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An integrated method for the enrichment and selective isolation of *Actinokineospora* spp. in soil and plant litter

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Aims: To devise and evaluate a method for isolating the rare, zoosporic actinomycetes, *Actinokineospora* spp. in soil and plant litter.

Methods and Results: The newly developed method consists of two enrichment stages followed by plating on a selective medium. The source material is initially incubated with calcium carbonate to multiply the population of *Actinokineospora* spp., and is then air-dried. The second stage consists of rehydration-centrifugation, in which the amended substrate is immersed in phosphate buffer-soil extract to liberate actinomycete zoospores, and nonmotile microbial associates are then eliminated by centrifugation. Portions of the supernatant enriched with zoospores are plated on humic-acid vitamin agar supplemented with fradiomycin, kanamycin, nalidixic acid and trimethoprim. We examined 39 soil and plant-litter samples taken from fields, forests and stream banks. The proposed method consistently enriched and selectively isolated *Actinokineospora* spp. in 17 samples. Evidence for antimicrobial activity was found in most of the isolates.

Conclusions: A combination of enrichment and a medium containing selective antibiotics can be used successfully for efficient isolation of certain rare actinomycete taxa.

Significance and Impact of the Study: The development of new methodologies with which to isolate rare actinomycetes is of great importance to extend our understanding of their ecology, taxonomy and bioactivity.

INTRODUCTION

Besides their ecological significance to biodegradation (Williams *et al*. 1984), interest in actinomycetes concerns their capacity to produce industrially useful metabolites such as antibiotics and enzymes (Okami and Hotta 1988; Peczynska-Czoch and Mordarski 1988). Much effort has long been focused upon the genus *Streptomyces*, which is the most abundant and a recoverable actinomycete group in soil. Extensive screening of this taxon has led to the discovery of many novel strains that produce useful secondary metabolites (Tanaka and Omura 1990). Although *Streptomyces* spp. continue to provide new bioactive products, reliable methodologies are required to isolate rare and unusual actinomycetes, to reduce the re-isolation of strains producing known bioactive compounds and to improve the quality of natural products screened (Goodfellow and Williams 1986).

Members of the genus *Actinokineospora* (Hasegawa 1988) are aerobic, mesophilic actinomycetes that characteristically produce a long branching substrate mycelium and a relatively short, flexuous aerial mycelium with chains of rod-shaped spores. These spores become motile through lophotrichous flagella under aqueous conditions. This genus currently accommodates five validated species, of which *Actinokineospora riparia* (strain C-39162) produces a macromolecular compound with antimycoplasmal activity (Hasegawa 1991). *Actinokineospora* spp. are associated in nature with soil and plant litter (Tamura *et al*. 1995), but they typically represent only a minor component of the microbial population. Therefore, isolation of this rare actinomycete group using conventional dilution plating procedures is precluded by the presence of numerically dominant and faster growing microbial associates on the plates.

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More recently, an effective enrichment technique, designated rehydration and centrifugation (RC), has been developed for the isolation of motile actinomycetes (Hayakawa *et al*. 2000). The RC method consists of accumulating actinomycete zoospores in a favourable flooding solution above the substrate, centrifuging the fluid, then plating the supernatant on humic acid–vitamin (HV) agar supplemented with nalidixic acid and trimethoprim (Hayakawa and Nonomura 1987). *Actinokineospora* spp. can be recovered on isolation plates depending on the natural sample source. However, they are still largely outnumbered by *Actinoplanes* spp. and related associates, causing problems in recognizing and isolating *Actinokineospora* colonies.

The present study aimed to develop an efficient strategy with which to isolate *Actinokineospora* spp. by combining the RC method with HV agar containing highly selective agents for this rare actinomycete group and to exploit their bioactivity with respect to antibiotic synthesis. A preliminary survey revealed that all species of the genus *Actinokineospora* have the ability to grow in the presence of the aminoglycoside antibiotics, fradiomycin and kanamycin. This resistance was utilized to construct a novel isolation method. The applicability of using calcium carbonate (Tsao *et al*. 1960) for pre-enrichment was also tested to determine whether or not the likelihood of *Actinokineospora* spp. appearing on isolation plates is increased.

MATERIALS AND METHODS

Strains and culture conditions

Forty-two strains of motile bacteria (Table 1), including five of the actinomycete genus *Actinokineospora*, were stored on oatmeal-YGG agar (Hayakawa *et al*. 1982) or nutrient agar slopes, and their capability to resist antibacterial agents was investigated. Their origins and culture conditions were as described (Hayakawa *et al*. 1991a).

Sensitivity test to antibacterial agents

Basal HV-N agar (Hayakawa and Nonomura 1989) was autoclaved, then supplemented with fradiomycin (40 mg l^{-1}; Wako Pure Chemical Ind., Osaka, Japan), kanamycin (40 mg l^{-1}; Wako), or with a mixture of nalidixic acid (10 mg l^{-1}: Sigma Chemical Co., St Louis, USA) and trimethoprim (20 mg l^{-1}; Wako) (Hayakawa *et al*. 1996a). When appropriate, these four antibacterial agents were all incorporated into autoclaved HV-N agar. Dense suspensions of actinomycetes and other bacteria, prepared as described (Hayakawa and Nonomura 1989), were streaked onto plate surfaces and growth was scored relative to that on basal medium alone, at appropriate intervals up to 14 d.

Recovery of *Actinokineospora* strains from zoospore suspensions

Basal HV agar was supplemented after being autoclaved with the same mixture of the four antibacterial agents described above. Zoospore suspensions of the test motile actinomycetes were prepared in 10 mmol l^{-1} phosphate buffer (pH 7·0) containing 10% soil extract under the described conditions (Hayakawa *et al*. 2000). The suspension was diluted with sterile tap water and 0·2 ml aliquots were plated in triplicate onto HV agar and onto the same agar supplemented with the antibacterial agents. The plates were incubated for 14 d, then appearing colonies were counted. Experiments were performed in triplicate to obtain mean colony counts.

