esters**	
iso-Leucine-TFA-7AMC	Bachem	4MU-(protected) Acetate	AJ
L-Leucine-7AMC	Bachem	4MU-Eicosanoate	AJ
L-Methionine-7AMC	Bachem	4MU-Elaidate	KL
L-Proline-HBr-7AMC	CRB	4MU-Heptanoate	KL
L-Pyroglutamate-7AMC	Bachem	4MU-Laurate	KL
L-Serine-HCl-7AMC	Bachem	4MU-Lignocerate	NBS
L-Tyrosine-7AMC	Bachem	4MU-Myristate	AJ
L-Valine-7AMC	Bachem	4MU-Palmitate	Sigma
L-Glycine-L-proline-HBr-7AMC	Bachem	4MU-Pentadecanoate	AJ
L-Arginine-L-arginine-3HCl-7AMC	CRB	4MU-Stearate	Sigma
		4MU-Octadecanoate	AJ

Boc, tert-butyloxycarbonyl; Bz, benzoyl; Bzl, benzyl; HBr, hydrogen bromide; HCl, hydrochloride; TFA, trifluoroacetate; Z, benzyloxycarbonyl.

AJ, A.L.James, School of Chemistry and Life Science, University of Northumbria at Newcastle, Newcastle upon Tyne, NE1 8ST, U.K.; Bachem, Bachem Feinchemikalien AG Ltd., Hauptstrasse 144, CH-4416 Bubendorf, Switzerland; CRB, Cambridge Research Biochemicals Ltd., 3 Heathcoat Building, Highfields Science Park, Nottingham, NG7 2QJ; KL, Koch-Light Ltd., Rookwood Way, Haverhill, Suffolk, CB9 8PB, U.K.; NBS, New Brunswick Scientific Ltd., Edison House, 163 Dixons Hill Road, North Mymms, Hatfield AL9 7JE, U.K.; Nova, Calbiochem-Novabiochem Ltd., 3 Heathcoat Building, Highfields Science Park, Nottingham, NG7 2QJ, U.K.

The tests strains were examined for their ability to degrade the 71 conjugated fluorogenic substrates. The organisms were inoculated onto the centre of sterile cellulose nitrate membrane filters (0.45µm pore size, 47mm diameter; Whatman Ltd., Maidstone, England, U.K.) that had been aseptically placed in the centre of modified Bennett's agar plates (Agrawal, unpublished data); the membranes were allowed to absorb surface water and the resultant plates incubated at 30°C for 7 days.

After incubation, growth was removed from the surface of the cellulose nitrate membrane filters and transferred to universal bottles containing 15 ml of 0.1M MOPS buffer (pH 7.5) and about ten sterile glass beads (Jencon Scientific Ltd., Leighton Buzzard, Bedfordshire, England, U.K.; gauge 5mm diameter). The preparations were agitated on a Vortex mixer (Fisons Scientific Apparatus Ltd., Loughborough, Leicestershire, England, U.K.) and the homogeneous suspensions obtained adjusted to 0.2 turbidity on a colorimeter at 600 nm. This procedure yielded suspensions of between *ca.* 6.2×10^7 to 10^8 viable colony forming units per ml.

Homogenised suspensions of each test strain (100µl) were inoculated into wells of the microtitre plates (Sensititre Ltd.) using an eight channel automatic pipette. The plates contained negative controls, that is, wells with only inoculum and substrate with buffer, respectively. Inoculated plates were resealed and incubated at 30°C for 24 hours and the results read using an automatic fluorescent plate reader (Sensititre Ltd.) at a wavelength of 366 nm. The reader was allowed to warm-up for 30 minutes and calibrated against an internal standard before the analyses. A negative control plate inoculated with MOPS buffer (pH7.5) was read both at the beginning and at the end of the analyses in order to provide readings for background fluorescence and autofluorescence of the compounds.

The results were collected on a PC-AT microcomputer *via* dedicated software (Sensititre Ltd.).

3. DATA ANALYSIS

a) Automatic Data Collection

The results of the enzyme tests were collected on an IBM PC computer and analysed using Quattropro 4.0 (Borland International Inc.). The tests were coded positive when the difference in fluorescent intensities between test and negative control wells that contained only cell inoculum and substrate with buffer was more than 0 [$R_p = V_r - V_c - V_{s+b}$ (R_p,positive result; V_r, resultant reaction between test strain and enzyme substrate; V_c, value of cell inoculum alone; V_{s+b}, value of substrate with 0.1Mol MOPS buffer)].

b) Numerical Classification

All of the tests were scored + for a positive and - for a negative result. The binary test data were typed in a +/- format as input into the TAXON program (Ward, unpublished data; Appendix A). The +/- results in the final data matrix were converted to a binary format (1/0) and written to a DOS text file using the TAXON program and analysed using the CLUSTAN 2.1 statistical computer package (Clustan Ltd., Scotland, U.K.). The CLUSTAN procedure HIERARCHY was used to examine the data using the D_p, S_J and S_{sm} coefficients. Clustering was achieved using the unweighted pair group method with arithmetic averages (UPGMA; Sneath and Sokal, 1973) algorithm.

c) Test Error

Seventeen strains were examined in duplicate (Table 17, pages 114 to 116) and an estimate of test variance calculated (formula 15; Sneath and Johnson,

1972); this was used to estimate the average probability (p) of an erroneous test result (Formula 4; Sneath and Johnson, 1972).

RESULTS

A. SELECTIVE ISOLATION, ENUMERATION AND CHARACTERISATION OF STREPTOSPORANGIA

1. ENUMERATION

The numbers of presumptive microbisporae, microtetrasporae, streptosporangia and unidentified sporoactinomycetes isolated from the composite soil samples on humic acid vitamins agar supplemented with actidione (50mg/l) and nalidixic acid (30mg/l) are shown in Table 19, pages 123 to 129. Typical selective isolation plates supporting the growth of streptosporangia are shown in Figure 5, page 130.

It is evident from the information in Table 19 that *Microbispora*, *Microtetraspora* and *Streptosporangium* strains are widely distributed in the soils examined though they were not isolated from the composite soil sample collected from around Mount Bromo in Indonesia or from the sample of Brazilian rainforest soil, that is, from the soils with bulk pH values of 3.8. The highest actinomycete counts were consistently recorded from the soil samples that were subject to the less extreme pretreatment regimes, namely when 10^{-1} dilutions were heated in the presence of the germicide sodium dodecyl sulphate (0.05%, w/v) or the spore germinant yeast extract (6%, w/v). The highest counts were recorded for the composite 8 sample (soils 579-581) that was pretreated with yeast extract (6%, w/v) for 20 minutes at 40°C prior to dilution and plating out onto HV agar supplemented with actidione (50mg/l) and nalidixic acid (30mg/l) and incubated for 4 weeks at 30°C.

In nearly all cases, streptosporangia were isolated from dried soil samples pretreated at 120°C for an hour and from 10^{-1} dilutions of soil treated with yeast extract at 40°C for 20 minutes. In general, the higher counts were recorded using

Table 19 Number of *Microbispora*, *Microtetraspora*, *Streptosporangium* and other soil actinomycetes (colony forming units per gram dry weight soil) isolated from diverse soil samples after pretreatment and dilution onto HV agar supplemented with actidione (50mg/l) and nalidixic acid (30mg/l) and incubated at 30°C for 4 weeks

a) Comparison of pretreatment regimes

Pretreatment	Primary Target Organism(s)	Colony Forming Units Per Gram Dry Weight Soil							
		Microbispora		*Microtetraspora*		*Streptosporangium*		Other Actinomycetes	
		$X10^3$	±SD	$X10^3$	±SD	$X10^3$	±SD	$X10^3$	±SD
Pretreatment with dry heat at 120°C for 1hour	*Microbispora*, *Streptosporangium*	16.4	1.2	3.5	0.3	21.1	2.5	582.2	39.6
Pretreatment with dry heat at 120°C for 1hour and phenol (1.5%, w/v) at 30°C for 30 minutes	*Microbispora*	23.0	1.14	0.7	0.1	5.8	0.7	394	20.6
Pretreatment with sodium dodecyl sulphate (0.05%, w/v) at 40°C for 20 minutes	actinomycetes	29.4	5.8	8.0	0.6	26.9	5.1	1358.4	56.1
Pretreatment with yeast extract (6%, w/v) at 40°C for 20minutes	actinomycetes	216.3	16.4	94.9	6.9	166.6	20.4	3246.7	126

b) Comparison of composite soil samples pretreated with dry heat at 120°C for 1hour

Composite Soil Sample	Primary Target Organism(s)	Colony Forming Units Per Gram Dry Weight Soil							
		Microbispora		*Microtetraspora*		*Streptosporangium*		Other Actinomycetes	
		$X10^3$	±SD	$X10^3$	±SD	$X10^3$	±SD	$X10^3$	±SD
1. Composite soil, 433-434, Hibuya Park, Tokyo, Japan (pH 6.3)	*Microbispora, Streptosporangium*	0.3	0.3	0.1	0.1	6.5	10.6	45	20
2. Composite soil, 435-436, Tsukuba University, Tsukuba, Japan (pH 4.6)		1.1	0.9	0.1	0.1	0.4	0.4	35	12
3. Composite soil, 443-444, Garden soil, IMTECH, Chandigarh, India (pH 6.4)		1.1	0.9	0.1	0.1	0.1	0.1	63	33
4. Composite soil, 482-489, Rainforest soil, Meru Betini, Indonesia (pH 6.2)		1.9	1.9	0.3	0.3	7.2	10.2	56	28

Table 19 b) continued

Composite Soil Sample	Primary Target Organism(s)	Colony Forming Units Per Gram Dry Weight Soil							
		Microbispora		*Microtetraspora*		*Streptosporangium*		Other Actinomycetes	
		$X10^3$	±SD	$X10^3$	±SD	$X10^3$	±SD	$X10^3$	±SD
5. Composite soil, 512-513, Rim of crater, Mount Bromo, Indonesia (pH 3.8)	*Microbispora, Streptosporangium*	0	0	0	0	0	0	0.2	0.2
6. Composite soil, 515-516, Garden soil, Yogyakarta, Indonesia (pH 6.2)		0.9	1.0	0.3	0.3	1.0	0.9	43	7.0
7. Composite soil, 576-577, Soils rich in humus, Keswick, England, U.K. (pH 5.9)		1.0	0.9	0.1	0.1	0	0	23	1.0
8. Composite soil, 579-581, Ginseng soil (post harvest), Kumsan, Republic of Korea (pH 5.9)		4.6	4.7	1.0	0.9	3.5	3.8	83	53

Table 19 b) continued

Composite Soil Sample	Primary Target Organism(s)	Colony Forming Units Per Gram Dry Weight Soil							
		Microbispora		*Microtetraspora*		*Streptosporangium*		Other Actinomycetes	
		$X10^3$	±SD	$X10^3$	±SD	$X10^3$	±SD	$X10^3$	±SD
9. Composite soil, 583-584, Ginseng soil (post harvest), Kumsan, Republic of Korea (pH 6.2)	*Microbispora, Streptosporangium*	2.6	2.9	0.3	0.3	1.1	0.9	157	160
10. Composite soil, 585-587, Ginseng soil (young plant), Kumsan, Republic of Korea (pH 5.8)		1.8	1.9	0.3	0.3	1.0	0.9	42	19
11. Composite soil, 604-605, Woodland soil, Mount Sorak, Republic of Korea (pH 5.8)		1.1	0.9	0.9	1.0	0.3	0.3	32	12
12. Soil, A2, Rainforest soil, Brazil (pH 3.8)		0	0	0	0	0	0	3.0	3.0

c) Comparison of composite soil samples pretreated with yeast extract (6%, w/v) at 40°C for 20 minutes

Composite Soil Sample	Primary Target Organism(s)	Colony Forming Units Per Gram Dry Weight Soil							
		Microbispora		*Microtetraspora*		*Streptosporangium*		Other Actinomycetes	
		$X10^3$	±SD	$X10^3$	±SD	$X10^3$	±SD	$X10^3$	±SD
1. Composite soil, 433-434, Hibuya Park, Tokyo, Japan (pH 6.3)	soil actinomycetes	21.9	16.2	13.8	6.5	3.8	2.2	265	35
2. Composite soil, 435-436, Tsukuba University, Tsukuba, Japan (pH 4.6)		8.9	9.6	1.1	0.9	3.8	2.2	303	157
3. Composite soil, 443-444, Garden soil, IMTECH, Chandigarh, India (pH 6.4)		7.5	4.3	5.6	3.2	3.8	2.2	285	95
4. Composite soil, 482-489, Rainforest soil, Meru Betini, Indonesia (pH 6.2)		9.3	9.3	1.8	1.9	13.8	6.5	316	19

Table 19 c) continued

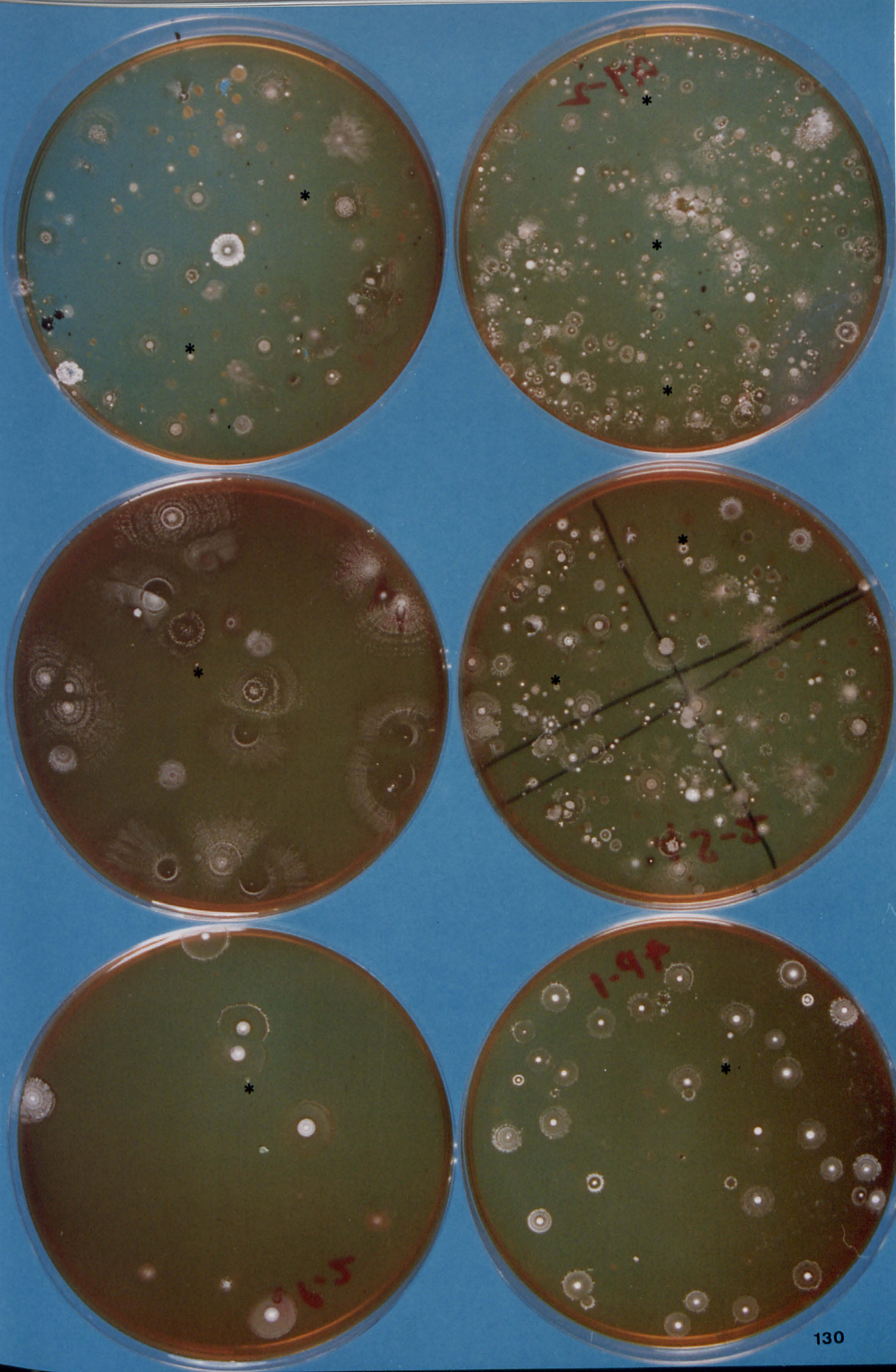
Composite Soil Sample	Primary Target Organism(s)	Colony Forming Units Per Gram Dry Weight Soil							
		Microbispora		*Microtetraspora*		*Streptosporangium*		Other Actinomycetes	
		$X10^3$	±SD	$X10^3$	±SD	$X10^3$	±SD	$X10^3$	±SD
5. Composite soil, 512-513, Rim of crater, Mount Bromo, Indonesia (pH 3.8)	soil actinomycetes	0	0	0	0	0	0	0.7	0.9
6. Composite soil, 515-516, Garden soil, Yogyakarta, Indonesia (pH 6.2)		20.0	17.3	8.1	9.7	16.3	19.5	332	112
7. Composite soil, 576-577, Soils rich in humus, Keswick, England, U.K. (pH 5.9)		15.6	5.4	13.8	6.5	11.9	7.6	233	39
8. Composite soil, 579-581, Ginseng soil (post harvest), Kumsan, Republic of Korea (pH 5.9)		61.8	7.6	23.8	15.2	79.4	11.9	428	143

Table 19 c) continued

Composite Soil Sample	Primary Target Organism(s)	Colony Forming Units Per Gram Dry Weight Soil							
		Microbispora		*Microtetraspora*		*Streptosporangium*		Other Actinomycetes	
		$X10^3$	±SD	$X10^3$	±SD	$X10^3$	±SD	$X10^3$	±SD
9. Composite soil, 583-584, Ginseng soil (post harvest), Kumsan, Republic of Korea (pH 6.2)	soil actinomycetes	35.6	22.7	5.6	3.2	11.9	7.6	423	204
10. Composite soil, 585-587, Ginseng soil (young plant), Kumsan, Republic of Korea (pH 5.8)		23.8	15.2	13.8	65.0	10.0	86.6	369	89
11. Composite soil, 604-605, Woodland soil, Mount Sorak, Republic of Korea (pH 5.8)		11.9	7.6	7.5	4.3	11.9	7.6	247	60
12. Soil, A2, Rainforest soil, Brazil (pH 3.8)		0	0	0	0	0	0	45	48

Figure 5 Streptosporangia growing on humic acid vitamins agar plates supplemented with actidione (50mg/l) and nalidixic acid (30mg/l) and incubated for 4 weeks at 30°C.

Representative colonies of streptosporangia are indicated by an asterisk.



the latter procedure. The highest count, 7.94 ± 1.19 x 10^4 colony forming units per gram dry weight soil, was recorded from a sample of composite soil 8 which had been the subject of the pretreatment regime involving yeast extract prior to plating out onto HV agar and incubation at 30°C for 4 weeks. In this instance, the streptosporangia accounted for almost 40% of the sporoactinomycetes growing on the isolation plates. However, in most cases, streptosporangia accounted for less than 5% of the actinomycetes growing on isolation plates (Table 19, pages 123-129). Indeed, the vast majority of the colonies growing on isolation plates were assigned to the "catch-all" group, namely the "other actinomycetes". It was subsequently shown that most of the organisms belonging to this group were streptomycetes.

The highest numbers of microbisporae and microtetrasporae were also recorded from soil suspensions treated with yeast extract and heated for 20 minutes at 40°C. It is also evident (Table 19, pages 123 to 129) that heat pretreated soil subsequently treated with phenol (1.5%, w/v) at 30°C for 30 minutes favoured the growth of microbisporae as opposed to microtetrasporae and streptosporangia. Nevertheless, the highest counts of microbisporae, 6.18 ± 0.76 x 10^4 colony forming units per gram dry weight soil, were recorded from suspensions of composite soil 8 treated with yeast extract (6%, w/v) for 20 minutes at 40°C. The highest counts of microtetrasporae, 2.38 ± 1.52 x 10^4 colony forming units per gram dry weight soil, were also recorded from suspensions of composite soil 8 treated with yeast extract (6%, w/v) at 40°C for 20 minutes.

2. SELECTION AND PURIFICATION

Presumptive streptosporangial colonies growing on humic acid vitamins (HV) agar supplemented with actidione (50mg/l) and nalidixic acid (30mg/l) were

examined for the presence of spore vesicles (sporangia) using a Nikon Optiphot binocular light microscope fitted with a long distance objective (X400 magnification). One hundred and fifty-three presumptively identified streptosporangia were picked from the isolation plates using sterile tooth-picks and inoculated onto HV agar plates which were incubated for two weeks at 30°C. The resultant cultures were examined for purity both by eye and using the Nikon Optiphot binocular light microscope (X400 magnification) and single colonies used to inoculate further HV plates which were also incubated for two weeks at 30°C. This procedure was repeated until all of the isolates were in pure culture. The sources and procedures used to isolate all of the test strains are given in Table 20, pages 133 to 136. In subsequent studies, 136 of the presumptive streptosporangia were examined for the presence of isomers of diaminopimelic acid in whole-organism hydrolysates, screened against diagnostic tests included in a computer-assisted procedure designed for the identification of streptosporangia and examined using a battery of rapid enzyme tests to determine their enzymatic profiles.

3. CHARACTERISATION

a. Morphological Studies

All of the isolates presumptively identified as streptosporangia produced spore vesicles (Figures 6a to 6f, pages 137 to 139). Scanning electron micrographs of three of the test strains (HJ 047, HJ 084 and HJ 094) are shown in Figure 7, pages 140 to 141.

b. Diaminopimelic Acid Analysis of Soil Isolates

The LL- and *meso*-diaminopimelic acid standards were clearly separated on the cellulose TLC plates. All of the presumptive streptosporangia contained

Table 20 Source of presumptive streptosporangia isolated on HV agar incubated at 30°C for 4 weeks following various pretreatment regimes

Strain Number (HJ)	Pretreatment Regime	Soil Number	Source
001*, 002*, 003, 004	Dried soil heated at 120°C for 1 hour	585-587	Ginseng field (young plant), Kumsan, Republic of Korea
005*, 006*, 007	Dried soil heated at 120°C for 1 hour and treated with phenol (1.5%, w/v) at 30°C for 30 minutes		
008*	Dried soil treated with sodium dodecyl sulphate (0.05%, w/v) at 40°C for 20 minutes		
009*, 010*, 011*	Dried soil treated with yeast extract (6%, w/v) at 40°C for 20 minutes		
012*, 013*, 014*,**, 015*, 016*,**, 017*,**, 018, 019*,**, 020*,**, 021*,**, 022*, 023*,**, 024*	Dried soil heated at 120°C for 1 hour	579-581	Ginseng field (post harvest), Kumsan, Republic of Korea
025*,**, 026*,**, 027*,**, 028*,**, 029*, 030*, 031*,**, 032*,**, 033*	Dried soil heated at 120°C for 1 hour and treated with phenol (1.5%, w/v) at 30°C for 30 minutes		
034*, 035*,**, 036*,**, 037*, 038*, 039*, 040*,**, 041*, 042*,**, 043*, 044*,**, 045*,**, 046*, 047*,+, 048*,**, 049*, 050*, 051*	Dried soil treated with sodium dodecyl sulphate (0.05%, w/v) at 40°C for 20 minutes		

Table 20 continued

Strain Number (HJ)	Pretreatment Regime	Soil Number	Source
052*, 053*,**, 054*,**, 055*, 056*, 057*, 058*,**, 059*, 060*, 061*, 062*, 063*, 064*, 065*, 066*, 067*, 068*,**, 069*,**, 070*, 071*, 072*, 073*, 074*, 075*, 076*, 077*, 078*, 079*, 080*, 081*, 082*, 083*, 084*,+, 085*, 086*, 087*	Dried soil treated with yeast extract (6%, w/v) at 40°C for 20 minutes	579-581	Ginseng field (post harvest), Kumsan, Republic of Korea
088, 089, 090*, 091*, 092*	Dried soil heated at 120°C for 1 hour	583-584	Ginseng field (post harvest), Kumsan, Republic of Korea
093*, 094*,+, 095, 096*, 097*	Dried soil heated at 120°C for 1 hour and treated with phenol (1.5%, w/v) at 30°C for 30 minutes		
098*, 099*,**	Dried soil treated with sodium dodecyl sulphate (0.05%, w/v) at 40°C for 20 minutes		
100*, 101*, 102*, 103*	Dried soil treated with yeast extract (6%, w/v) at 40°C for 20 minutes		
104*	Dried soil heated at 120°C for 1 hour	443-444	Garden soil, IMTECH, Chandigarh, India
105*, 106*	Dried soil treated with yeast extract (6%, w/v) at 40°C for 20 minutes		
107*, 108*, 109*	Dried soil heated at 120°C for 1 hour	433-434	Garden soil, Hibuya Park, Tokyo, Japan
110, 111*	Dried soil treated with yeast extract (6%, w/v) at 40°C for 20 minutes		

Table 20 continued

Strain Number (HJ)	Pretreatment Regime	Soil Number	Source
112*, 113*,**, 114*	Dried soil heated at 120°C for 1 hour	435-436	Garden soil, Tsukuba University, Tsukuba, Japan
115*,**, 116*,**	Dried soil treated with yeast extract (6%, w/v) at 40°C for 20 minutes		
117*, 118*,**, 119, 120, 121	Dried soil heated at 120°C for 1 hour	482-489	Tropical rainforest soil, Meru Betini, Indonesia
122*	Dried soil heated at 120°C for 1 hour and treated with phenol (1.5%, w/v) at 30°C for 30 minutes		
123*, 124*,**	Dried soil treated with sodium dodecyl sulphate (0.05%, w/v) at 40°C for 20 minutes		
125*,**, 126*,**, 127, 128*, 129*	Dried soil treated with yeast extract (6%, w/v) at 40°C for 20 minutes		
130*,**, 131*, 132*,**, 133*	Dried soil heated at 120°C for 1 hour	515-516	Garden soil, Yogyakarta, Indonesia
134, 135*,**, 136	Dried soil heated at 120°C for 1 hour and phenol (1.5%, w/v) at 30°C for 30 minutes		
137	Dried soil treated with sodium dodecyl sulphate (0.05%, w/v) at 40°C for 20 minutes		
138*, 139*, 140*, 141*	Dried soil treated with yeast extract (6%, w/v) at 40°C for 20 minutes		
142, 143*	Dried soil heated at 120°C for 1 hour	604-605	Woodland soil, Mount Sorak, Republic of Korea
144*, 145, 146*, 147*	Dried soil treated with yeast extract (6%, w/v) at 40°C for 20 minutes		

Table 20 continued

Strain Number (HJ)	Pretreatment Regime	Soil Number	Source
148*, 149*	Dried soil treated with sodium dodecyl sulphate (0.05%, w/v) at 40°C for 20 minutes	576-577	Soil rich in humus, Keswick, England, U.K.
150*, 151*, 152*, 153*	Dried soil treated with yeast extract (6%, w/v) at 40°C for 20 minutes		

* Strains examined for the presence of diaminopimelic acid and screened against the diagnostic and rapid enzyme tests.

** Strains examined for the presence of spore vesicles.

+ Strains examined in the SEM studies.

Figure 6a Spore vesicles of isolate HJ 14 growing on HV agar after 4 weeks at 30° (X400 magnification)

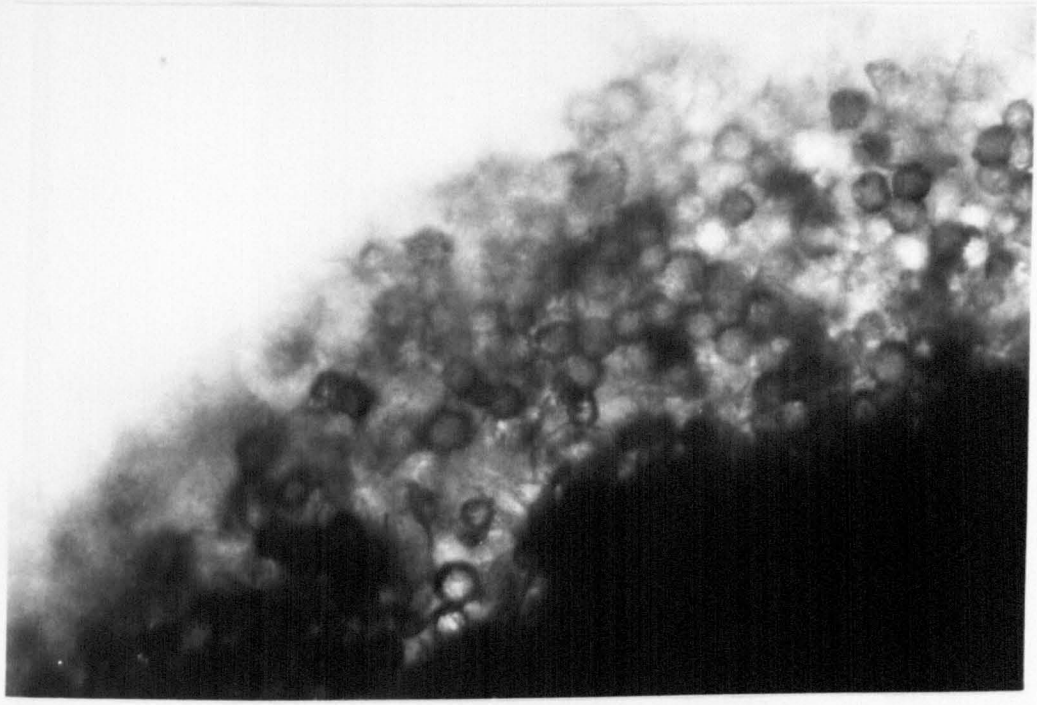


Figure 6b Spore vesicles of isolate HJ 45 growing on HV agar after 4 weeks at 30° (X400 magnification)

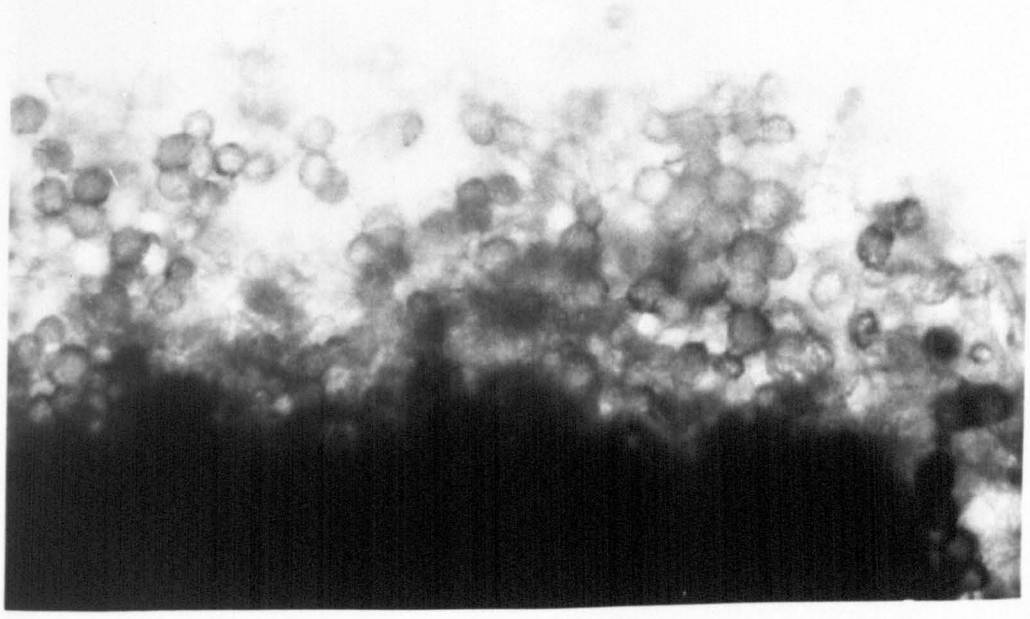


Figure 6c Spore vesicles of isolate HJ 48 growing on HV agar after 4 weeks at 30° (X400 magnification)

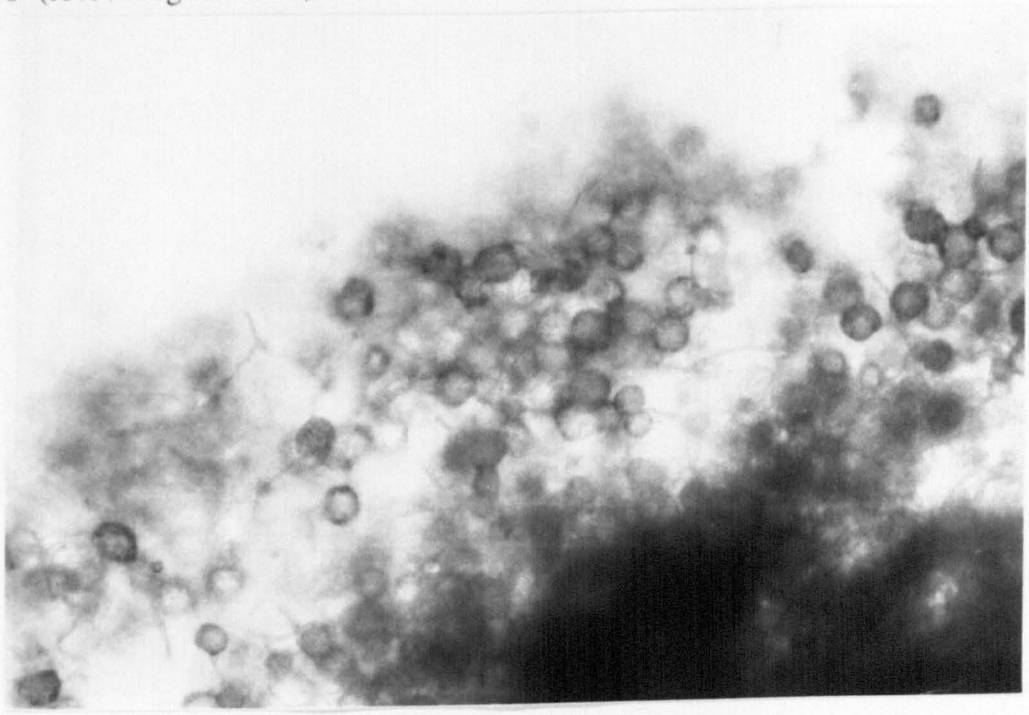


Figure 6d Spore vesicles of isolate HJ 69 growing on HV agar after 4 weeks at 30° (X400 magnification)

