Invited Review

Isolation of new actinomycete strains for the screening of new bioactive compounds

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In order to facilitate the discovery of novel bioactive compounds from microorganisms, various techniques for isolation of new actinomycete strains have been attempted. Studies of the vertical distribution of actinomycetes in soil, isolation of actinomycetes from desert soils or fallen leaves, selective isolation of *Kitasatospora* strains using novobiocin or *Actinoplanes* strains using the chemotactic method, and the use of gellan gum as a solidifying agent were carried out. We discovered 9 novel bioactive compounds from actinomycete strains isolated under unusual conditions, and proposed two new genera, five new species and one new subspecies.

Key Words——*actinobacteria*; isolation of actinomycetes; new microorganisms; novel bioactive metabolites

1. Introduction

Since the discoveries of penicillin and streptomycin, a large number of antibiotics have been isolated from culture broths of microorganisms, such as actinomycetes. About 70% of these novel bioactive metabolites were derived from actinomycetes (Miyado, 1993). One of the efficient ways of discovering novel bioactive metabolites is through isolating new microorganisms, and numerous approaches have been used for this purpose. Table 1 shows various methods and habitats for isolating actinomycetes.

Over the past year, we have attempted various methods to isolate new actinomycetes. In this paper, we will focus on our use of these isolation methods and will describe the following in greater detail: 1. the vertical distribution of microorganisms in soil; 2. the habitat variation of actinomycetes; 3. unusual isolation methods; and 4. further prospects of microbial re-

sources.

2. Vertical Distribution of Microorganisms in Soils

Few studies have investigated the vertical distribution of actinomycetes in soil (Hagedorn, 1976). We were able to study distribution and variation of microorganisms living in 0 to 40 m depths of underground soils at the Kitasato Institute Medical Center in Kitamoto City, which is located 40 km northwest of the center of Tokyo, through the use of test borings for soil analyses of the grounds (Takahashi et al., 1990). Three kinds of agar media, heart infusion agar, glycerol starch agar and potato dextrose agar, were used for detection and isolation of bacteria, actinomycetes and fungi respectively. The number of microbial colonies was counted by using serial dilution method. The soft colonies were considered as bacterial strains. Actinomycete strains were distinguished from fungal strains by microscopic observation. The largest population of bacteria (10^8 /g of dried soil sample), actinomycetes (10^7 /g) and fungi (10^5 /g) were observed in soils from the top layer (0–0.3 m) of black loam. Fungi

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Table 1. Variation in isolation methods for actinomycetes.

Habitat	Actinomycete genus isolated
Cultivated field	<i>Streptomyces</i>
Cultivated paddy field	<i>Micromonospora</i>
Plant matter	<i>Microbispora</i>
Pasture	<i>Micromonospora</i>
Lake sediment	<i>Micromonospora</i>
Mangrove rhizosphere soil	<i>Actinobacteria</i>
Tea field soil	Acidophilic and acid tolerant actinomycetes
Desert soil, Marine sediment, Sea water and Activated sludge	
Pretreatment	
Yeast extract (6%, 20 min)	Enrichment of actinomycetes
Heating (100°C, 1 h)	<i>Streptosporangium</i> , <i>Microbispora</i> , <i>Microtetraspora</i>
Phenol (1.0–1.5%, 30 min)	<i>Dactylosporangium</i> , <i>Microbispora</i> , <i>Micromonospora</i>
Preculture with CaCO ₃	Enrichment of actinomycetes
Chemotactic method (KCl, γ -collidin, xylose)	Motile actinomycetes, <i>Actinoplanes</i> , <i>Dactylosporangium</i>
Medium for isolation	
Addition of antibiotics	
Novobiocin	<i>Actinoplanes</i> , <i>Kitasatospora</i>
Tunicamycin	<i>Micromonospora</i>
Rifampicin	<i>Actinomadura</i>
Chlortetracycline	<i>Nocardia</i>
Macrolide or aminoglycoside	Macrolide or aminoglycoside producer
Addition of humic acid	Rare actinomycetes
Addition of proline	Enrichment of actinomycetes
Gellan gum (substitute for agar)	<i>Actinobispora</i>
Condition for isolation	
High temperature	Thermophilic actinomycetes

Nonomura and Hayakawa (1988), Huck et al. (1991), Iwai and Takahashi (1992), Nioh et al. (1995), Jiang and Xu (1996), Suzuki et al. (1998), Takeuchi and Hatano (1999).

were observed only in the upper layer. Reduced numbers of bacteria and actinomycetes were detected in soils of the second upper layer (0.5–5 m). Bacteria ($10^6/\text{g}$) were also found in silty and sandy soils at 40 m (Figs. 1 and 2). The vertical distribution of actinomycetes was investigated in detail in soils taken at 10-cm intervals between 0 and 1 m. A comparative survey of all isolates demonstrated that the actinomycete population was the largest in soils of the surface layer, and although the number decreased gradually as depth increased, individual actinomycete strains were present in all soil layers (Fig. 3).