Isolation procedure for *Actinokineospora* spp. from natural substrates

Isolation media and natural samples. The isolation media were HV agar with or without a mixture of fradiomycin (40 mg l^{-1}), kanamycin (40 mg l^{-1}), nalidixic acid (10 mg l^{-1}) and trimethoprim (20 mg l^{-1}). The media also contained cycloheximide (50 mg l^{-1}; Wako) to suppress fungal growth (Williams and Davies 1965).

Thirty-five soil samples and four leaf-litter samples (decaying leaves) were collected from several locations in Yamanashi and Nagano prefectures (Japan). Soil samples, as well as leaf-litter samples previously ground with a blender, were sieved and air-dried, then actinomycetes were isolated. The chemical nature (pH, moisture content, and loss on ignition as a function of humus content) of these samples was determined as described (Hayakawa and Nonomura 1987).

Sample preparation, inoculation and isolation. Calcium carbonate as proposed by Tsao *et al*. (1960) was applied with slight procedure modifications. Samples of air-dried soil (5 g) or leaf-litter (3 g) were mixed in a mortar with 0·5 g or 0·3 g, respectively, of powdered calcium carbonate and spread over the surface of a glass-fibre filter (70 mm in diameter, 0·44 mm thickness; ADVANTEC Inc, Tokyo, Japan) that was moistened with 3 ml of sterilized tap water and placed in a Petri dish. Where necessary, 1–2 ml of sterilized tap water was added to the sample to give a moisture content of about 20% (w/w). The Petri dish functioned as a moist chamber, and was maintained at 26°C for 14 d. The sample was then air-dried at room temperature to a constant weight.

Samples (0·5 g) processed or not (control) with calcium carbonate, were placed in a glass vessel and flooded with 50 ml of 10 mmol l^{-1} phosphate buffer (pH 7·0) containing 10% soil extract at 30°C for 2 h to liberate actinomycete

Table 1 Ability of a range of motile actinomycetes and other bacteria to grow in the presence of antibacterial agents

Strains	Growth on HV-N agar containing				
	No anti-bacterial agents	FM	KM	NA + TP	FM + KM + NA + TP
Actinomycetes					
Actinokineospora diospyrosa IFO 15665^T	+	+	+	+	+
Actinokineospora globicatena IFO 15664^T	+	+	+	+	+
Actinokineospora inagensis IFO 15663^T	+	+	+	+	+
Actinokineospora riparia IFO 14541^T	+	+	+	+	+
Actinokineospora terrae IFO 15668^T	+	+	+	+	+
Actinoplanes auranticolor IFO 13992	+	−	−	+	−
Actinoplanes brasiliensis JCM 3196^T	+	−	−	+	−
Actinoplanes 'garbadinensis' JCM 3248	+	−	−	+	−
Actinoplanes italicus JCM 3165^T	+	−	−	+	−
Actinoplanes missouriensis JCM 3121^T	+	±	±	+	±
Actinoplanes 'nipponensis' JCM 3264	+	−	−	+	−
Actinoplanes 'pallidoaurantiacus' JCM 3242	+	−	−	+	−
Actinoplanes rectilineatus JCM 3194^T	+	−	−	+	−
Actinoplanes roseosporangius JCM 3243^T	+	−	−	+	−
Actinoplanes rutilosporangius JCM 3244^T	+	−	−	+	−
Actinoplanes utahensis IFO 13244^T	+	−	−	+	−
Actinosynnema mirum IFO 14064^T	+	+	+	+	+
Catenuloplanes atrovinosus IFO 15579^T	+	−	−	+	−
Catenuloplanes castaneus IFO 15584^T	+	±	±	+	±
Catenuloplanes indicus IFO 15575^T	+	−	−	+	−
Catenuloplanes japonicus IFO 14176^T	+	−	−	+	−
Catenuloplanes nepalensis IFO 15583^T	+	−	−	+	−
Catenuloplanes niger IFO 14177^T	+	−	−	+	−
Dactylosporangium aurantiacum JCM 3083^T	+	−	−	+	−
Dactylosporangium fulvum IFO 14381^T	+	−	−	+	−
Dactylosporangium matsuzakiense IFO 14259^T	+	−	+	+	−
Dactylosporangium roseum IFO 14352^T	+	−	−	+	−
Dactylosporangium thailandense JCM 3084^T	+	−	−	+	−
Dactylosporangium vinaceum IFO 14181^T	+	−	+	+	−
Other bacteria					
Agrobacterium rhizogenes IFO 14554	+	+	+	−	−
Bacillus cereus IAM 1072	+	±	−	−	−
Bacillus coagulans ATCC 8038	+	−	−	−	−
Bacillus megaterium IAM 1166	+	−	−	−	−
Bacillus subtilis IAM 1069	+	−	−	−	−
Bacillus subtilis IFO 3134	+	−	−	−	−
Pseudomonas chlororaphis IFO 3904^T	+	+	−	+	−
Pseudomonas fluorescens IFO 14160^T	+	+	+	+	−
Pseudomonas mendocina IFO 14162^T	+	−	−	−	−
Pseudomonas putida IAM 1506	+	−	−	−	−
Pseudomonas saccharophila IAM 1504	+	+	−	−	−
Pseudomonas stutzeri IFO 14165^T	+	±	−	+	−
Rhizobium leguminosarum IFO 14778^T	+	+	+	−	−

FM, fradiomycin (40 mg l^{-1}); KM, kanamycin (40 mg l^{-1}); NA, nalidixic acid (10 mg l^{-1}); TP, trimethoprim (20 mg l^{-1}).
±, weak reaction.
T, type strain.

zoospores. A portion (8 ml) of the flooding mixture was transferred into a screw-cap test tube and centrifuged at $1500 \times g$ for 20 min in a swinging bucket rotor. After settling for 30 min, a portion of the supernatant enriched with zoospores was serially diluted with sterile tap water, then 0·2 ml aliquots were plated in triplicate onto plates of HV agar with or without antibacterial agents (Hayakawa *et al*. 2000).