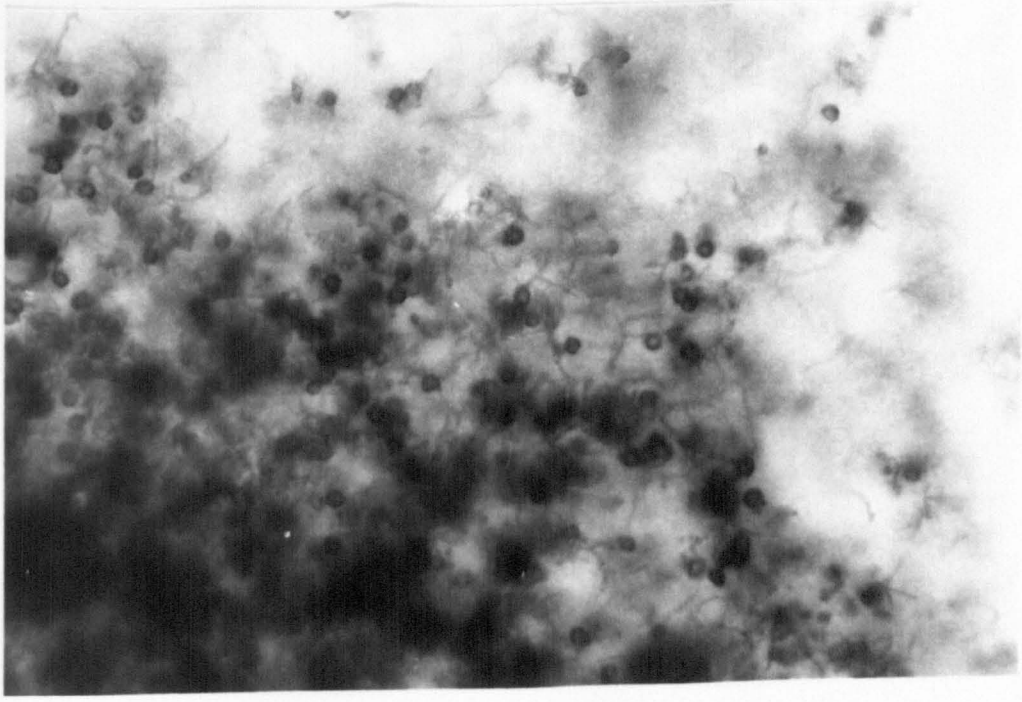


Figure 6e Spore vesicles of isolate HJ 99 growing on HV agar after 4 weeks at 30° (X400 magnification)

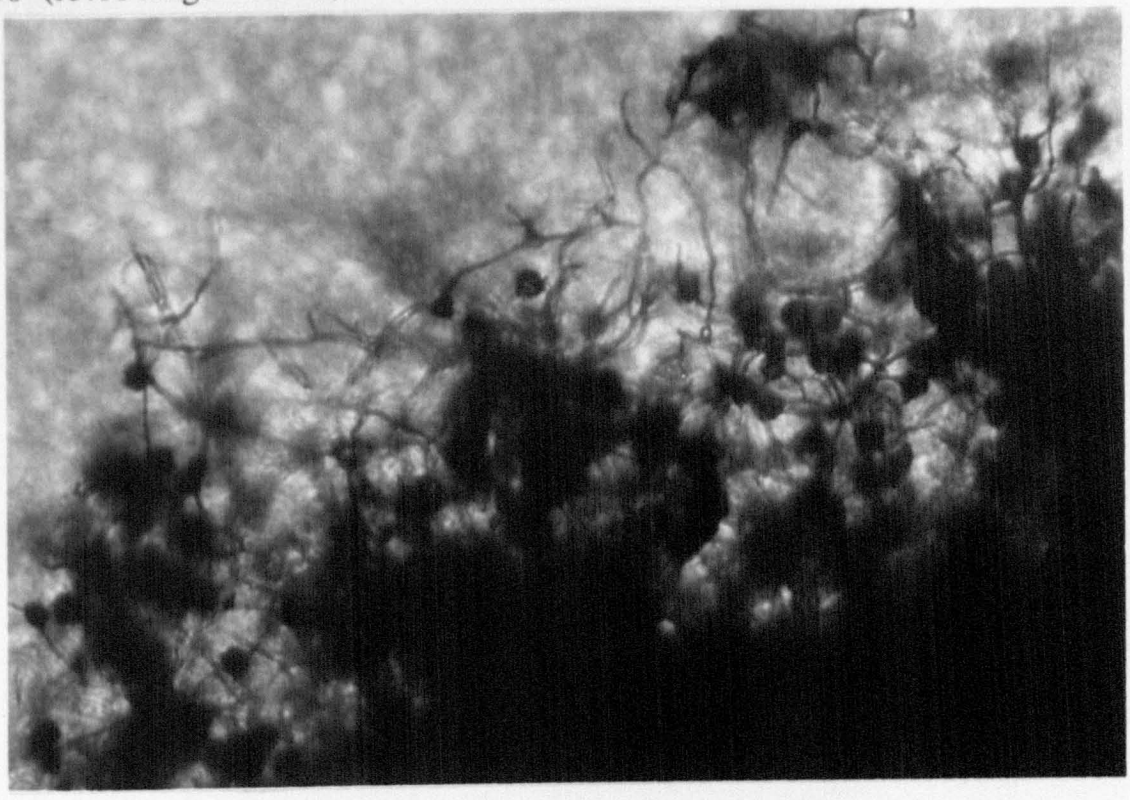


Figure 6f Spore vesicles of isolate HJ 135 growing on HV agar after 4 weeks at 30° (X400 magnification)

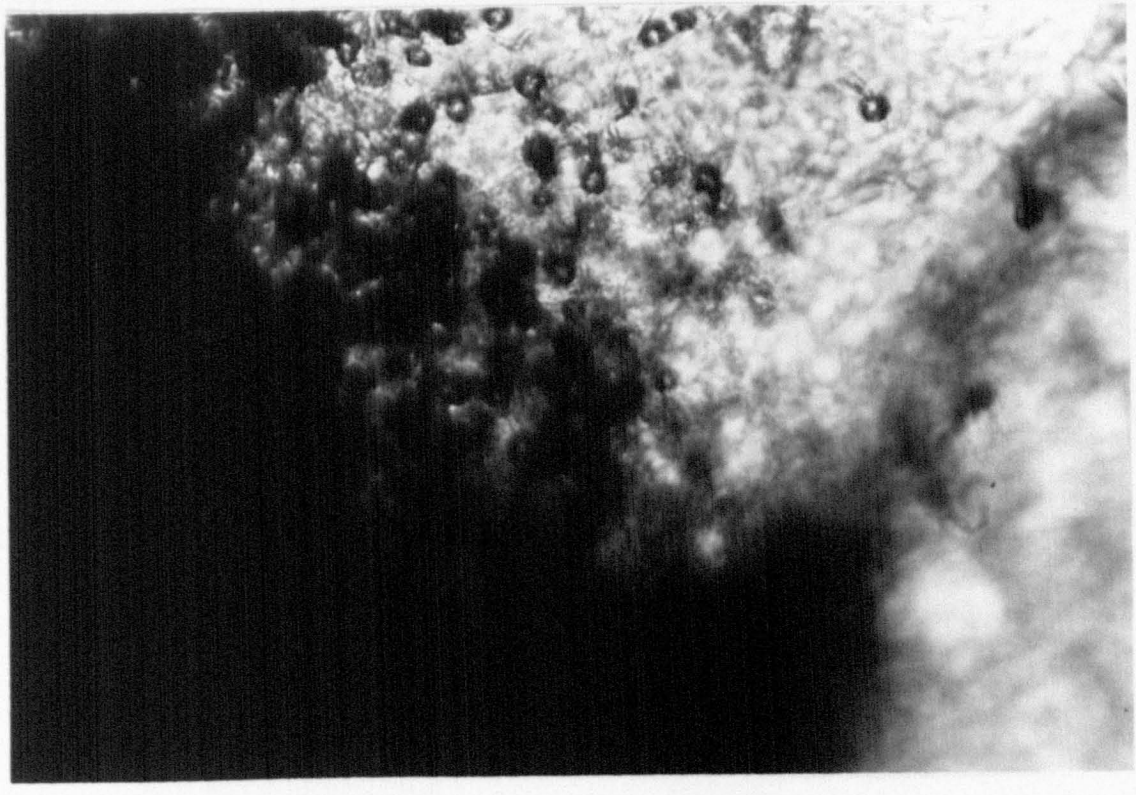


Figure 7a Morphology of spore vesicles of *Streptosporangium* isolates HJ 047 (11,4KX)

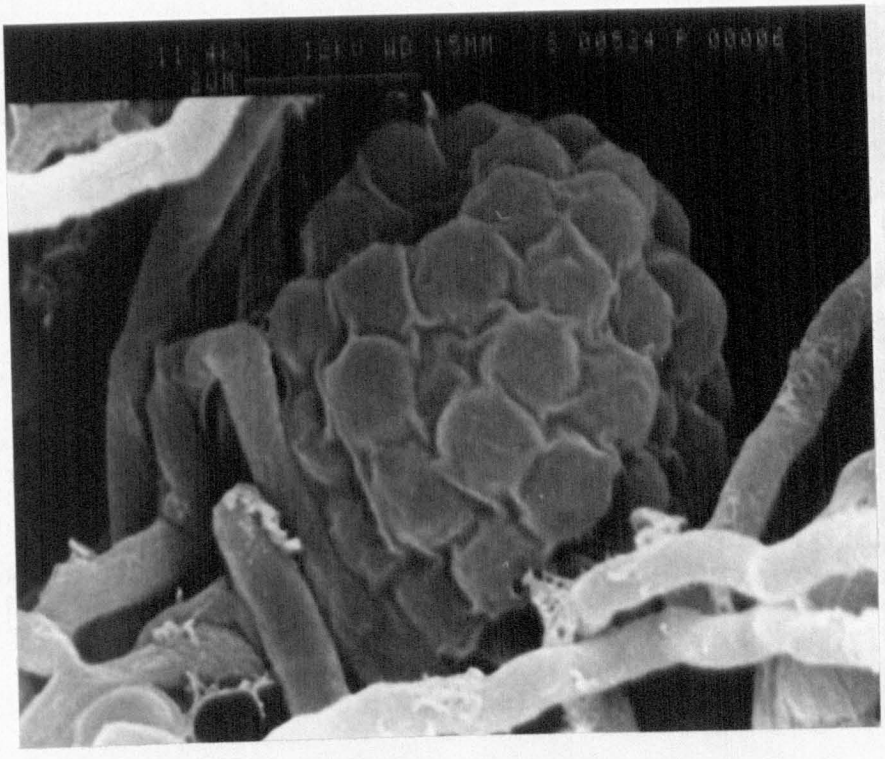


Figure 7b Morphology of spore vesicles of *Streptosporangium* isolates HJ 084 (683KX)

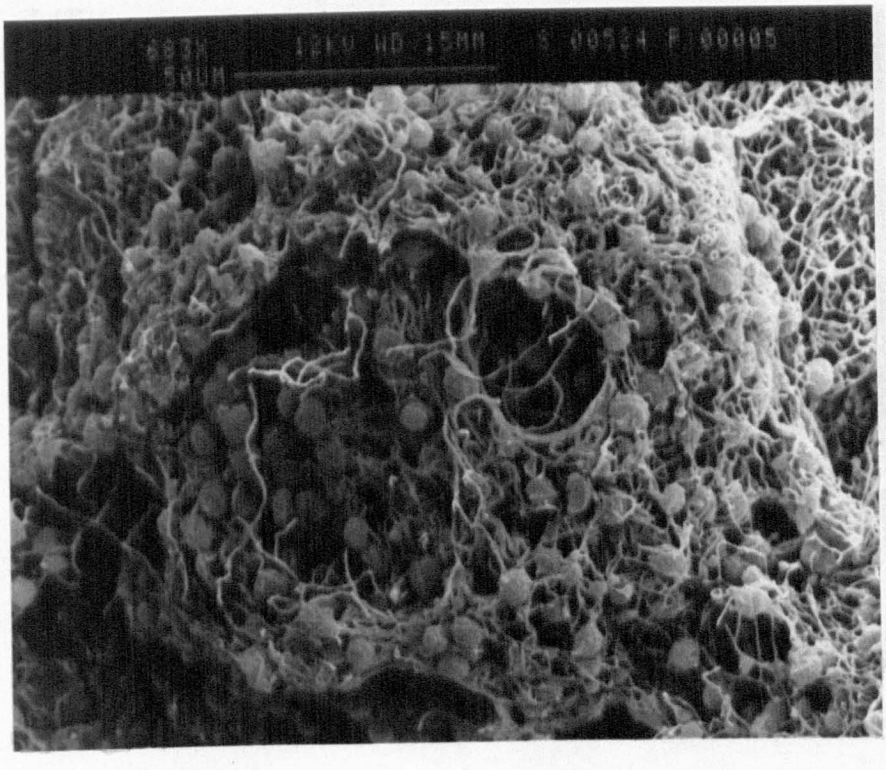


Figure 7c Morphology of spore vesicles of *Streptosporangium* isolates HJ 094 (6,57KX)

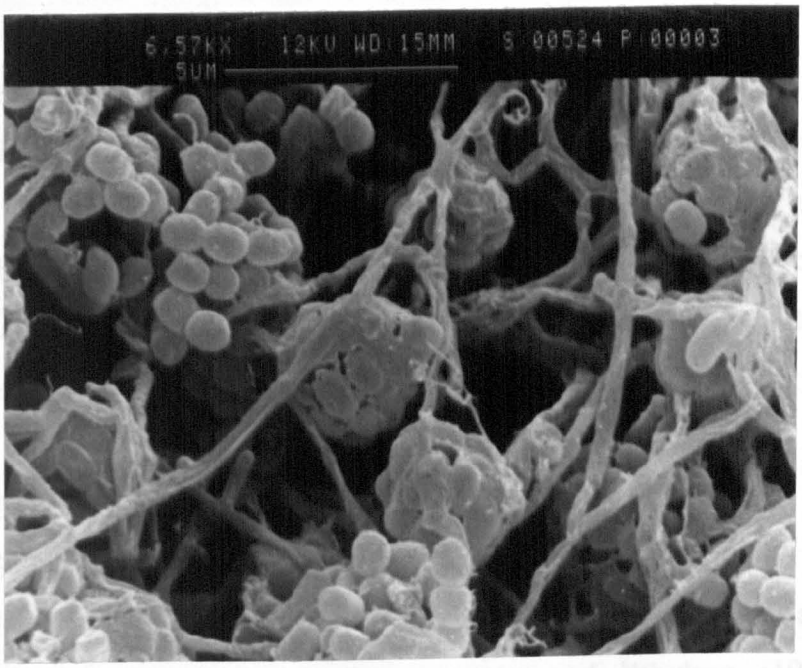
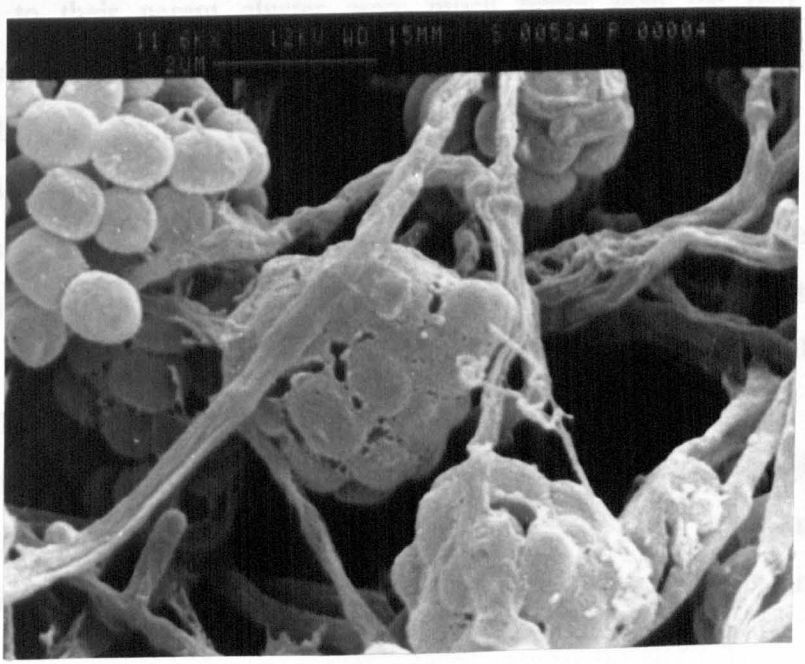


Figure 7d Morphology of spore vesicles of *Streptosporangium* isolates HJ 094 (11,6KX)



meso-diaminopimelic acid, and all of the 46 unknown actinomycetes LL-diaminopimelic acid (Figure 8, page 143).

c. Conclusions

It is evident from the chemical and morphological studies that strains presumptively identified as streptosporangia can be classified in this genus with considerable confidence. Similarly, most of the unknown actinomycetes have chemical and morphological properties consistent with their classification in the genus *Streptomyces* (Williams *et al.*, 1989).

B. NUMERICAL IDENTIFICATION OF STREPTOSPORANGIA

1. PRACTICAL EVALUATION OF THE FREQUENCY MATRIX

All seventy representatives of the twelve major streptosporangial clusters circumscribed in the numerical phenetic survey of Whitham (1988) were unambiguously assigned to their parent cluster with high identification scores (Table 21, pages 144 to 156). In all cases the identification scores of strains assigned to their parent cluster were much better than the two next best alternatives. The ten centrotype strains, namely TW 166 (cluster 1), TW 292 (cluster 2), TW 116 (cluster 4), TW 141(cluster 6), TW 226 (cluster 7), TW 213 (cluster 8), TW 005 (cluster 9), TW 002 (cluster 10), TW 126 (cluster 11) and TW 182 (cluster 12) had high Willcox probabilities (> 0.9999 in all but one instance), taxonomic distances smaller than the 95% taxonomic radius and high Gaussian distance probability values (range 51.2-100, apart from strain TW 005 with a value of 1.98).

Similarly, all thirty representatives of cluster 1 showed high Willcox probabilities (range 0.9844-0.9999), taxonomic distances smaller than the 95% taxonomic radius and high Gaussian distance probability values (range 7.561-

Figure 8 Identification of diaminopimelic acid isomers by one dimensional thin layer chromatography of whole-organism hydrolysates of test strains using the solvent system methanol:water:10N HCl:pyridine = 80:26.25:3.75:10, v/v.

Plate A

tracks (from left to right)

1. α, ε-diaminopimelic acid
2. HJ 104
3. A 001
4. HJ 105
5. A 003
6. α, ε-diaminopimelic acid
7. HJ 106
8. HJ 107
9. A 004
10. HJ 108
11. α, ε-diaminopimelic acid

Plate B

tracks (from left to right)

1. α, ε-diaminopimelic acid
2. HJ 109
3. A 005
4. A 007
5. A 009
6. α, ε-diaminopimelic acid
7. HJ 111
8. HJ 112
9. A 010
10. A 011
11. α, ε-diaminopimelic acid

Plate C

tracks (from left to right)

1. α, ε-diaminopimelic acid
2. HJ 034
3. HJ 035
4. HJ 036
5. HJ 037
6. HJ 038
7. HJ 039
8. HJ 040
9. HJ 041
10. HJ 042
11. α, ε-diaminopimelic acid

Plate D

tracks (from left to right)

1. α, ε-diaminopimelic acid
2. HJ 143
3. HJ 144
4. HJ 146
5. HJ 147
6. HJ 148
7. HJ 149
8. HJ 150
9. HJ 151
10. HJ 152
11. α, ε-diaminopimelic acid

Plate A

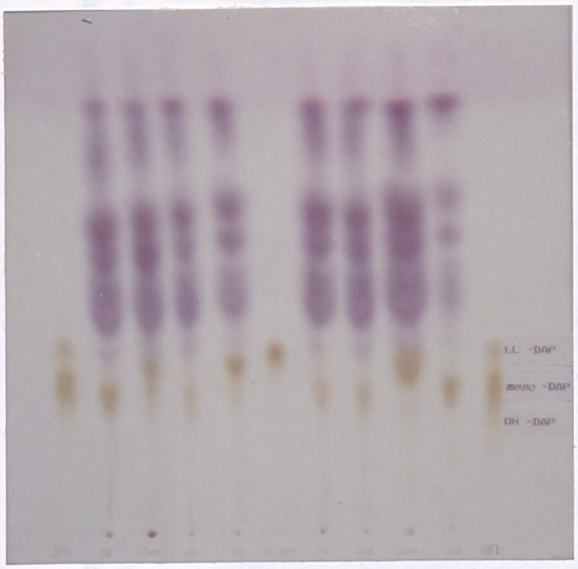


Plate B

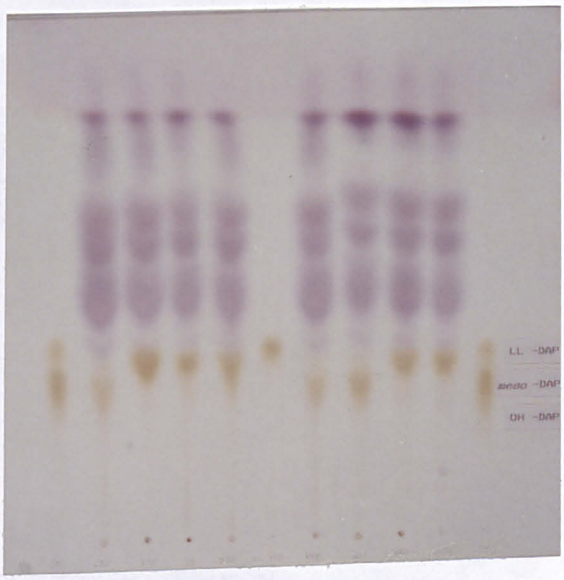


Plate C

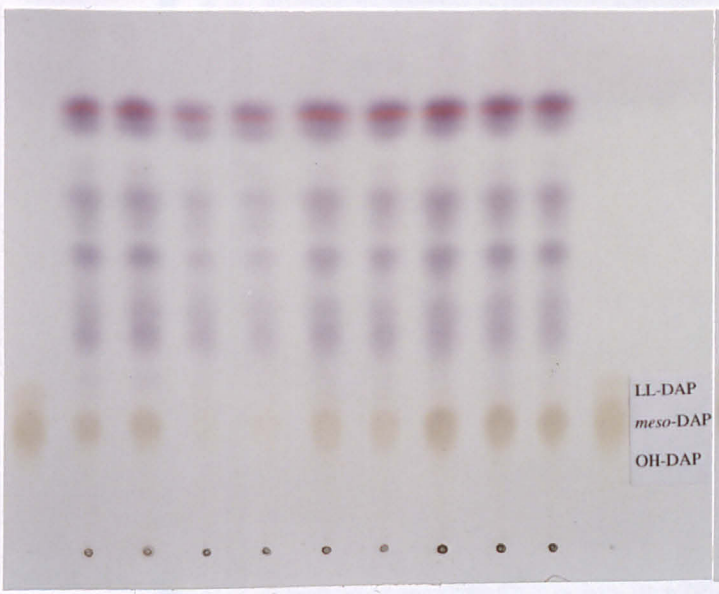
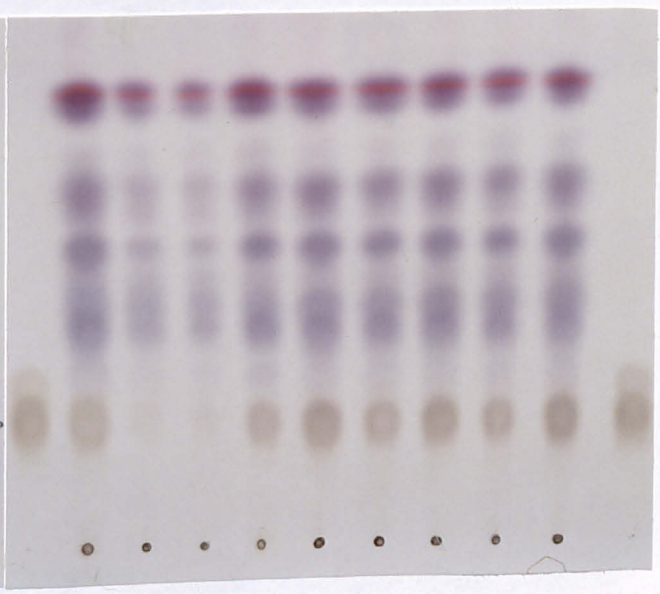


Plate D



LL-DAP, LL-diaminopimelic acid; *meso*-DAP, *meso*-diaminopimelic acid; OH-DAP, 2,6-diamino-3-hydroxypimelic acid.

Table 21 Identification scores recorded for representatives of the major clusters of streptosporangia using the frequency matrix of Whitham (1988)

Cluster		Strain Number (TW)	Best Three Identifications		Identification Scores				
			Cluster						
Number	Name of Strain		Name	Number	Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability	Strain Identified
1	*Streptosporangium* sp.	101	*Streptosporangium* sp.	1	0.9999	0.2293	0.3629	94.420	Yes
			Streptosporangium sp.	2	0.0000	0.4268	0.3575	0.041	
			Streptosporangium sp.	5	0.0000	0.4682	0.3687	0.003	
1	*Streptosporangium* sp.	104	*Streptosporangium* sp.	1	0.9986	0.2692	0.3629	73.395	Yes
			Streptosporangium sp.	2	0.0014	0.3742	0.3575	1.997	
			Streptosporangium sp.	4	0.0000	0.4351	0.3186	0.000	
1	*Streptosporangium* sp.	106	*Streptosporangium* sp.	1	0.9998	0.2143	0.3629	97.459	Yes
			Streptosporangium sp.	2	0.0002	0.3839	0.3575	1.096	
			Streptosporangium sp.	5	0.0000	0.4260	0.3687	0.130	
1	*Streptosporangium* sp.	117	*Streptosporangium* sp.	1	0.9999	0.3039	0.3629	41.463	Yes
			Streptosporangium sp.	2	0.0000	0.4925	0.3575	0.000	
			Streptosporangium sp.	5	0.0000	0.4543	0.3687	0.000	
1	*Streptosporangium* sp.	121	*Streptosporangium* sp.	1	0.9990	0.3229	0.3629	24.932	Yes
			Streptosporangium sp.	2	0.0009	0.4341	0.3575	0.021	
			Streptosporangium sp.	5	0.0000	0.5069	0.3687	0.000	
1	*Streptosporangium* sp.	127	*Streptosporangium* sp.	1	0.9983	0.3409	0.3629	13.309	Yes
			Streptosporangium sp.	2	0.0016	0.4159	0.3575	0.104	
			Streptosporangium sp.	4	0.0000	0.5145	0.3186	0.000	

Table 21 continued

Cluster Number	Cluster Name of Strain	Strain Number (TW)	Best Three Identifications Cluster Name	Cluster Number	Identification Scores Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability	Strain Identified
1	*Streptosporangium* sp.	128	*Streptosporangium* sp.	1	0.9999	0.2016	0.3629	98.815	Yes
			Streptosporangium sp.	5	0.0000	0.3619	0.3687	6.890	
			Streptosporangium sp.	2	0.0000	0.4374	0.3575	0.016	
1	*Streptosporangium* sp.	129	*Streptosporangium* sp.	1	0.9615	0.3310	0.3629	19.133	No
			Streptosporangium sp.	2	0.0384	0.3801	0.3575	1.395	
			Streptosporangium sp.	5	0.0000	0.5544	0.3687	0.000	
1	*Streptosporangium* sp.	143	*Streptosporangium* sp.	1	0.9844	0.3039	0.3629	41.465	Yes
			Streptosporangium sp.	4	0.0133	0.3259	0.3186	3.248	
			Streptosporangium sp.	11	0.0023	0.3364	0.1212	0.000	
1	*Streptosporangium* sp.	144	*Streptosporangium* sp.	1	0.9999	0.2692	0.3629	73.395	Yes
			Streptosporangium sp	2	0.0000	0.4268	0.3575	0.041	
			Streptosporangium sp.	5	0.0000	0.4408	0.3687	0.038	
1	*Streptosporangium* sp.	145A	*Streptosporangium* sp.	1	0.9998	0.2591	0.3629	80.727	Yes
			Streptosporangium sp.	5	0.0001	0.3792	0.3687	2.896	
			Streptosporangium sp.	4	0.0000	0.4086	0.3186	0.002	
1	*Streptosporangium* sp.	153	*Streptosporangium* sp.	1	0.9997	0.3448	0.3629	11.399	Yes
			Streptosporangium sp.	2	0.0002	0.4782	0.3575	0.000	
			Streptosporangium sp.	4	0.0000	0.4524	0.3186	0.000	

Table 21 continued

Cluster Number	Cluster Name of Strain	Strain Number (TW)	Best Three Identifications Cluster Name	Cluster Number	Identification Scores Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability	Strain Identified
1	*Streptosporangium* sp.	159	*Streptosporangium* sp.	1	0.9928	0.3188	0.3629	28.212	Yes
			Streptosporangium sp.	2	0.0068	0.3618	0.3575	4.018	
			Streptosporangium sp.	5	0.0002	0.4117	0.3687	0.381	
1	*Streptosporangium* sp.	165	*Streptosporangium* sp.	1	0.9999	0.3813	0.3629	1.831	No
			Streptosporangium sp.	2	0.0000	0.5072	0.3575	0.000	
			Streptosporangium sp.	5	0.0000	0.5069	0.3687	0.000	
1	*Streptosporangium* sp.	166*	*Streptosporangium* sp.	1	0.9994	0.2566	0.3629	82.386	Yes
			Streptosporangium sp.	2	0.0006	0.3839	0.3575	1.096	
			Streptosporangium sp.	5	0.0000	0.4817	0.3687	0.001	
1	*Streptosporangium* sp.	179	*Streptosporangium* sp.	1	0.9999	0.2566	0.3629	82.391	Yes
			Streptosporangium sp.	2	0.0000	0.4832	0.3575	0.000	
			Streptosporangium sp.	4	0.0000	0.4262	0.3186	0.000	
1	*Streptosporangium* sp.	218	*Streptosporangium* sp.	1	0.9997	0.2642	0.3629	77.192	Yes
			Streptosporangium sp.	2	0.0001	0.4159	0.3575	0.104	
			Streptosporangium sp.	5	0.0000	0.3958	0.3687	1.100	
1	*Streptosporangium* sp.	220	*Streptosporangium* sp.	1	0.9985	0.2591	0.3629	80.727	Yes
			Streptosporangium sp.	4	0.0012	0.3259	0.3186	3.249	
			Streptosporangium sp.	5	0.0001	0.3792	0.3687	2.896	

Table 21 continued

Cluster Number	Cluster Name of Strain	Strain Number (TW)	Best Three Identifications: Cluster Name	Cluster Number	Identification Scores: Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability	Strain Identified
1	*Streptosporangium* sp.	222	*Streptosporangium* sp.	1	0.9996	0.3310	0.3629	19.133	Yes
			Streptosporangium sp.	2	0.0002	0.4353	0.3575	0.019	
			Streptosporangium sp.	4	0.0001	0.4086	0.3186	0.002	
1	*Streptosporangium* sp.	224	*Streptosporangium* sp.	1	0.9999	0.3848	0.3629	1.486	No
			Streptosporangium sp.	7	0.0000	0.4558	0.2052	0.000	
			Streptosporangium sp.	6	0.0000	0.5098	0.3161	0.000	
1	*Streptosporangium* sp.	235	*Streptosporangium* sp.	1	0.9999	0.3167	0.3629	29.943	Yes
			Streptosporangium sp.	2	0.0000	0.4677	0.3575	0.001	
			Streptosporangium sp.	3	0.0000	0.4809	0.2514	0.000	
1	*Streptosporangium* sp.	245	*Streptosporangium* sp.	1	0.9999	0.2617	0.3629	78.995	Yes
			Streptosporangium sp.	2	0.0000	0.4457	0.3575	0.007	
			Streptosporangium sp.	4	0.0000	0.4351	0.3186	0.000	
1	*Streptosporangium* sp.	253	*Streptosporangium* sp.	1	0.9877	0.3146	0.3629	31.734	Yes
			Streptosporangium sp.	2	0.0122	0.3717	0.3575	2.307	
			Streptosporangium sp.	4	0.0000	0.4764	0.3186	0.000	
1	*Streptosporangium* sp.	256	*Streptosporangium* sp.	1	0.9868	0.2433	0.3629	88.459	Yes
			Streptosporangium sp.	5	0.0130	0.3055	0.3687	44.507	
			Streptosporangium sp.	2	0.0001	0.4048	0.3575	0.251	

Table 21 continued

Cluster Number	Cluster Name of Strain	Strain Number (TW)	Best Three Identifications Cluster Name	Cluster Number	Identification Scores Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability	Strain Identified
1	*Streptosporangium* sp.	263	*Streptosporangium* sp.	1	0.9999	0.2765	0.3629	67.3021	Yes
			Streptosporangium sp.	2	0.0000	0.4416	0.3575	0.010	
			Streptosporangium sp.	4	0.0000	0.3794	0.3186	0.045	
1	*Streptosporangium* sp.	266	*Streptosporangium* sp.	1	0.9999	0.2882	0.3629	56.513	Yes
			Streptosporangium sp.	2	0.0000	0.4268	0.3575	0.041	
			Streptosporangium sp.	5	0.0000	0.5193	0.3687	0.000	
1	*Streptosporangium* sp.	320	*Streptosporangium* sp.	1	0.9996	0.2765	0.3629	67.302	Yes
			Streptosporangium sp.	5	0.0003	0.3619	0.3687	6.890	
			Streptosporangium sp.	2	0.0000	0.4457	0.3575	0.007	
1	*Streptosporangium* sp.	353	*Streptosporangium* sp.	1	0.9998	0.3543	0.3629	7.561	Yes
			Streptosporangium sp.	2	0.0001	0.4716	0.3575	0.000	
			Streptosporangium sp.	5	0.0000	0.4948	0.3687	0.000	
1	*Streptosporangium* sp.	354	*Streptosporangium* sp.	1	0.9992	0.2995	0.3629	45.652	Yes
			Streptosporangium sp.	2	0.0004	0.4048	0.3575	0.251	
			Streptosporangium sp.	5	0.0004	0.3792	0.3687	2.896	
1	*Streptosporangium* sp.	369	*Streptosporangium* sp.	1	0.9998	0.2264	0.3629	95.170	Yes
			Streptosporangium sp.	5	0.0001	0.3619	0.3687	6.890	
			Streptosporangium sp.	2	0.0001	0.3933	0.3575	0.581	