3. Habitat Variation of the Actinomycetes

In most cases, soil samples collected in temperate

environments, such as a farm, a garden or a forest, were used for isolation of microbes. However, it was suggested that the number and variety of the isolates depend upon the environmental or regional microflora of the collected soils (Lacey, 1973). It is believed that diversity of microorganisms leads to diversity of secondary metabolites.

(i) Isolation of actinomycete strains from desert soils

We are interested in isolation of actinomycetes living in desert soils (Takahashi et al., 1996). Nine samples of the desert soils used were collected from the Mojave Desert, California and 335 actinomycete strains were isolated from these samples. The effects of temperature on actinomycete colonies were investigated

Fig. 2. Vertical distribution of microorganisms in soil.

Fig. 3. Vertical distribution of different actinomycetes in soil.
 □ Number of actinomycetes after elimination of duplicate strains in each layer. ■ Number of individual actinomycetes after comparison with strains from other layer.

at 27°C and 37°C. The number of colonies on media cultured at 37°C was about twice that at 27°C. Furthermore, the size of colonies grown at 37°C was much larger than those at 27°C (Fig. 4). These data indicate that the optimum temperature for growth of actinomycetes isolated from desert soils is higher than that for actinomycetes isolated from temperate zones, which can be reasonably understood in connection with the high temperature and dryness of the desert environment.

From 1 g of dried soil samples, 1×10^3 to 1×10^6 actinomycete colonies were counted. The number of actinomycetes is generally estimated to be over 10^7 /g of dried soils collected from a normal environment (Alan and Stanley, 1990; Goodfellow and Williams, 1983). Therefore, our data showed that the number of actinomycetes in desert soils was lower than that in ordinary soils. Elwan et al. (1985) also observed 1×10^3 to 1×10^7 colonies of *Streptomyces* from rhizosphere or non-rhizosphere desert soils. We isolated 335 actinomycete strains from 9 desert soil samples and culture broths of these strains were tested for isolation of novel metabolites. Novel bioactive panosialins (Ya-

mada et al., 1995) possessing glycosidase inhibitory activity, and 4-chlorothreonine (Yoshida et al., 1994) possessing herbicidal activity were isolated from two streptomycete strains.

The diaminopimelic acid (DAP) isomer of about 50 actinomycete strains randomly selected from the 335 strains was analyzed and 41 strains contained LL-DAP, while 9 strains contained *meso*-DAP (Lechevalier and Lechevalier, 1970). Further taxonomical study was conducted on the 9 *meso*-DAP containing strains which were regarded as non-*Streptomyces* actinomycetes (rare actinomycetes). The light and scanning electron micrographs as well as the chemotaxonomic characteristics are shown in Fig. 5 and Table 2, respectively. The study demonstrated that six genera of rare actinomycetes, *Microbispora*, *Nocardia*, *Microtetraspora*, *Amycolatopsis*, *Actinomadura* and *Saccharothrix*, were included among the 50 strains randomly selected from the 335 strains isolated from the 9 desert soils. This suggests that a large number of rare actinomycetes exist in desert soils. Lechevalier and Lechevalier (1967) reported that 95% of 5,000 cultures isolated from 16 general soils were *Streptomyces*, 2% were *Nocardia*, and 1.4% were *Micromonospora*. From the desert soil samples used in our study, no *Micromonospora* was isolated, and this may be because strains of *Micromonospora* are sensitive to high temperature or dryness. Six strains were found to grow in a wide range of temperatures (Table 2). Five of these strains were even able to grow at 50°C. In fact, the growth range of strain OH-5250 was from 15 to 53°C. The ground temperature in the desert can reach 70°C during the day, while it falls to below freezing at night. The extensive temperature range for growth might be due to this environment, and thus thermotolerant and mesophilic actinomycetes seem to be living in desert

Fig. 1. Growth of microorganisms in soils taken at different depths.