All plates were incubated at 30°C for 2–3 weeks before counting actinomycete colonies, and all experiments were performed in triplicate. Actinomycetes were examined by eye and by using a light microscope equipped with a 40 × long working distance objective (model ULWDCDPlan; Olympus, Tokyo, Japan) and tentatively identified up to genus rank based on morphological criteria (Labeda 1987; Cross 1989; Lechevalier 1989). *Actinokineospora* strains were identified as those isolates forming thin, flat colonies with sparse white aerial hyphae. Microscopic observation subsequently confirmed the formation of long branching substrate hyphae and relatively short, tufted aerial hyphae with chains of rod-shaped arthrospores (Hasegawa 1988). Spore motility was confirmed in hanging drops by light microscopy.

Taxonomic analyses

Strains. Twenty-nine representative isolates with the morphology typical of the genus *Actinokineospora* according to light microscopy, were subcultured and their taxonomic properties examined in greater detail. Type strains of *Actinokineospora diospyrosa*, *A. globicatena*, *A. inagensis*, *A. riparia* and *A. terrae* were simultaneously compared. Distilled water suspensions of spores and hyphae, prepared from stock culture slants, were used as inocula (Shirling and Gottlieb 1966). All cultures were incubated at 28°C for 14 d.

Morphological, cultural and physiological characterization. Cultures were grown on HA agar (Nonomura *et al*. 1979), or on oatmeal-YGG agar supplemented with soil extract (10%, v/v; Henrich 1947), and morphology was observed by light and scanning electron microscopy (Hayakawa *et al*. 1996b). Spore motility and flagellation were observed as described (Hayakawa *et al*. 2000). Substrate mycelial colour and soluble pigment production were observed on yeast extract-malt extract agar (Shirling and Gottlieb 1966). Colours were identified with reference to the *Guide to Color Standard* (Japan Color Research Institute 1954).

Carbohydrate utilization was tested by the method of Shirling and Gottlieb (1966) using C2 (Nonomura and Ohara 1971) agar. The degradation of starch (0·5 g l^{-1}) and calcium malate (0·5 g l^{-1}) in Bennett's agar (Jones 1949) was detected as described by Williams *et al*. (1983). Gelatin hydrolysis and nitrate reduction were examined as described by Nonomura *et al*. (1979) and Gordon (1968), respectively.

Chemotaxonomic analyses. Cell-wall sugars, polar lipids, isoprenoid quinones, and diaminopimelic acid isomer were analysed as described (Hayakawa *et al*. 2000).

16S rDNA sequence analysis. Genomic DNA was extracted as described by Saito and Miura (1963) and amplification of 16S ribosomal DNA (rDNA) genes was PCR (Saiki *et al*. 1988) mediated using TaKaRa *Taq* polymerase (Takara Shuzo, Kyoto, Japan) and the primer pairs, 9F (5′-GAGTTTGATCCTGGCTCAG) and 1541R (5′-AAGGAGGTGATCCAGCC). Amplified 16S rDNA (1·5 kb) was purified and directly sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, Calif., USA) as specified by the manufacturer. The sequencing primers were: 9F (5′-GAGTTTGATCCTGGCTCAG), 515F (5′-GTGCCAGCAGCCGCCGCGGT), 536R (5′-GTATTACCGCGGCTGCTG), 785F (5′-GGATTAGATACCCTGGTAGTC), 802R (5′-TACCAGGGTATCTAAT CC), 1099F (5′-GCAACGAGCGCAACCC), 1115R (5′-AGGGTTGCGCTCGTTG), and 1541R (5′-AAGGAGGTGATCCAGCC). An Applied Biosystems PRISM 310 Genetic Analyser performed electrophoresis of the sequencing reaction mixtures.

The 16S rDNA sequences determined in this study were manually aligned with the published sequences of reference strains available from the EMBL/GenBank/DDBJ databases. The CLUSTAL W software package (Thompson *et al*. 1994) generated evolutionary distances (the K_{nuc} value of Kimura 1980) and similarity values. A phylogenetic tree was constructed by neighbour-joining (Saitou and Nei 1987) from K_{nuc} values. The topology of the phylogenetic tree was evaluated by bootstrap re-sampling as described by Felsenstein (1985) with 1000 replicates.

Nucleotide sequence accession numbers. The 16S rDNA sequences of strains YU 873-1 and YU 923-201 are available from the EMBL/GenBank/DDBJ databases under accession numbers AB048863 and AB048864, respectively.

Determination of antimicrobial activity

The ability to inhibit the growth of Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi was observed using an overlay method (Williams *et al*. 1983). The tested bacteria were *Bacillus subtilis* IFO 13719, *Micrococcus luteus* IFO 12708, *Staphylococcus aureus* IFO 3061, *Agrobacterium rhizogenes* IFO 14554, *Escherichia coli* IFO 3044 and *Pseudomonas fluorescens* IFO 14106. The

yeasts examined were *Candida krusei* (laboratory strain) and *Saccharomyces cerevisiae* IFO 10217. The filamentous fungi included *Aspergillus niger* ATCC 9642 and *Aspergillus oryzae* (laboratory strain). Bennett's agar supplemented with humic acid (0·5 g l^{-1}) (Hayakawa *et al.* 1995) was the antibiotic production medium. Spot-inoculated, 10-d-old colonies on the plates were inverted over 1·5 ml chloroform for 40 min. Killed colonies were overlaid with 5 ml of sloppy Bennett's agar inoculated with the test organisms. Zones of inhibition around the colonies were recorded after 24 h at 30°C.