Table 21 continued

Cluster Number	Cluster Name of Strain	Strain Number (TW)	Best Three Identifications Cluster Name	Cluster Number	Identification Scores Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability	Strain Identified
2	*Streptosporangium* sp.	115	*Streptosporangium* sp.	2	0.9999	0.1955	0.3575	99.026	Yes
			Streptosporangium sp.	5	0.0000	0.4551	0.3687	0.011	
			Streptosporangium sp.	1	0.0000	0.4564	0.3629	0.005	
2	*Streptosporangium* sp.	269	*Streptosporangium* sp.	2	0.9999	0.1705	0.3575	99.842	Yes
			Streptosporangium sp.	1	0.0000	0.4130	0.3629	0.213	
			Streptosporangium sp.	5	0.0000	0.4408	0.3687	0.038	
2	*Streptosporangium* sp.	270	*Streptosporangium* sp.	2	0.9999	0.1705	0.3575	99.842	Yes
			Streptosporangium sp.	1	0.0000	0.4130	0.3629	0.213	
			Streptosporangium sp.	5	0.0000	0.4408	0.3687	0.038	
2	*Streptosporangium* sp.	271	*Streptosporangium* sp.	2	0.9999	0.2454	0.3575	86.678	Yes
			Streptosporangium sp.	1	0.0000	0.4529	0.3629	0.007	
			Streptosporangium sp.	5	0.0000	0.4551	0.3687	0.011	
2	*Streptosporangium* sp.	274	*Streptosporangium* sp.	2	0.9889	0.2835	0.3575	56.952	Yes
			Streptosporangium sp.	5	0.0109	0.3449	0.3687	14.058	
			Streptosporangium sp.	1	0.0001	0.4153	0.3629	0.179	
2	*Streptosporangium* sp.	282	*Streptosporangium* sp.	2	0.9999	0.1955	0.3575	99.026	Yes
			Streptosporangium sp.	1	0.0000	0.4226	0.3629	0.101	
			Streptosporangium sp.	5	0.0000	0.3958	0.3687	1.100	

Table 21 continued

Cluster Number	Name of Strain	Strain Number (TW)	Best Three Identifications Cluster Name	Cluster Number	Identification Scores Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability	Strain Identified
2	*Streptosporangium* sp.	286	*Streptosporangium* sp.	2	0.9872	0.2899	0.3575	50.724	Yes
			Streptosporangium sp.	5	0.0126	0.3449	0.3687	14.058	
			Streptosporangium sp.	1	0.0001	0.4425	0.3629	0.018	
2	*Streptosporangium* sp.	292*	*Streptosporangium* sp.	2	0.9999	0.1705	0.3575	99.842	Yes
			Streptosporangium sp.	1	0.0000	0.4130	0.3629	0.213	
			Streptosporangium sp.	5	0.0000	0.4408	0.3687	0.038	
2	*Streptosporangium* sp.	303	*Streptosporangium* sp.	2	0.9999	0.1705	0.3575	99.841	Yes
			Streptosporangium sp.	1	0.0000	0.4130	0.3629	0.213	
			Streptosporangium sp.	5	0.0000	0.4408	0.3687	0.038	
2	*Streptosporangium* sp.	366	*Streptosporangium* sp.	2	0.9999	0.1955	0.3575	99.026	Yes
			Streptosporangium sp.	5	0.0000	0.4551	0.3687	0.011	
			Streptosporangium sp.	1	0.0000	0.4564	0.3629	0.005	
3	*Streptosporangium* sp.	541	*Streptosporangium* sp.	3	0.9999	0.2069	0.2514	46.365	Yes
			Streptosporangium sp.	1	0.0000	0.4279	0.3629	0.065	
			Streptosporangium sp.	4	0.0000	0.4852	0.3186	0.000	
3	*Streptosporangium* sp.	547	*Streptosporangium* sp.	3	0.9999	0.1732	0.2514	86.161	Yes
			Streptosporangium sp.	1	0.0000	0.4416	0.3629	0.019	
			Streptosporangium sp.	5	0.0000	0.5193	0.3687	0.000	

Table 21 continued

Cluster Number	Cluster Name of Strain	Strain Number (TW)	Best Three Identifications: Cluster Name	Cluster Number	Identification Scores: Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability	Strain Identified
4	*Streptosporangium* sp.	116*	*Streptosporangium* sp.	4	0.9999	0.2322	0.3186	77.000	Yes
			Streptosporangium sp.	1	0.0000	0.4056	0.3629	0.370	
			Streptosporangium sp.	5	0.0000	0.4551	0.3687	0.011	
4	*Streptosporangium* sp.	161	*Streptosporangium* sp.	4	0.9999	0.2150	0.3186	88.747	Yes
			Streptosporangium sp.	1	0.0000	0.4425	0.3629	0.000	
			Streptosporangium sp.	6	0.0000	0.4997	0.3161	0.000	
4	*Streptosporangium* sp.	163	*Streptosporangium* sp.	4	0.9999	0.2482	0.3186	61.690	Yes
			Streptosporangium sp.	1	0.0000	0.4178	0.3629	0.147	
			Streptosporangium sp.	11	0.0000	0.4342	0.1212	0.000	
4	*Streptosporangium* sp.	254	*Streptosporangium* sp.	4	0.9999	0.2322	0.3186	77.000	Yes
			Streptosporangium sp.	1	0.0000	0.4826	0.3629	0.000	
			Streptosporangium sp.	6	0.0000	0.5361	0.3161	0.000	
4	*Streptosporangium* sp.	375	*Streptosporangium* sp.	4	0.9999	0.3039	0.3186	10.762	Yes
			Streptosporangium sp.	1	0.0000	0.4659	0.3629	0.002	
			Streptosporangium sp.	3	0.0000	0.4252	0.2514	0.000	
5	*Streptosporangium* sp.	168	*Streptosporangium* sp.	5	0.9999	0.2616	0.3687	81.782	Yes
			Streptosporangium sp.	1	0.0000	0.4365	0.3629	0.031	
			Streptosporangium sp.	2	0.0000	0.4793	0.3575	0.000	

Table 21 continued

Cluster Number	Cluster Name of Strain	Strain Number (TW)	Best Three Identifications: Cluster Name	Cluster Number	Identification Scores: Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability	Strain Identified
6	*Streptosporangium* sp.	136	*Streptosporangium* sp.	6	0.9999	0.2079	0.3161	91.348	Yes
			Streptosporangium sp.	7	0.0000	0.4558	0.2052	0.000	
			Streptosporangium sp.	8	0.0000	0.4633	0.2743	0.000	
6	*Streptosporangium* sp.	141*	*Streptosporangium* sp.	6	0.9999	0.1636	0.3161	99.529	Yes
			Streptosporangium sp.	8	0.0000	0.4207	0.2743	0.000	
			Streptosporangium sp.	5	0.0000	0.4817	0.3687	0.000	
6	*Streptosporangium* sp.	148	*Streptosporangium* sp.	6	0.9998	0.2858	0.3161	21.113	Yes
			Streptosporangium sp.	5	0.0001	0.3958	0.3687	1.100	
			Streptosporangium sp.	8	0.0000	0.4207	0.2743	0.000	
6	*Streptosporangium* sp.	169	*Streptosporangium* sp.	6	0.9999	0.3545	0.3161	0.336	No
			Streptosporangium sp.	7	0.0000	0.4558	0.2052	0.000	
			Streptosporangium sp.	8	0.0000	0.4724	0.2743	0.000	
6	*Streptosporangium* sp.	227	*Streptosporangium* sp.	6	0.9999	0.2554	0.3161	51.756	Yes
			Streptosporangium sp.	5	0.0000	0.4107	0.3687	0.407	
			Streptosporangium sp.	1	0.0000	0.4470	0.3629	0.012	
6	*Streptosporangium* sp.	251	*Streptosporangium* sp.	6	0.9999	0.1458	0.3161	99.900	Yes
			Streptosporangium sp.	5	0.0000	0.4408	0.3687	0.038	
			Streptosporangium sp.	7	0.0000	0.4558	0.2052	0.000	

Table 21 continued

Cluster Number	Cluster Name of Strain	Strain Number (TW)	Best Three Identifications Cluster Name	Cluster Number	Identification Scores Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability	Strain Identified
6	*Streptosporangium* sp.	393	*Streptosporangium* sp.	6	0.9999	0.3044	0.3161	9.343	Yes
			Streptosporangium sp.	5	0.0000	0.4817	0.3687	0.001	
			Streptosporangium sp.	8	0.0000	0.4876	0.2743	0.000	
7	*Streptosporangium* sp.	226*	*Streptosporangium* sp.	7	0.9999	0.1390	0.2052	88.300	Yes
			Streptosporangium sp.	6	0.0000	0.4100	0.3161	0.001	
			Streptosporangium sp.	12	0.0000	0.4342	0.1212	0.000	
7	*Streptosporangium* sp.	232	*Streptosporangium* sp.	7	0.9999	0.1390	0.2052	88.300	Yes
			Streptosporangium sp.	6	0.0000	0.4714	0.3161	0.000	
			Streptosporangium sp.	12	0.0000	0.5138	0.1212	0.000	
8	*Streptosporangium* sp.	170	*Streptosporangium* sp.	8	0.9999	0.1620	0.2743	97.466	Yes
			Streptosporangium sp.	6	0.0000	0.3676	0.3161	0.106	
			Streptosporangium sp.	5	0.0000	0.4817	0.3687	0.001	
8	*Streptosporangium* sp.	209	*Streptosporangium* sp.	8	0.9999	0.2221	0.2743	51.176	Yes
			Streptosporangium sp.	6	0.0000	0.4157	0.3161	0.001	
			Streptosporangium sp.	5	0.0000	0.5193	0.3687	0.000	

Table 21 continued

Cluster Number	Name of Strain	Strain Number (TW)	Best Three Identifications Cluster Name	Cluster Number	Identification Scores Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability	Strain Identified
8	*Streptosporangium* sp.	213*	*Streptosporangium* sp.	8	0.9999	0.2221	0.2743	51.176	Yes
			Streptosporangium sp.	6	0.0000	0.4772	0.3161	0.000	
			Streptosporangium sp.	5	0.0000	0.5193	0.3687	0.000	
8	*Streptosporangium* sp.	276	*Streptosporangium* sp.	8	0.9999	0.1620	0.2743	97.466	Yes
			Streptosporangium sp.	6	0.0000	0.4475	0.3161	0.000	
			Streptosporangium sp.	5	0.0000	0.4817	0.3687	0.001	
8	*Streptosporangium* sp.	355	*Streptosporangium* sp.	8	0.9999	0.2543	0.2743	15.749	Yes
			Streptosporangium sp.	2	0.0000	0.4613	0.3575	0.001	
			Streptosporangium sp.	6	0.0000	0.4772	0.3161	0.000	
9	*Streptosporangium roseum*	005*,**	*Streptosporangium roseum/ S. vulgare*	9	0.9999	0.2761	0.2637	1.977	No
			Streptosporangium sp.	8	0.0000	0.5871	0.2743	0.000	
			Streptosporangium sp.	6	0.0000	0.5931	0.3161	0.000	
9	*Streptosporangium vulgare*	007**	*Streptosporangium roseum/ S. vulgare*	9	0.9999	0.1963	0.2637	72.566	Yes
			Streptosporangium sp.	2	0.0000	0.5630	0.3575	0.000	
			Streptosporangium sp.	5	0.0000	0.5881	0.3687	0.000	

Table 21 continued

Cluster Number	Name of Strain	Strain Number (TW)	Best Three Identifications Cluster Name	Cluster Number	Identification Scores Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability	Strain Identified
10	*Streptosporangium amethystogenes*	001**	*Streptosporangium amethystogenes/ S.corrugatum/ S.longisporum*	10	0.9999	0.3270	0.3687	25.776	Yes
			Streptosporangium sp.	6	0.0000	0.5605	0.3161	0.000	
			Streptosporangium sp.	7	0.0000	0.5664	0.2052	0.000	
10	*Streptosporangium corrugatum*	002*,**	*Streptosporangium amethystogenes/ S. corrugatum/S.longisporum*	10	0.9999	0.2851	0.3687	63.615	Yes
			Streptosporangium sp.	7	0.0000	0.5988	0.2052	0.000	
			Streptosporangium sp.	6	0.0000	0.6159	0.3161	0.000	
11	*Streptosporangium* sp.	126*	*Streptosporangium* sp.	11	0.9999	0.0100	0.1212	100	Yes
			Streptosporangium sp.	4	0.0000	0.4360	0.3186	0.000	
			Streptosporangium sp.	1	0.0000	0.4491	0.3629	0.010	
11	*Streptosporangium* sp.	155	*Streptosporangium* sp.	11	0.9999	0.0100	0.1212	100	Yes
			Streptosporangium sp.	4	0.0000	0.4360	0.3186	0.000	
			Streptosporangium sp.	1	0.0000	0.4491	0.3629	0.010	

Table 21 continued

Cluster Number	Cluster Name of Strain	Strain Number (TW)	Best Three Identifications: Cluster Name	Cluster Number	Identification Scores: Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability	Strain Identified
12	*Streptosporangium* sp.	182*	*Streptosporangium* sp.	12	0.9999	0.1944	0.1212	100	Yes
			Streptosporangium sp.	7	0.0000	0.4955	0.2052	0.000	
			Streptosporangium sp.	6	0.0000	0.5653	0.3161	0.000	
12	*Streptosporangium* sp.	194	*Streptosporangium* sp.	12	0.9999	0.0944	0.1212	100	Yes
			Streptosporangium sp.	7	0.0000	0.4955	0.2052	0.000	
			Streptosporangium sp.	6	0.0000	0.5653	0.3161	0.000	

*, Centrotype strain. **, Type strain.

98.815), apart from strain TW 224 which had a high Willcox probability score (0.9999) but a taxonomic distance larger than 95% taxonomic radius and a low Gaussian distance probability. The raw data obtained for all of the test strains are given in Appendix C.

Given the high identification scores obtained with the marker strains it was decided in the first instance that stringent criteria should be set for a positive identification of both known and unknown strains, namely:

(i) a Willcox probability score of 0.9700 or above.
(ii) a taxonomic distance score below the 95% taxonomic radius score.
(iii) high Gaussian probability scores signifying that there was not any significant overlap between the cluster strains were assigned to and the immediate next best alternatives.
(iv) the best identification scores to be much better than the two next best alternatives

Sixty-five of the seventy marker strains fulfilled these cut-off criteria for a positive identification. Three of the four exceptions, namely strains TW 224 (cluster 1), TW 165 (cluster 1), TW 169 (cluster 6) and TW 005 (cluster 9) had Willcox probabilities above 0.9999 but showed taxonomic distance values somewhat above the 95% taxonomic radius scores. The remaining organism, strain TW 129 showed similar identification scores but in this case the Willcox probability was relatively low at 0.9615.

2. IDENTIFICATION OF UNKNOWN STREPTOSPORANGIA

Twelve out of the one hundred and thirty-six unknown streptosporangia were identified to known clusters using the stringent cut-off criteria mentioned above. Ten of the strains were identified to cluster 1 (*Streptosporangium* sp.) and

two to cluster 2 (*Streptosporangium* sp.; Table 22, pages 159 to 177). The raw data recorded for all of these strains are given in Appendix D.

A further nineteen organisms were identified to known clusters using less stringent cut-off criteria, namely strains HJ 005, HJ 010, HJ 011, HJ 012, HJ 020, HJ 021, HJ 032, HJ 036, HJ 053, HJ 055, HJ 056, HJ 068, HJ 087, HJ 092, HJ 096, HJ 097, HJ 113, HJ 126 and HJ 129. These strains showed high Willcox probabilities (>0.9700) and had taxonomic distances just above the 95% taxonomic radius (Taxonomic distance - 95% taxonomic radius < 0.13). Twelve of the nineteen strains were identified to cluster 1 (*Streptosporangium* sp.) and the remaining seven to cluster 2 (*Streptosporangium* sp.). The balance of one hundred and five strains were not identified using either of the cut-off points chosen for a positive identification.

Table 22 Identification scores recorded for streptosporangia isolated from diverse soils

Strain Number (HJ)	Soil Numbers Comprising Composite Soil samples and Pretreatment Regimes	Best Three Identifications: Cluster Name	Cluster Number	Identification Scores: Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability
A. STRAINS MEETING CRITERIA FOR A POSITIVE IDENTIFICATION							
090	583-584, Ginseng field (post harvest) Kumsan, Republic of Korea Heat[a]	*Streptosporangium* sp.	1	0.9999	0.2460	0.3629	88.213
		Streptosporangium sp.	2	0.0000	0.4395	0.3575	0.013
		Streptosporangium sp.	5	0.0000	0.4682	0.3687	0.003
091		*Streptosporangium* sp.	2	0.9999	0.1705	0.3575	99.842
		Streptosporangium sp.	1	0.0000	0.4130	0.3629	0.213
		Streptosporangium sp.	5	0.0000	0.4408	0.3687	0.038
098	583-584, Ginseng field (post harvest) Kumsan, Republic of Korea Sodium dodecyl sulphate[b]	*Streptosporangium* sp.	1	0.9999	0.2460	0.3629	88.213
		Streptosporangium sp.	2	0.0000	0.4395	0.3575	0.013
		Streptosporangium sp.	5	0.0000	0.4682	0.3687	0.003
105	443-444, Garden soil, IMTECH Chandigarh, India Yeast extract[c]	*Streptosporangium* sp.	2	0.9999	0.1705	0.3575	99.842
		Streptosporangium sp.	1	0.0000	0.4130	0.3629	0.213
		Streptosporangium sp.	5	0.0000	0.4408	0.3687	0.038
107	433-434, Garden soil, Hibuya Park Tokyo, Japan Heat	*Streptosporangium* sp.	1	0.9996	0.2765	0.3629	67.302
		Streptosporangium sp.	5	0.0004	0.3619	0.3687	6.890
		Streptosporangium sp.	2	0.0000	0.4457	0.3575	0.007
108		*Streptosporangium* sp.	1	0.9996	0.2765	0.3629	67.302
		Streptosporangium sp.	5	0.0004	0.3619	0.3687	6.890
		Streptosporangium sp.	2	0.0000	0.4457	0.3575	0.007
109		*Streptosporangium* sp.	1	0.9996	0.2765	0.3629	67.302
		Streptosporangium sp.	5	0.0004	0.3619	0.3687	6.890
		Streptosporangium sp.	2	0.0000	0.4457	0.3575	0.007

Table 22 continued

Strain Number (HJ)	Soil Numbers Comprising Composite Soil samples and Pretreatment Regimes	Best Three Identifications: Cluster Name	Cluster Number	Identification Scores: Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability
112	435-436, Garden soil, Tsukuba	*Streptosporangium* sp.	1	0.9991	0.3229	0.3629	24.932
	University, Tsukuba, Japan	*Streptosporangium* sp.	2	0.0009	0.4341	0.3575	0.021
	Heat	*Streptosporangium* sp.	5	0.0000	0.5069	0.3687	0.000
116	435-436, Garden soil, Tsukuba	*Streptosporangium* sp.	1	0.9999	0.3039	0.3629	41.463
	University, Tsukuba, Japan	*Streptosporangium* sp.	2	0.0000	0.4925	0.3575	0.000
	Yeast extract	*Streptosporangium* sp.	5	0.0000	0.4543	0.3687	0.011
118	482-489, Tropical rainforest soil,	*Streptosporangium* sp.	1	0.9998	0.2264	0.3629	95.170
	Meru Betini, Indonesia	*Streptosporangium* sp.	5	0.0001	0.3619	0.3687	6.890
	Heat	*Streptosporangium* sp.	2	0.0001	0.3933	0.3575	0.581
123	482-489, Tropical rainforest soil,	*Streptosporangium* sp.	1	0.9999	0.2692	0.3629	73.395
	Meru Betini, Indonesia	*Streptosporangium* sp.	2	0.0001	0.4268	0.3575	0.041
	Sodium dodecyl sulphate	*Streptosporangium* sp.	4	0.0000	0.4262	0.3186	0.000
125	482-489, Tropical rainforest soil,	*Streptosporangium* sp.	1	0.9991	0.3229	0.3629	24.932
	Meru Betini, Indonesia	*Streptosporangium* sp.	2	0.0009	0.4341	0.3575	0.021
	Yeast extract	*Streptosporangium* sp.	5	0.0000	0.5069	0.3687	0.000
B. STRAINS PRESUMPTIVELY IDENTIFIED ON THE BASIS OF HIGH WILLCOX PROBABILITIES WITH TAXONOMIC DISTANCES JUST ABOVE THE 95% TAXONOMIC RADIUS							
005	585-587, Ginseng field (young plant)	*Streptosporangium* sp.	1	0.9986	0.4440	0.3629	0.016
	Kumsan, Republic of Korea	*Streptosporangium* sp.	2	0.0014	0.5118	0.3575	0.000
	Heat and phenol[d]	*Streptosporangium* sp.	6	0.0000	0.5405	0.3161	0.000
010	585-587, Ginseng field (young plant)	*Streptosporangium* sp.	1	0.9975	0.4673	0.3629	0.002
	Kumsan, Republic of Korea	*Streptosporangium* sp.	2	0.0025	0.5020	0.3575	0.000
	Yeast extract	*Streptosporangium* sp.	5	0.0000	0.5315	0.3687	0.000

Table 22 continued

Strain Number (HJ)	Soil Numbers Comprising Composite Soil samples and Pretreatment Regimes	Best Three Identifications: Cluster Name	Cluster Number	Identification Scores: Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability
011	585-587, Ginseng field (young plant)	*Streptosporangium* sp.	1	0.9987	0.4887	0.3629	0.000
	Kumsan, Republic of Korea	*Streptosporangium* sp.	3	0.0006	0.5061	0.2514	0.000
	Yeast extract	*Streptosporangium* sp.	2	0.0005	0.5751	0.3575	0.000
012	579-581, Ginseng field (post harvest)	*Streptosporangium* sp.	1	0.9993	0.4693	0.3629	0.001
	Kumsan, Republic of Korea	*Streptosporangium* sp.	2	0.0007	0.5287	0.3575	0.000
	Heat	*Streptosporangium amethystogenes/ S.corrugatum/S.longisporum*	10	0.0000	0.5551	0.3687	0.000
020		*Streptosporangium* sp.	1	0.9959	0.4630	0.3629	0.002
		Streptosporangium sp.	2	0.0028	0.4797	0.3575	0.000
		Streptosporangium sp.	8	0.0008	0.4884	0.2743	0.000
021		*Streptosporangium* sp.	2	0.9912	0.4419	0.3575	0.010
		Streptosporangium roseum/S. vulgare	9	0.0087	0.4765	0.2637	0.000
		Streptosporangium sp.	5	0.0001	0.5435	0.3687	0.000
032	579-581, Ginseng field (post harvest)	*Streptosporangium* sp.	1	0.9999	0.4535	0.3629	0.006
	Kumsan, Republic of Korea	*Streptosporangium* sp.	2	0.0000	0.5474	0.3575	0.000
	Heat and phenol	*Streptosporangium* sp.	3	0.0000	0.5421	0.2514	0.000
036	579-581, Ginseng field (post harvest)	*Streptosporangium* sp.	1	0.9867	0.4446	0.3629	0.015
	Kumsan, Republic of Korea	*Streptosporangium* sp.	2	0.0133	0.4440	0.3575	0.008
	Sodium dodecyl sulphate	*Streptosporangium* sp.	4	0.0001	0.4844	0.3186	0.000
053	579-581, Ginseng field (post harvest)	*Streptosporangium* sp.	2	0.9739	0.4280	0.3575	0.037
	Kumsan, Republic of Korea	*Streptosporangium* sp.	1	0.0211	0.5073	0.3629	0.000
	Yeast extract	*Streptosporangium* sp.	5	0.0039	0.4817	0.3687	0.001

Table 22 continued

Strain Number (HJ)	Soil Numbers Comprising Composite Soil samples and Pretreatment Regimes	Best Three Identifications					
		Cluster		Identification Scores			
		Name	Number	Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability
055	579-581, Ginseng field (post harvest) Kumsan, Republic of Korea Yeast extract	*Streptosporangium* sp.	1	0.9997	0.4558	0.3629	0.005
		Streptosporangium sp.	2	0.0003	0.5057	0.3575	0.000
		Streptosporangium sp.	4	0.0000	0.5436	0.3186	0.000
056		*Streptosporangium* sp.	1	0.9997	0.4558	0.3629	0.005
		Streptosporangium sp.	2	0.0003	0.5057	0.3575	0.000
		Streptosporangium sp.	4	0.0000	0.5436	0.3186	0.000
068		*Streptosporangium* sp.	1	0.9994	0.4644	0.3629	0.002
		Streptosporangium sp.	2	0.0005	0.5129	0.3575	0.000
		Streptosporangium sp.	4	0.0001	0.5001	0.3186	0.000
087		*Streptosporangium* sp.	2	0.9986	0.4481	0.3575	0.006
		Streptosporangium sp.	1	0.0013	0.5565	0.3629	0.000
		Streptosporangium roseum/S.vulgare	9	0.0001	0.5145	0.2637	0.000
092	583-584, Ginseng field (post harvest) Kumsan, Republic of Korea Heat	*Streptosporangium* sp.	1	0.9997	0.4729	0.3629	0.001
		Streptosporangium sp.	2	0.0002	0.5210	0.3575	0.000
		Streptosporangium sp.	5	0.0000	0.5435	0.3687	0.000
096	583-584, Ginseng field (post harvest) Kumsan, Republic of Korea Heat and phenol	*Streptosporangium* sp.	2	0.9994	0.3890	0.3575	0.781
		Streptosporangium sp.	5	0.0005	0.4948	0.3687	0.000
		Streptosporangium sp.	1	0.0001	0.5395	0.3629	0.000
097		*Streptosporangium* sp.	2	0.9994	0.3890	0.3575	0.781
		Streptosporangium sp.	5	0.0005	0.4948	0.3687	0.000
		Streptosporangium sp.	1	0.0001	0.5395	0.3629	0.000
113	435-436, Garden soil, Tsukuba University, Tsukuba, Japan Heat	*Streptosporangium* sp.	1	0.9995	0.3916	0.3629	0.966
		Streptosporangium sp.	2	0.0005	0.5098	0.3575	0.000
		Streptosporangium sp.	4	0.0000	0.4772	0.3186	0.000

Table 22 continued

Strain Number (HJ)	Soil Numbers Comprising Composite Soil samples and Pretreatment Regimes	Best Three Identifications: Cluster Name	Cluster Number	Identification Scores: Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability
126	482-489, Tropical rainforest soil, Meru Betini, Indonesia Yeast extract	*Streptosporangium* sp.	2	0.9992	0.4672	0.3575	0.001
		Streptosporangium sp.	5	0.0006	0.5551	0.3687	0.000
		Streptosporangium sp.	1	0.0002	0.5745	0.3629	0.000
129		*Streptosporangium* sp.	2	0.9778	0.3693	0.3575	2.659
		Streptosporangium sp.	5	0.0195	0.4269	0.3687	0.121
		Streptosporangium sp.	1	0.0027	0.4721	0.3629	0.001
	C. UNIDENTIFIED STRAINS						
001	585-587, Ginseng field (young plant) Kumsan, Republic of Korea Heat	*Streptosporangium* sp.	5	0.8259	0.4690	0.3687	0.003
		Streptosporangium sp.	1	0.0889	0.5202	0.3629	0.000
		Streptosporangium sp.	2	0.0849	0.4700	0.3575	0.001
002		*Streptosporangium* sp.	5	0.8259	0.4690	0.3687	0.003
		Streptosporangium sp.	1	0.0889	0.5202	0.3629	0.000
		Streptosporangium sp.	2	0.0849	0.4700	0.3575	0.001
006	585-587, Ginseng field (young plant) Kumsan, Republic of Korea Heat and phenol	*Streptosporangium* sp.	2	0.7856	0.4207	0.3575	0.070
		Streptosporangium sp.	1	0.2096	0.4859	0.3629	0.000
		Streptosporangium sp.	5	0.0048	0.4948	0.3687	0.000
008	585-587, Ginseng field (young plant) Kumsan, Republic of Korea Sodium dodecyl sulphate	*Streptosporangium* sp.	1	0.9361	0.5015	0.3629	0.000
		Streptosporangium sp.	2	0.0639	0.5010	0.3575	0.000
		Streptosporangium sp.	4	0.0000	0.5364	0.3186	0.000
009	585-587, Ginseng field (young plant) Kumsan, Republic of Korea Yeast extract	*Streptosporangium* sp.	12	0.4401	0.5138	0.1212	0.000
		Streptosporangium sp.	1	0.4342	0.5764	0.3629	0.000
		Streptosporangium sp.	5	0.1044	0.5544	0.3687	0.000

Table 22 continued

Strain Number (HJ)	Soil Numbers Comprising Composite Soil samples and Pretreatment Regimes	Best Three Identifications					
		Cluster		Identification Scores			
		Name	Number	Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability
013	579-581, Ginseng field (post harvest) Kumsan, Republic of Korea Heat	*Streptosporangium* sp.	2	0.9804	0.5964	0.3575	0.000
		Streptosporangium sp.	1	0.0193	0.6690	0.3629	0.000
		Streptosporangium sp.	4	0.0003	0.6152	0.3186	0.000
014		*Streptosporangium* sp.	1	0.7520	0.5866	0.3629	0.000
		Streptosporangium sp.	8	0.2406	0.5603	0.2743	0.000
		Streptosporangium sp.	4	0.0042	0.5838	0.3186	0.000
015		*Streptosporangium* sp.	2	0.9934	0.5517	0.3575	0.000
		Streptosporangium sp.	1	0.0051	0.6365	0.3629	0.000
		Streptosporangium sp.	12	0.0013	0.5492	0.1212	0.000
016		*Streptosporangium* sp.	1	0.8300	0.5861	0.3629	0.000
		Streptosporangium sp.	12	0.1532	0.5138	0.1212	0.000
		Streptosporangium sp.	2	0.0165	0.5754	0.3575	0.000
017		*Streptosporangium* sp.	1	0.9900	0.5536	0.3629	0.000
		Streptosporangium amethystogenes/ S.corrugatum/S.longisporum	10	0.0094	0.5551	0.3687	0.000
		Streptosporangium sp.	2	0.0005	0.5902	0.3575	0.000
019		*Streptosporangium* sp.	6	0.7158	0.4656	0.3161	0.000
		Streptosporangium sp	1	0.2820	0.5158	0.3629	0.000
		Streptosporangium sp.	12	0.0017	0.4757	0.1212	0.000
022		*Streptosporangium* sp.	2	0.9934	0.5517	0.3575	0.000
		Streptosporangium sp.	1	0.0051	0.6365	0.3629	0.000
		Streptosporangium sp.	12	0.0013	0.5492	0.1212	0.000
023		*Streptosporangium* sp.	2	0.5673	0.5023	0.3575	0.000
		Streptosporangium sp.	1	0.2628	0.5576	0.3629	0.000
		Streptosporangium sp.	5	0.1698	0.5435	0.3687	0.000

Table 22 continued

Strain Number (HJ)	Soil Numbers Comprising Composite Soil samples and Pretreatment Regimes	Best Three Identifications: Cluster Name	Cluster Number	Identification Scores: Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability
024	579-581, Ginseng field (post harvest) Kumsan, Republic of Korea Heat	*Streptosporangium* sp.	1	0.8447	0.5138	0.3629	0.000
		Streptosporangium sp.	2	0.1553	0.4984	0.3575	0.000
		Streptosporangium sp.	5	0.0002	0.5544	0.3687	0.000
025	579-581, Ginseng field (post harvest) Kumsan, Republic of Korea Heat and phenol	*Streptosporangium* sp.	1	0.8447	0.5138	0.3629	0.000
		Streptosporangium sp.	2	0.1553	0.4984	0.3575	0.000
		Streptosporangium sp.	5	0.0002	0.5544	0.3687	0.000
026		*Streptosporangium* sp.	1	0.9505	0.5390	0.3629	0.000
		Streptosporangium sp.	2	0.0476	0.5321	0.3575	0.000
		Streptosporangium sp.	4	0.0005	0.5364	0.3186	0.000
027		*Streptosporangium* sp.	1	0.9818	0.5517	0.3629	0.000
		Streptosporangium sp.	2	0.0181	0.5510	0.3575	0.000
		Streptosporangium sp.	5	0.0000	0.6089	0.3687	0.000
028		*Streptosporangium* sp.	2	0.5172	0.5314	0.3575	0.000
		Streptosporangium sp.	1	0.2415	0.5546	0.3629	0.000
		Streptosporangium roseum/S.vulgare	9	0.2369	0.5145	0.2637	0.000
029		*Streptosporangium* sp.	1	0.9689	0.4791	0.3629	0.000
		Streptosporangium sp.	2	0.0311	0.4777	0.3575	0.000
		Streptosporangium sp.	4	0.0000	0.5364	0.3186	0.000
030		*Streptosporangium* sp.	1	0.9288	0.5289	0.3629	0.000
		Streptosporangium roseum/S.vulgare	9	0.0495	0.5145	0.2637	0.000
		Streptosporangium sp.	2	0.0208	0.5583	0.3575	0.000
031		*Streptosporangium* sp.	5	0.7963	0.4690	0.3687	0.003
		Streptosporangium sp.	2	0.2035	0.4452	0.3575	0.007
		Streptosporangium sp.	1	0.0001	0.5498	0.3629	0.000

Table 22 continued

Strain Number (HJ)	Soil Numbers Comprising Composite Soil samples and Pretreatment Regimes	Best Three Identifications: Cluster Name	Cluster Number	Identification Scores: Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability
033	579-581, Ginseng field (post harvest) Kumsan, Republic of Korea Heat and phenol	*Streptosporangium* sp.	2	0.6921	0.5031	0.3575	0.000
		Streptosporangium sp.	1	0.2900	0.5326	0.3629	0.000
		Streptosporangium sp.	5	0.0177	0.5435	0.3687	0.000
034	579-581, Ginseng field (post harvest) Kumsan, Republic of Korea Sodium dodecyl sulphate	*Streptosporangium* sp.	5	0.8222	0.4551	0.3687	0.011
		Streptosporangium sp.	2	0.1707	0.4347	0.3575	0.020
		Streptosporangium sp.	1	0.0064	0.5143	0.3629	0.000
035		*Streptosporangium* sp.	5	0.8222	0.4551	0.3687	0.011
		Streptosporangium sp.	2	0.1707	0.4347	0.3575	0.020
		Streptosporangium sp.	1	0.0064	0.5143	0.3629	0.000
037		*Streptosporangium* sp.	2	0.9998	0.4955	0.3575	0.000
		Streptosporangium sp.	5	0.0001	0.5551	0.3687	0.000
		Streptosporangium sp.	1	0.0001	0.6011	0.3629	0.000
038		*Streptosporangium* sp.	2	0.6921	0.5031	0.3575	0.000
		Streptosporangium sp.	1	0.2900	0.5326	0.3629	0.000
		Streptosporangium sp.	5	0.0177	0.5435	0.3687	0.000
039		*Streptosporangium* sp.	2	0.9998	0.4955	0.3575	0.000
		Streptosporangium sp.	5	0.0001	0.5551	0.3687	0.000
		Streptosporangium sp.	1	0.0001	0.6011	0.3629	0.000
040		*Streptosporangium* sp.	5	0.4226	0.5881	0.3687	0.000
		Streptosporangium sp.	2	0.3342	0.5731	0.3575	0.000
		Streptosporangium sp.	1	0.2432	0.5988	0.3629	0.000
041		*Streptosporangium* sp.	2	0.9885	0.4968	0.3575	0.000
		Streptosporangium sp.	1	0.0107	0.5745	0.3629	0.000
		Streptosporangium sp.	5	0.0008	0.5771	0.3687	0.000