The vertical distribution of microorganisms was investigated between 0 and 40 m in soil samples taken at appropriate depths. Date of soil collection: December 2nd, 1985. Place of soil collection: Farmer agricultural station, 121-1 Arai, Kitamoto-shi, Saitama Prefecture, Japan.

Fig. 4. Growth of microorganisms from desert soil.

Soil (1 g) collected from under a tree in the Mojave Desert, California, was suspended in 9 ml of Winogradsky's salt solution. The supernatant (300 μ l) was mixed with modified glucose-asparagine agar containing 1% proline and was cultured at 27°C for 2 weeks or at 37°C for 1 week.

Fig. 6. Scanning electron micrograph of sporangium with hairy surface (A) and transmission electron micrograph of flagellated spore stained with uranyl acetate (B) of *Actinoplanes capillaceus* K93-5561^T.

Fig. 8. Growth of microorganisms in a preserved soil sample.

One hundred microliters of a soil sample diluted 100,000-fold with distilled water was spread on the glycerol starch agar medium. The plates were cultured at 27°C for 1 week. A, Used immediately after collection; B, preserved at room temperature for 1 week; C, preserved at room temperature for 4 weeks; D, preserved at 5°C for 1 week; E, preserved at 5°C for 4 weeks.

Fig. 5. Light and scanning electron micrographs of rare actinomycetes from desert soil.

1, Paired spores of *Microbispora* sp. strain OH-5039; 2, fragmented substrate mycelium of *Nocardia* sp. strain OH-5044; 3, aerial growth of strain OH-5090 belonging to *Actinomadura* or *Microtetraspora*; 4, spiral spore chain of strain OH-5102 belonging to *Actinomadura* or *Microtetraspora*; 5, aerial spore chain of *Amycolatopsis* sp. strain OH-5119; 6, short spore chain of strain OH-5147 belonging to *Actinomadura* or *Microtetraspora*; 7, fragmented substrate mycelium of *Nocardia* sp. OH-5229; 8, long straight spore chain of *Amycolatopsis* sp. strain OH-5250; 9, long flexuous spore chain of *Saccharothrix* sp. strain OH-5302.

Table 2. Temperature range for growth and chemotaxonomic characteristics of 9 strains containing *meso*-DAP from the desert soil samples and their generic assignments.

Strain No.	Temp. range for growth	Sugar pattern ^a	Mycolic acid	Acyl type ^b	Menaquinone	Genus
OH-5039	17–50°C	B	–	Ace	MK-9 (H ₀ , H ₂ & H ₄)	<i>Microbispora</i>
OH-5044	19–50°C	A	+	Gly	MK-8 (H ₄)	<i>Nocardia</i>
OH-5090	17–43°C	B	–	Ace	MK-9 (H ₄ , H ₆ & H ₂)	<i>Microtetraspora</i> or <i>Actinomadura</i>
OH-5102	17–50°C	B	–	Ace	MK-9 (H ₀ , H ₂ & H ₄)	<i>Microtetraspora</i> or <i>Actinomadura</i>
OH-5119	16–50°C	A	–	Ace	MK-9 (H ₄)	<i>Amycolatopsis</i>
OH-5147	16–44°C	B	–	Gly	MK-8 (H ₄)	<i>Nocardia</i>
OH-5250	15–53°C	A	–	Ace	MK-9 (H ₄)	<i>Amycolatopsis</i>
OH-5302	14–47°C	C	–	Ace	MK-9 (H ₄) or MK-10 (H ₄)	<i>Saccharothrix</i>

^a According to classification of Lechevalier and Lechevalier (1970).

^b *N*-Acyl types of muramic acid: Ace, *N*-acetylated; Gly, *N*-glycolated.

soils.

Such microorganisms are thought to be potential producers of unknown and useful secondary metabolites.

(ii) Isolation of actinomycete strains from fallen leaves

Matsukuma et al. (1994) and Okazaki et al. (1995) reported that numerous *Streptomyces* and *Microbispora* strains were isolated from the fallen or fresh leaves of various plants. We attempted to isolate actinomycetes from fallen leaves using the following three methods: the topping method, in which samples were put onto the surface of an agar plate; the agar layered method, in which samples put onto the agar surface were covered with the same agar medium; and the extraction method, in which samples cut in 1- to 2-mm squares were intensively mixed in an inorganic-salt solution, and were then mixed with agar medium (Matsumoto et al., 1998). We isolated 77 actinomycete strains from 15 samples. We subsequently observed the morphological characteristics and DAP-isomers of all of these strains. More than 50% of total actinomycete strains isolated were found to be rare actinomycetes containing *meso*-DAP in the cell wall (Table 3). The genus *Microbispora* occurred with high frequency in the samples of bamboo (*Sasa* and *Pleioblastus*) and loosestrife (*Lythrum*). Identification of other rare actinomycetes has not yet been carried out. Diketopiperazine possessing antitumor activity was obtained from the culture broth of *Streptomyces* sp. K96-5794 isolated from pine needle, though this

was a known compound.