RESULTS

Susceptibility to fradiomycin and kanamycin

Using dense cell suspensions as inocula, we investigated the growth of a range of motile actinomycetes (29 strains: five genera and 29 species) and nonfilamentous bacteria (13 strains: four genera and 12 species), that represent types found in soil, in the presence of antibacterial agents (Table 1). Fradiomycin (40 mg l^{-1}) and kanamycin (40 mg l^{-1}) had no effect on the growth of all the test *Actinokineospora* strains. In contrast, the growth of other test strains was inhibited or restricted by fradiomycin and/or kanamycin, except for *Actinosynnema mirum*, *Agrobacterium rhizogenes*, *Pseudomonas fluorescens* and *Rhizobium leguminosarum* strains which were less sensitive to both antibiotics. Growth of the Gram-negative bacteria, *Agrobacterium rhizogenes*, *Pseudomonas fluorescens* and *Rhizobium leguminosarum*, was suppressed when a mixture of fradiomycin and kanamycin was combined with that of nalidixic acid (10 mg l^{-1}) and trimethoprim (20 mg l^{-1}), which are selective inhibitors of Gram-negative bacteria and bacilli (Hayakawa *et al.* 1996a).

Improvement to the isolation medium

Diluted zoospore suspensions of the actinomycete strains listed in Table 1 were plated onto HV agar with or without a mixture of fradiomycin (40 mg l^{-1}), kanamycin (40 mg l^{-1}), nalidixic acid (10 mg l^{-1}) and trimethoprim (20 mg l^{-1}) to improve medium selectivity. We then confirmed the recovery of each strain on the two media. Viable counts, as well as colony sizes and aerial hyphae formation of all the *Actinokineospora* reference strains tested were not affected by the antibacterial agents (data not shown). Virtually no colonies of other actinomycete strains appeared on the HV agar containing antibacterial agents, except for *Actinosynnema mirum* and *Actinoplanes missouriensis*. Colony counts of these strains on the amended HV agar were 82% and 7% of those on the HV agar control, respectively.

Table 2 Effects of calcium carbonate and antibacterial agents on isolation of *Actinokineospora* spp. from soil*

Soil treatment‡	Antibacterial agents§ in HV agar	cfu† g^{-1} of air-dried soil					*Actinokineospora*/ total counts
		Actinokineospora	*Actinoplanes*	*Catenuloplanes*	*Dactylosporangium*	Other actinomycetes	Other bacteria	
None	None	$2·9\ (0·7) \times 10^{4a}$	$1·1\ (0·4) \times 10^{6a}$	$8·3\ (7·2) \times 10^{3a}$	$2·1\ (0·7) \times 10^{4a}$	$5·0\ (1·3) \times 10^{4a}$	$1·0\ (0·1) \times 10^{6a}$	0·01
$CaCO_3$	None	$2·5\ (0·6) \times 10^{5b}$	$4·1\ (1·4) \times 10^{6b}$	$6·7\ (5·8) \times 10^{4b}$	$5·4\ (1·4) \times 10^{4b}$	$1·1\ (0·2) \times 10^{5b}$	$1·6\ (0·2) \times 10^{6b}$	0·04
$CaCO_3$	FM + KM + NA + TP	$2·9\ (0·2) \times 10^{5b}$	$2·2\ (0·4) \times 10^{5c}$	ND	$6·7\ (1·4) \times 0^{3c}$	$9·2\ (2·9) \times 10^{3c}$	$1·4\ (0·2) \times 10^{5c}$	0·44

* Rehydration and centrifugation was used to isolate actinomycetes (see Materials and Methods). Soil sample no. 838 (level-land forest, pH 6·1; 15·5% loss on ignition).
† Colony forming unit. Counts represent mean of triplicate experiments (triplicate plate counts for each experiment) with standard deviations given in parentheses. Within each column, means having the same superscript are not significantly different ($P < 0·05$) by Duncan's multiple range test.
‡ $CaCO_3$, calcium carbonate treatment.
§ FM, fradiomycin (40 mg l^{-1}); KM, kanamycin (40 mg l^{-1}); NA, nalidixic acid (10 mg l^{-1}); TP, trimethoprim (20 mg l^{-1}).
ND, none detected.

Enrichment and selective isolation of *Actinokineospora* spp.

Sample preparation and selective media were used to preferentially isolate *Actinokineospora* spp. from a sample of forest soil no. 838 (Table 2). *Actinokineospora* spp. were recovered on HV agar by rehydration-centrifugation (RC) (Hayakawa *et al.* 2000). However, microbes, including undesirable zoosporic actinomycetes and motile unicellular bacteria, highly contaminated the isolation plates and *Actinokineospora* spp. ($2{\cdot}9 \times 10^4$ cfu g^{-1} of dried soil) constituted only 1% of the total microbial population recovered. We therefore attempted to increase the quantitative recovery of *Actinokineospora* spp. The number of *Actinokineospora* colonies on HV agar significantly increased when the calcium carbonate procedure (Tsao *et al.* 1960; El-Nakeeb and Lechevalier 1963) was applied before the RC method. Although this protocol concomitantly and moderately increased the number of undesirable motile bacteria including actinoplanetes such as *Actinoplanes* spp., further growth was severely curtailed by incorporating a mixture of fradiomycin, kanamycin, nalidixic acid and trimethoprim into the HV agar. These antibacterial agents had no adverse effect on the emergence and development of *Actinokineospora* colonies. Thus, the integrated procedure consisting of calcium carbonate soil treatment, the RC technique, and plating on the HV agar supplemented with antibacterial agents, yielded a high colony count ($2{\cdot}9 \times 10^5$ cfu g^{-1} of dried soil) of *Actinokineospora* spp. that accounted for 44% of the total colonies recovered.

The efficiency of the integrated method for isolating *Actinokineospora* spp. was confirmed when applied to 39 different soil and leaf-litter samples collected from fields, forests and stream banks (Table 3). From 17 samples (15 of soils and two of leaf-litter materials), the integrated method selectively isolated *Actinokineospora* spp., constituting 4–86% of the total microbial population recovered. Among the samples examined, the most favourable isolation sources for *Actinokineospora* spp. were forest soils that were rich in humus and not too acidic.

A typical isolation plate prepared by the integrated method is shown in Fig. 1. The *Actinokineospora* colonies that developed were thin and flat with sparse white aerial hyphae. Microscopic observation revealed long, straight substrate hyphae with branching and relatively short, tufted aerial hyphae.