Table 22 continued

Strain Number (HJ)	Soil Numbers Comprising Composite Soil samples and Pretreatment Regimes	Best Three Identifications: Cluster Name	Cluster Number	Identification Scores: Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability
042	579-581, Ginseng field (post harvest) Kumsan, Republic of Korea Sodium dodecyl sulphate	*Streptosporangium* sp.	2	0.9885	0.4968	0.3575	0.000
		Streptosporangium sp.	1	0.0107	0.5745	0.3629	0.000
		Streptosporangium sp.	5	0.0008	0.5771	0.3687	0.000
043		*Streptosporangium* sp.	2	0.9975	0.5543	0.3575	0.000
		Streptosporangium sp.	5	0.0010	0.6089	0.3687	0.000
		Streptosporangium sp.	1	0.0006	0.6605	0.3629	0.000
044		*Streptosporangium amethystogenes/ S. corrugatum/S. longisporum*	10	0.7126	0.5201	0.3687	0.000
		Streptosporangium sp.	1	0.1591	0.5666	0.3629	0.000
		Streptosporangium sp.	6	0.1257	0.5361	0.3161	0.000
045		*Streptosporangium amethystogenes/ S.corrugatum/S.longisporum*	10	0.6798	0.4825	0.3687	0.001
		Streptosporangium sp.	1	0.3202	0.5285	0.3629	0.000
		Streptosporangium sp.	2	0.0000	0.6217	0.3575	0.000
046		*Streptosporangium* sp.	1	0.6614	0.5246	0.3629	0.000
		Streptosporangium sp.	2	0.3282	0.5075	0.3575	0.000
		Streptosporangium sp.	4	0.0104	0.5226	0.3186	0.000
047		*Streptosporangium* sp.	2	0.9600	0.5118	0.3575	0.000
		Streptosporangium sp.	1	0.0369	0.5770	0.3629	0.000
		Streptosporangium sp.	4	0.0002	0.5364	0.3186	0.000
048		*Streptosporangium* sp.	2	0.6921	0.5031	0.3575	0.000
		Streptosporangium sp.	1	0.2900	0.5326	0.3629	0.000
		Streptosporangium sp.	5	0.0177	0.5435	0.3687	0.000

Table 22 continued

Strain Number (HJ)	Soil Numbers Comprising Composite Soil samples and Pretreatment Regimes	Best Three Identifications: Cluster Name	Cluster Number	Identification Scores: Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability
049	579-581, Ginseng field (post harvest) Kumsan, Republic of Korea Sodium dodecyl sulphate	*Streptosporangium* sp.	1	0.9818	0.5517	0.3629	0.000
		Streptosporangium sp.	2	0.0181	0.5510	0.3575	0.000
		Streptosporangium sp.	5	0.0000	0.6089	0.3687	0.000
050		*Streptosporangium* sp.	4	0.7595	0.4447	0.3186	0.000
		Streptosporangium sp.	1	0.2371	0.5007	0.3629	0.000
		Streptosporangium sp.	2	0.0024	0.5075	0.3575	0.000
051		*Streptosporangium* sp.	1	0.9567	0.5118	0.3629	0.000
		Streptosporangium sp.	2	0.0362	0.5103	0.3575	0.000
		Streptosporangium sp.	4	0.0000	0.5684	0.5226	0.000
052	579-581, Ginseng field (post harvest) Kumsan, Republic of Korea Yeast extract	*Streptosporangium* sp.	1	0.6614	0.5246	0.3629	0.000
		Streptosporangium sp.	2	0.3282	0.5075	0.3575	0.000
		Streptosporangium sp.	4	0.0104	0.5226	0.3186	0.000
054		*Streptosporangium* sp.	1	0.7910	0.5291	0.3629	0.000
		Streptosporangium sp.	2	0.2089	0.5189	0.3575	0.000
		Streptosporangium sp.	4	0.0001	0.5364	0.3186	0.000
057		*Streptosporangium* sp.	1	0.7090	0.5512	0.3629	0.000
		Streptosporangium amethystogenes/ S. corrugatum/S. longisporum	10	0.2882	0.5323	0.3687	0.000
		Streptosporangium sp.	11	0.0020	0.5138	0.1212	0.000
058		*Streptosporangium* sp.	2	0.9690	0.5182	0.3575	0.000
		Streptosporangium sp.	1	0.0286	0.5844	0.3629	0.000
		Streptosporangium sp.	4	0.0024	0.5364	0.3186	0.000
059		*Streptosporangium* sp.	1	0.8386	0.5228	0.3629	0.000
		Streptosporangium sp.	4	0.1177	0.4852	0.3186	0.000
		Streptosporangium sp.	2	0.0436	0.5277	0.3575	0.000

Table 22 continued

Strain Number (HJ)	Soil Numbers Comprising Composite Soil samples and Pretreatment Regimes	Best Three Identifications					
		Cluster		Identification Scores			
		Name	Number	Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability
060	579-581, Ginseng field (post harvest) Kumsan, Republic of Korea Yeast extract	*Streptosporangium* sp.	2	0.9893	0.5434	0.3575	0.000
		Streptosporangium sp.	1	0.0107	0.6114	0.3629	0.000
		Streptosporangium sp.	12	0.0000	0.5825	0.1212	0.000
061		*Streptosporangium* sp.	2	0.9978	0.5964	0.3575	0.000
		Streptosporangium sp.	1	0.0012	0.6734	0.3629	0.000
		Streptosporangium sp.	5	0.0010	0.6296	0.3687	0.000
062		*Streptosporangium* sp.	1	0.8446	0.5493	0.3629	0.000
		Streptosporangium sp.	2	0.1553	0.5349	0.3575	0.000
		Streptosporangium sp.	12	0.0001	0.5492	0.1212	0.000
063		*Streptosporangium* sp.	2	0.9214	0.4846	0.3575	0.000
		Streptosporangium sp.	1	0.0429	0.5553	0.3629	0.000
		Streptosporangium sp.	5	0.0357	0.5193	0.3687	0.000
064		*Streptosporangium* sp.	2	0.5293	0.5593	0.3575	0.000
		Streptosporangium sp.	1	0.4703	0.5848	0.3629	0.000
		Streptosporangium sp.	5	0.0004	0.6193	0.3687	0.000
065		*Streptosporangium* sp.	1	0.9703	0.5184	0.3629	0.000
		Streptosporangium sp.	2	0.0248	0.5049	0.3575	0.000
		Streptosporangium sp.	6	0.0033	0.5145	0.3161	0.000
066		*Streptosporangium* sp.	2	0.9930	0.5393	0.3575	0.000
		Streptosporangium sp.	5	0.0063	0.5989	0.3687	0.000
		Streptosporangium sp.	1	0.0006	0.6279	0.3629	0.000
067		*Streptosporangium* sp.	5	0.6200	0.4948	0.3687	0.000
		Streptosporangium roseum/S. vulgare	9	0.3400	0.4765	0.2637	0.000
		Streptosporangium sp.	1	0.0203	0.5505	0.3629	0.000

Table 22 continued

Strain Number (HJ)	Soil Numbers Comprising Composite Soil samples and Pretreatment Regimes	Best Three Identifications: Cluster Name	Cluster Number	Identification Scores: Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability
069	579-581, Ginseng field (post harvest) Kumsan, Republic of Korea Yeast extract	*Streptosporangium roseum/S.vulgare*	9	0.6874	0.4765	0.2637	0.000
		Streptosporangium sp.	2	0.2886	0.5235	0.3575	0.000
		Streptosporangium sp.	1	0.0239	0.5534	0.3629	0.000
070		*Streptosporangium* sp.	2	0.6921	0.5031	0.3575	0.000
		Streptosporangium sp.	1	0.2900	0.5326	0.3629	0.000
		Streptosporangium sp.	5	0.0177	0.5435	0.3687	0.000
071		*Streptosporangium* sp.	2	0.7856	0.4207	0.3575	0.070
		Streptosporangium sp.	1	0.2096	0.4859	0.3629	0.000
		Streptosporangium sp.	5	0.0048	0.4948	0.3687	0.000
072		*Streptosporangium* sp.	1	0.8722	0.4840	0.3629	0.000
		Streptosporangium sp.	2	0.1204	0.4661	0.3575	0.001
		Streptosporangium sp.	5	0.0075	0.4948	0.3687	0.000
073		*Streptosporangium amethystogenes/ S. corrugatum/S. longisporum*	10	0.5176	0.4948	0.3687	0.000
		Streptosporangium sp.	2	0.2904	0.5380	0.3575	0.000
		Streptosporangium sp.	1	0.1920	0.5607	0.3629	0.000
074		*Streptosporangium* sp.	1	0.8545	0.5536	0.3629	0.000
		Streptosporangium sp.	4	0.0980	0.5001	0.3186	0.000
		Streptosporangium sp.	2	0.0413	0.5474	0.3575	0.000
075		*Streptosporangium* sp.	1	0.7853	0.5548	0.3629	0.000
		Streptosporangium amethystogenes/ S. corrugatum/S. longisporum	10	0.1439	0.5323	0.3687	0.000
		Streptosporangium sp.	2	0.0701	0.5597	0.3575	0.000

Table 22 continued

Strain Number (HJ)	Soil Numbers Comprising Composite Soil samples and Pretreatment Regimes	Best Three Identifications: Cluster Name	Cluster Number	Identification Scores: Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability
076	579-581, Ginseng field (post harvest) Kumsan, Republic of Korea Yeast extract	*Streptosporangium* sp.	2	0.8053	0.4641	0.3575	0.001
		Streptosporangium sp.	1	0.1943	0.4965	0.3629	0.000
		Streptosporangium sp.	5	0.0004	0.5315	0.3687	0.000
077		*Streptosporangium* sp.	1	0.5368	0.4879	0.3629	0.000
		Streptosporangium sp.	2	0.4626	0.4689	0.3575	0.001
		Streptosporangium sp.	4	0.0006	0.5001	0.3186	0.000
078		*Streptosporangium* sp.	1	0.5045	0.5339	0.3629	0.000
		Streptosporangium sp.	2	0.4951	0.5175	0.3575	0.000
		Streptosporangium roseum/S. vulgare	9	0.0004	0.5499	0.2637	0.000
079		*Streptosporangium* sp.	2	0.6921	0.4641	0.3575	0.001
		Streptosporangium sp.	1	0.2900	0.4960	0.3629	0.000
		Streptosporangium sp.	5	0.0177	0.5076	0.3687	0.000
080		*Streptosporangium* sp.	1	0.9897	0.5248	0.3629	0.000
		Streptosporangium amethystogenes/ S. corrugatum/S. longisporum	10	0.0099	0.5076	0.3687	0.000
		Streptosporangium sp.	6	0.0003	0.5513	0.3161	0.000
081		*Streptosporangium* sp.	2	0.8053	0.4641	0.3575	0.001
		Streptosporangium sp.	1	0.1943	0.4965	0.3629	0.000
		Streptosporangium sp.	5	0.0004	0.5315	0.3687	0.000
082		*Streptosporangium* sp.	5	0.7963	0.4690	0.3687	0.003
		Streptosporangium sp.	2	0.2035	0.4452	0.3575	0.007
		Streptosporangium sp.	1	0.0001	0.5489	0.3629	0.000

Table 22 continued

Strain Number (HJ)	Soil Numbers Comprising Composite Soil samples and Pretreatment Regimes	Best Three Identifications: Cluster Name	Cluster Number	Identification Scores: Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability
083	579-581, Ginseng field (post harvest) Kumsan, Republic of Korea Yeast extract	*Streptosporangium* sp.	1	0.9587	0.4935	0.3629	0.000
		Streptosporangium amethystogenes/ S. corrugatum/S. longisporum	10	0.0403	0.4956	0.3687	0.000
		Streptosporangium sp.	2	0.0011	0.5448	0.3575	0.000
084		*Streptosporangium* sp.	2	0.5539	0.4582	0.3575	0.002
		Streptosporangium sp.	1	0.4098	0.5034	0.3629	0.000
		Streptosporangium sp.	5	0.0231	0.4948	0.3687	0.000
085		*Streptosporangium* sp.	5	0.8227	0.4117	0.3687	0.381
		Streptosporangium sp.	2	0.1709	0.3890	0.3575	0.781
		Streptosporangium sp.	1	0.0064	0.4763	0.3629	0.001
086		*Streptosporangium* sp.	2	0.7181	0.4939	0.3575	0.000
		Streptosporangium sp.	1	0.2818	0.5251	0.3629	0.000
		Streptosporangium sp.	5	0.0001	0.5771	0.3687	0.000
093	583-584, Ginseng field (post harvest) Kumsan, Republic of Korea Heat and phenol	*Streptosporangium* sp.	5	0.4226	0.5551	0.3687	0.000
		Streptosporangium sp.	2	0.3342	0.5393	0.3575	0.000
		Streptosporangium sp.	1	0.2432	0.5664	0.3629	0.000
094		*Streptosporangium* sp.	1	0.9154	0.5505	0.3629	0.000
		Streptosporangium roseum/S. vulgare	9	0.0492	0.5145	0.2637	0.000
		Streptosporangium sp.	2	0.0336	0.5501	0.3575	0.000
099	583-584, Ginseng field (post harvest) Kumsan, Republic of Korea Sodium dodecyl sulphate	*Streptosporangium* sp.	1	0.5191	0.4895	0.3629	0.000
		Streptosporangium sp.	6	0.4549	0.4351	0.3161	0.000
		Streptosporangium sp.	2	0.0216	0.4758	0.3575	0.000

Table 22 continued

Strain Number (HJ)	Soil Numbers Comprising Composite Soil samples and Pretreatment Regimes	Best Three Identifications					
		Cluster		Identification Scores			
		Name	Number	Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability
100	583-584, Ginseng field (post harvest) Kumsan, Republic of Korea Yeast extract	*Streptosporangium* sp.	12	0.7419	0.5138	0.1212	0.000
		Streptosporangium sp.	5	0.1760	0.5544	0.3687	0.000
		Streptosporangium sp.	6	0.0745	0.5647	0.3161	0.000
101		*Streptosporangium* sp.	6	0.9852	0.5361	0.3161	0.000
		Streptosporangium sp.	5	0.0143	0.5308	0.3687	0.000
		Streptosporangium sp.	1	0.0006	0.6038	0.3629	0.000
102		*Streptosporangium* sp.	6	0.9852	0.5361	0.3161	0.000
		Streptosporangium sp.	5	0.0142	0.5308	0.3687	0.000
		Streptosporangium sp.	1	0.0006	0.6038	0.3629	0.000
103		*Streptosporangium* sp.	1	0.5191	0.4895	0.3629	0.000
		Streptosporangium sp.	6	0.4549	0.4351	0.3161	0.000
		Streptosporangium sp.	2	0.0216	0.4758	0.3575	0.000
104	443-444, Garden soil, IMTECH, Chandigarh, India Heat	*Streptosporangium* sp.	1	0.8156	0.5850	0.3629	0.000
		Streptosporangium sp.	8	0.0738	0.5603	0.2743	0.000
		Streptosporangium sp.	2	0.0000	0.4342	0.1212	0.000
106	443-444, Garden soil, IMTECH, Chandigarh, India Yeast extract	*Streptosporangium* sp.	2	0.6762	0.4947	0.3575	0.000
		Streptosporangium roseum/*S. vulgare*	9	0.3097	0.4765	0.2637	0.000
		Streptosporangium sp.	1	0.0084	0.5388	0.3629	0.000
111	433-434, Garden soil, Hibuya Park, Tokyo, Japan. Yeast extract	*Streptosporangium* sp.	5	0.5686	0.5659	0.3687	0.000
		Streptosporangium sp.	2	0.2969	0.5569	0.3575	0.000
		Streptosporangium sp.	1	0.1296	0.6164	0.3629	0.000
114	435-436, Garden soil, Tsukuba University, Tsukuba, Japan Heat	*Streptosporangium* sp.	5	0.8083	0.3958	0.3687	1.100
		Streptosporangium sp.	1	0.1775	0.4455	0.3629	0.014
		Streptosporangium sp.	4	0.0122	0.4360	0.3186	0.000

Table 22 continued

Strain Number (HJ)	Soil Numbers Comprising Composite Soil samples and Pretreatment Regimes	Best Three Identifications: Cluster Name	Cluster Number	Identification Scores: Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability
115	435-436, Garden soil, Tsukuba University, Tsukuba, Japan Yeast extract	*Streptosporangium* sp.	4	0.7351	0.4360	0.3186	0.000
		Streptosporangium sp.	5	0.2450	0.4551	0.3687	0.011
		Streptosporangium sp.	1	0.0198	0.5125	0.3629	0.000
117	482-489, Tropical rainforest soil, Meru Betini, Indonesia Heat	*Streptosporangium* sp.	1	0.7090	0.5512	0.3629	0.000
		Streptosporangium sp. *amethystogenes/ S.corrugatum/S.longisporum*	10	0.2882	0.5323	0.3687	0.000
		Streptosporangium sp.	11	0.0020	0.5138	0.1212	0.000
122	482-489, Tropical rainforest soil, Meru Betini, Indonesia Heat and Phenol	*Streptosporangium* sp.	12	0.7419	0.5138	0.1212	0.000
		Streptosporangium sp.	5	0.1760	0.5544	0.3687	0.000
		Streptosporangium sp.	6	0.0745	0.5647	0.3161	0.000
124	482-489, Tropical rainforest soil, Meru Betini, Indonesia Sodium dodecyl sulphate	*Streptosporangium* sp.	4	0.7351	0.4360	0.3186	0.000
		Streptosporangium sp.	5	0.2450	0.4551	0.3687	0.011
		Streptosporangium sp.	1	0.0198	0.5125	0.3629	0.000
128	482-489, Tropical rainforest soil, Meru Betini, Indonesia Yeast extract	*Streptosporangium* sp.	2	0.7856	0.4207	0.3575	0.070
		Streptosporangium sp.	1	0.2096	0.4859	0.3629	0.000
		Streptosporangium sp.	5	0.0048	0.4948	0.3687	0.000
130	515-516, Garden soil, Yokyakarta, Indonesia Heat	*Streptosporangium* sp.	1	0.7381	0.5524	0.3629	0.000
		Streptosporangium sp.	8	0.1160	0.5256	0.2743	0.000
		Streptosporangium sp.	2	0.0949	0.5023	0.3575	0.000
131		*Streptosporangium* sp.	12	0.7419	0.5138	0.1212	0.000
		Streptosporangium sp.	5	0.1760	0.5544	0.3687	0.000
		Streptosporangium sp.	6	0.0745	0.5647	0.3161	0.000
132		*Streptosporangium* sp.	1	0.6155	0.5985	0.3629	0.000
		Streptosporangium sp.	4	0.2339	0.5506	0.3186	0.000
		Streptosporangium sp.	8	0.0896	0.5671	0.2743	0.000

Table 22 continued

Strain Number (HJ)	Soil Numbers Comprising Composite Soil samples and Pretreatment Regimes	Best Three Identifications: Cluster Name	Cluster Number	Identification Scores: Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability
133	515-516, Garden soil, Yokyakarta, Indonesia Heat	*Streptosporangium* sp.	1	0.9779	0.5825	0.1212	0.000
		Streptosporangium sp.	11	0.0160	0.5492	0.1212	0.000
		Streptosporangium sp.	2	0.0038	0.6184	0.3575	0.000
135	515-516, Garden soil, Yokyakarta, Indonesia Heat and Phenol	*Streptosporangium* sp.	2	0.5958	0.5085	0.3575	0.000
		Streptosporangium sp.	1	0.2077	0.5456	0.3629	0.000
		Streptosporangium sp.	5	0.1960	0.5069	0.3687	0.000
138	515-516, Garden soil, Yokyakarta, Indonesia Yeast extract	*Streptosporangium* sp.	6	0.4073	0.5647	0.3161	0.000
		Streptosporangium sp.	5	0.2405	0.5771	0.3687	0.000
		Streptosporangium sp.	1	0.1932	0.6077	0.3629	0.000
139		*Streptosporangium* sp.	6	0.4073	0.5647	0.3161	0.000
		Streptosporangium sp.	5	0.2405	0.5771	0.3687	0.000
		Streptosporangium sp.	1	0.1932	0.6077	0.3629	0.000
140		*Streptosporangium* sp.	2	0.8251	0.5255	0.3575	0.000
		Streptosporangium sp.	1	0.1548	0.5985	0.3629	0.000
		Streptosporangium sp.	6	0.0180	0.5647	0.3161	0.000
141		*Streptosporangium* sp.	3	0.6317	0.5538	0.2514	0.000
		Streptosporangium sp.	1	0.2312	0.6217	0.3629	0.000
		Streptosporangium sp.	12	0.1241	0.5492	0.1212	0.000
143	604-605, Woodland soil, Mount Sorak, Republic of Korea Heat	*Streptosporangium amethystogenes/ S.corrugatum/S.longisporum*	10	0.7033	0.5881	0.3687	0.000
		Streptosporangium sp.	1	0.2727	0.6292	0.3629	0.000
		Streptosporangium sp.	3	0.0123	0.5868	0.2514	0.000
144	604-605, Woodland soil, Mount Sorak, Republic of Korea Yeast extract	*Streptosporangium* sp.	2	0.8251	0.5255	0.3575	0.000
		Streptosporangium sp.	1	0.1548	0.5985	0.3629	0.000
		Streptosporangium sp.	6	0.0180	0.5647	0.3161	0.000

Table 22 continued

Strain Number (HJ)	Soil Numbers Comprising Composite Soil samples and Pretreatment Regimes	Best Three Identifications: Cluster Name	Cluster Number	Identification Scores: Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability
146	604-605, Woodland soil, Mount Sorak, Republic of Korea Yeast extract	*Streptosporangium* sp.	3	0.6317	0.5538	0.2514	0.000
		Streptosporangium sp.	1	0.2312	0.6217	0.3629	0.000
		Streptosporangium sp.	12	0.1241	0.5492	0.1212	0.000
147		*Streptosporangium amethystogenes/ S.corrugatum/S.longisporum*	10	0.7033	0.5881	0.3687	0.000
		Streptosporangium sp.	1	0.2727	0.6292	0.3629	0.000
		Streptosporangium sp.	3	0.0123	0.5868	0.2514	0.000
148	576-577, Soil rich in humus Keswick, England, U.K. Sodium dodecyl sulphate	*Streptosporangium*	1	0.5732	0.4914	0.3629	0.000
		Streptosporangium amethystogenes/ S.corrugatum/S.longisporum	10	0.4265	0.4825	0.3687	0.001
		Streptosporangium sp.	2	0.0003	0.5758	0.3575	0.000
149		*Streptosporangium* sp.	12	0.5915	0.4342	0.1212	0.000
		Streptosporangium sp.	6	0.4085	0.4942	0.3161	0.000
		Streptosporangium sp.	1	0.0000	0.5764	0.3629	0.000
150	576-577, Soil rich in humus Keswick, England, U.K. Yeast extract	*Streptosporangium* sp.	12	0.5533	0.4757	0.1212	0.000
		Streptosporangium sp.	1	0.4391	0.5383	0.3629	0.000
		Streptosporangium sp.	2	0.0044	0.5808	0.3575	0.000
151		*Streptosporangium* sp.	2	0.5906	0.4038	0.3575	0.270
		Streptosporangium sp.	1	0.4092	0.4558	0.3629	0.005
		Streptosporangium sp.	5	0.0002	0.4948	0.3687	0.000
152		*Streptosporangium* sp.	12	0.5533	0.4757	0.1212	0.000
		Streptosporangium sp.	1	0.4391	0.5383	0.3629	0.000
		Streptosporangium sp.	2	0.0044	0.5808	0.3575	0.000

Table 22 continued

Strain Number (HJ)	Soil Numbers Comprising Composite Soil samples and Pretreatment Regimes	Best Three Identifications: Cluster Name	Cluster Number	Identification Scores: Willcox Probability	Taxonomic Distance	95% Taxonomic Radius	Gaussian Distance Probability
153	576-577, Soil rich in humus	*Streptosporangium* sp.	2	0.7856	0.3732	0.3575	2.122
	Keswick, England, U.K.	*Streptosporangium* sp.	1	0.2096	0.4455	0.3629	0.014
	Yeast extract	*Streptosporangium* sp.	5	0.0048	0.4551	0.3687	0.011

[a], Dry soil heated at 120°C for 1 hour; [b], Dry soil suspension (10^{-1}) treated with sodium dodecyl sulphate (0.05%, w/v) at 40°C for 20 minutes; [c], Dry soil suspension treated with yeast extract (6%, w/v) at 40°C for 20 minutes; [d], Dry soil heated at 120°C for 1 hour and then treated with phenol (1.5%, w/v) at 30°C for 30 minutes.

C. PYROLYSIS MASS SPECTROMETRY

The pyrolysis mass spectral data support the taxonomic integrity of streptosporangial clusters 1 and 2 (Figure 9, page 179). It is also evident that cluster 1 accommodates more variation than cluster 2 (Figures 9 and 10, pages 179 and 180) and that representatives of clusters 1 and 2 had little in common with the type strains of validly described species of *Streptosporangium* (Figures 11 and 12, pages 181 and 182), apart from *Streptosporangium roseum* TW 005 which was loosely associated with representatives of these clusters. It is also evident that the type strain of *Streptosporangium viridogriseum* subspecies *viridogriseum* has little in common with *bona fide* members of the genus *Streptosporangium.*

Six of the twelve isolates identified to cluster 1 using the stringent cut-off criteria were recovered in the group corresponding to this cluster. It is even more encouraging, however, that four of the ten organisms identified to cluster 1 in the computer-assisted identification using less stringent criteria, namely strains a020, a036, a055 and a056, were closely associated with the representatives of this taxon. Similarly, organisms identified to cluster 2 using the less stringent criteria, namely strains HJ 021, HJ 126 and HJ 129, were associated with the representatives of this taxon. The unidentified strains were assigned to two groups (Figures 13 and 14, pages 183 and 184). The group comprising strains c009, c131 and c149 was sharply separated from all of the remaining test strains (Figure 14, page, 184).

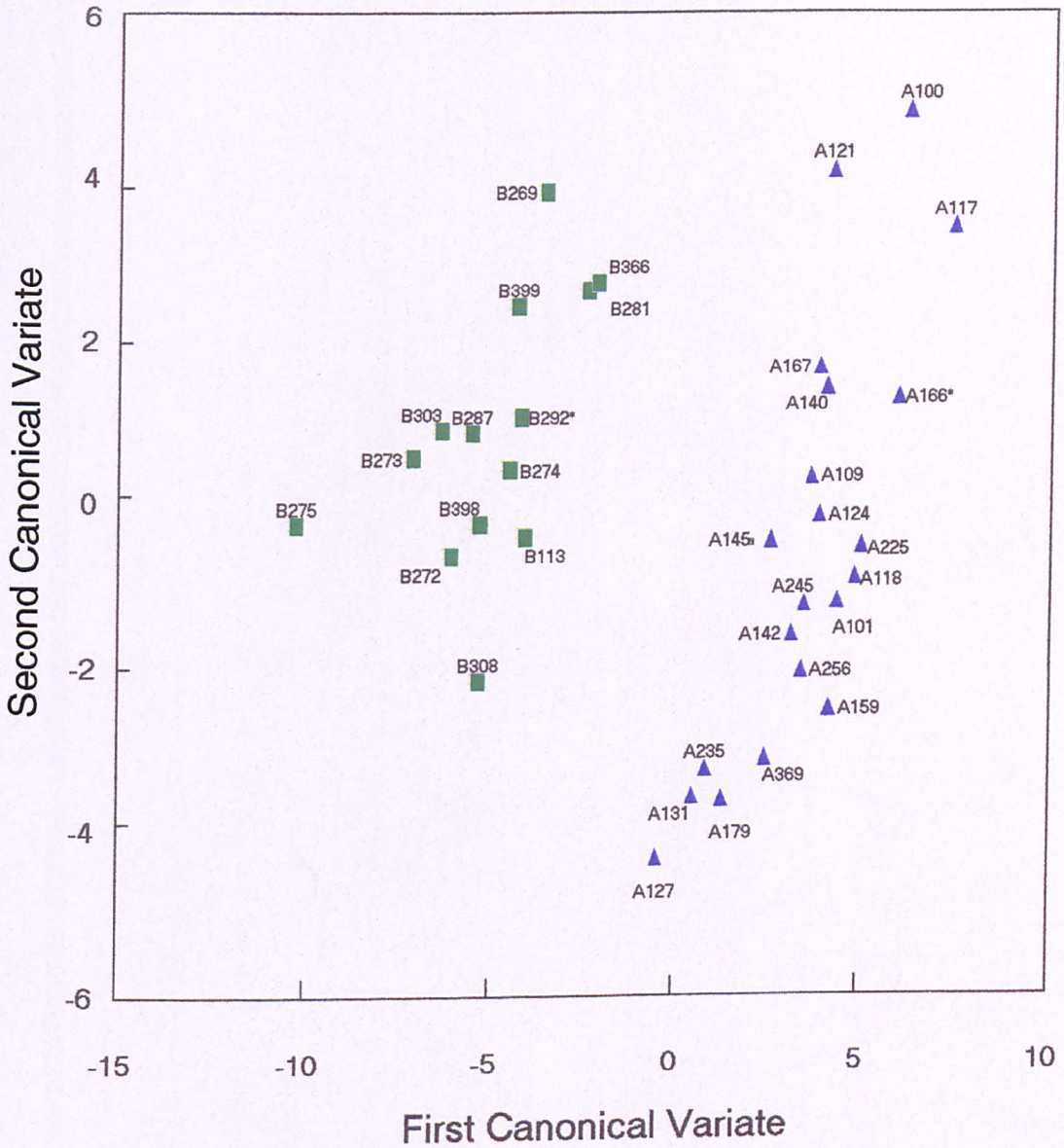


Figure 9 Ordination plot along the first two canonical variate axes showing the mean position of the representatives of streptosporangial clusters 1 and 2 (see Table 16, pages 105 to 108; Whitham, 1988; Whitham *et al.*, 1993). The first two axes accounted for 76% of the variation between strains.

▲ Representatives of cluster 1; ■ Representatives of cluster 2; * Centrotype strains.

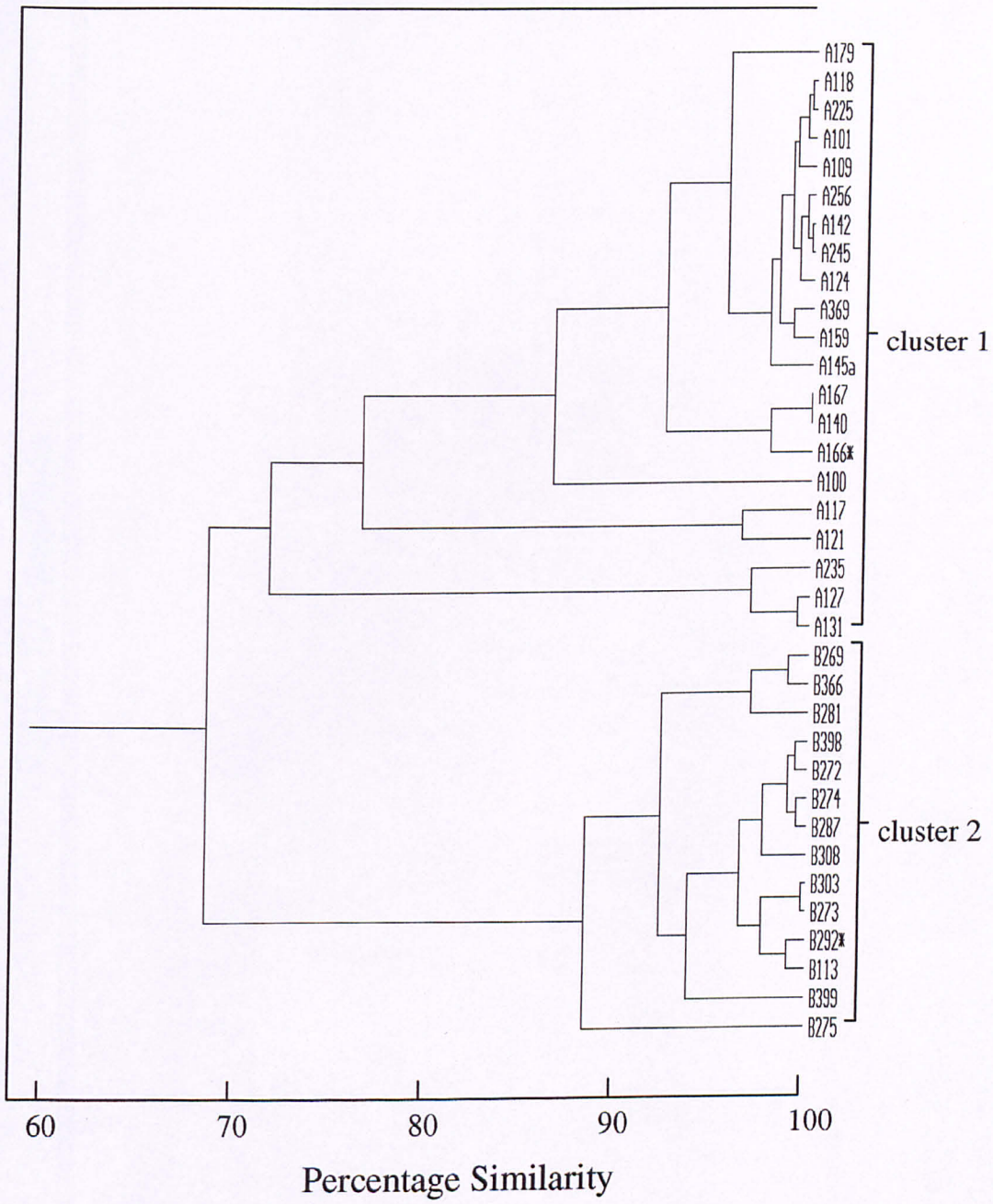


Figure 10 Dendrogram representing the relationships found between representative *Streptosporangium* strains from clusters 1 and 2 (see Table 16, pages 105 to 108; Whitham, 1988; Whitham *et al.*, 1993). The dendrogram is based on similarity values derived from Mahalanobis distances with clustering achieved using the unweighted pair group method with arithmetic averages algorithm.

*, Centrotype strains.