4. Variation of Isolation Methods

In view of the microbiological aspect, one of the epoch-making studies in the history of antibiotic screening was Weinstein's discovery of gentamicin (Weinstein et al., 1963) from a *Micromonospora* strain. After this turning point, various strains of rare actinomycetes were isolated. We attempted to isolate rare actinomycete strains in search of novel bioactive compounds, and decided to use selective isolation methods for particular genus strains.

(i) Selective isolation of *Kitasatospora* strains by the use of novobiocin

The genus *Kitasatospora* was proposed as a new genus by the authors in 1982 (Ômura et al., 1982, 1985). *Kitasatospora* strains produce long spore chains on aerial mycelia and contain both LL-DAP and *meso*-DAP in the cell wall. Although *Kitasatospora* resembles the genus *Streptomyces* in its morphology, it is clearly different in its cell wall composition. The aerial spores on solid culture and the submerged spores in liquid culture contain LL-DAP, while the mycelia in both cultures mainly contain *meso*-DAP. These properties are unique characteristics of *Kitasatospora* strains (Ômura et al., 1981, 1982; Takahashi et al., 1983, 1984b).

Following our proposal, the genus has been noted in the actinomycete classification and has been investigated as a source of bioactive metabolites (Takahashi

Table 3. Number of actinomycetes isolated from fallen leaves.

Source		Rare actinomycetes		
Vernacular name	Genus	<i>Streptomyces</i>	<i>Microbispora</i>	Others
Pine	<i>Pinus</i>	5	4	3
Pine	<i>Pinus</i>	1	0	0
Pine	<i>Pinus</i>	10	2	1
Pine	<i>Pinus</i>	8	1	2
Pine	<i>Pinus</i>	2	3	0
Bamboo	<i>Sasa</i>	0	1	0
Bamboo	<i>Sasa</i>	0	5	0
Bamboo	<i>Pleioblastus</i>	2	3	0
Japanese cypress	<i>Chamaecyparis</i>	0	1	0
Cherry tree	<i>Prunus</i>	0	1	0
Japan cedar	<i>Cryptomeria</i>	3	1	0
Loosestrife	<i>Lythrum</i>	0	11	3
Camphor tree	<i>Neolitsea</i>	0	1	1
Japanese rose	<i>Kerria</i>	0	3	0
Maple tree	<i>Acer</i>	2	0	0
Total		32	33	10

Table 4. *Kitasatospora* strains and new metabolites.

Strain	Compound	Activity	Reference
<i>Kitasatospora setae</i> KM-6054 ^T	Setamycin	Antitrichomonal	Omura et al. (1982)
<i>Kitasatospora griseola</i> AM-9660 ^T	Setamycin	Antifungal	Takahashi et al. (1984a)
<i>Kitasatospora phosalacinea</i> KA-338 ^T	Phosalacine	Herbicidal	Takahashi et al. (1984a)
" <i>Kitasatospora melanogena</i> " K55-G-32	NT		Shimazu et al. (1984)
<i>Kitasatospora setae</i> SANK60684	Propioxatins	Enkephalinase B inhibitor	Inaoka et al. (1986)
<i>Kitasatospora griseola</i> MF730-N6	Terpentecin	Antitumor	Tamamura et al. (1985)
<i>Kitasatospora setae</i> S-9	Microbial transformation of azacarbazole		Peczynska-Czoch et al. (1987)
" <i>Kitasatospora brunne</i> " 1719-SV3	NT		Shimazu et al. (1987)
" <i>Kitasatospora kifunense</i> " No. 9482	FR-900494	Immunomodulator	Iwami et al. (1987)
<i>Kitasatospora cystarginea</i> RK-419 ^T	Cystargin	Antifungal	Kusakabe and Isono (1988)
<i>Kitasatospora mediocidica</i> NRRL B-16109 ^T	NT		Labeda (1988)
<i>Kitasatospora</i> sp. No. 55	Tyrostatin	Proteinase inhibitor	Oda et al. (1989)
" <i>Kitasatospora kyotoensis</i> " SAM 0170	SUAM-20007-20012	Proteinase inhibitor	Maeda et al. (1989)
<i>Kitasatospora</i> sp. SK-60	Phospholipase D		Sawada et al. (1989)
<i>Kitasatospora</i> sp. F-0368	F-0368	Agrochemical	Taguchi et al. (1989)
<i>Kitasatospora cochleata</i> M-5 ^T	NT		Nakagaito et al. (1992)
<i>Kitasatospora paracochleata</i> M-13 ^T	NT		Nakagaito et al. (1992)
<i>Kitasatospora azatica</i> IFO-13803 ^T	NT		Nakagaito et al. (1992)
" <i>Kitasatospora kimorexae</i> " 90-GT-302	Kimorexins	Antifungal	Yao et al. (1994)
<i>Kitasatospora cheerisanensis</i> YC75 ^T	Bafilomycin like	Antifungal	Chung et al. (1999)
<i>Kitasatospora cineracea</i> SK-3255 ^T	NT		Tajima et al. (2001)
<i>Kitasatospora niigatensis</i> SK-3406 ^T	NT		Tajima et al. (2001)