Taxonomic evaluation of the isolates

Confirmation of generic identification. To validate the presumptive generic identification according to light microscopy, 29 putative *Actinokineospora* strains isolated from 17 samples and randomly selected, were further tested by scanning electron microscopy (SEM) and chemical analyses. In addition, two representative isolates were characterized by 16S rDNA sequence analysis to establish their phylogenetic positions.

SEM showed that all 29 test isolates produced branched, flexuous aerial hyphae that divided to form the chains of rod-shaped arthrospores. The spores of all the test strains were smooth-surfaced and became motile through lophotrichous flagella under aqueous conditions. Detailed spore-chain morphology of typical strains is illustrated in Fig. 2. Spore chains of strain YU 924-201 at maturity tended to aggregate into clusters resembling sporodochia. Chemical analyses on the other hand, revealed that the test 29 strains all contained meso-diaminopimelic acid as the cell-wall diamino acid. The principal whole-cell sugars were

Table 3 Selective isolation of *Actinokineospora* spp. from various soil and plant-litter samples using newly developed method*

Sample type	No. of samples examined	*Actinokineospora*-positive samples	Sample pH†	Organic matter content‡	cfu† g^{-1} of air-dried sample		
					Actinokineospora	Other actinomycetes	Other bacteria
Soil from							
Cultivate field	15	4	7·3	13·5	$8{\cdot}2 \times 10^3$ (27·4%)‡	$1{\cdot}1 \times 10^4$	$2{\cdot}1 \times 10^4$
Paddy field	5	2	5·8	12·8	$5{\cdot}9 \times 10^3$ (4·8%)	$1{\cdot}1 \times 10^5$	$1{\cdot}3 \times 10^5$
Level-land forest	8	5	6·0	19·6	$2{\cdot}6 \times 10^5$ (25·4%)	$2{\cdot}0 \times 10^5$	$3{\cdot}9 \times 10^5$
Mountainous forest	3	2	6·9	19·7	$1{\cdot}1 \times 10^3$ (19·5%)	$4{\cdot}2 \times 10^5$	$2{\cdot}7 \times 10^5$
Stream bank	4	2	6·4	8·7	$5{\cdot}9 \times 10^4$ (9·6%)	$6{\cdot}5 \times 10^4$	$2{\cdot}5 \times 10^6$
Plant-leaf litter from							
Stream bank	2	1	6·5	39·9	$7{\cdot}4 \times 10^4$ (18·2%)	$2{\cdot}1 \times 10^4$	$3{\cdot}1 \times 10^5$
Level-land forest	2	1	5·2	66·0	$4{\cdot}4 \times 10^4$ (9·0%)	$1{\cdot}1 \times 10^5$	$4{\cdot}8 \times 10^5$

* Refer to Materials and Methods.

† Mean value of *Actinokineospora* positive samples.

‡ % of total counts.