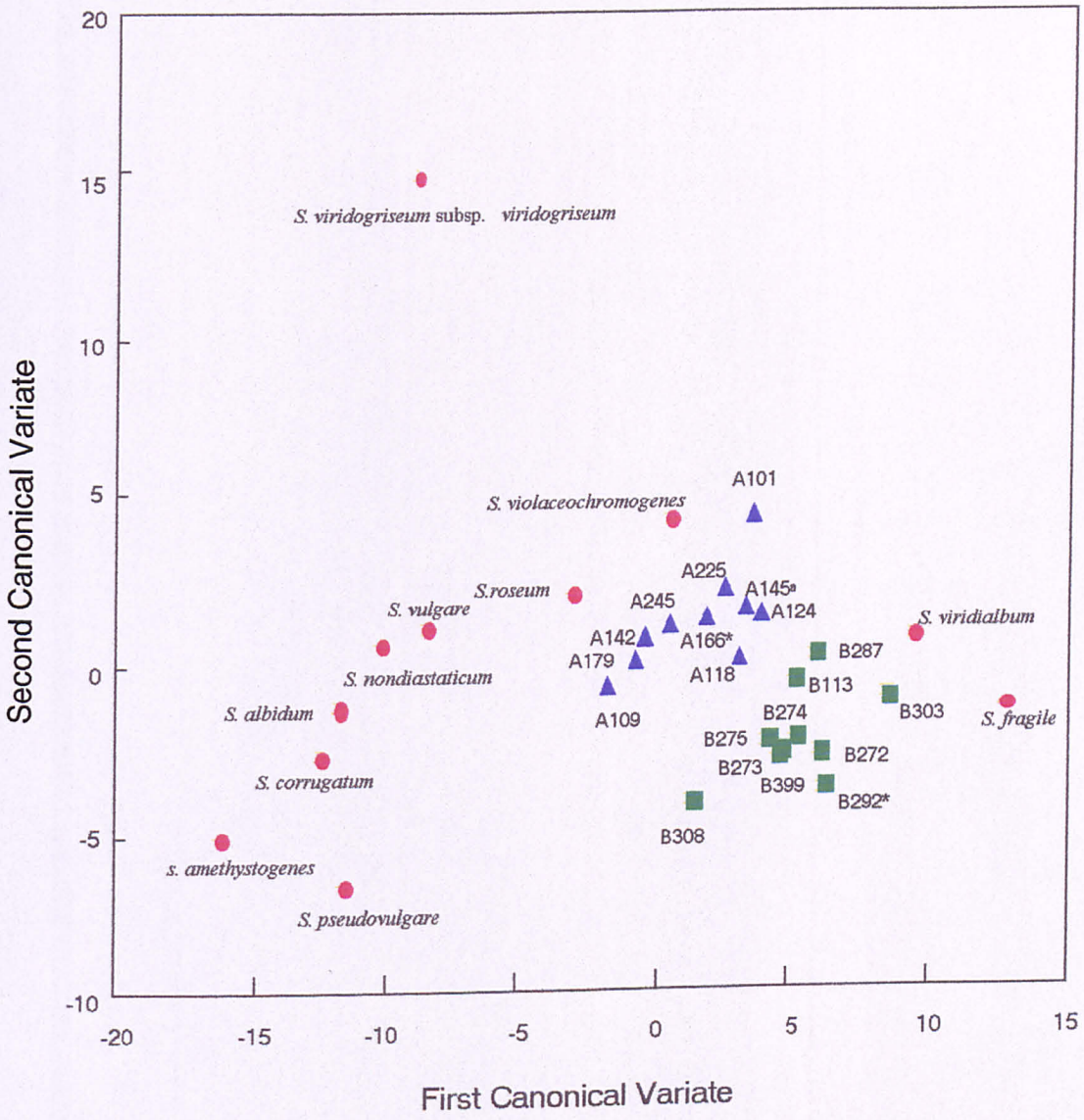


Figure 11 Ordination plot along the first two canonical variate axes showing the mean position of the representatives of streptosporangial clusters 1 and 2 (Whitham, 1988; Whitham *et al.*, 1993) and type strains of validly described *Streptosporangium* species (see Table 16, pages 105 to 108). The first two axes accounted 67% of the variation between strains.

▲ Representatives of cluster 1, ■ Representatives of cluster 2; *, Centrotype strains and ⬭ Type strains.

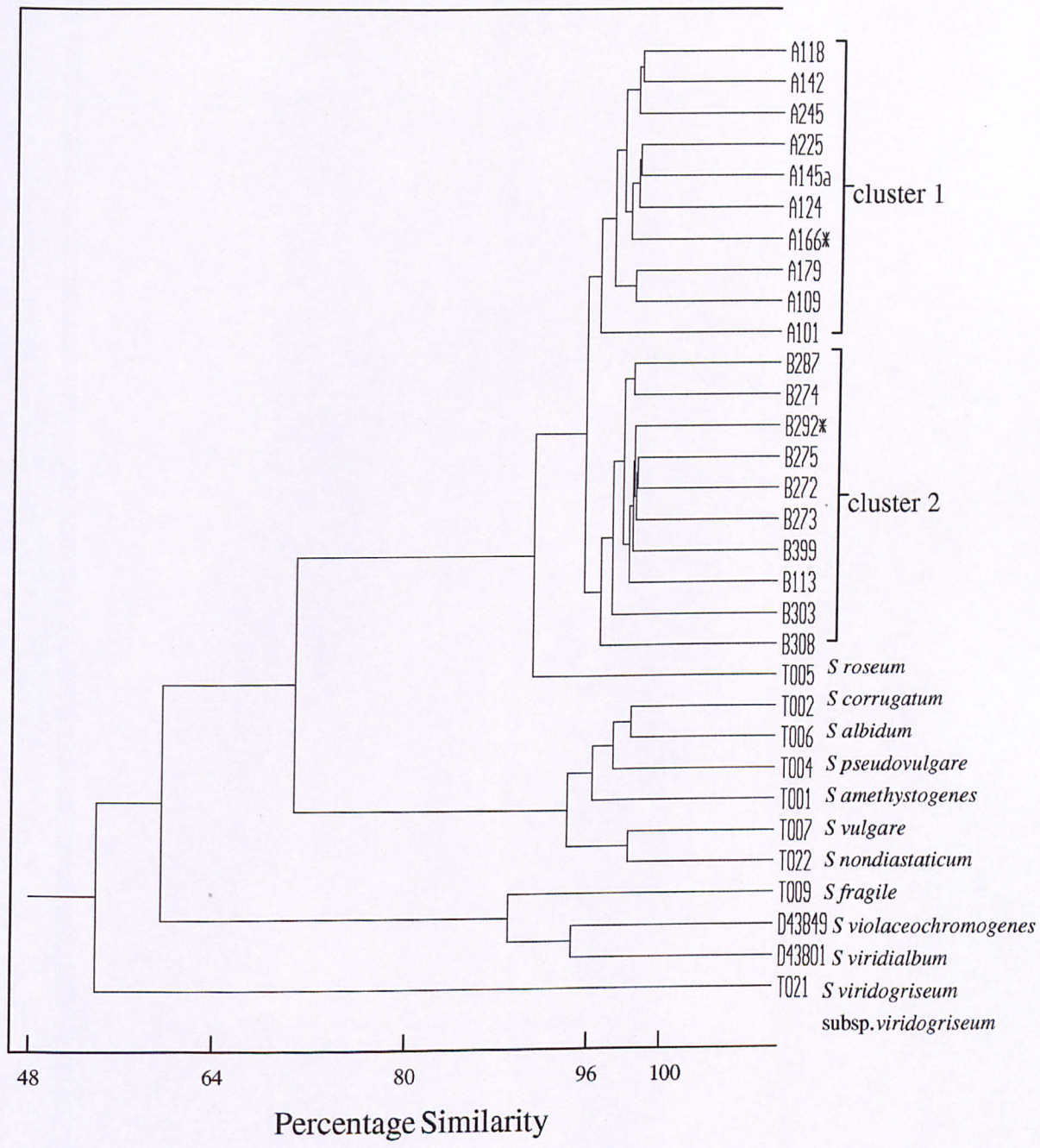


Figure 12 Dendrogram representing the relationships found between representatives of streptosporangial clusters 1 and 2 (Whitham, 1988; Whitham *et al.*, 1993) and the type strains of validly described *Streptosporangium* species (see Table 16, pages 105-108). The dendrogram is based on similarity values derived from Mahalanobis distances with clustering achieved using the unweighted pair group method with arithmetic average algorithm.
S, Streptosporangium; *, Centrotype strains.

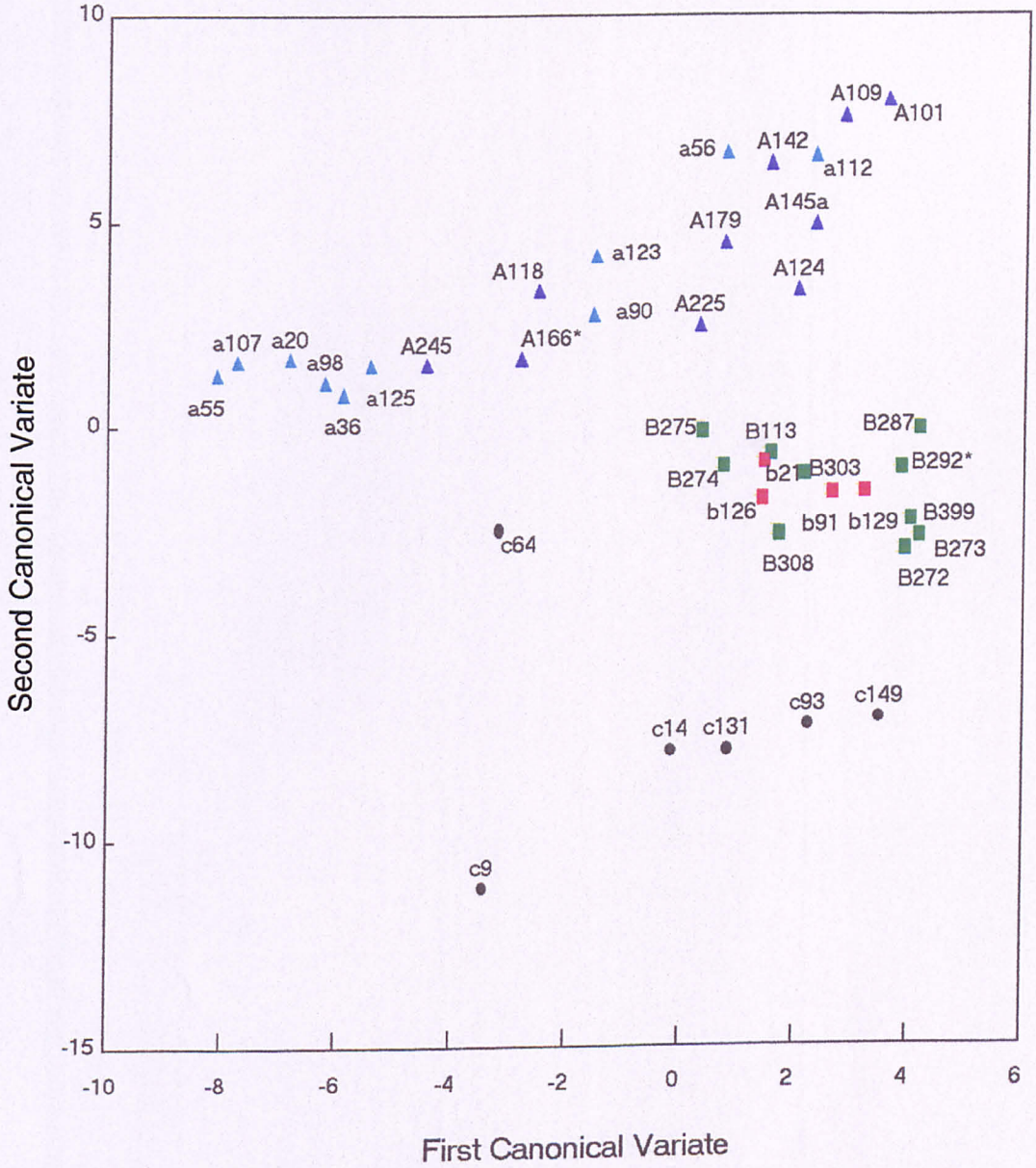


Figure 13 Ordination plot along the first two canonical variate axes showing the mean position of representatives of streptosporangial clusters 1 and 2 (Whitham, 1988; Whitham *et al.*, 1993) and isolates identified to clusters 1 and 2 together with unidentified strains (see Table 16, pages 105 to 108). The first two axes accounted for 71% of the variation between strains.

▲ Representatives of cluster 1; ■ Representatives of cluster 2; ▲ Strains identified to cluster 1; ■ Strains identified to cluster 2; ● Unidentified strains; * Centrotype strains.

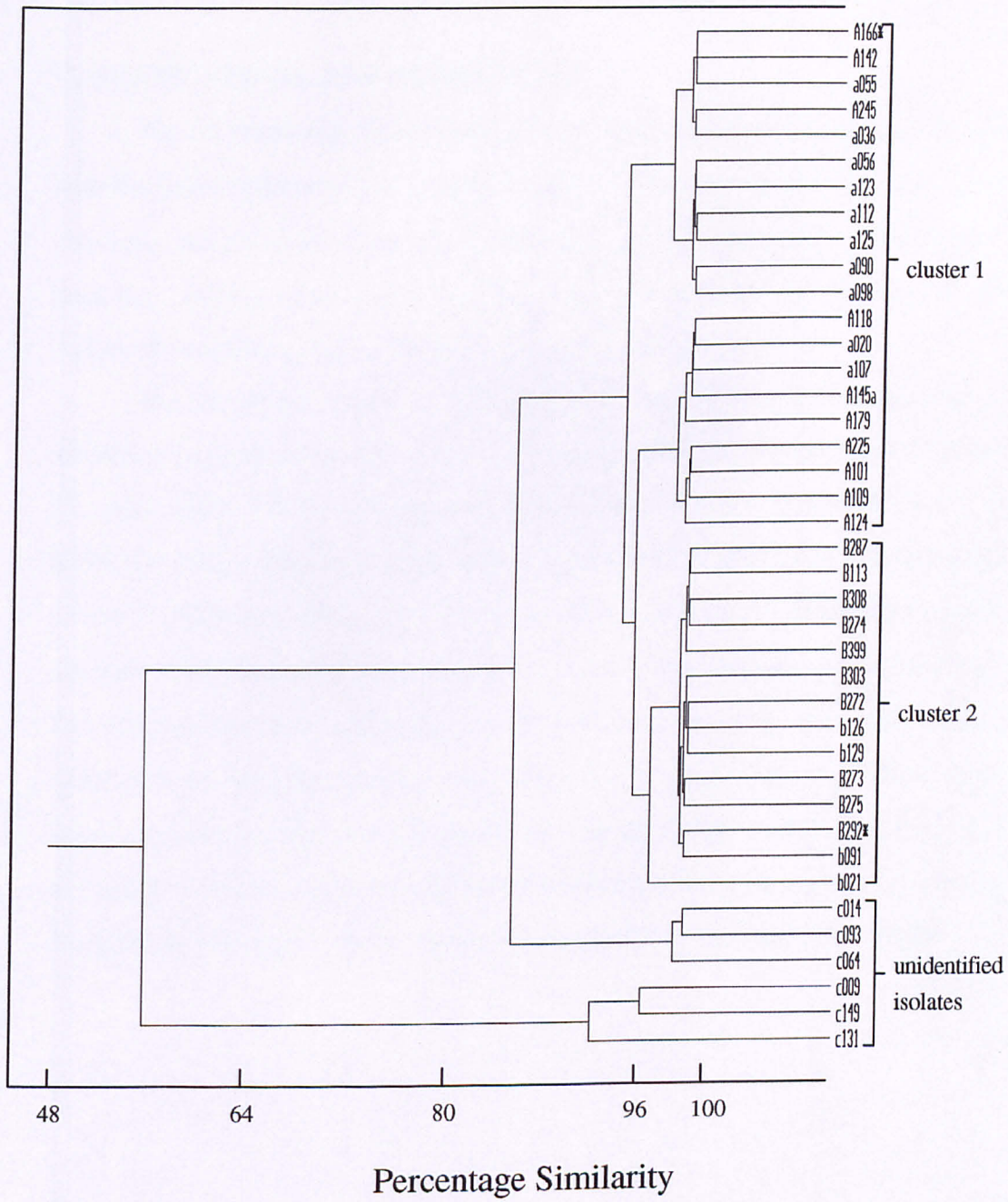


Figure 14 Dendrogram representing the relationships found between representatives of streptosporangial clusters 1 and 2 (Whitham, 1988; Whitham *et al.*, 1993) and isolates identified to clusters 1 and 2 together with unidentified strains (see Table 16, pages 105 to 108). The dendrogram is based on similarity values derived from Mahalanobis distances with clustering achieved using the unweighted pair group method with arithmetic averages algorithm.
A, representatives of cluster 1; B, representatives of cluster 2; a, representative isolates identified to cluster 1; b, representative isolates identified to cluster 2; c, representative unidentified isolates; *, centrotype strains.

D. 5S RIBOSOMAL RNA SEQUENCING

The 5S ribosomal RNA of all nine test strains consisted of 120 nucleotides with the same sequences being found in some of the loop regions of the secondary structure, namely regions bLc, c'Lb', b'Ld (Figure 15, page 186). The secondary structure models that were obtained are exemplified by the model for *Streptosporangium vulgare* TW 007 (Figure 16, page 187).

The 5S rRNA sequences of the strains were aligned by juxtaposing the defined secondary structures which were then divided into fifteen regions (Figure 15, page 186). *Streptosporangium* strain HJ 090 (cluster 1) had the same 5S rRNA nucleotide sequence as the centrotype strain (TW 292) of streptosporangial cluster 2 (Whitham, 1988; Table 23, page 188). In contrast, *Streptosporangium albidum* TW 006 and *Streptosporangium viridogriseum* subspecies *viridogriseum* TW 021 showed low homology values with the remaining test strains. It is evident from the phylogenetic tree (Figure 17, page 189) that these latter organisms form a distinct evolutionary line. Strain HJ 011, which was identified to cluster 1 in the computer-assisted identification exercise based on the less stringent identification criteria, showed a low similarity with the other strains.

STRAIN NAME	NUMBER	10		20		30	40
Streptosporangium albidum	TW 006*	UU UCGGUGGU	GUUG	GCGGAGGG	GAAAC	GCCCGG	UCCCAUUCCGAAC
Streptosporangium pseudovulgare	TW 004*	UUUACGGCGGU	UAUG	GCGAAGGG	GAAAC	ACCCGG	UUACAUUCCGAAC
Streptosporangium isolate	HJ 011	UU UCGGUGGU	CAUA	GCGUGAGG	GAAAC	GCCCGG	UUACAUUCCGAAC
Streptosporangium isolate	HJ 090	UUCACGGCGGU	UAUG	GCGAAGGG	GAAAC	ACCCGG	UUACAUUCCGAAC
Streptosporangium amethystogenes	TW 001*	UUCACGGCGGU	UAUG	GCGAAGGG	GAAAC	ACCCGG	UUACAUUCCGAAC
Streptosporangium vulgare	TW 007*	AUUACGGCGGU	UAUG	GCGAAGGG	GAAAC	ACCCGG	UUACAUUCCGAAC
Streptosporangium sp.	TW 166	UU ACGGCGGU	UAUG	GCGAAGGG	GAAAC	ACCCGG	UUACAUUCCGAAC
Streptosporangium sp.	TW 292	UUCACGGCGGU	UAUG	GCGAAGGG	GAAAC	ACCCGG	UUACAUUCCGAAC
Streptosporangium viridogriseum subsp. *viridogriseum*	TW 021*	UU CGGUGGU	GUUG	GCGGAGGG	GAAAC	GCCCGG	UCCCAUUCCGAAC
		A	**aLb**	**B**	**bLc**	**C**	**cLc'**

50		60	70	80	90		100	110	120
CCGGAAGC	UAAG	CCCUCCUGC	GCCGAUGGUA	CUGCACUC	GUGA	GGGUGUGG	GAGAGUAGGAC	GCUGCCGAACA	
CCGGAAGU	UAAG	CUCUUCAGC	GCCGAUGGUA	CUGCACCG	GGGA	CGGUGUGG	GAGAGUAGGUC	GCCGCCGGACA	
CCGGAAGC	UAAG	CCUUACAGC	GCCGAUGGUA	CUGCAGGG	GGGA	CCCUGUGG	GAGAGUAGGAC	GCCGCCGAACA	
CCGGAAGU	UAAG	CUCUUCAGC	GCCGAUGGUA	CUGCACCG	GGGA	CGGUGUGG	GAGAGUAGGUC	GCCGCCGGACA	
CCGGAAGU	UAAG	CUCUUCAGC	GCCGAUGGUA	CUGCACCG	GGGA	CGGUGUGG	GAGAGUAGGUC	ACCGCCGGACA	
CCGGAAGU	UAAG	CUCUUCAGC	GCCGAUGGUA	CUGCACCG	GGGA	CGGUGUGG	GAGAGUAGGUC	GCCGCCGGACA	
CCGGAAGU	UAAG	CUCUUCAGC	GCCGAUGGUA	CUGCACCG	GGGA	CGGUGUGG	GAGAGUAGGUC	GCCGCCGGACA	
CCGGAAGU	UAAG	CUCUUCAGC	GCCGAUGGUA	CUGCACCG	GGGA	CGGUGUGG	GAGAGUAGGUC	GCCGCCGGACA	
CCGGAAGC	UAAG	CCCUCCUGC	GCCGAUGGUA	CUGCACUC	GUGA	GGGUGUGG	GAGAGUAGGAC	GCUGCCGAACA	
C'	**c'Lb'**	**B'**	**b'Ld**	**D**	**dLd'**	**D'**	**d'La'**	**A'**	

Figure 15 Sequence alignment of 5S rRNAs from 5 marker and 4 unidentified strains of *Streptosporangium*.

Base paired regions: A, A', B, B', C, C', D and D'; loop regions: aLb, bLc, cLc', c'Lb', b'Ld, dLd' and d'La'; *, type strain.

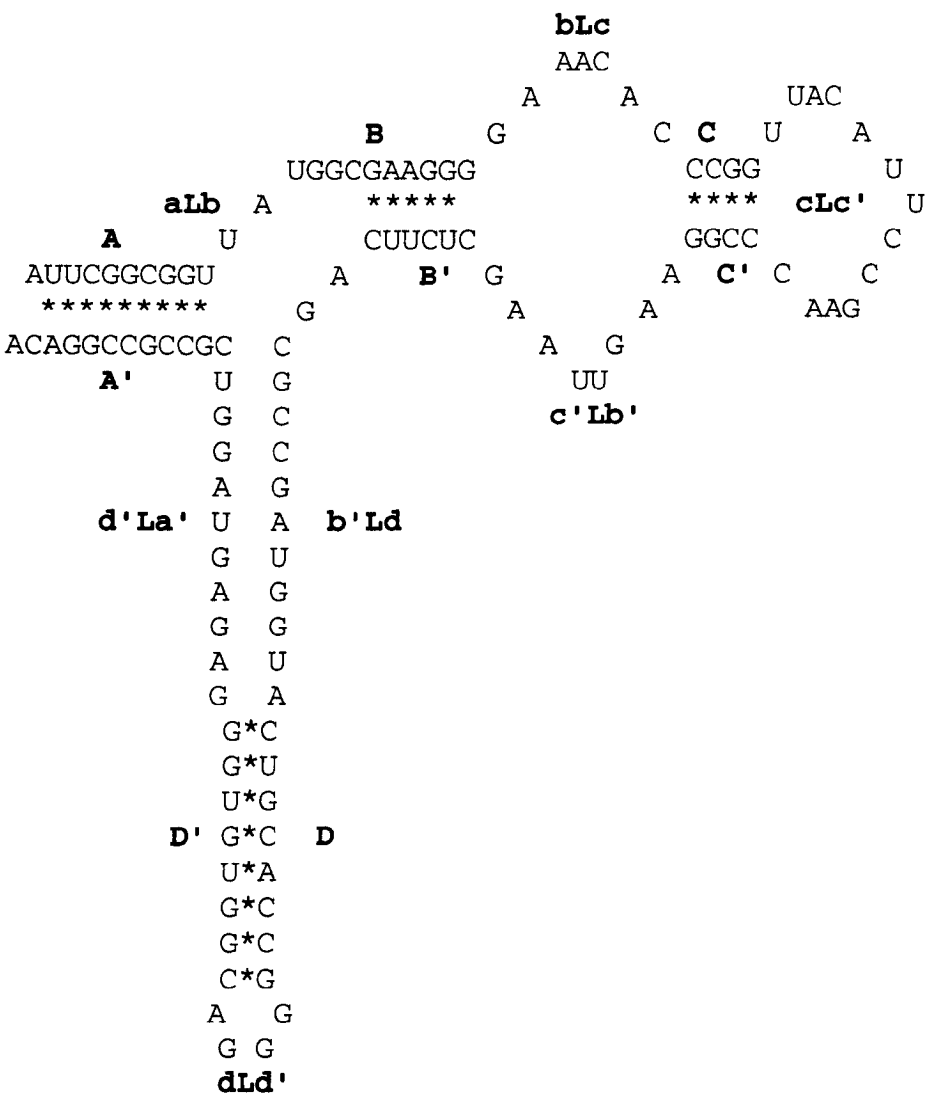


Figure 16 Secondary structure model of the 5S rRNA of *Streptosporangium vulgare* TW 007.

*, Base pairing.

Table 23 Homology percentage matrix of 5S rRNA sequences of *Streptosporangium* strains

Strain Name	Number	1	2	3	4	5	6	7	8	9
1. *Streptosporangium albidum*	TW 006*	100								
2. *Streptosporangium pseudovulgare*	TW 004*	60	100							
3. *Streptosporangium* isolate	HJ 011	72	62	100						
4. *Streptosporangium* isolate	HJ 090	59	98	60	100					
5. *Streptosporangium amethystogenes*	TW 001*	57	97	59	98	100				
6. *Streptosporangium vulgare*	TW 007*	59	98	60	97	95	100			
7. *Streptosporangium* sp.	TW 166**	72	87	73	87	85	87	100		
8. *Streptosporangium* sp.	TW 292**	59	98	60	100	98	97	87	100	
9. *Streptosporangium viridogriseum* subsp. *viridogriseum*	TW 021*	86	60	58	60	58	58	60	60	100

*, Type strain; **, centrotype strain.

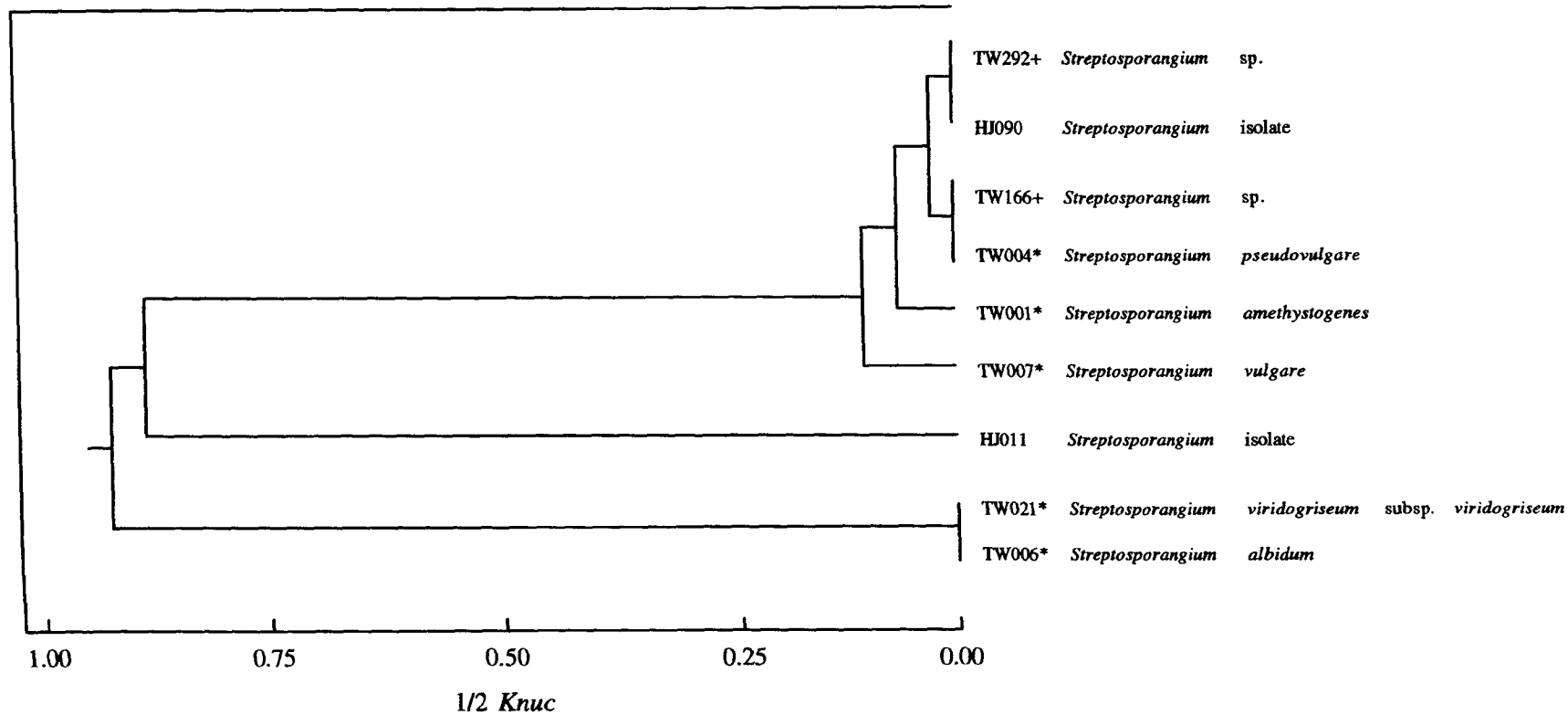


Figure 17 Phylogenetic tree showing relationships among selected streptosporangia based on 5S rRNA sequence data.

*, Type strains; +, centrotype strains (Whitham, 1988; Whitham *et al.*, 1993).

E. RAPID ENZYME TESTS

Inclusion of the seventeen duplicated strains in the fluorogenic enzyme tests enabled experimental test error to calculated (Table 24, pages 191 to 193). The average probability of an erroneous test result (p) calculated from the pooled variance ($Si^{2)}$ for all of the strains was 0.29%. The percentage positive frequencies for each of the conjugated fluorogenic substrates for all of the test strains is given in Table 24, pages 191 to 193. Twenty-two 7-amino-4-methylcoumarin and seven 4-methylumbelliferone substrates were deleted from the final data matrix as they were not of any differential value. The final data matrix, therefore, contained information on 142 strains and 42 tests. The raw enzymatic data for all of the test strains is given in Appendix D.

Very little taxonomic structure was obtained when the information in the final database was examined using the D_p, S_J and S_{sm} coefficients and the UPGMA algorithm. This somewhat disappointing result can be attributed to the small number of unit characters involved. It was, however, encouraging that some of the test strains can be distinguished by their capacity to cleave particular conjugated substrates (Table 25, pages 194 to 196).

Table 24 Test error calculated from comparison of the rapid enzyme test data from the seventeen duplicated cultures together with the percentage positive values for all of the test strains

Substrate	Agreement between Duplicated Strains (%)	Test Variance (Si^2)	% of Strains Positive+
A. 7-amino-4-methylcoumarins (7AMC)			
Endopeptidase substrates			
Boc-*iso*-L-Leucine-L-glutamine-glycine-L-arginine-HCl-7AMC*	100	0.000	100
Boc-L-Leucine-glycine-L-arginine-7AMC*	100	0.000	100
Boc-L-Valine-L-leucine-L-lysine-7AMC*	100	0.000	100
Boc-L-Valine-L-proline-L-arginine-HCl-7AMC*	100	0.000	100
Bz-L-Valine-glycine-L-arginine-HCl-7AMC*	100	0.000	100
Glutaryl-glycine-glycine-L-phenylalanine-7AMC	100	0.000	98
Succinyl-L-alanine-L-alanine-L-phenylalanine-7AMC*	100	0.000	100
Succinyl-glycine-L-proline-7AMC	100	0.000	99
Succinyl-L-leucine-L-leucine-L-valine-L-tyrosine-7AMC*	100	0.000	100
Succinyl-L-leucine-L-tyrosine-7AMC*	100	0.000	100
Z-L-Arginine-L-arginine-7AMC	100	0.000	98
Z-Glycine-glycine-L-leucine-7AMC*	100	0.000	100
Z-Glycine-L-proline-7AMC*	100	0.000	100
Exopeptidase substrates			
D-Alanine-TFA-7AMC*	100	0.000	100
L-Alanine-7AMC*	100	0.000	100
β-Alanine-TFA-7AMC	100	0.000	99
L-Arginine-7AMC*	100	0.000	100
L-Arginine-L-arginine-3HCl-7AMC*	100	0.000	100
L-Asparagine-TFA-7AMC	100	0.000	96
Asparate-7AMC	100	0.000	52
L-Cysteine(Bzl)-7AMC*	100	0.000	100
L-Glutamine-HCl-7AMC*	100	0.000	100
Glycine-HBr-7AMC	100	0.000	91

Table 24 continued

Substrate	Agreement between Duplicated Strains (%)	Test Variance (Si^2)	% of Strains Positive+
Glycine-L-proline-HBr-7AMC	100	0.000	99
L-Histidine-7AMC	100	0.000	99
iso-L-Leucine-TFA-7AMC	100	0.000	94
L-Leucine-7AMC*	100	0.000	100
L-Methionine-7AMC*	100	0.000	100
L-Proline-HBr-7AMC*	100	0.000	100
L-Pyroglutamate-7AMC	100	0.000	76
L-Serine-HCl-7AMC	100	0.000	97
L-Tyrosine-7AMC*	100	0.000	100
L-Valine-7AMC	100	0.000	99
Other peptidase substrates			
L-Alanine-L-phenylalanine-L-lysine-2TFA-7AMC*	100	0.000	100
L-Lysine-L-alanine-7AMC*	100	0.000	100
B. 4-Methylumbebelliferones (4MU)			
Glycosides			
4MU-2-Acetamido-4,6-o-benzylidene-2-deoxy-β-D-glucopyranoside	100	0.000	97
4MU-2-Acetamido-2-deoxy-β-D-galactopyranoside	93.72	0.059	83
4MU-2-Acetamido-2-deoxy-β-D-glucopyranoside	100	0.000	98
4MU-N-Acetyl-β-D-galactosamine	100	0.000	95
4MU-N-Acetyl-β-D-glucosamine	100	0.000	89
4MU-β-D-Cellobiopyranoside	100	0.000	99
4MU-α-L-Fucopyranoside	100	0.000	95
4MU-β-D-Fucoside	100	0.000	99
4MU-β-L-Fucoside	100	0.000	99
4MU-α-D-Galactoside	100	0.000	94
4MU-β-D-Galactoside	100	0.000	99
4MU-α-D-Glucoside*	100	0.000	100
4MU-β-D-Glucoside*	100	0.000	100
4MU-α-D-Glucuronide	96.97	0.029	82

Table 24 continued

Substrate	Agreement between Duplicated Strains (%)	Test Variance (Si^2)	% of Strains Positive+
4MU-β-D-Maltoside	96.97	0.029	96
4MU-α-D-Mannopyranoside	100	0.000	97
4MU-β-D-Mannopyranoside	100	0.000	57
4MU-β-D-Ribofuranoside	100	0.000	62
4MU-2,3,5-Trio-o-benzyl-α-L-arabinofuranoside	100	0.000	99
4MU-β-D-Xylopyranoside	100	0.000	98
4MU-β-D-Xyloside	100	0.000	99
Inorganic esters			
Bis-(4MU)-Phosphate	100	0.000	96
4MU-Phosphate*	100	0.000	100
4MU-Pyrophosphate	100	0.000	99
4MU-Sulphate	100	0.000	94
Organic esters			
4MU-Eicosanoate*	100	0.000	100
4MU-Elaidate*	100	0.000	100
4MU-Heptanoate*	100	0.000	100
4MU-Laurate	100	0.000	97
4MU-Lignocerate	96.97	0.029	94
4MU-Myristate	96.97	0.029	77
4MU-Octadecanoate	100	0.000	74
4MU-Palmitate	100	0.000	97
4MU-Pentadecanoate	100	0.000	60
4MU-Protected acetate*	100	0.029	100
4MU-Stearate	100	0.000	98

+, Data for duplicated strains not included.