NT: not reported.

Superscript T shows type strain and valid name.

Table 5. Antibiotic sensitivities of *Kitasatospora* and *Streptomyces*.

	MIC (µg/ml)				
	Ampicillin	Gentamicin	Novobiocin	Tetracycline	Tylosin
<i>Kitasatospora setae</i> KM-6054 ^T	>100	6.25	100	50	100
<i>Kitasatospora cineracea</i> SK-3255 ^T	3.12	12.5	>100	50	100
<i>Kitasatospora griseola</i> AM-9660 ^T	12.5	3.12	100	12.5	25
<i>Kitasatospora griseola</i> OM-5023	6.25	6.25	100	25	25
<i>Kitasatospora phosalacinea</i> KA-338 ^T	6.25	6.25	>100	100	100
<i>Kitasatospora niigatensis</i> SK-3406 ^T	3.12	6.25	>100	100	>100
<i>Kitasatospora niigatensis</i> SK-3412	6.25	3.12	>100	50	50
<i>Kitasatospora niigatensis</i> SK-3421	6.25	12.5	>100	100	>100
<i>Streptomyces griseus</i> IFO12875 ^T	>100	6.25	0.8	25	>100
<i>Streptomyces albus</i> IFO13014 ^T	25	1.6	20	100	>100
<i>Streptomyces hygroscopicus</i> IFO13472 ^T	<0.8	1.6	<0.8	12.5	0.8
<i>Streptomyces tanashiensis</i> IFO12919 ^T	100	12.5	1.6	50	12.5

et al., 1999). Several new species have been reported as producers of novel bioactive compounds with varying structures and interesting activities (Table 4). Therefore, in order to isolate additional *Kitasatospora* strains we investigated selective isolation methods and found that the *Kitasatospora* strains are resistant to novobiocin (Table 5) (Iwai and Takahashi, 1992).

Two hundred seventy actinomycete strains were isolated from soil samples using agar medium containing novobiocin (50 µg/ml). Among these, 5 strains were found to belong to the genus *Kitasatospora*. We proposed two of them as new species, *Kitasatospora cineracea* SK-3255^T and *K. niigatensis* SK-3406^T, on the basis of DNA-DNA hybridization in comparison with known species and physiological characteristics (Tajima et al., 2001). As a result, we have proposed 5 new species of *Kitasatospora*, *K. setae*, *K. griseola*, *K. phosalacinea*, *K. cineracea* and *K. niigatensis*, and have found two novel bioactive compounds among their metabolites (Table 4). One of these compounds is setamycin, which possesses antimicrobial and nematocidal activities, and the other is phosalacin, which possesses herbicidal activity. To date, eleven species of the genus *Kitasatospora* have been recognized in accordance with the Bacteriological Code (Table 4).

(ii) Selective isolation of *Actinoplanes* strains using the chemotactic method

Palleroni (1980) found that xylose, chloride and bromide ions were effective attractants for assembling motile spores. Hayakawa et al. (1991) also reported

that γ -collidine was an effective attractant for selective isolation of *Actinoplanes* and *Dactylosporangium*.

We used capillary-containing xylose as a chemoattractant and isolated two motile actinomycete strains, K95-5561^T and K95-5562, from a soil sample, and found that these strains produced a novel antimicrobial agent, 2-hydroxyethyl-3-methyl-1,4-naphthoquinone (Fukami et al., 2000).