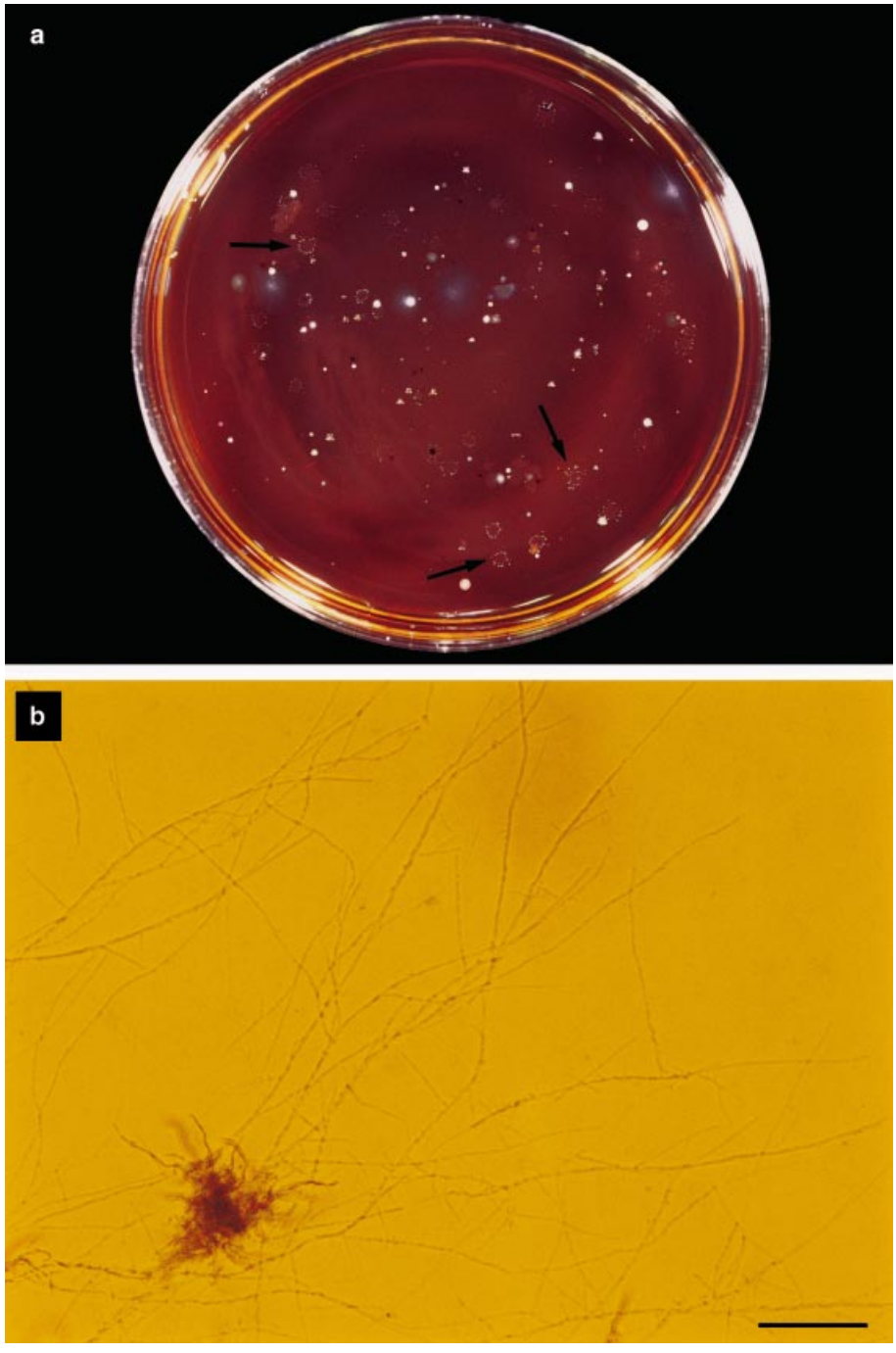

Fig. 1 Isolation plate prepared from soil no. 924 (level-land forest, pH 5·5; 14·8% loss on ignition) using newly developed method (a) after 2 weeks incubation at 28°C (arrows indicate representative *Actinokineospora* colonies), and light microscopy of *Actinokineospora* colony on the plate (b) showing long branching substrate hyphae, from which aerial hyphae arise in tufts. Bar, 10 μm

arabinose, galactose and rhamnose. The predominant menaquinone component of the test strains was MK-9(H_4), and they also contained phosphatidylethanolamine (PE) as well as hydroxy-PE (phospholipid type PII *sensu* Lechevalier *et al*. 1981). These morphological and chemical properties are consistent with the classification of the 29 test isolates into *Actinokineospora*.

The 16S rDNA sequences (> 1400 bases; positions 28–1524, according to the *Escherichia coli* numbering system of Brosius *et al*. 1978) of the isolates YU 873-1 and YU 923-

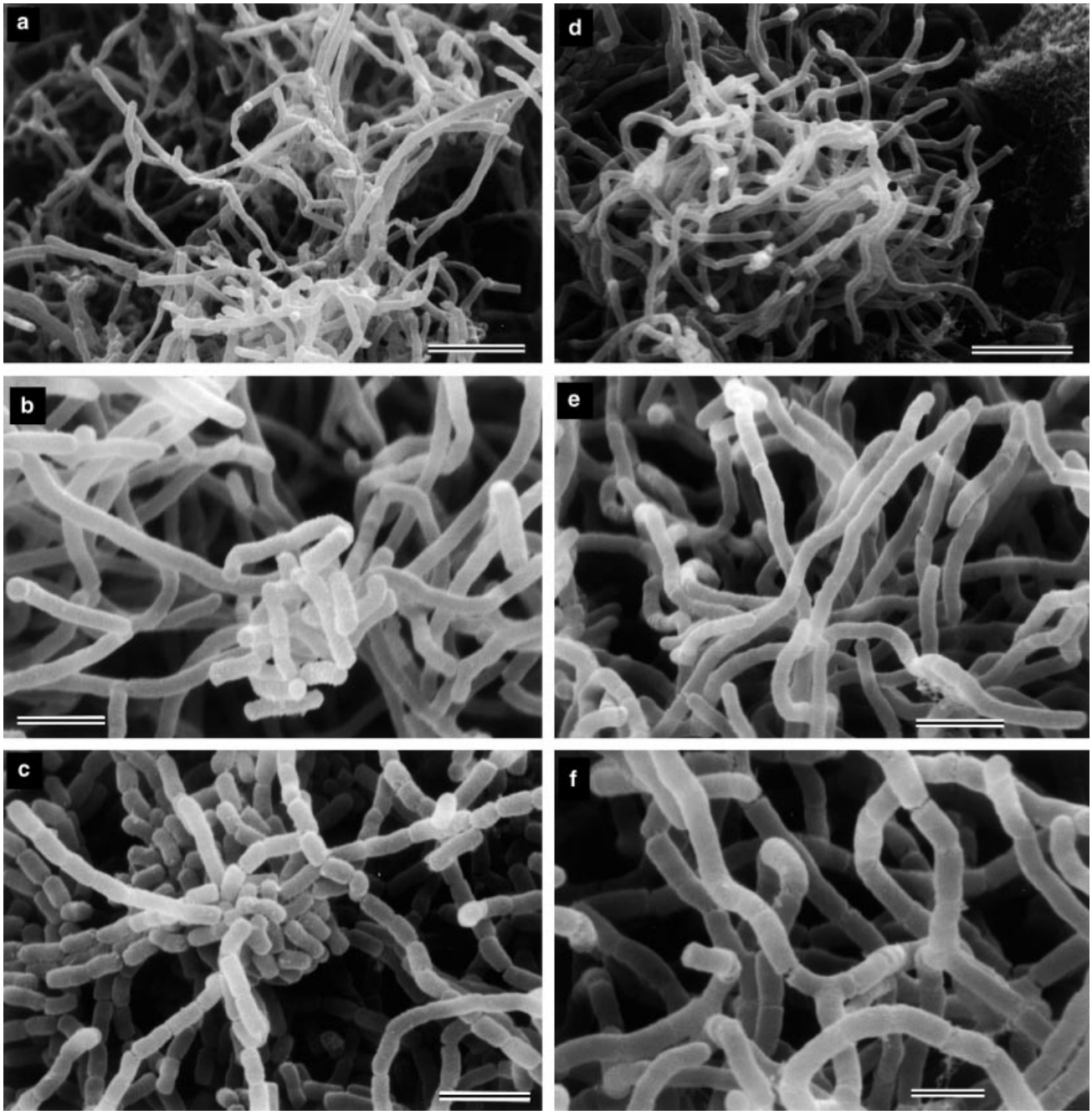

Fig. 2 Isolates of *Actinokineospora*, sporulating aerial hyphae. Scanning electron microscopy. (a–c) Strain YU 923-201. (d–f) Strain YU 961–201. Bar: a, d, 5 μm; b, c, e, 2 μm; f, 1 μm

201 were almost completely determined and compared with the sequences of selected members of the family *Actinosynnemataceae* (Labeda and Kroppenstedt 2000). The phylogenetic dendrogram derived from evolutionary distances determined by neighbour-joining is shown in Fig. 3. A total of 1339 nucleotides were analysed after eliminating all sites that were not determined in any sequence. Isolates YU 873-1 and YU 923-201 were closely related to *Actinokineospora riparia* NRRL B-16432 (= IFO 14541; type strain of the genus *Actinokineospora*) and these three strains formed a coherent cluster supported by bootstrap analysis at a confidence level of 88%. The values of 16S rDNA sequence similarity between the test isolates and *Actinokineospora riparia* NRRL B-16432 were 96·7–98·6%.