*, Tests for which all of test strains gave positive results.

Abbreviations: Boc, tert-butyloxycarbonyl; Bz, benzoyl; Bzl, benzyl; HBr, hydrogen bromide; HCl, hydrochloride; TFA, trifluoroacetate; Z, benzyloxycarbonyl.

Table 25 Ability of centrotype strains of the streptosporangial clusters (Whitham, 1988; Whitham *et al.*, 1993) and type strains of validly described species of the genera *Microbispora*, *Microtetraspora*, *Planobispora*, *Streptomyces* and *Streptosporangium* to cleave 7-amino-4-methylcoumarin and 4-methylumbelliferone derivatives.

	*Streptosporangium albidum**	*Streptosporangium album**	*Streptosporangium amethystogenes**	*Streptosporangium corrugatum**	*Streptosporangium fragile**	*Streptosporangium nondiastaticum**	*Streptosporangium pseudovulgare**	*Streptosporangium roseum**	*Streptosporangium viridogriseum* subsp. *kofuense**	*Streptosporangium viridogriseum* subsp. *viridogriseum**	*Streptosporangium vulgare**	*Streptosporangium* sp.** (TW166)	*Streptosporangium* sp.** (TW292)	*Streptosporangium* sp.** (TW116)	*Streptosporangium* sp.** (TW141)	*Streptosporangium* sp.** (TW213)	*Microbispora chromogenes**	*Microbispora rosea**	*Microtetraspora fusca**	*Microtetraspora glauca**	*Microtetraspora niveoalba**	*Planobispora longispora**	*Streptomyces indiaensis**
Strain Numbers	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
A. 7-amino-4-methylcoumarins (7AMC)																							
Endopeptidase substrates																							
Glutaryl-glycine-glycine-L-phenylalanine-7AMC	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Succinyl-glycine-L-proline-7AMC	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Z-L-Arginine-L-arginine-7AMC	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Table 25 continued

Strain Numbers	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Exopeptidase substrates																						
Asparate-7AMC	0	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0
Glycine-HBr-7AMC	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1
iso-L-Leucine-TFA-7AMC	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1
L-Pyroglutamate-7AMC	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1
L-Serine-HCl-7AMC	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
B. 4-Methylumbelliferone (4MU)																							
Glycosides																							
4MU-2-Acetamido-2-deoxy-β-D-galactopyranoside	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
4MU-N-Acetyl-β-D-glucosamine	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
4MU-α-D-Galactoside	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1
4MU-α-D-Glucuronide	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	0	0	1	1	1	1	1	1
4MU-β-D-Mannopyranoside	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1	1
4MU-β-D-Ribofuranoside	0	1	1	1	0	0	0	0	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1
Inorganic ester																							
4MU-Sulphate	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1
Organic esters																							
4MU-Laurate	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4MU-Lignocerate	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	0

Table 25 continued

Strain Numbers	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4MU-Myristate	0	1	1	1	1	1	1	0	1	1	1	1	1	1	0	0	1	0	0	1	1	0	1
4MU-Octadecanoate	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	0
4MU-Pentadecanoate	1	1	1	1	1	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	1

Boc, tert-butyloxycarbonyl; Bz, benzoyl; Bzl, benzyl; HBr, hydrogen bromide; HCl, hydrochloride; TFA, trifluoroacetate; Z, benzyloxycarbonyl.

*, Type strains.

**, Centrotype strains: TW166 (cluster 1), TW292 (cluster 2), TW116 (cluster 4), TW141 (cluster 6) and TW213 (cluster 8) (Whitham, 1988; Whitham *et al.*, 1993).

DISCUSSION

A. SELECTIVE ISOLATION

There is compelling evidence to show that the discovery of previously unknown bioactive compounds occurs when rare and novel microorganisms, notably actinomycetes, are examined using new and existing screening systems (Nolan and Cross, 1988; Okami and Hotta, 1988). Members of the order *Actinomycetales* have been the most widely exploited group of microorganisms in terms of biotechnological applications (Cross, 1982; Goodfellow and O'Donnell, 1989; Labeda and Shearer, 1991). Antibiotics, enzymes and enzyme inhibitors of commercial importance have been produced by large-scale cultivation of members of this taxon for the past 50 years with new products being discovered, patented and marketed every year. There is, therefore, a strong incentive to use natural, in addition to genetically engineered, microorganisms in pharmacological screening programmes designed to discover replacement antibiotics and biopharmaceutics needed to meet growing consumer demand for natural products (Bull *et al.*, 1992).

The numerical predominance of streptomycetes in soils explains why the majority of secondary metabolites from actinomycetes discovered and developed in the 1950s and 1960s were from these organisms. The capacity of streptomycetes to produce new natural products remains unsurpassed though members of other actinomycete genera are becoming increasingly important as a source of novel products (Okami and Hotta, 1988; Goodfellow and O'Donnell, 1989; Labeda and Shearer, 1991). It is, therefore, important that new isolation procedures are developed to ensure a steady supply of novel and uncommon actinomycetes for both high and low throughput screens.

It is difficult to know which kinds of actinomycetes should be selected for screening since commercially important products such as antibiotics and enzymes

are produced by members of taxonomically diverse genera. It can be useful to know whether there is any relationship between the class of compound sought and specific taxa. *Actinoplanes* strains, for instance, are known to be a source of polyether ionophore antibiotics, *Actinomadura* strains produce depsipeptides and *Micromonospora* strains aminoglycoside, ansamacrolide and macrolide antibiotics (Labeda and Shearer, 1991). It may, however, be much more productive, in terms of the discovery of novel compounds, to screen actinomycete groups that have received relatively little attention. Members of the family *Streptosporangiaceae*, including the type genus, *Streptosporangium*, can be cited to exemplify this point. Little is known about the occurrence, numbers, kinds or activities of such organisms in natural habitats (Goodfellow, 1991).

There are several reasons why the extent of actinomycete diversity in natural environments is underestimated. These include difficulties in achieving a representative sample of actinomycetes from heterogeneous substrates such as soil (Hopkins *et al.*, 1991), absence of accepted criteria for delineating species and genera (Goodfellow and O'Donnell, 1993), and a lack of objective and effective selective isolation procedures (Williams *et al.*, 1984a; Goodfellow and O'Donnell, 1989; Bull *et al.*, 1992). However, given developments in actinomycete systematics it is now possible to develop reliable strategies for the selective isolation, classification and identification of members of specific taxa and to recognise and characterise novel organisms.

It is important that taxonomy reflects the extent of the natural diversity of actinomycetes in natural habitats. In practice, the number of species in a genus is still markedly influenced by the aims of the taxonomist, the extent to which the taxon has been studied, the criteria used to define the species, and the ease by which strains can be brought into pure culture (Williams *et al.*, 1984a; Goodfellow and O'Donnell, 1989, 1993). The fact that there are over 150

described taxospecies of *Streptomyces* (Williams *et al.*, 1989) but only 15 validly described species of *Streptosporangium* may merely be due to the current pre-eminence of the former in actinomycete biology.

One of the primary aims of the present investigation was to further unravel the extent of variation encompassed by the genus *Streptosporangium* by evaluating the taxonomic databases generated by Whitham (1988) for the classification and identification of these organisms. In particular, Curie-point pyrolysis mass spectrometry was used to evaluate the taxonomic integrity of representative numerically circumscribed clusters of streptosporangia and to check the identity of strains examined using the numerical identification procedure. Experiments were also carried out to determine the value of fluorogenic enzyme tests in the rapid circumscription of streptosporangia isolated from natural habitats.

The selectivity of isolation procedures for actinomycetes is influenced by factors that include the nature of pretreatment regimes and the selectivity of isolation media and incubation conditions. Innumerable procedures have been recommended for the selective isolation of specific actinomycete genera from natural habitats (Cross, 1982; Williams and Wellington, 1982; Nolan and Cross, 1988) but little attempt has been made to determine their effectiveness. The importance of evaluating how effective selective isolation procedures are was underlined in the present investigation when it was demonstrated that the vast majority of the actinomycetes from pretreated soil growing on HV agar supplemented with actidione (50mg/l) and nalidixic acid (30mg/l) and incubated for 4 weeks at 30°C were streptomycetes.

In the present investigation streptosporangia were isolated from ten of the twelve composite soil samples using selective isolation procedures (Nonomura and Ohara, 1969a; Nonomura, 1984; Hayakawa and Nonomura, 1987a; Nonomura

and Hayakawa, 1988) that involved the application of various pretreatment regimes prior to plating soil dilutions onto HV agar supplemented with actidione and nalidixic acid. Streptosporangial colonies were recognised by their capacity to produce spore vesicles on an abundant aerial mycelium. The numbers of streptosporangia fell within the range $3.8 \pm 2.2 \times 10^3$ to $7.9 \pm 1.19 \times 10^4$ colony forming units per gram dry weight soil, but there was no obvious correlation between the counts and the pH, moisture content or organic matter content of the composite soil samples. Counts within the recorded range are similar to those reported by Nonomura and his colleagues for Japanese soils (Nonomura and Ohara, 1960, 1969a, b; Hayakawa and Nonomura, 1987a, b; Nonomura and Hayakawa, 1988; Hayakawa *et al.*, 1991). The failure to isolate streptosporangia from the acidic Mount Bromo (Indonesia) and Brazilian rainforest soils suggests that these organisms cannot cope with low pH regimes. Slightly acidic, humic rich garden soils are considered to offer favourable habitats for streptosporangia (Nonomura, 1984; Nonomura and Hayakawa, 1988).

The highest actinomycete counts, including those for streptosporangia, were consistently found with the composite soil samples that were subject to the less extreme pretreatment regimes, namely the heat pretreatment of 10^{-1} dilutions of air dried soil in the presence of either sodium dodecyl sulphate or yeast extract. The highest actinomycete counts were recorded for composite soil 8, that is, post-harvested Ginseng soil, that was pretreated with yeast extract for 20 minutes at 40°C prior to dilution and plating onto HV agar supplemented with actidione and nalidixic acid and incubation at 30°C for 4 weeks. Previous studies have also shown that there is a marked decrease in streptosporangial numbers when dried soil is heated at 120°C for an hour (Nonomura and Ohara, 1969a, b; Whitham, 1988). The results of the present study show that it is no longer necessary to use

such drastic pretreatment regimes for the selective isolation of streptosporangia and related organisms from environmental samples.

The highest numbers of microbisporae and microtetrasporae were also observed on isolation plates seeded with soil suspensions containing yeast extract and heated for 20 minutes at 40°C. It was also evident that heat pretreated soil (120°C for an hour) treated with phenol at 30°C for half an hour favoured the growth of microbisporae as opposed to microtetrasporae and streptosporangia; similar results were reported by Nonomura and Hayakawa (1988). Nevertheless, the highest counts of microbisporae, $6.18 \pm 0.76 \times 10^4$ colony forming units per gram dry weight soil, were obtained with suspensions of composite soil 8 pretreated with yeast extract. It is evident from the present study that this latter procedure is the most effective one of those studied for isolating microbisporae, microtetrasporae and streptosporangia from soil.

It is evident both from this and earlier investigations (Couch, 1955a; Nonomura and Ohara, 1960; Hayakawa and Nonomura, 1987a, b; Nonomura and Hayakawa, 1988; Hayakawa *et al.*, 1991; Whitham *et al.*, 1993) that streptosporangia are more common and widely distributed in soil than was suggested by early studies (Van Brummelen and Went, 1957; Potekhina, 1965). Nevertheless, it is evident from the present study that the vast majority of actinomycetes growing on HV isolation plates, irrespective of the pretreatment regime, are almost invariably streptomycetes. Clearly more effective procedures are needed for the selective isolation of streptosporangia from natural habitats. It is possible that treating dried soil with a solution of benzethonium chloride prior to preparing a dilution series and plating onto HV agar will reduce the number of streptomycetes and thereby foster the recovery of streptosporangia (Hayakawa *et al.*, 1991).

B. CLASSIFICATION

The family *Streptosporangiaceae* forms a distinct phyletic line (Stackebrandt and Schleifer, 1984; Stackebrandt, 1986; Kudo *et al.*, 1993; Ochi *et al.*, 1993) that encompasses six genera defined primarily by a few judiciously chosen morphological and chemical properties. Stackebrandt *et al.* (1993) found that while streptosporangia share a number of chemical and morphological features they fell into two groups based on the discontinuous distribution of some chemical markers known to be a value in actinomycete systematics (Goodfellow, 1989; Suzuki *et al.*, 1993). All of their test strains were known to have a wall chemotype III (Lechevalier and Lechevalier, 1970), a fatty acid pattern 3c (Kroppenstedt, 1985), 2-hydroxy fatty acids (Kroppenstedt and Goodfellow, 1991) and DNA rich in guanine plus cytosine (Nonomura, 1989). The majority of species, including *Streptosporangium roseum*, the type species, had a phospholipid pattern type IV (Lechevalier *et al.*, 1977) and predominant menaquinones of the MK-9 (H_2) and MK-9 (II, VIII-H_4), MK-9 and /or MK-9 (H_6) type whereas the type strains of *Streptosporangium albidum*, *Streptosporangium viridogriseum* subspecies *kofuense* and *Streptosporangium viridogriseum* subspecies *viridogriseum* had a phospholipid pattern type II and principal menaquinones of the MK-9 (II, III-H_4) type. The division of the genus into two groups is supported by scanning electron microscopy studies on the morphology of spores and spore vesicles (Nonomura, 1989), electrophoretic mobility of ribosomal protein AT-L30 (Ochi and Miyadoh, 1992) and the results of 16S rDNA (Kemmerling *et al.*, 1993) and 5S rRNA sequencing analyses (Kudo *et al.*, 1993).

The 5S rRNA sequencing and Curie-point pyrolysis mass spectrometric analyses carried out in the present investigation provided further evidence of the heterogeneity of the genus *Streptosporangium*. Bacterial 5S rRNAs can be

assigned to three groups based on differences in their primary and secondary structures (Hori and Osawa, 1986; Park *et al.*, 1987a, b, 1991, 1993). Those from Gram-positive bacteria with a low genomic G+C content (< 55 mol %) usually have 116 nucleotides, five base-pairs and a U-U mismatch in the D-D' helix. Ribosomal RNAs from actinomycetes and Gram-negative bacteria have around 120 nucleotides and eight base-pairs in the D-D' region. It was clear from the primary and secondary structures of the 5S rRNAs that all nine test strains had sequences belonging to the 120 nucleotide group typical of actinomycetes (Simoncsits, 1980; Dekio *et al.*, 1984; Dams *et al.*, 1987; Park *et al.*, 1987a, b, 1991, 1993).

The phylogenetic tree generated from the 5S rRNA sequence data showed that representatives of the five validly described species of *Streptosporangium* formed two phyletic lines, one containing the type strains of *Streptosporangium amethystogenes*, *Streptosporangium pseudovulgare* and *Streptosporangium vulgare* and the other the type strains of *Streptosporangium albidum* and *Streptosporangium viridogriseum* subspecies *viridogriseum*. These findings confirm and extend the earlier 5S rRNA sequencing studies of Kudo *et al.* (1993). They also provide further evidence that small rRNA sequencing studies can be used to establish fine evolutionary relationships between prokaryotes (Hori and Osawa, 1986; Van den Eynde *et al.*, 1990), including actinomycetes (Dekio *et al.*, 1984; Park *et al.*, 1987a, b, 1991, 1993).

The type strain of *Streptosporangium viridogriseum* was found as an outlier when representative strains of *Streptosporangium* were analysed by pyrolysis mass spectrometry. Nine of the remaining ten type strains were assigned to two broad groups, one containing *Streptosporangium albidum*, *Streptosporangium amethystogenes*, *Streptosporangium corrugatum*, *Streptosporangium nondiastaticum*, *Streptosporangium pseudovulgare* and

Streptosporangium vulgare, and the second *Streptosporangium fragile*, *Streptosporangium violaceochromogenes* and *Streptosporangium viridialbum*. The recovery of the type strain of *Streptosporangium albidum* in the first group is puzzling as it is clear from other studies that this organism is closely related to *Streptosporangium viridogriseum* (Mertz and Yao, 1990; Ochi and Miyadoh, 1992; Stackebrandt *et al.*, 1993). The remaining organism, the type strain of *Streptosporangium roseum*, formed a single membered cluster. These data provide further evidence that pyrolysis mass spectrometry can be used as a quick and effective way of evaluating the taxonomic integrity of actinomycete taxa (Hindmarch *et al.*, 1990; Sanglier *et al.*, 1992).

The clustering of *Streptosporangium* species according to the chemotaxonomic and morphological features outlined above is in excellent agreement with the phylogenetic analysis of representatives of the two clusters (Kemmerling *et al.*, 1993). 16S rDNA analysis indicated a close similarity between *Streptosporangium roseum*, *Streptosporangium nondiastaticum* and *Streptosporangium pseudovulgare*, *Streptosporangium corrugatum* was also associated with this group. In contrast, the type strain of *Streptosporangium viridogriseum* subspecies *viridogriseum* showed a closer similarity to members of the family *Pseudonocardiaceae* than to other *Streptosporangium* species.

It is evident from the present and earlier studies (Nonomura, 1989; Ochi and Miyadoh, 1992; Kemmerling *et al.*, 1993; Kudo *et al.*, 1993; Stackebrandt *et al.*, 1993; Whitham *et al.*, 1993) that *Streptosporangium albidum* and *Streptosporangium viridogriseum* cannot be retained within the genus *Streptosporangium*. Differences in the primary structure of 5S and 16S rRNA, as well as in chemotaxonomic properties, between members of the two streptosporangial groups are greater than those found to separate genera of the family *Streptosporangiaceae*. In light of these findings the genus

Streptosporangium should be restricted to *Streptosporangium roseum* and its relatives, namely *Streptosporangium album*, *Streptosporangium amethystogenes*, *Streptosporangium carneum*, *Streptosporangium corrugatum*, *Streptosporangium fragile*, *Streptosporangium longisporum*, *Streptosporangium nondiastaticum*, *Streptosporangium pseudovulgare*, *Streptosporangium violaceochromogenes*, *Streptosporangium viridialbum* and *Streptosporangium vulgare*. It is, however, possible that additional studies will support the exclusion of *Streptosporangium corrugatum* from the genus as substantial differences exist between this organism and *Streptosporangium roseum* in the primary structure of 16S rRNA (Kemmerling *et al.*, 1993; Stackebrandt *et al.*, 1993) and in the amino acid sequence of the AT-L30 protein (Ochi and Miyadoh, 1992).

It is clear from both phenotypic and genotypic data that *Streptosporangium viridogriseum* should be classified in the family *Pseudonocardiaceae*, adjacent to but distinct from *Saccharothrix australiensis* (Kemmerling *et al.*, 1993; Kudo *et al.*, 1993; Stackebrandt *et al.*, 1993; Whitham *et al.*, 1993). *Streptosporangium albidum* and *Streptosporangium viridogriseum* form a distinct taxon but can be separated on the basis of chemical and DNA relatedness data (Stackebrandt *et al.*, 1993; Whitham *et al.*, 1993). Consequently, there is a wealth of evidence to support the proposal that *Streptosporangium albidum* and *Streptosporangium viridogriseum* be classified in the genus *Kutzneria* as proposed by Stackebrandt *et al.* (1993).

The proposal for the genus *Kutzneria* leaves the genus *Streptosporangium* as a relatively homogeneous taxon with characteristic genotypic and phenotypic properties. The exclusion of the two species from the genus *Streptosporangium* together with additional information on *bona fide* members of the taxon justify an emendation of this genus.

Emended description of *Streptosporangium* Couch 1955, 148[AL]

Strep.to.spo(an'gi.um. Gr.adj. *streptos* twisted; Gr. n. *spora* a seed; Gr.n. *angeion* a vessel; M.L.neut.n. *Streptosporangium* spore coiled within a sporangium.

The description is based on the characteristics given by Nonomura (1989), Stackebrandt *et al.* (1993) and Whitham *et al.* (1993) together with information from the present study.

Aerobic, Gram-positive, non-acid fast actinomycetes that form a stable branched mycelium. Globose spore vesicles, up to 10 μm in diameter, are formed on aerial hyphae. Sporangiophores are produced by septation of a coiled, unbranched hypha within the spore vesicle; they are oval, spherical or rod shaped (0.2-1.3 x 0.2-1.5 μm) and non-motile. Some strains require B vitamins for growth. All are mesophilic and chemoorganotrophic with an oxidative type of metabolism.

Streptosporangia degrade casein and gelatin, produce hydrogen sulphide, use cellobiose as a sole carbon source and grow in the presence of crystal violet (0.001%, w/v). They also cleave a range of conjugated substrates that include Boc-*iso*-L-leucine-L-glutamine-glycine-L-arginine-HCl-7AMC, Boc-L-leucine-glycine-L-arginine-7AMC, Boc-L-valine-L-leucine-L-lysine-7AMC, Boc-L-valine-L-proline-L-arginine-HCl-7AMC, Bz-L-valine-glycine-L-arginine-HCl-7AMC, succinyl-L-alanine-L-alanine-L-phenylalanine-7AMC, succinyl-L-leucine-L-leucine-L-valine-L-tyrosine-7AMC, succinyl-L-leucine-L-tyrosine-7AMC, Z-glycine-glycine-L-leucine-7AMC, Z-glycine-L-proline-7AMC (endopeptidases); D-alanine-TFA-7AMC, L-alanine-7AMC, L-arginine-7AMC, L-arginine-L-arginine-3HCl-7AMC, L-cysteine(Bzl)-7AMC, L-glutamine-HCl-7AMC, L-leucine-7AMC, L-methionine-7AMC, L-proline-HBr-7AMC, L-tyrosine-7AMC (exopeptidases); L-alanine-L-phenylalanine-L-lysine-2TFA-7AMC, L-lysine-L-alanine-7AMC (other peptidases); 4MU-β-D-fucopyranoside, 4MU-

galactopyranoside, 4MU-α-D-glucopyranoside, 4MU-β-D-glucopyranoside, 4MU-α-D-glucoside, 4MU-β-D-glucoside (glycosides); 4MU-phosphate (inorganic ester); 4MU-eicosanoate, 4MU-elaidate, 4MU-heptanoate and 4MU-protected acetate (organic esters).

Cell walls contain N-acetylated muramic acid, *meso*-diaminopimelic acid but no characteristic sugars. Whole-organism hydrolysates contain madurose. Major phospholipids include diphosphatidylglycerol, hydroxy-phosphatidylethanolamine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides and a n-acetylglucosamine containing phospholipid but no phosphatidylcholine. Predominant menaquinones are MK-9 (H_2) and MK-9 (II, VIII-H_4), MK-9 and/ or MK-9 (H_6). Complex mixtures of straight and branched chain fatty acids are formed. The mol % G+C of the DNA is 69-71 (Tm). The primary habitat is soil.

The type species is *Streptosporangium roseum* Couch 1955, 151[AL].

All twelve of the validly described species of *Streptosporangium* mentioned earlier have properties consistent with their assignment to this revised taxon. Numerical and chemical data also support the taxonomic integrity of these species (Mertz and Yao, 1990; Stackebrandt *et al.*, 1993; Whitham *et al.*, 1993).

The species concept remains a difficult theme in prokaryotic systematics (Goodfellow and O'Donnell, 1993). Early definitions of actinomycete species were often based on monothetic groups described on the basis of a few morphological, pigmentation and biochemical features. This concept of speciation is clearly flawed as strains which vary in key characters will be misclassified. It is, however, now good practice to distinguish a taxospecies, a group of strains that share a high proportion of properties from a genomic species, a group of organisms which share high DNA relatedness values. It can be anticipated that the application of polyphasic taxonomy, that is, the use of a comprehensive set of

phenetic and genomic data for the circumscription of species will lead to better classifications and hence to more reliable methods for the identification of prokaryotic species (Goodfellow and O'Donnell, 1993; O'Donnell *et al.*, 1993).

The emended genus *Streptosporangium* is well circumscribed given an impressive set of data derived from extensive chemical, molecular and numerical taxonomic studies (Ochi and Miyadoh, 1992; Kemmerling *et al.*, 1993; Stackebrandt *et al.*, 1993; Whitham *et al.*, 1993). In contrast, the circumscription and definition of *Streptosporangium* species is still weak with the majority of species regarded as taxospecies (Whitham *et al.*, 1993). However, the extensive numerical taxonomic survey of streptosporangia carried out by Whitham and his colleagues was never meant to be an end in itself but as a means to an end.

It is widely acknowledged that relationships expressed in numerical taxonomies can be distorted by factors such as test and strain selection, test error and by the genetic instability of the organisms under examination (Goodfellow and O'Donnell, 1993). It is important, therefore, that numerical taxonomies are checked in light of independent taxonomic features derived from the application of other modern taxonomic techniques, that is, by adopting the polyphasic approach to classification (Colwell, 1970; Murray *et al.*, 1990). In the present study, the pyrolysis mass spectral data support the taxonomic integrity of streptosporangial clusters 1 and 2 as defined by Whitham *et al.* (1993). These data, taken together with those from an earlier study on streptomycetes (Sanglier *et al.*, 1992) indicate that pyrolysis mass spectrometry can be used as a rapid and reliable way of determining the taxonomic status of numerically defined clusters.

C. IDENTIFICATION

Good classification is a prerequisite of accurate identification. This means that the quality of a frequency matrix is only as good as the classification from

which it was derived. It is necessary in practice to have at least as many tests as taxa in frequency matrices (Sneath and Chater, 1978; Priest and Williams, 1993). The frequency matrix generated by Whitham (1988) fulfils this latter criterion. This matrix, which is based on twelve numerically defined clusters and twenty-six diagnostic tests, was shown to be theoretically sound. In addition, it was used together with the MATIDEN program (Sneath, 1979a) in the identification of a small number of unknown streptosporangia from soil (Whitham, 1988). One of the primary aims of the present investigation was to extend these preliminary studies.

It has repeatedly been stressed that experience is needed to interpret identification scores based on Willcox probabilities, taxonomic distances and standard errors of taxonomic distances (Lapage *et al.*, 1973; Sneath, 1978; Williams *et al.*, 1983b; Priest and Williams, 1993). Thus, use of Willcox probabilities alone can lead to false positive results for unknown strains when data for the unknown taxon/ taxa are not included in the frequency matrix (Willcox *et al.*, 1973; Priest and Williams, 1993). Such anomalous results can be attributed to the normalisation process in the calculation of this coefficient. Values for taxonomic distance and its standard error are not deceptive in this way (Sneath, 1979a). In the present study, the application of the 95% taxonomic radius and Gaussian probability coefficients proved useful in the definition of acceptable identification scores.

The need to evaluate frequency matrices using strains that were not used in their construction has also been emphasised (Sneath and Sokal, 1973; Williams *et al.*, 1983b; Langham *et al.*, 1989; Priest and Williams, 1993). The percentage of unknown strains identified partly reflects the choice of cut-off points and the state of the taxonomy of the organisms under study. Consequently, the criteria chosen for a successful identification tend to be somewhat arbitrary (Williams *et al.*,

1985a; Priest and Williams, 1993). As stated earlier, the widely adopted criteria for the identification of streptomycetes are Willcox probabilities greater than 0.850, low scores for taxonomic distance and its standard error, all scores significantly better than those for the next best alternatives, and a small number of characters of the unknown cited as being atypical of those of the cluster to which it has been assigned. More stringent criteria have been recommended for the identification of aerobic endospore-forming bacilli (Priest and Alexander, 1988), "coryneform" bacteria (Hill *et al.*, 1978), mycobacteria (Wayne *et al.*, 1980) and Gram-negative bacteria (Lapage *et al.*, 1973; Dawson and Sneath, 1985; Homes, 1986a, b).

The MATIDEN program (Sneath, 1979a) and the frequency matrix of Williams *et al.* (1983b) have been used to identify unknown neutrophilic streptomycetes from several distinct natural habitats and to evaluate media formulated for the selective isolation of uncommon and rare streptomycetes (Vickers *et al.*, 1984; Williams *et al.*, 1984a; Goodfellow and O'Donnell, 1989). Thus, 81% of neutrophilic streptomycetes from soil (Williams *et al.*, 1983b; Langham *et al.*, 1989), 44% from freshwater habitats (Stanton, 1984), 70% from marine sediments (Goodfellow and Haynes, 1984) and 30% of alkalitolerant streptomycetes have been identified using these procedures (Saddler, 1988). Corresponding studies have been carried out on bacteria other than streptomycetes.

Probabilistic identification of Gram-negative isolates has been especially effective. Of more than nine hundred strains of fermentative Gram-negative bacteria and over six hundred strains of non-fermenters, 98% (Holmes *et al.*, 1986a) and 92% (Holmes *et al.*, 1986b) respectively were identified using the Willcox probability coefficient at > 0.999. Similarly, of nearly two hundred and fifty vibrios isolated from freshwater habitats, most (72% at a Willcox probability > 0.999 or 79% at Willcox probability > 0.990) were identified using a 50-test

matrix (Dawson and Sneath, 1985). Results from similar studies on Gram-positive bacteria have not been so encouraging. Hill *et al.* (1978) only identified half of nearly three hundred strains of "coryneform" bacteria using a Willcox probability of > 0.999. Similarly, mycobacteria (47% of 298 strains at > 0.99; Wayne *et al.*, 1980) and aerobic, endospore-forming bacilli (70% of 58 strains at > 0.95; Priest and Alexander, 1988) have proved difficult to identify.

Two related explanations can be offered to account for the low identification values cited above. First, the nature of the unidentified isolates. It seems probable that almost all of these isolates are representatives of undescribed species that were not included in the frequency matrix. It is, of course, possible that some will be atypical strains of established species but these are probably a minority. Genera such as *Bacillus* and *Streptomyces* have been overclassified in the past but are now underclassified with many new species awaiting description (Sanglier *et al.*, 1992; Labeda and Lyons, 1991a, b; Atalan, 1993; Priest, 1993).

A second reason is that some taxospecies, such as *Bacillus megaterium* (Hunger and Claus, 1981), *Bacillus polymyxa* (Nakamura, 1987a), *Bacillus subtilis* (Nakamura, 1987b, 1989), *Streptomyces cyaneus* (Labeda and Lyons, 1991a), *Streptomyces hygroscopicus* and *Streptomyces violaceusniger* (Labeda and Lyons, 1991b) are species-groups. Probabilistic identification to such taxa will almost invariably result in low scores given the variation encompassed by the taxon and overlap with neighbouring taxa. Thus, poor identification is a function of inadequate classification as mentioned earlier. In contrast, Gram-negative genera of medical interest have been extensively studied with relatively few species awaiting description. Such species are homogeneous and in most cases comprehensive phenotypic descriptions have been supported by DNA homology studies.

In the present investigation, the frequency matrix recommended by Whitham (1988) was comprehensively evaluated using seventy representatives of the twelve major *Streptosporangium* clusters defined in the initial numerical taxonomic survey (Whitham *et al.*, 1993). The identification scores obtained for these organisms were critically examined and cut-off points set for the identification of both marker strains and unknown isolates derived from the selective isolation studies. Sixty-five of the seventy marker strains were assigned to their parent clusters using stringent identification criteria, namely Willcox probability scores of 0.9700 or above, taxonomic distance scores below the corresponding 95% taxonomic radius scores, high Gaussian probability scores signifying that there was no significant overlap between the cluster strains were assigned to with the best identification scores being much better than the two next best alternatives. These cut-off criteria for a positive identification of streptosporangia are similar to those recommended by Whitham (1988).

The final logical step in the practical evaluation of a frequency matrix is to isolate new strains and attempt to identify them. In the present investigation, twelve of the one hundred and thirty-six unknown streptosporangia, isolated from a range of composite soils using several isolation procedures, were identified to known clusters using the stringent cut-off criteria mentioned earlier. Ten of the isolates were identified to cluster 1 (*Streptosporangium* sp.) and two to cluster 2 (*Streptosporangium* sp.). An additional twelve strains were assigned to cluster 1 and seven to cluster 2 using less stringent cut-off points. The remaining one hundred and five strains, that is, 77% of the isolates were not identified.

Surprisingly little attention has been given to validating identifications derived from probabilistic methods. In most cases identifications based on Willcox probabilities, either alone or in combination with other identification coefficients, have been accepted at face value. However, as mentioned earlier,

Willcox probability scores can sometimes be erroneously high for an unknown strain, when data for the appropriate taxon are not in the identification matrix. It is, therefore, important to obtain some information on the validity of the identifications. In the investigations carried out by Holmes *et al.* (1986a, b) identifications were validated using conventional identification schemes. This approach is sound for well classified taxa, such as constituents of the family *Enterobacteriaceae*, but is not practicable in the case of actinomycetes and aerobic, endospore-forming bacilli which are underclassified.