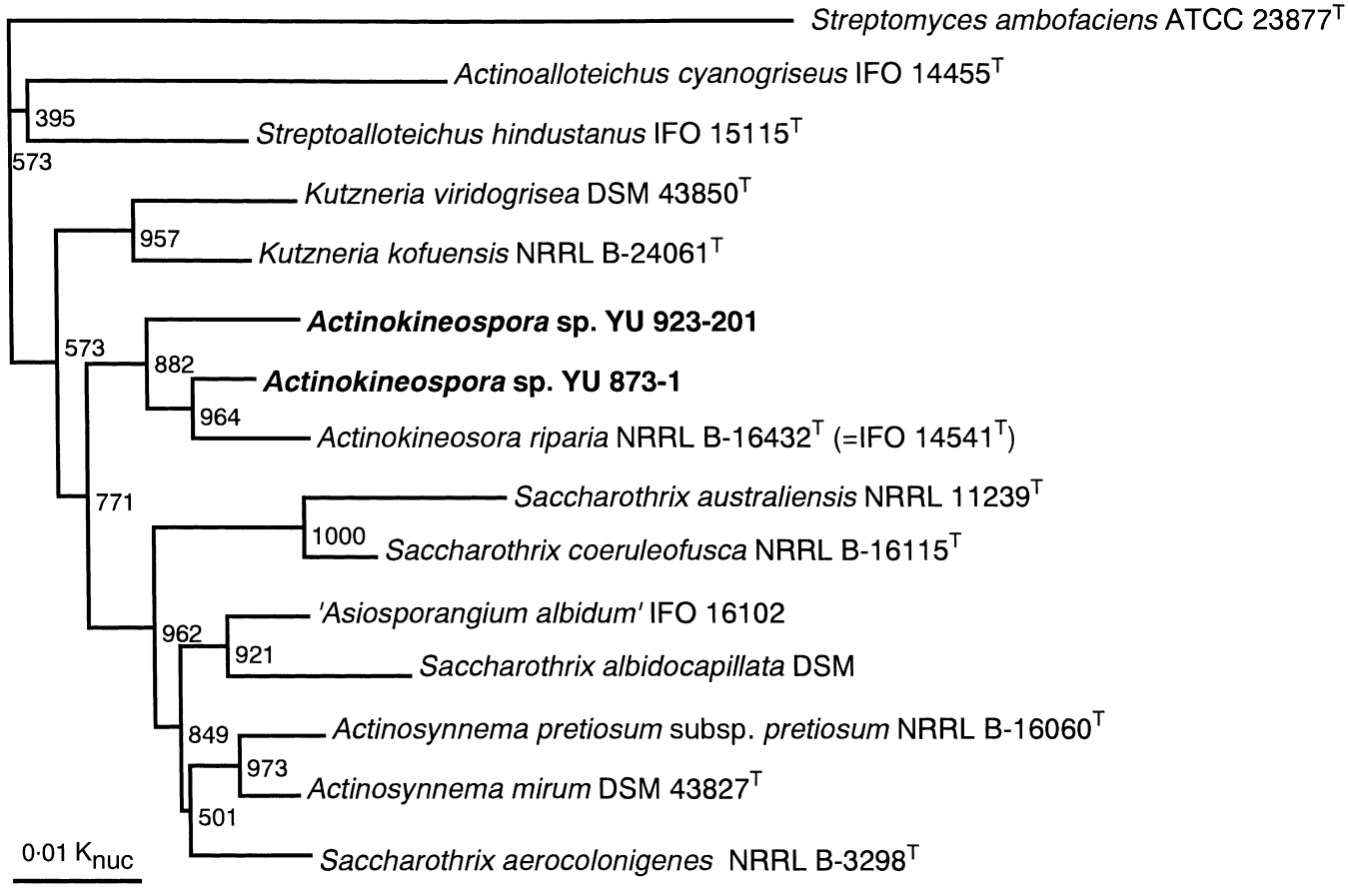

Fig. 3 Neighbour-joining tree based on 16S rDNA gene sequences, showing relationships among strains YU 873-1 and YU 923-201 and representatives of the family *Actinosynnemataceae*. Bar, 0·01 nucleotides substitutions per site. Numbers on branches are confidence limits estimated from bootstrap analysis of 1000 replicates

Characterization based on phenotypic properties. The 29 test isolates were classified according to the phenotypic criteria recommended by Tamura *et al.* (1995) as being useful for differentiating *Actinokineospora* species (Table 4). The results divided the isolates into 10 phenotypic groups including four groups of single members. Of the 29 test isolates, 15 shared all test morphological, cultural and physiological characteristics with *Actinokineospora diospyrosa* IFO 15665, *Actinokineospora globicatena* IFO 15664, or *Actinokineospora terrae* IFO 15668. However, the remaining 14 strains, which represented seven phenotypic groups, were differentiated from any known *Actinokineospora* strain by some taxonomic properties.

Antimicrobial activity

The *Actinokineospora* isolates were tested for antimicrobial activity. Of the 29 test strains, 28 (97%) exhibited inhibitory activity. Most of this activity was directed against Gram-positive bacteria, which were inhibited by 25 (86%) of the isolates. Within this bacterial group, *Micrococcus luteus* was the most susceptible, followed closely by *Staph. aureus*. Thirteen isolates (45%) inhibited Gram-negative bacteria. *Agrobacterium rhizogenes* was the least susceptible of the Gram-negative bacteria tested, as it was inhibited by only one isolate. Among the 13 active isolates, nine strains also inhibited Gram-positive bacteria. Antimycotic activity was found in 11 isolates (38%), all of which inhibited *Aspergillus oryzae*, whereas only four had anti *Candida* activity. These 11 strains were also active against Gram-positive and/or Gram-negative bacteria.

DISCUSSION

Methodologies that favour the selective isolation of actinomycetes have been extensively reviewed (Cross 1982; Williams and Wellington 1982; McCarthy 1985; Goodfellow and O'Donnel 1989; Labeda and Shearer 1990). One reasonable approach is to multiply actinomycete propagules (enrichment) in the source materials prior to plating. The

Table 4 Some morphological and physiological characteristics of *Actinokineospora* isolates (29 strains)

No. of isolates	9* *A. globicatena*	5 *A. diospyrosa*					1 *A. terrae*				*A. inagensis*	*A. riparia*
Reference strains	IFO 15664^T	IFO 15665^T	4	3	2	2	IFO 15668^T	1	1	1	IFO 15663^T	IFO 14541^T
Morphology												
Sporodochium-like structures	+	–	–	–	–	–	–	–	–	–	–	–
Pigmentation												
Substrate mycelium colour†	y. brown	y. black	g. grey	b. purple	y. brown	b. black	y. brown	y. brown	y. brown	d. brown	y. brown	y. brown
Growth on sole carbon source												
Fructose	+	+	–	–	–	–	+	–	–	–	–	±
Maltose	+	+	–	+	–	–	+	–	+	+	±	–
D-Mannose	+	+	–	–	–	–	+	+	–	–	–	±
Sucrose	+	+	–	+	–	+	+	+	–	+	–	–
Salt tolerance (%, w/v)												
Sodium chloride (2·0)	+	+	+	+	+	+	+	+	+	+	–	+
Sodium chloride (3·0)	+	+	–	–	–	–	+	–	+	+	–	–
Degradation of:												
Calcium malate	+	–	+	+	+	–	+	–	–	–	–	–
Gelatin	+	+	+	+	+	+	+	+	+	+	–	–
Starch	+	+	–	+	–	+	+	+	+	–	–	–
Enzyme activity												
Nitrate reduction	+	+	+	+	–	–	+	+	–	–	+	–

*Includes strain YU 923-201 (refer to Fig. 2 and Fig. 3).

†On yeast extract-malt extract agar.

±, weak reaction.

y, yellowish; g, greenish; b, brownish; d, dark.

prior incubation of soil samples with calcium carbonate (Tsao *et al.* 1960), or with organic materials such as chitin (Porter and Wilhelm 1961; Williams *et al.* 1972), significantly increases the actinomycete population, thereby yielding higher total and relative plate counts. Enrichment can also be used for the isolation of specific actinomycete taxa that rarely appear on spread plates prepared by conventional dilution procedures. The most popular means of isolating motile actinoplanetes such as *Actinoplanes* spp. rely upon baiting with pollen and hair (Couch 1954; Nonomura and Takagi 1977; Hayakawa *et al.* 1991c) and accumulating zoospores in glass capillaries containing chemoattractants (Palleroni 1980; Hayakawa *et al.* 1991b). Rehydration-centrifugation (RC) is a novel isolation technique that universally favours diverse motile actinomycetes. The procedure involves an enrichment stage that promotes the liberalization of zoospores from a substrate by flooding (Hayakawa *et al.* 2000).

Successful isolation of *Actinokineospora* spp. depends on combining the calcium carbonate and RC procedures. Incubating source materials moistened with calcium carbonate prior to RC significantly increased the number of *Actinokineospora* spp. on HV plates, thus offering a considerable advantage for yielding a high number and greater diversity of subcultures. A subsequent taxonomic study has found that *Actinokineospora* isolates can be categorized into diverse phenotypes. Although the rationale behind the calcium carbonate effect remains to be studied, Tsao *et al.* (1960) have stressed that the pH of the isolation sources after mixing with powdered calcium carbonate would be altered in favour of the growth of actinomycete propagules. Calcium ions reportedly stimulate the formation of aerial mycelia by several actinomycete cultures (Natsume *et al.* 1989).

Since enrichment usually allows for the concomitant increase of impeding microbes, selective media must be used for isolation. In the present study, undesirable zoosporic actinomycetes and motile unicellular bacteria that arose on the HV isolation plates were significantly reduced by supplementing the medium with specific antibacterial agents. Fradiomycin and kanamycin were included because they inhibit the growth of a wide range of motile actinomycetes apart from *Actinokineospora* strains. Nalidixic acid and trimethoprim were also included, since *Actinokineospora* strains were entirely insensitive to these inhibitors of Gram-negative bacteria and bacilli (Hayakawa *et al.* 1996a). The efficiency of using all four of these antibacterial agents in reducing a wide range of contaminating bacteria may be due to differences in their antimicrobial spectra. Their synergistic antibacterial effect may also account for the efficient de-contamination. Tests of pure cultures showed a synergistic association of the four antibacterials with *Pseudomonas fluorescens*, a commonly encountered motile bacterium (Table 1).

The development of new methodologies with which to isolate actinomycetes has altered understanding about their ecology, taxonomy and bioactivity (Goodfellow 1992; Williams *et al.* 1993). While the isolation procedure described here cannot be used to estimate the actual population of *Actinokineospora* spp. in natural substrates, it has already revealed considerable information about their distribution. *Actinokineospora* spp. are more common in soil and plant-litter than was previously estimated. Evidence for the frequent occurrence of *Actinokineospora* spp. in forest soils and decaying leaves suggests that they play roles in the degradation of plant remains, but more detailed studies are required to determine their significance in such ecosystems. The proposed isolation method has provided various *Actinokineospora* cultures, which can be clearly differentiated from known species in terms of phenotypic properties. Further investigation, including numerical phenetic and molecular systematic analyses, could reveal their exact taxonomic status at the species rank. *Actinokineospora* isolates may also be worthy of investigation in natural product screening programmes, since most of the test strains have significant antibiotic activity against diverse microbes.

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