In an elegant study, DNA reassociation was used as an absolute measure of relatedness by which to assess phenetic identifications of unknown, aerobic endospore-forming bacilli (Priest and Williams, 1993). Representative isolates identified to various *Bacillus* species, namely *Bacillus amylolyticus*, *Bacillus circulans*, *Bacillus glucanolyticus*, *Bacillus lautus*, *Bacillus pabuli* and *Bacillus validus*, were compared against labelled DNA from reference strains. In all cases, the test strains showed more than 70% sequence similarity to DNA from the appropriate reference strain and less than 30% similarity to DNA from other reference strains. Thus, the phenotypic identifications were thoroughly validated. Other taxonomic methods, such as whole-organism protein electrophoresis, which are congruous with DNA similarity, could be used in place of DNA reassociation.

In the present study, fourteen of the thirty-one isolates identified to either clusters 1 or 2, and five unidentified streptosporangia, were compared with representatives of the two clusters using Curie-point pyrolysis mass spectrometry. All six of the representative isolates identified to cluster 1 using the stringent cut-off criteria grouped with the representative strains of this cluster. In addition, the four representative isolates assigned to cluster 1 using the less stringent cut-off criteria were also recovered with the cluster 1 strains. Similarly, the identifications of the four isolates assigned to cluster 2 were validated by the PyMS data. In

contrast, the five unidentified isolates were assigned to two groups, one of which was sharply separated from clusters 1 and 2. These preliminary results are most encouraging as they suggest that Curie-point pyrolysis mass spectrometry can be used to validate phenotypic identifications and to set cut-off points for positive identification of strains in computer-assisted identification systems. It can also be concluded that the ability to analyse small amounts of bacterial growth with minimal sample preparation to obtain, in minutes, fingerprint data that can be used to validate phenotypic identifications is unparalleled by other taxonomic methods, including molecular fingerprinting techniques.

Rapid and reproducible tests are needed to distinguish between validly described species of *Streptosporangium* and to classify novel isolates prior to the examination of representative strains using more exacting taxonomic methods that cannot readily be used to classify large numbers of environmental isolates. In the present investigation, known and unidentified streptosporangia were screened against seventy-one fluorogenic enzyme tests using an automated procedure that had been successfully used to classify representatives of closely related fast growing species of mycobacteria (Hamid *et al.*, 1993) and to assign streptomycetes taken from selective isolation plates to three putatively novel species (Atalan, 1993). The low test error of 0.29% recorded for the streptosporangia compared favourably with the corresponding figures from the earlier studies (p 5.8%, Hamid *et al.*, 1993; p 4.9%, Atalan, 1993).

The results of the present study are in good agreement with those from previous investigations as they show that rapid enzyme tests based on the fluorophores 7-amino-4-methylcoumarin (7AMC) and 4-methylumbelliferone (4MU) provided data of value for the classification and identification of actinomycetes (Goodfellow *et al.*, 1987b, c, 1988, 1990b, 1991; O'Donnell *et al.*, 1993; Whitham *et al.*, 1993). It has already been pointed out that some of the

rapid enzyme tests provide data that can be used to strengthen the definition of the genus *Streptosporangium*, others have potential for the circumscription of streptosporangial species. It was, however, disappointing that only forty-two of the seventy-one tests, that is, 59%, gave data of differential value; consequently there was insufficient information in the database to assign the unidentified isolates to artificial groups. Clearly additional enzymatic tests are needed for artificial grouping of streptosporangia isolated from natural habitats. Possible areas of development have been considered in recent review articles (Manafi *et al.*, 1991; Goodfellow and James, 1993; James, 1993).

D. FUTURE STUDIES

The results of the present study show that the emended genus *Streptosporangium* is a well defined taxon. It is evident from the numerical phenetic data that the revised genus is grossly underspeciated and that minimal standards are needed to delineate both existing and putatively novel species. Additional comparative taxonomic studies need to be carried out on well chosen representative strains to determine the most appropriate methods likely to be of value. A number of powerful chemical and molecular techniques, such as ribotyping and quantitative analyses of cellular fatty acids and whole-organism proteins, can be expected to yield high quality data for improved classification. In addition, representatives of existing and presumptively new taxospecies should be the subject of DNA relatedness studies.

The application of suitable chemical and molecular methods on representatives of the genus *Streptosporangium* should help unravel the diversity encompassing by this taxon. This is turn will help in the development of improved methods for the identification of unknown streptosporangia and thereby facilitate the taxonomic approach to selective isolation of specific fractions of the

streptosporangial community from natural habitats. In the first instance, selective media for this purpose should be formulated using information in the taxonomic database generated by Whitham (1988).

The current procedures used to determine the number and types of streptosporangia in soil invariably involve plating out tenfold dilutions of pretreated environmental samples onto selective media. The results of such experiments are influenced by the properties of the environmental samples, the effect of extraction and recovery procedures, competition on isolation plates and difficulties in identifying isolates. It seems likely, however, that more effective representative sampling of streptosporangia would be achieved using the dispersion and differential centrifugation technique introduced by Hopkins *et al.* (1991). This technique has been shown to be three to twelve times more effective in extracting streptomycete propagules from a range of soil samples than the conventional reciprocal shaking procedure (Atalan, 1993). These results suggest that actinomycete-soil interactions may be major limitations to quantitatively sampling and that the use of sodium cholate, Tris buffer and mild ultrasonication are effective in breaking down such interactions. It may also be possible to directly identify streptosporangia on isolation plates by examining small samples of colonial growth using pyrolysis mass spectrometry and analysing the resultant data using neural networks.

The results of the fluorogenic enzyme tests, although disappointing, were sufficiently encouraging to suggest that streptosporangia might show species specific patterns. However, additional studies on representative strains and further conjugated substrates are needed to prove the point. Fortunately, there is still scope for the design and synthesis of additional 7AMC and 4MU derivatives in order to extend the range of enzymatic activities detectable (Goodfellow and James, 1993; James, 1993). Thus, the short chain esters of 4MU could be of

considerable taxonomic value but their use is currently limited by stability problems. Recently, a protected 4MU-acetate was synthesised by James and found to be more stable than 4MU-acetate. Its value in actinomycete systematics has also been demonstrated. Additional protected esters, including ones for butyrate and propionate, are being synthesised. The range of peptidase substrates that can be obtained is even greater with 7AMC derivatives available for most of the known aminopeptidases and many endopeptidases.

The fluorescence generated by 7AMC and 4MU is intense and usually easily detected. Fluorescence in the blue region has, however, certain disadvantages, particular where organisms produce pigments which fluoresce. Such endogenous fluorescence is generally in this blue region. Alternative fluorophores are available which fluoresce in other regions of the visible spectrum (James, 1993). These include 6-aminoquinoline, resorufin and trifluoroethyl coumarins. These fluoresce in yellow, orange-red and green regions, respectively. The number of substrates based on such fluorophores is currently limited but it is possible that improved methods of synthesis and greater demand may lead to greater availability.

Another potentially valuable area of development could be the search for taxonomically useful enzymatic activities that are not normally included in identification protocols. An example of one such activity has been demonstrated using the compound anthranilonitrile (Whitehead, 1989). This non-fluorescent molecule is converted to the highly fluorescent amide or to the acid by the activity of nitrile hydratases and/ or nitrilases (Figure 18, page 218). Within the family *Pseudonocardiaceae*, *Amycolata* strains were separated from *Amycolatopsis*, *Pseudonocardia* and *Saccharopolyspora* strains by their capacity to form strong blue fluorescence due to the conversion of anthranilonitrile to the amide or acid. Similarly, the conversion of Haloxon (3-chloro-7-hydroxy-4-methylcoumarin-bis-

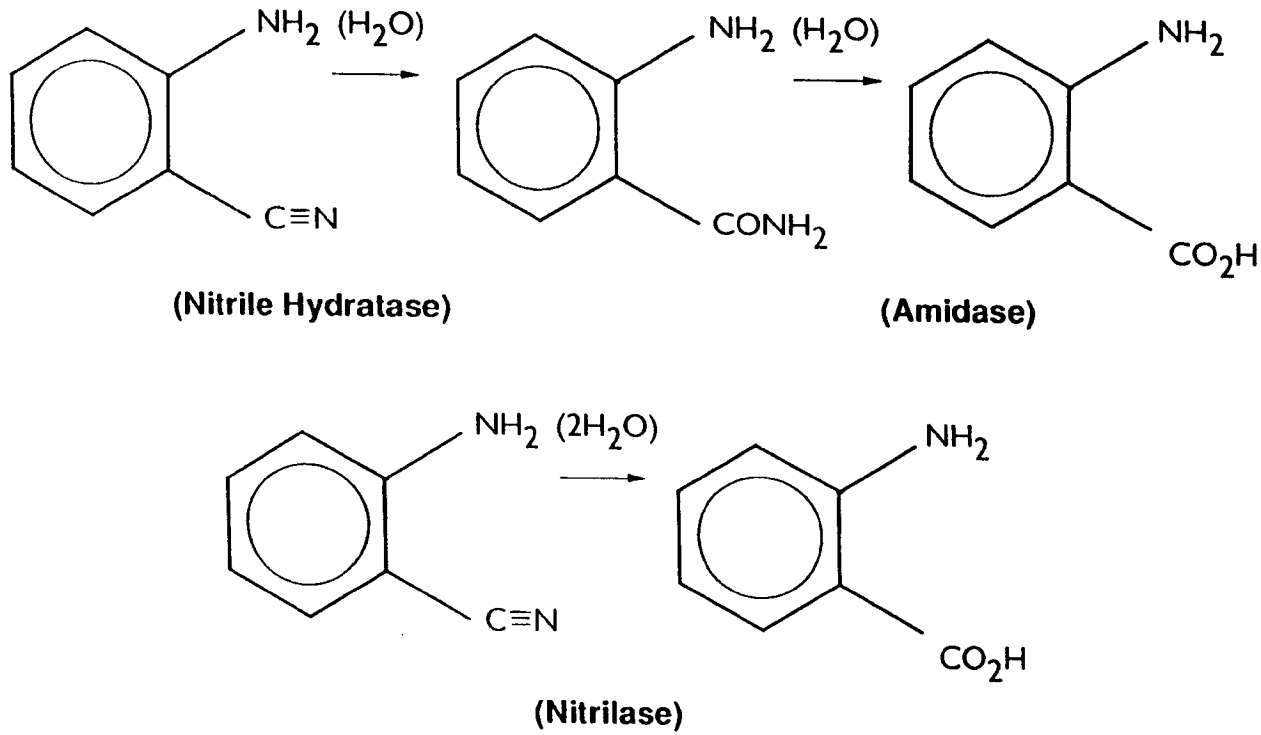


Figure 18 Possible metabolic fate of antranilonitrile (2-aminobenzonitrile) showing fluorescent products.

[2-chloroethyl]-phosphate) to 3-chloro-7-hydroxy-4-methylumbelliferone causes an intense sky-blue fluorescence under ultra-violet light. Most representatives of the family *Pseudonocardiaceae* are characterised by their ability to degrade Haloxon, exceptions include *Amycolata autotrophica*, *Amycolatopsis rugosa* and *Saccharomonospora viridis* (Whitehead, 1989).

The application of the strategy outlined above should help highlight minimal standards for the delineation of *Streptosporangium* species. It will not be possible to effectively apply the taxonomic approach to selective isolation in any comprehensive way until the subgeneric classification of the genus *Streptosporangium* is clarified. In the meantime representatives of the genus should be isolated from natural habitats using the more "gentle" isolation procedures highlighted in the present investigation and examined in pharmacological screening programmes. In conclusion, it should be remembered that objectively designed isolation procedures and target directed screening techniques still have a pivotal role to play in the search and discovery of novel bioactive compounds even in an era dominated by genetic engineering and molecular biology. Actinomycetes remain good friends with many secrets to share should we take the time and care to study them properly.

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APPENDIX A

TAXON PROGRAM

The TAXON computer program was written in UCSD Pascal for the Apple IIe computers by Dr. A.C. Ward of the Department of Microbiology, University of Newcastle upon Tyne. The program has been transferred to IBM PC, written in standard Pascal for the Propas compiler running under MSDOS. The program allows for:

(a) a simple data entry system for binary data (+/-) derived from numerical taxonomic studies,

(b) pre-processing of data,

(c) on-line transfer of data from IBM PC/AT microcomputers to the Northumbria Universities Multiple Access Computer (NUMAC) AMDAHL 5860 in a form suitable for direct analysis using the CLUSTAN suite of programs (Wishart, 1978),

(d) analysis of data for clusters of strains defined by the numerical analysis and

(e) identification of unknown strains to clusters of strains defined in numerical analyses.

(a) Entry of Data

Initially, a screen editor is employed to generate a text file containing information on organism names (up to five alphanumeric characters), test names (up to three alphanumeric characters) and organisms and test groupings. The definition of groups of organisms and tests is necessary due to the limited size of the screen; the largest matrix that can be displayed on the screen is 70 tests by 18 organisms.

The program is completely menu driven and incorporates full error checking. Upon running the TAXON program, the text file is read in and an empty data matrix of the appropriate size created and held in random access memory (RAM). Using the organism and test groups defined in the text file a screen sized "window" of part of the data matrix is displayed. The contents of each window can be set to contain positive, negative or blank values depending on the nature of the results. Thus, if the results are predominantly positive then the window can be completely filled with positive or negative values, respectively. The minority results can then be entered where appropriate saving the overall time required for data entry. Data are entered directly as single key strokes (+,- or space). After all the results for a "window" have been entered, the data are saved to RAM and another "window" displayed. The data matrix can be saved onto hard disc once the whole data matrix has been filled or during data entry. The TAXON program can handle a data matrix containing up to 512 unit characters for 512 organisms.

A facility exists for the addition of further organisms and/or tests to an established database. Thus, a new matrix file is created with additional organisms and/or test names and organism/ test groups. This amended text file is read in by the TAXON program and superimposed on the existing data matrix. The latter is expanded accordingly. Organisms and test names can be deleted from the matrix using a similar procedure. Once the matrix has been altered, it may be stored in hard disc with a new, or the existing file name.

(b) Pre-Processing of Data

Raw data can be examined to determine the overall percentage distribution of positive characters and the reproducibility of individual tests prior to numerical analysis. Percentage positive results for each character can be determined so that

any test that is positive or negative for all of the organisms within the matrix can be identified. In addition, by entering the names of duplicated strains an output is obtained giving information on the individual test variances and percentage agreement between duplicated strains, and also the average probability of an erroneous result, the test error as defined by Sneath and Johnston (1972).

Tests considered to have little if any differential value or which show poor reproducibility can be removed from a data matrix by the creation of a new text file from which the appropriate tests have been deleted. The results corresponding to the missing tests are automatically discarded from the matrix when the new text file is read by TAXON. The reduced data matrix is then saved on hard disc.

(C) Transfer of Data

CLUSTAN requires databases to exist in a pre-determined format. The first 8 columns may contain a label, 9 and 10 are left blank. Columns 11 to 80 are available for data in a binary format (1/0). Data are written on multiple lines using the same format when more than 70 tests are used. A facility within TAXON allows the conversion of +/- results of a data matrix into the 1/0 format which are then saved to a text file. A second text file containing the names of the organisms in the dataset can be generated to contain the organism names in a format suitable for reading by CLUSTAN procedure LABELS. Finally, a batch-file is generated containing the information required for the execution of the numerical analysis on CLUSTAN. The formation of the batch file is facilitated by a simple question and answer procedure where many of the parameters are commonly available as default options. This is also saved to a text file. The three text files are then transferred to the mainframe computer to run a better CLUSTAN job.

(d) Analysis of Defined Clusters

The clusters of test strains defined during the numerical analysis can be superimposed onto the data matrix by a simple modification of the organism groups in the text file. Post-cluster analysis data processing facilities for a given cluster include:

(i) determination of average similarity, or dissimilarity of each strain to the other strains in the same cluster,

(ii) calculation of mean inter-cluster similarity or dissimilarity,

(iii) designation of centrotype strain,

(iv) calculation of percentage of strains in the cluster which are positive for each cluster for each test,

(v) calculation of the observed similarities, or dissimilarities, between pairs of duplicated, or defined groups of strains expressed by either the D_p, S_J or S_{sm} coefficients and

(vi) analysis of percentage positive data for clusters using procedures taken from the DIACHAR program (Sneath, 1980a)

The inclusion of the DIACHAR procedure within the TAXON program allows the selection of characters either for an identification matrix or for the design of isolation media selective for the recovery of representatives of chosen clusters, or groups of clusters, from natural habitats. The organism and the test grouping in post-analysis text files may be used to define the size of data matrices to be examined using DIACHAR. The output lists several properties for each cluster examined:

(i) the difference values and corresponding diagnostic scores for each test,

(ii) the sum of diagnostic scores for tests in each of the tests groupings examined and

(iii) a total sum of scores for all test sets examined.

The output is listed in descending order of difference values. Thus, tests are chosen that have both a high difference score and for which a high percentage of strains in a cluster are positive. The percentage positive values of the selected tests can be either displayed on the screen or printed out. The data for clusters in which all the strains are negative for one or more of the chosen tests are removed from the data matrix. The reduced data matrix is then subjected to a second analysis when more tests are selected to distinguish between the target cluster and remaining clusters. The data matrix may be reduced in this way until no further tests are highlighted that were scored negative for all members of any of the remaining clusters. Using this facility, a minimum number of characters can be chosen which enable each and every cluster to be distinguished from the others.

(e) Identification of Unknown Organisms

Unknown organisms can be examined for the appropriate diagnostic tests once an identification matrix has been constructed. The unknown organisms can be added to the TAXON data matrix by the creation of a new text file which contains the names of the organisms. This new file is read in the TAXON program and the binary data for unidentified strains entered into a data matrix as described previously. Identification scores are then calculated for each of the unknown strains to every one of the clusters defined in the data matrix, using the procedure IDENTIFY which is based on the MATIDEN program (Sneath, 1980a, b). The identification coefficients calculated include:

(i) Willcox probability,

(ii) taxonomic distance of each known strain to the centroid of every cluster,

(iii) the 95% taxonomic radius of each cluster and

(iv) the Gaussian distance probability, that is, the percentage probability of a member of the cluster to which the unknown strain is being identified lying further

away from the cluster centroid that the unknown organism itself. The derivation and values required for a good identification are described in detail on page 31.

The procedure COMPARE, also included in the TAXON program, calculates the same identification scores for centrotype strains, hypothetical median organisms, and outer most member of each cluster; these values can be used to measure the degree of confidence that can be placed in the identification of unknown organisms to defined clusters. The identification of large numbers of strains can be performed in batch and the results either displayed on the screen or printed out.

APPENDIX B
MEDIA AND REAGENTS

AV MEDIUM (Nonomura and Ohara, 1969a)

Basal medium: L-arginine, 0.3 g; glucose, 1.0 g; glycerol, 1.0 g; K_2HPO_4, 0.3 g; $MgSO_4.7H_2O$, 0.2 g; NaCl, 0.3 g; agar, 15 g; distilled water, 1.0 litre. To this basal medium add 1.0 ml per litre of a trace salt solution (i) and adjust to pH 6.8. Autoclave at 120°C for 15 minutes. Following autoclaving, add 1.0 ml per litre of a filter sterilised B-vitamins solution (ii) just prior to pouring the medium.

(i) Trace salt solution (Nonomura and Ohara, 1969a)

$CuSO_4.5H_2O$, 0.1 g; $ZnSO_4.7H_2O$, 0.1 g; $MnSO_4.7H_2O$, 0.1 g; distilled water, 100 ml.

(ii) B-vitamins solution (Nonomura and Ohara, 1969a)

Thiamine (aneurine) hydrochloride, 50.0 mg; riboflavin, 50.0 mg; niacin (nicotinic acid), 50 mg; pyridoxine hydrochloride, 50.0mg; inositol, 50.0 mg; calcium pantothenate, 50.0 mg; *para*-aminobenzoic acid, 50.0 mg; biotin, 25.0 mg; distilled water, 100 ml.

MODIFIED BENNETT'S MEDIUM (Agrawal, unpublished data)

Lab lemco, 10.0 g; peptone, 2.0 g; yeast extract, 2.0 g; tryptose, 2.0 g; $CaCo_3$, 100 mg; starch, 100 mg; D-glucose, 10.0 g; agar, 15.0 g; $CoCl_2$, trace; ferric ammonium citrate, trace; distilled water, 1 litre and adjust to pH 7.0. Autoclave at 120°C for 15 minutes.

CARBON SOURCE UTILISATION (Shirling and Gottlieb, 1966)

ISP 9 medium (modified from Pridham and Gottlieb, 1948)

Basal mineral salts agar: $(NH_4)SO_4$, 2.64 g; KH_2PO, 2.38 g; K_2HPO, 4.68 g; $MgSO_4.7H_2O$, 1.00 g. To this basal medium add 1.0 ml per litre of a Pridham

and Gottlieb trace salts solution (ii) and adjust to pH 6.8. Autoclave at 120°C for 15 minutes. Following autoclaving, add each of the Tyndallised carbon sources (i) just prior to pouring the medium.

(i) Sterile carbon sources

D-glucose used as positive control; solutions of each carbon source sterilised by Tyndallisation.

(ii) Pridham and Gottlieb trace salts

$CuSO_4.5H_2O$, 0.64 g; $FeSO_4.7H_2O$, 0.11 g; $Mncl_2.4H_2O$, 0.79 g; $ZnSO_4.7H_2O$, 0.15 g; distilled water, 100 ml.

DEGRADATION TESTS

AV agar was supplemented with keratin (5 g/l, Aldrich) prior to autoclaving at 120°C for 15 minutes.

DNA DEGRADATION

Bacto-DNase test agar (Difco, 0632-01), 42.0 g; distilled water, 1.0 litre, adjust to pH 7.3. Autoclaved at 120°C for 15minutes.

HV MEDIUM (Nonomura, 1984; Hayakawa and Nonomura, 1987a)

Basal medium: humic acid, 1.0 g; $CaCO_3$, 0.02 g; $FeSO_4.7H_2O$, 0.01 g; KCl, 1.7 g; $MgSO_4.7HO$, 0.05 g; Na_2HPO_4, 0.5 g; agar, 18 g; distilled water, 1.0 litre and adjust to pH 6.8. Autoclave at 120°C for 15 minutes. Following autoclaving, add 1.0 ml per litre of a filter sterilised B-vitamins solution (i) just prior to pouring the medium.

(i) B-vitamins solution (Nonomura and Ohara, 1969a)

Thiamine (aneurine) hydrochloride, 50.0 mg; riboflavin, 50.0 mg; niacin (nicotinic acid), 50 mg; pyridoxine hydrochloride, 50.0 mg; inositol, 50.0 mg; calcium

pantothenate, 50.0 mg; *para*-aminobenzoic acid, 50.0 mg; biotin, 25.0 mg; distilled water, 100 ml.

OATMEAL AGAR (Küster, 1959)

Oatmeal, 20.0 g; agar, 18.0 g; trace salts solution, 1.0 ml (i); distilled water, 1.0 litre. Steam the oatmeal in 1.0 litre of distilled water for one hour, filter and make up the volume to 1.0 litre with more distilled water. Add trace salts solution and agar, adjust to pH 7.0 and autoclave at 120°C for 15 minutes.

(i) Trace salts solution

$FeSO_4.7H_2O$, 0.1 g; $Mncl_2.4H_2O$, 0.1 g; $ZnSO_4.7H_2O$, 0.1 g; distilled water, 100 ml.

UREA DEGRADATION (Gordon, 1966)

Basal broth: KH_2PO_4, 9.1 g; Na_2HPO_4 (anhydrous), 9.5 g; yeast extract, 0.1 g; phenol red, 0.01 g; distalled water, 1.0 litre and adjust to pH 6.8. Add 10 ml of a filter sterilised solution (150 g/l) of urea to 75 ml of sterilised basal broth and dispense into sterile tubes (2.0 ml).

APPENDIX C

Table 26 Practical evaluation of the *Streptosporangium* frequency matrix: Results of the diagnostic tests*

	Tests																								
Strains	G M T S T B P T A A K S D U A C C M C G N N S F F R A T U C P V E A E E E T N R M P R F A N E E T U U F L L R 4 2 T 3 2 1 2 R D A E 2 3 1 2 2 5 4 6 4 5 6 6
TW001	-	+	+	-	-	+	+	+	+	-	-	+	+	+	-	-	-	-	-	+	-	+	-	-	-	+
TW002	-	-	-	-	-	-	-	+	+	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-
TW005	+	-	+	-	+	+	+	+	+	-	+	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-
TW007	+	-	+	-	-	+	+	+	-	+	-	-	-	-	+	+	+	+	-	+	+	+	+	-	-	-
TW101	+	+	+	-	-	-	-	-	-	+	-	+	+	-	+	-	-	-	+	+	-	+	+	-	-	+
TW104	+	+	-	-	+	-	-	-	-	+	-	+	+	-	+	-	-	-	+	+	-	+	+	-	+	+
TW106	+	+	+	-	+	-	-	-	-	+	-	+	+	-	+	-	-	-	+	-	-	+	+	-	+	+
TW115	+	-	+	-	+	-	+	-	-	+	-	+	+	-	+	+	-	+	+	+	+	+	+	-	+	+
TW116	-	+	-	-	-	+	+	-	-	+	-	+	+	-	+	+	-	-	+	-	-	+	+	-	+	+
TW117	+	+	-	-	-	-	-	-	-	+	-	+	+	-	+	-	-	-	+	-	-	+	-	-	-	+
TW121	+	-	+	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	+	+	-	+	+	-	-	+
TW126	-	+	+	-	-	-	+	+	-	+	-	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+
TW127	+	+	+	-	+	-	+	-	-	+	-	+	-	-	+	-	-	-	+	+	-	+	+	-	-	+
TW128	+	+	+	-	-	-	-	-	-	+	-	-	+	-	+	-	-	-	+	-	-	+	+	-	+	+
TW129	+	-	+	-	+	-	-	-	-	+	-	+	-	-	+	-	-	-	+	+	-	+	+	-	-	+
TW136	+	+	+	-	-	+	-	+	+	-	-	-	+	-	+	+	-	-	+	-	-	+	-	-	-	+
TW141	+	+	+	-	+	+	-	+	+	-	-	-	+	-	+	+	-	-	+	-	-	+	-	-	-	+
TW143	-	+	+	-	-	-	+	-	-	+	-	+	+	-	+	-	-	-	+	-	-	+	+	-	+	+
TW144	-	+	+	-	+	-	-	-	-	+	-	+	+	-	+	-	-	-	+	-	-	+	+	-	+	+
T145A	-	+	+	-	-	-	-	-	-	+	-	-	+	-	+	-	-	-	+	-	-	+	+	-	+	+
TW148	-	+	+	-	+	+	-	-	+	-	-	-	+	-	+	+	-	+	+	-	-	+	-	-	+	+
TW153	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	-	-	-	+	-	-	+	+	-	-	+
TW155	-	+	+	-	-	-	+	+	-	+	-	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+
TW159	-	+	+	-	+	-	-	-	-	+	-	+	+	-	+	+	-	-	+	+	-	+	+	-	+	+
TW161	-	+	-	-	-	-	+	-	+	-	-	+	+	-	+	+	-	-	+	-	-	+	+	-	+	+
TW163	-	+	-	-	-	-	+	+	-	+	-	+	+	-	+	+	-	-	+	+	-	+	+	-	+	+
TW165	-	+	+	-	+	-	-	-	-	+	-	+	+	-	-	-	-	-	+	-	-	+	-	-	-	+
TW166	+	+	+	-	+	-	-	-	-	+	-	+	+	-	+	-	-	-	+	+	-	+	+	-	-	+
TW168	-	+	+	-	-	-	-	-	-	+	-	-	+	-	+	+	-	+	-	-	-	+	-	-	+	+
TW169	+	+	+	-	+	+	+	-	+	-	-	-	+	-	+	-	-	-	+	-	-	+	-	-	-	-
TW170	+	+	+	-	+	+	-	-	+	-	-	-	+	+	+	+	-	+	+	-	-	+	+	-	-	+
TW179	+	+	-	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-	+	-	-	+	+	-	+	+
TW182	+	+	+	+	+	-	+	+	+	-	-	-	+	+	-	-	-	-	-	-	-	+	-	+	+	-
TW194	+	+	+	+	+	-	+	+	+	-	-	-	+	+	-	-	-	-	-	-	-	+	-	+	+	-
TW209	+	+	+	-	+	+	-	-	+	-	-	-	-	+	+	+	-	+	+	-	-	+	+	-	-	+
TW213	+	+	-	-	+	-	-	-	+	-	-	-	+	+	+	+	-	+	+	-	-	+	+	-	-	-
TW218	+	+	-	-	+	-	-	-	-	+	-	-	+	-	+	-	-	-	+	-	-	+	+	-	+	+
TW220	+	+	-	-	-	-	-	-	-	+	-	+	+	-	+	+	-	-	+	-	-	+	+	-	+	+
TW222	+	+	+	-	-	-	+	-	-	+	-	-	-	-	+	+	-	-	+	-	-	+	+	-	+	+
TW224	+	+	+	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	+	-	-	+	+	-	+	+
TW226	+	+	+	-	-	+	-	+	+	-	-	-	+	-	-	-	-	-	+	-	-	+	-	+	+	-
TW227	+	+	+	-	-	-	-	+	+	-	-	-	+	-	+	+	-	-	+	-	-	+	-	-	+	+
TW232	+	+	+	-	-	+	-	-	+	-	-	+	+	-	-	-	-	-	+	-	-	+	-	+	+	-
TW235	+	+	-	-	+	-	-	-	-	+	-	+	+	-	-	-	-	-	+	-	-	+	+	-	-	+
TW245	+	+	-	-	-	-	-	-	-	+	-	+	+	-	+	-	-	-	+	+	-	+	+	-	-	+
TW251	+	+	+	-	+	+	-	+	+	-	-	-	+	-	+	+	-	-	+	-	-	+	-	-	+	+
TW253	+	+	+	-	+	-	+	-	-	+	-	+	+	-	+	-	-	-	+	+	-	+	+	-	-	+
TW254	-	+	-	-	-	-	+	-	+	-	-	+	+	-	+	+	-	-	+	-	-	+	-	-	+	+
TW256	+	+	+	-	-	-	-	-	-	+	-	-	+	-	+	+	-	-	+	-	-	+	+	-	+	+
TW263	+	+	-	-	-	-	-	-	-	+	-	+	+	-	+	+	-	-	+	-	-	+	+	-	-	+
TW266	+	+	+	-	+	-	-	-	-	+	-	+	-	-	+	-	-	-	+	+	-	+	+	-	-	+

Table 26 continued

Strains	Tests																									
	GAL	MTL	TUR	SC4	TP2	BVT	PE3	TA2	AE1	AE2	KER	STD	DNA	URE	AM2	CP3	CR1	MF2	CA2	GN5	NE4	NE6	ST4	FU4	FU6	RF6
TW269	+	-	+	-	+	-	+	-	-	+	-	+	+	-	+	+	-	+	+	+	-	+	+	-	+	+
TW270	+	-	+	-	+	-	+	-	-	+	-	+	+	-	+	+	-	+	+	+	-	+	+	-	+	+
TW271	+	-	+	-	+	-	+	-	-	+	-	+	+	-	+	+	-	+	-	+	-	+	+	-	+	+
TW274	+	-	+	-	+	-	-	-	-	+	-	-	+	-	+	+	-	+	+	-	+	+	+	-	+	+
TW276	+	+	-	-	+	-	-	-	+	-	-	-	+	+	+	+	-	+	+	-	-	+	+	-	-	+
TW282	+	-	+	-	+	-	+	-	-	+	-	-	+	-	+	+	-	+	+	+	-	+	+	-	+	+
TW286	+	-	-	-	+	-	-	-	-	+	-	-	+	-	+	+	-	+	+	+	-	+	-	-	+	+
TW292	+	-	+	-	+	-	+	-	-	+	-	+	+	-	+	+	-	+	+	+	-	+	+	-	+	+
TW303	+	-	+	-	+	-	+	-	-	+	-	+	+	-	+	+	-	+	+	+	-	+	+	-	+	+
TW320	+	+	+	-	-	-	-	-	-	+	-	-	-	-	+	+	-	-	+	-	-	+	+	-	+	+
TW353	+	+	+	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	+	+	-	-	+
TW354	+	+	+	-	+	-	-	-	-	+	-	-	-	-	+	+	-	-	+	-	-	+	+	-	+	+
TW355	+	+	+	-	+	-	+	-	+	-	-	-	+	+	+	+	-	+	+	+	-	+	+	-	-	+
TW366	+	-	+	-	+	-	+	-	-	+	-	+	+	-	+	+	-	+	+	+	+	+	+	-	+	+
TW369	+	+	+	-	-	-	-	-	-	+	-	+	+	-	+	+	-	-	+	-	-	+	+	-	+	+
TW375	-	+	-	-	-	-	+	-	-	+	-	-	+	-	+	+	-	-	-	+	-	-	+	-	+	+
TW393	+	+	+	-	+	+	+	+	+	-	+	-	+	-	+	+	-	+	+	-	-	+	-	-	+	+
TW541	+	+	+	-	-	-	+	-	-	+	+	-	+	-	+	-	-	-	-	-	-	-	+	-	+	+
TW547	+	+	-	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	+	-	+	+

* Tests recommended by Whitham (1988).

TW, Representatives representing the twelve major *Streptosporangium* clusters (see Table 13, pages 86 to 90; Whitham, 1988; Whitham *et al.*, 1993).

Key for the diagnostic tests: GAL, Galactose as a sole carbon source; MTL, Mannitol as a sole carbon source; TUR, Turanose as a sole carbon source; SC4, Growth in the presence of sodium chloride; TP2, Growth at 37°C; BVT, Growth in the absence of B-vitamins; PE3, Growth in the presence of phenyl ethanol; TA2, Growth in the presence of thallous acetate; AE1, Aerial mycelium colour-pink; AE2, Aerial mycelium colour-white; KER, Degradation of keratin; STD, Degradation of starch; DNA, Degradation of DNA; URE, Urease production; AM2, Resistance to amoxicillin (250µg/ml); CP3, Resistance to cephaloridine (50µg/ml); CR1, Resistance to cephradine (500µg/ml); MF2, Resistance to cefoxitin (250µg/ml); CA2, Resistance to clavulanic acid (250µg/ml); GN5, Resistance to gentamycin sulphate(5µg/ml); NE4, Resistance to neomycin sulphate(25µg/ml); NE6, Resistance to neomycin sulphate(0.5µg/ml); ST4, Resistance to streptomycin sulphate(25µg/ml); FU4, Resistance to fusidic acid (5µg/ml); FU6, Resistance to fusidic acid (0.5µg/ml); RF6, Resistance to rifampicin (0.5µg/ml).

APPENDIX D

Table 27 Identification of the *Streptosporangium* isolates: Results of the diagnostic tests*

	Tests																									
Strains	GAL	MTL	TUR	SC4	TP2	BVT	PE3	TA2	AE1	AE2	KER	STD	DNA	URE	AM2	CP3	CR1	MF2	CA2	GN5	NE4	NE6	ST4	FU5	FU6	RF6
HJ001	-	+	+	-	+	-	+	+	-	+	-	-	-	-	+	+	+	+	+	+	-	+	+	-	+	+
HJ002	-	+	+	-	+	-	+	+	-	+	-	-	-	-	+	+	+	+	+	+	-	+	+	-	+	+
HJ005	+	+	+	-	+	-	+	+	-	+	-	+	-	-	-	-	-	-	+	-	-	+	-	-	+	+
HJ006	+	+	+	-	+	-	+	+	-	+	-	+	-	-	+	+	+	+	+	+	-	+	+	-	+	+
HJ008	+	+	-	-	+	-	+	+	-	+	-	+	-	-	-	+	-	-	+	+	-	+	-	-	-	+
HJ009	+	+	+	+	-	-	+	+	-	+	-	+	+	+	-	+	-	-	-	-	-	+	-	+	+	+
HJ010	+	+	+	-	+	-	+	+	-	+	-	+	-	-	-	+	-	-	+	-	-	+	+	+	+	+
HJ011	+	+	+	+	-	-	+	-	-	+	-	+	+	-	-	-	-	-	-	-	-	+	-	+	+	+
HJ012	+	+	+	-	+	-	+	+	-	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+
HJ013	-	-	-	+	+	-	+	-	+	-	-	-	-	-	-	+	-	-	+	+	+	+	-	+	+	+
HJ014	+	+	+	-	+	-	+	+	+	-	-	+	-	+	+	+	+	-	+	+	-	+	+	+	+	+
HJ015	+	-	+	+	+	-	+	-	+	-	-	-	-	-	-	+	-	-	+	+	+	+	-	+	+	+
HJ016	+	-	+	-	+	-	+	+	+	-	-	+	-	+	-	-	-	-	+	+	-	+	+	+	+	+
HJ017	-	+	+	+	+	-	+	+	-	-	-	+	-	-	-	-	-	-	+	+	-	+	-	-	+	+
HJ019	+	+	+	-	+	-	+	+	+	-	-	+	-	-	-	-	-	-	+	-	-	+	-	-	+	+
HJ020	+	+	+	-	+	-	+	+	-	-	-	-	-	-	+	+	-	-	+	+	-	+	+	-	-	+
HJ021	+	-	+	-	+	-	+	+	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+
HJ022	+	-	+	+	+	-	+	-	+	-	-	-	-	-	-	+	-	-	+	+	+	+	-	+	+	+
HJ023	+	+	+	-	+	-	+	+	-	+	-	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+
HJ024	+	+	+	-	+	-	+	+	-	+	-	+	-	+	-	+	-	-	+	+	-	+	-	-	+	+
HJ025	+	+	+	-	+	-	+	+	-	+	-	+	-	+	-	+	-	-	+	+	-	+	-	-	+	+
HJ026	-	+	+	-	+	-	+	+	-	+	-	+	-	+	-	+	-	-	+	+	-	+	-	-	+	+
HJ027	+	+	+	+	+	-	+	+	-	+	-	+	-	-	-	+	+	-	+	+	-	+	+	+	+	+
HJ028	+	+	+	-	-	-	+	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+
HJ029	+	+	+	-	+	-	+	+	-	+	-	+	-	+	-	+	-	-	+	+	-	+	+	-	+	+
HJ030	+	+	+	-	-	-	+	+	-	+	-	-	-	+	+	+	+	-	+	+	+	+	+	-	-	+
HJ031	+	+	+	-	+	-	+	+	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+
HJ032	+	+	+	+	-	-	+	+	-	+	-	-	-	-	-	-	-	-	+	+	-	+	+	-	+	+
HJ033	+	+	+	-	+	-	+	+	-	+	-	-	-	+	+	+	+	-	+	+	+	+	+	-	+	+
HJ034	+	+	+	-	+	-	+	+	-	+	-	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
HJ035	+	+	+	-	+	-	+	+	-	+	-	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
HJ036	+	+	+	-	+	-	+	-	-	-	-	+	-	-	+	+	+	-	+	+	-	+	+	-	+	+
HJ037	-	-	-	+	+	-	+	-	-	+	-	-	+	-	-	+	-	-	+	+	+	+	-	+	+	+
HJ038	+	+	+	-	+	-	+	+	-	+	-	-	-	+	+	+	+	-	+	+	+	+	+	-	+	+
HJ039	-	-	-	+	+	-	+	-	-	+	-	-	+	-	-	+	-	-	+	+	+	+	-	+	+	+
HJ040	+	+	+	+	+	-	+	+	-	+	-	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+
HJ041	+	-	+	+	+	-	+	+	-	+	-	-	+	+	+	+	+	-	+	+	-	+	+	+	+	+
HJ042	+	-	+	+	+	-	+	+	-	+	-	-	+	+	+	+	+	-	+	+	-	+	+	+	+	+
HJ043	-	-	+	+	+	-	+	+	-	+	-	+	-	+	+	+	+	+	+	+	-	+	-	+	+	+
HJ044	+	+	+	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
HJ045	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+
HJ046	+	-	-	-	-	-	+	+	-	+	-	-	-	+	+	+	+	-	+	+	-	+	+	-	+	+
HJ047	-	-	-	-	+	-	+	+	-	+	-	+	-	+	-	+	-	-	+	+	-	+	-	-	+	+
HJ048	+	+	+	-	+	-	+	+	-	+	-	-	-	+	+	+	+	-	+	+	+	+	+	-	+	+
HJ049	+	+	+	+	+	-	+	+	-	+	-	+	-	-	-	+	+	-	+	+	-	+	+	+	+	+
HJ050	-	+	-	-	+	-	+	+	-	+	-	-	-	-	+	+	+	-	+	+	-	+	+	-	+	+
HJ051	+	+	-	-	+	-	+	+	-	+	-	-	-	+	+	+	+	-	+	+	-	+	+	-	+	+
HJ052	+	-	-	-	-	-	+	+	-	+	-	-	-	+	+	+	+	-	+	+	-	+	+	-	+	+
HJ053	+	-	+	-	-	-	+	+	-	+	-	-	-	-	+	+	+	+	+	+	-	+	+	-	+	+
HJ054	-	-	-	+	+	-	+	+	-	+	-	+	-	-	-	-	-	-	+	+	-	+	+	-	+	+
HJ055	+	+	+	-	+	-	+	+	-	-	-	+	-	-	-	-	-	-	+	+	-	+	+	-	+	+

Table 27 continued

Strains	Tests																								
	GAL	MLR	TUR	SC4	TP2	BVT	PE3	TA2	AE1	AE2	KER	STD	DAN	UER	AM2	CP3	CR1	MF2	CA2	GN5	NE4	NE6	SE4	FU5	FU6	RF6
HJ056	+	+	+	-	+	-	+	+	-	-	-	+	-	-	-	-	-	-	+	+	-	+	+	-	+	+
HJ057	-	+	+	-	+	-	+	+	-	+	-	-	-	+	-	-	-	-	-	-	-	+	-	-	+	+
HJ058	-	-	-	-	+	-	+	+	-	+	-	+	-	+	+	+	+	-	+	+	-	+	-	-	+	+
HJ059	-	-	-	-	-	-	+	+	-	+	-	-	-	-	+	+	+	-	+	+	-	+	+	-	-	+
HJ060	+	-	+	+	+	-	+	-	-	+	-	-	-	+	-	+	+	-	+	+	-	+	-	+	+	+
HJ061	-	-	-	+	+	-	+	+	-	+	-	-	-	-	-	+	+	-	+	+	+	+	-	+	+	+
HJ062	+	+	+	+	+	-	+	+	-	+	-	+	-	+	-	+	-	-	+	+	-	+	-	-	+	+
HJ063	+	+	+	+	+	-	+	+	-	+	-	+	-	+	+	+	-	+	+	+	-	+	-	-	+	+
HJ064	+	+	+	+	+	-	+	+	-	+	-	+	-	-	-	+	+	-	+	+	+	+	+	+	+	+
HJ065	+	+	+	-	+	-	+	+	-	-	-	+	-	-	+	+	-	-	+	+	-	+	-	+	+	+
HJ066	-	-	+	+	+	-	+	+	-	+	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+
HJ067	+	+	+	-	-	-	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	+	+	
HJ068	+	+	+	-	-	-	+	+	-	+	-	+	-	-	-	+	-	-	+	+	-	+	+	+	+	+
HJ069	+	-	+	-	-	-	+	+	-	+	-	-	-	+	+	+	+	-	+	+	+	+	+	-	-	+
HJ070	+	+	+	-	+	-	+	+	-	+	-	-	-	+	+	+	+	-	+	+	+	+	+	-	+	+
HJ071	+	+	+	-	+	-	+	+	-	+	-	+	-	+	+	+	-	+	+	+	-	+	+	-	+	+
HJ072	+	+	+	-	+	-	+	+	-	+	-	+	-	-	+	+	-	-	+	+	-	+	-	+	+	+
HJ073	-	-	-	-	+	-	+	+	-	+	-	+	-	+	-	-	-	-	+	+	-	+	-	-	+	+
HJ074	-	+	-	-	+	-	+	+	-	+	-	+	-	+	-	+	-	-	+	+	-	+	-	-	+	+
HJ075	-	+	+	-	+	-	+	+	-	+	-	-	-	-	-	+	-	-	+	+	-	+	-	-	-	-
HJ076	+	+	+	-	+	-	+	-	-	+	-	-	-	+	+	+	+	-	+	+	+	+	+	-	+	+
HJ077	+	-	-	-	-	-	+	-	-	+	-	-	-	+	+	+	+	-	+	+	-	+	+	-	+	+
HJ078	+	+	+	-	+	-	+	+	-	+	-	+	-	+	+	+	+	-	+	+	+	+	+	-	-	+
HJ079	+	+	+	-	+	-	+	+	-	+	-	-	-	-	+	+	+	-	+	+	+	+	+	-	+	+
HJ080	-	+	+	-	+	-	+	-	+	-	-	+	-	-	-	-	-	-	+	+	-	+	-	-	-	+
HJ081	+	+	+	-	+	-	+	-	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+
HJ082	+	+	+	-	+	-	+	+	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+
HJ083	-	+	+	-	+	-	+	+	-	+	-	+	-	-	-	-	-	-	+	+	-	+	-	-	-	+
HJ084	+	+	+	-	+	-	+	+	-	+	-	-	-	-	+	+	+	+	+	+	-	+	+	-	-	+
HJ085	+	+	+	-	+	-	+	+	-	+	-	-	+	-	+	+	+	+	+	+	-	+	+	-	+	+
HJ086	+	+	+	+	+	-	+	+	-	+	-	+	-	-	+	+	+	-	+	+	+	+	+	-	+	+
HJ087	+	-	+	-	+	-	+	+	-	+	-	+	-	+	+	+	+	+	+	+	-	+	+	-	-	+
HJ090	+	+	+	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-	+	+	-	+	+	-	+	+
HJ091	+	-	+	-	+	-	+	-	-	+	-	+	+	-	+	+	-	+	+	+	-	+	+	-	+	+
HJ092	+	+	+	-	+	-	+	+	-	-	-	-	-	-	+	-	-	-	+	+	-	+	+	+	+	+
HJ093	+	+	+	+	+	-	+	+	-	+	-	-	-	+	+	+	-	-	+	+	+	+	+	+	+	+
HJ094	+	-	+	-	-	-	+	+	-	-	-	-	-	+	+	+	+	-	+	+	-	+	+	-	-	+
HJ096	+	-	+	-	+	-	+	+	-	+	-	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
HJ097	+	-	+	-	+	-	+	+	-	+	-	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
HJ098	+	+	+	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-	+	+	-	+	+	-	+	+
HJ099	+	+	+	-	+	-	+	+	-	-	-	-	-	-	+	+	-	-	+	+	-	+	-	-	+	+
HJ100	+	+	+	+	+	-	+	+	-	-	-	-	-	+	+	+	+	+	+	-	-	+	-	+	+	+
HJ101	+	+	+	+	-	-	+	+	-	-	-	-	-	-	+	+	+	+	+	-	-	+	-	-	+	-
HJ102	+	+	+	+	-	-	+	+	-	-	-	-	-	-	+	+	+	+	+	-	-	+	-	-	+	-
HJ103	+	+	+	-	+	-	+	+	-	-	-	-	-	-	+	+	-	-	+	+	-	+	-	-	+	+
HJ104	+	+	+	-	+	-	+	+	+	-	-	+	-	-	+	+	+	+	+	+	-	+	+	+	+	+
HJ105	+	-	+	-	+	-	+	-	-	+	-	+	+	-	+	+	-	+	+	+	-	+	+	-	+	+
HJ106	+	+	+	-	-	-	+	+	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	-	-	-
HJ107	+	+	+	-	-	-	-	-	-	+	-	-	-	-	+	+	-	-	+	-	-	+	+	-	+	+
HJ108	+	+	+	-	-	-	-	-	-	+	-	-	-	-	+	+	-	-	+	-	-	+	+	-	+	+
HJ109	+	+	+	-	-	-	-	-	-	+	-	-	-	-	+	+	-	-	+	-	-	+	+	-	+	+

Table 27 continued

Strains	Tests																								
	GAL	MTL	TUR	SC4	TP2	BVT	PE3	TA2	AE1	AE2	KER	STD	DNA	URE	AM2	CP3	CR1	CA2	GN5	NE4	NE6	ST4	FU4	FU6	RF6
HJ111	+	+	+	+	+	-	+	+	-	-	-	+	-	-	+	+	+	+	+	+	-	+	-	+	+	+
HJ112	+	-	+	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	+	+	-	+	+	-	-	+
HJ113	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	-	-	-	-	-	-	+	+	-	-	+
HJ114	+	+	-	-	-	-	+	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	-	+	+
HJ115	-	+	-	-	-	-	+	+	-	+	-	-	-	+	+	+	-	+	+	-	-	+	+	-	+	+
HJ116	+	+	-	-	-	-	-	-	-	+	-	+	+	-	+	-	-	-	+	-	-	+	-	-	-	+
HJ117	-	+	+	-	+	-	+	+	-	+	-	-	-	+	-	-	-	-	-	-	-	+	-	-	+	+
HJ118	+	+	+	-	-	-	-	-	-	+	-	+	+	-	+	+	-	-	+	-	-	+	+	-	+	+
HJ122	+	+	+	+	+	-	+	+	-	-	-	-	-	+	+	+	+	+	+	-	-	+	-	+	+	+
HJ123	-	+	+	-	+	-	-	-	-	+	-	+	+	-	+	-	-	-	+	-	-	+	+	-	+	+
HJ124	-	+	-	-	-	-	+	+	-	+	-	-	-	+	+	+	-	+	+	-	-	+	+	-	+	+
HJ125	+	-	+	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	+	+	-	+	+	-	-	+
HJ126	+	-	+	+	+	-	+	+	-	+	-	-	+	-	+	+	+	-	+	+	+	+	+	+	+	+
HJ128	+	+	+	-	+	-	+	+	-	+	-	+	-	-	+	+	+	+	+	+	-	+	+	-	+	+
HJ129	+	+	-	-	+	-	+	-	-	+	-	-	-	-	+	+	-	+	+	+	+	+	+	-	+	+
HJ130	+	+	+	-	+	-	+	+	+	-	-	+	-	-	+	+	+	+	+	+	-	+	+	-	+	+
HJ131	+	+	+	+	+	-	+	+	-	-	-	-	-	+	+	+	+	+	+	-	-	+	-	+	+	+
HJ132	+	+	-	-	+	-	+	+	+	-	-	+	-	-	+	+	+	+	+	+	-	+	+	+	+	+
HJ133	-	+	+	-	+	-	+	+	-	+	-	+	-	+	-	+	+	-	+	-	-	+	-	+	+	+
HJ135	+	+	+	+	+	-	+	+	-	+	-	+	-	-	+	+	+	+	+	-	-	+	-	-	+	+
HJ138	+	+	+	-	+	-	+	+	+	-	-	+	-	-	+	+	+	+	+	-	+	+	+	+	+	+
HJ139	+	+	+	-	+	-	+	+	+	-	-	+	-	-	+	+	+	+	+	-	+	+	+	+	+	+
HJ140	+	-	+	-	+	-	+	+	+	-	-	+	-	-	+	+	+	+	+	-	-	+	+	+	+	+
HJ141	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	
HJ143	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	
HJ144	+	-	+	-	+	-	+	+	+	-	-	+	-	-	+	+	+	+	+	-	-	+	+	+	+	+
HJ146	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	
HJ147	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	
HJ148	+	+	+	-	-	-	+	+	-	+	-	+	-	-	-	-	-	-	+	+	-	+	-	-	-	-
HJ149	+	+	+	+	+	-	+	+	+	-	-	+	+	-	+	-	-	-	+	-	-	-	-	-	+	-
HJ150	+	+	+	+	+	-	+	-	-	-	-	+	+	-	-	-	-	-	+	-	-	-	-	-	+	-
HJ151	+	+	+	-	+	-	+	+	-	+	-	+	-	-	+	+	-	+	+	+	-	+	+	-	-	+
HJ152	+	+	+	+	+	-	+	-	-	-	-	+	+	-	-	-	-	-	+	-	-	-	-	-	+	-
HJ153	+	+	+	-	+	-	+	+	-	+	-	+	-	-	+	+	-	+	+	+	-	+	+	-	+	+

* Tests recommended by Whitham (1988).

HJ, Presumptive *Streptosporangium* isolates (see Table 12, pages 82 to 83).

Key for the diagnostic tests: GAL, Galactose as a sole carbon source; MTL, Mannitol as a sole carbon source; TUR, Turanose as a sole carbon source; SC4, Growth in the presence of sodium chloride; TP2, Growth at 37°C; BVT, Growth in the absence of B-vitamins; PE3, Growth in the presence of phenyl ethanol; TA2, Growth in the presence of thallous acetate; AE1, Aerial mycelium colour-pink; AE2, Aerial mycelium colour-white; KER, Degradation of keratin; STD, Degradation of starch; DNA, Degradation of DNA; URE, Urease production; AM2, Resistance to amoxicillin (250µg/ml); CP3, Resistance to cephaloridine (50µg/ml); CR1, Resistance to cephradine (500µg/ml); MF2, Resistance to cefoxitin (250µg/ml); CA2, Resistance to clavulanic acid (250µg/ml); GN5, Resistance to gentamycin sulphate(5µg/ml); NE4, Resistance to neomycin sulphate(25µg/ml); NE6, Resistance to neomycin sulphate(0.5µg/ml); ST4, Resistance to streptomycin sulphate(25µg/ml); FU4, Resistance to fusidic acid (5µg/ml); FU6, Resistance to fusidic acid (0.5µg/ml); RF6, Resistance to rifampicin (0.5µg/ml).

APPENDIX E

Table 28 Data obtained from the fluorogenic enzyme tests for the 159 test strains including the seventeen duplicated strains

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                                                                Tests
        XXXXXXXXXXXXXXXXXXXXX00NNNNNNNNNNNNNNIIIIOOOOOOOOOOOOGGGGGGGGGGGGGGGGGGGGG
Strains0000000111111222222200000011111112200000000001111100000011111111122222 2
        1234568012457012456734467803567890212342457890345712345801234567901245 6
KH001++++++++++++++++++++++++++++++++++++++++++++++++++-+-++++++++++++++++--+++
KH002++++++++++++++-++++++++++++++++++++++++++++++++++++++++++++++++++++-+++-+++
KH005++++++++-++++++++++++++++++++++++++++++++++++++++-++++++++++++++++++++++++
KH006+++++++++++++-++++++++++++++++++++++++++++++++++---+----++++++++-++--+++
KH008++++-++++++++++++++++++++++++++++++++++++++++++++-++++++++++++++++-+++++++
KH009++++-++++++++++++++++++++++++++++++++++++++++++++-++++++++++++++++-+++++++
DH009++++-++++++++++++++++++++++++++++++++++++++++++++-++++++++++++++++-+++++++
KH010+++++++++++++--++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
KH011++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
KH012++++-++++++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
KH013++++++++++++++++++++++++++++++++++++++++++++++++++++++++-+++++++-+++-+++++++
KH014++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
KH015++++++++++++++++++++++++++++++++++++++++++++++++++++++++-++++++++++++++-+++
KH016++++-++++++++-++++++++++++++++++++++++++++++++++-+++-+-+++++++++--+--+++
DH016++++-++++++++-++++++++++++++++++++++++++++++++++-+++++-+++++++++--+--+++
KH017++++++++++++++++++++++++++++++++++++++++++++++++++-++++++++++++++++++++++
KH019++++-++++++++++++++++++++++++++++++++++++++++++++++++-+-++++++++++++++--+++
KH020+-++-++++++++-++++++++++++++++++++++++++++++++++++++++++-----++++++++-+-+++--
KH021++++-++++++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++-++--+++
DH021++++-++++++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++--+++
KH022++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
KH023++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
KH024++++-++++++++++++++++++++++++++++++++++++++++++++++-+-++++++++++++++++-++++
KH025++++++++++++++++++++++++++++++++++++++++++++++++++-+-++++++++++++++++++++++
KH026++++++++-++++++++++++++++++++++++++++++++++++++++-+++-++++++++++++++++-++++++
KH027++++-++++++++++-++-++++++++++++++++++++++++++++++++-+++-++++++++++++++++++++
KH028++++-++++++++++++-++++++++++++++++++++++++++++++++-++++++++++++++-++++-+--+++
DH028++++-++++++++++++-++++++++++++++++++++++++++++++++-++++++++++++++-++++-+--+++
KH029++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
KH030++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++--+++
KH031++++-++++++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++--+++
DH031++++-++++++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++--+++
KH032+++++-++++++++--++++++++++++++++++++++-++++++++++++++++++-++++++++++-+++
KH033+++++++++++++++-++++++++++++++++++++++-+++++++-+-+++-++-++++++++++++--+++
KH034++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++--+++
KH035++++-++++++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++--+++
DH035++++-++++++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++--+++
KH036++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++--+++
KH037++++++++++++++++++++++++++++++++++++++++-++++++++++-++++++++++++++++++++
KH038++++++++++++++++++++++++++++++++++++++++++++++++++++++++++-++++++++++++--+++
KH039++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++-++++++++++++++
KH040++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++++-++++++++++--+++
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KH042++++-++++++++-++++++++++++++++++++++++++-++++++++-+-++++++++++++++++++--+++
KH043++++++++++++++-++++++++++++++++++++++++++++++++++++++++++++++-+-+-+++++++++-++-++++
KH044++++-+++-+-++++++++++++++++++++++++++++++++++++++++++++++++++-+-++++++++++++--+++
KH045++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++-++++++++++++++++++
KH046++++-++++++++-++++++++++++++++++++++++++++++++++++++++++++++++-+++-++++++++++-++-++++
KH047++++-+++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++--+------++++++++--+-++++
KH048++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++-++++++++++++++++
KH049++++--++-+++++-++++++++++++-++++-+++++-++++++++-+++-+-+++++-++++++++++-
KH051++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++-+-++++++++++++++++-++++
KH052++++-++++++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++--+++
DH052++++-++++++++-++++++++++++++++++++++++++++-+++++-++++++++++++++++++++++--++++
KH053+++++++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++-++--+++
KH054++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++-+++-++++++++++-+++++++
```

Table 28 continued

	Tests
	XXXXXXXXXXXXXXXXXXXX00NNNNNNNNNNNNNIIIIOOOOOOOOOOOGGGGGGGGGGGGGGGGGGGGG
Strains	00000001111112222222000000111111122000000000011111000000111111111222222
	12345680124570124567344678035678902123424578903457123458012345679012456
KH055	++++-+++-+-++++++++++++++++++++++++++++++++++++-+-+-+++++++-+++-++--+++
KH056	++++-+++-+-++++++++++++++++++++++++++++++++++++-+-+-+++++++-+++-++--+++
KH057	++++-++-+++-+++++++++++-++-++++
KH058	++++-++--++-+++++++-+++
KH059	++++-+++-++++++++++++++++
KH060	++++-+++-++++++++++++++++
KH061	++-++++++++++-+++
KH062	++++--++-++++++++++++++++++++++++++++++++++++-+-+-+-+-+++++++++++++++++
KH063	++++++++++++++-++++++++++++++++++++++-++++++--+--+--+--++++-+++-+-+++++
DH063	++++++++++++++-++++++++++++++++++++++-+++++++-+--+-++--++++-+++-+-+++++
KH064	++++-++
KH065	++++-+++-++++++++++++++++
KH066	++++-+++-+++++++++--+++
KH067	++++-+++++++++-++-++--+++
KH068	+++
KH069	+++-++++++++++++++++--+++
KH070	+++-+++-++++++++++++++++--+++
KH071	+++
KH072	+++-+++++++++++++++++++++++++
KH073	++++-+++++++++-++
KH074	++++--+++-+++-+++++++++++-++-++++
KH075	++++-++++++++++++++++++++++++++++++++-+++++++-+-++-++++++++++++-+-+++++
KH076	++++-+++++-++++++++++++++++++++++++++++++++++++-+-+-++++++++++++++--+++
KH077	++++-++-+-+-++++++++++++++--+++
DH077	++++-++-+-+-++++++++++++++--+++
KH078	+++-++++++++++++++++++++--+++
KH079	++++-++-++++++++++++++++--+++
KH080	++++-++-+-++++++++++++++-++++
KH081	+++-++++++++++++++++--+++
KH082	++++++++++++++-++++++++++++++++++++++++++++++++++-++++++++++++++++--+++
KH083	++-++++
KH084	++++-+++--+++
KH085	++--+++
DH085	++--+++
KH086	+++-++++++++++++++++--+++
KH087	++++++++++++++-+++++++++++++++++++++++++++++-+++-+++++++++++++++-+----+
KH090	+++---++++++++++++++++++--+++
KH091	++++++++++++++-+++--+++
KH092	++++-++++++++++++++++++++++++++++++++-+++++++-+-++++++++-++++++++++++++
KH093	++++-++-++++++-++++++++++++-+++
KH094	++++-+++++++++-++-++++++++++++++-+++-+-++++++-+-+++-++++-+++++++-++++++
DH094	++++-+++++++++-++-++++++++++++++-+++-+-++++++-+-+++-++++-++++++++++++++
KH096	++++-+++++++++-++++++++++++++++++++++++++++++-++++++++++++++++++++-++++
KH097	++++++++++++++++++++++++++++++++++++++-++++++++-++++++++++++++++-+-++++
KH098	+++--+++++++++++++++++++--+++
KH099	++++--++++++++++++++++++++++++++++++++-++++++---+-+++++++++++++-++-++++
DH099	++++--++++++++++++++++++++++++++++++++-++++++---+-+++++++++++++-++-++++
KH100	++++-+++-++++++++++++++++++++++++++++++++++-+++-+++++++++++++++++++++++
KH101	+++-+-+++++++++++++++++++++++
KH102	+++-+-+++++++++++++++++-+++++
KH103	++++-+++++++++-++++++++++++++++++++++++++++++-+-++++++++-++++++++++++++
KH104	++++-++-+-+++++++++++++++++++++
KH105	++++++++++++++-+++--+++
KH106	++++-+++++++++-++++++++++++++++++++++++++++++++-+-++++++++++++++++--+++
DH106	++++-+++++++++-++++++++++++++++++++++++++++++++-+-++++++++++++++++--+++
KH107	++++-++
KH108	++++-++
KH109	++++-++
KH111	++-++++
KH112	+++-++++++++-++++++++++++-+++

Table 28 continued

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                                                                Tests
        XXXXXXXXXXXXXXXXXXXXX00NNNNNNNNNNNNNNIIIOOOOOOOOOOOGGGGGGGGGGGGGGGGGGGG
Strains0000000111111222222200000011111112200000000001111100000011111111222222
        1234568012457012456734467803567890212342457890345712345801234567901245 6
KH113++++++++-++++++++++++++++++++++++++++++++++++++++++-+-+-++++++++++++++++-++++
KH114++++++++++++++++++++++++++++++++++++++++++++++++++-+++++++-++++++++++++--++++++++++
KH115++++++++++++++++++++++++++++++++++++++++++++++++++++++++++-+-+-+-++-++++++++++++++++
KH116++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++-+-+-++++-++++++++++++-+++
KH117++++-+++++-++++++++++++++++++++++++++++++++++++++++++++++++-++++++++++++++++++++
KH118++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++-++++++++++++++++++++
KH122++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++-++++++++++++++++++++
KH123++++++++++++++++++++++++++++++++++++++++++++++++++++++++++-++++++++++++++++++++
KH124++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++--+++
KH125++++++++++++++++++++++++++++++++++++++++++++++++++++++++++-++++++++-++++++++++++-+++
KH126++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
KH128++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++-+-++++++++++++++++--+++
KH129++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++--+++
DH129++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++--+++
KH130++++++++++-+++-++++++++++++++++++++++++++++++++-+-+-+-++++++++++++++-++-++++
KH131++++++++-++++++++++++++++++++++++++++++++++++++++++++-++++++++++++++++++++++
KH132++++++++++++++++++++++++++++++++++++++++++++++++++-+++-+-++++++++++++++++-++++
KH133++++++++++++++++++++++++++++++++++++++++++++++++++++-+-+-++++++++++++++-++++
KH135++++++++++++++-++++++++++++++++++++++++++++++++++++++-+++++-++++++++++++++++
KH137++++++++-+++++-++++++++++++++++++++++++++++++++++++++-+-++++++++++++++++-++++
KH138++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
KH139++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++-+-++++++++++++++-++-++++
KH140++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++-+-++++++++++++++-++-++++
KH141++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++-+-++++++++++++++++++++
KH143++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
KH144++++-+++++++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++-++++
KH146++++++++++++++-++++++++++++++++++++++++++++++++++-+-++++++++++++++++++++++
KH147++++-+++++++++-++++++++++++++++++++++++++++++++++++-++++++++++++++++++++++
KH148++++-+++++++++-++++++++++++++++++++++++++++++++++++++++++++-++++++++++++++++
KH149++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++--+++++++++-
KH150++++--++++++++-++++++++++++-++++++++++++++++++++++++++++++++++++++--++++++++++++
KH151++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++-++++++++++++++++++++
KH152++++-+++++++++-++++++++++++++++++++++++++++++++++++++++++++++++-++++++++++++++++++++
KH153++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++++-+++-++++++++++++++++++++
KT001++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
DT001++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
KT002++++-++++++++++++++++++++++++++++++-++++++++++++++++++++++++++++++++++++++++++++++++
KT004++++++++++++++++++++++++++++++++--++++++++++++++++++++++++++++++++++++++++++++++-+++
KT005++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++-+++
KT006++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++-+++
DT006++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++-+++
KT007++++++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
KT008++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
KT009++++-+++-+-+++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++-+++
KT010++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
KT020++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
KT021++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
KT022++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++--+++
KT023++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
KT029++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
KT030++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
KT032++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
KT038++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
KT041++++++++++-++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
KT166++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
KT292++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
KT116++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
KT141++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
KT213++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
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DH and DT, duplicated strains; HJ, *Streptosporangium* isolates; TW, marker strains and centrotype strains of the streptosporangia clusters (See Table 17, pages 114 to 116)

N04	Boc-L-Leucine-glycine-L-arginine-7AMC
N06	Boc-L-Valine-L-leucine-L-lysine-7AMC
N07	Boc-L-Valine-L-proline-L-arginine-7AMC
N08	Boc-*iso*-L-Leucine-L-glutamine-glycine-L-arginine-HCl-7AMC
N10	Bz-L-Valine-glycine-L-arginine-HCl-7AMC
N13	Glutaryl-Glycine-glycine-L-phenylalanine-7AMC
N15	Succinyl-Glycine-L-proline-7AMC
N16	Succinyl-L-Leucine-L-tyrosine-7AMC
N17	Succinyl-L-alanine-L-alanine-L-phenylalanine-7AMC
N18	Succinyl-L-Leucine-L-leucine-L-valine-L-tyrosine-7AMC
N19	Z-L-Arginine-L-arginine-7AMC
N20	Z-Glycine-L-proline-7AMC
N22	Z-L-Glycine-glycine-L-leucine-7AMC
003	L-Lysine-L-alanine-7AMC
004	L-Alanine-L-phenylalanine-L-lysine-7AMC
X01	L-Alanine-7AMC
X02	β-Alanine-7AMC
X03	D-Alanine-7AMC
X04	L-Arginine-7AMC
X05	Asparate-7AMC
X06	L-Asparagine-7AMC
X08	L-Cysteine(Bzl)-7MAC
X10	L-Glutamine-HCl-7AMC
X11	L-Glycine-HBr-7AMC
X12	L-Histidine-7AMC
X14	*iso*-Leucine-7AMC
X15	L-Leucine-7AMC
X17	L-Methionine-7AMC
X20	L-Proline-HBr-7AMC
X21	L-Pyroglutamate-7AMC
X22	L-Serine-HCl-7AMC
X24	L-Tyrosine-7AMC
X25	L-Valine-7AMC
X26	L-Glycine-L-proline-HBr-7AMC
X27	L-Arginine-L-arginine-3HCl-7AMC
G01	4MU-2-Acetamido-4,6-o-benzylidene-2-deoxy-β-D-glucopyranoside
G02	4MU-2-Acetamido-2-deoxy-β-D-galactopyranoside
G03	4MU-2-Acetamido-2-deoxy-β-D-glucopyranoside
G04	4MU-N-Acetyl-β-D-galactosamine
G05	4MU-N-Acetyl-β-D-glucosamine
G08	4MU-β-D-Cellobiopyranoside
G10	4MU-α-L-Fucopyranoside
G11	4MU-β-D-Fucoside
G12	4MU-β-L-Fucoside
G13	4MU-α-D-Galactoside
G14	4MU-β-D-Galactoside
G15	4MU-α-D-Glucoside
G16	4MU-β-D-Glucoside
G17	4MU-α-D-Glucuronide
G19	4MU-β-D-Maltoside
G20	4MU-α-D-Mannopyranoside
G21	4MU-β-D-Mannopyranoside
G22	4MU-β-D-Ribofuranoside
G24	4MU-2,3,5-Trio-o-benzyl-α-L-arabinofuranoside
G25	4MU-β-D-Xyloside
G26	4MU-β-D-Xylopyranoside
O02	4MU-Phosphate
O04	4MU-Pyrophosphate
O05	4MU-Sulphate
O07	bis-(4MU)-phosphate
O08	4MU-(protected) Acetate
O09	4MU-Eicosanoate
O10	4MU-Elaidate
O13	4MU-Heptanoate
O14	4MU-Laurate
O15	4MU-Lignocerate
017	4MU-Myristate
I01	4MU-Palmitate
I02	4MU-Pentadecanoate
I03	4MU-Stearate
I04	4MU-Octadecanoate