These two strains did not form aerial mycelia, instead forming non-fragmenting substrate mycelia, which bore spore vesicles (sporangia) on short sporangiophores. The mature sporangia were bell-shaped with hairy surfaces (Fig. 6A). When released into water, the sporangiospores became motile by polar flagella (Fig. 6B). The two strains were regarded as belonging to the genus *Actinoplanes* on the basis of chemotaxonomic and morphological characteristics together with 16S rDNA sequence data. The two strains were recognized as a new species of the genus *Actinoplanes* and given the name *Actinoplanes capillaceus* K95-5561^T based on cultural, morphological and physiological characteristics, and DNA-DNA hybridization data (Matsumoto et al., 2000). The invalidly proposed species '*Ampullariella cylindrica*,' '*Ampullariella pekinensis*' and '*Ampullariella pilifera*' were assigned to *Actinoplanes capillaceus* on the basis of genotypic and phenotypic data.

(iii) Use of gellan gum as a solidifying agent

Suzuki et al. (1998) reported that gellan gum, instead of agar, was useful as a solidifying agent for se-

lective isolation of the genus *Actinobispora*. We applied this method with the intention of isolating rare actinomycete strains. As a result, we isolated strain K97-0003, which was classified as a new genus and new species, *Longispora albida* K97-0003^T (Matsumoto et al., 2003). Furthermore, actinohivin, a novel anti-HIV protein, was found in the culture broth of strain K97-0003 (Chiba et al., 2001).

Strain K97-0003 grew on various agar media, but aerial mycelia did not grow. When gellan gum was

substituted for agar as a solidifying agent, aerial mycelia grew slightly on a few media and spores were produced on 1/10 V8 gellan gum containing CaCl₂.

Spore chains were straight and had more than 20 spores on the tip of short sporophores that branched from the vegetative mycelia. The cell wall peptidoglycan contained *meso*-DAP, glycine and alanine, and arabinose, while xylose was detected in whole-cell hydrolysates. Phylogenetic analysis using 16S rDNA sequences showed that strain K97-0003 falls within the

Fig. 7. Phylogenetic tree showing the position of *Actinoplanes capillaceus* K97-0003^T based on 16S rDNA analysis.

Numbers at the nodes indicate the level (%) of bootstrap support based on neighbor-joining analysis of 1,000 resampled data sets. Only values greater than 50% are shown. The scale bar indicates one nucleotide substitution per 100 nucleotides.

cluster of the family *Micromonosporaceae* but is not clustered within any genera (Fig. 7). Strain K97-0003 was regarded as new genus on the basis of morphological and chemotaxonomic properties as well as phylogenetic analysis based on 16S rDNA sequence data.

5. Further Prospect of Microbial Resources

We believe that the scope of microbial resources should be expanded to bacterial strains other than actinomycetes in order to increase the likelihood of discovering new and useful bioactive compounds from microorganisms. We are focusing on Gram-positive bacteria with high DNA G+C contents, the *Actinobacteria* (Stackenbrandt et al., 1997), other than actinomycetes as a potential microbial resource, because these microorganisms have not yet been widely investigated. The class *Actinobacteria* consists of five subclasses, *Actinobacteridae*, *Acidimicrobidae*, *Coriobacteridae*, *Sphaerobacteridae* and *Rubrobacteridae*, and more than 120 genera. Subclass *Actinobacteridae* is the biggest group, containing actinomycetes and other genera that form bacteria-like colonies. While actinomycete strains form generally hard colonies on agar media, other *Actinobacteria* strains form soft colonies. The latter group of bacteria includes numerous strains that are morphologically indistinguishable from Gram-negative bacteria and Gram-positive bacteria with a low G+C content. We therefore established a simple PCR method for discriminating *Actinobacteria* strains from new isolates (Yu et al., 2002).

A stable 100-bp insertion within domain III (helix 54a) of the 23S ribosomal RNA gene has been identified as a phylogenetic marker for *Actinobacteria* (Roller et al., 1992). Based on this genetic characteristic, a PCR was designed to amplify a 380-bp fragment from *Actinobacteria* and a 270-bp fragment from other bacteria. A PCR-based survey of known strains as well as newly isolated soil bacteria confirmed that this PCR accurately selected *Actinobacteria* (Table 6). To isolate *Actinobacteria* strains, agar medium containing 25 µg/ml nalidixic acid and 25 µg/ml aztreonam for inhibition of Gram-negative and Gram-positive bacteria with low DNA G+C content, was used. We are currently evaluating a new bioactive compound possessing activity against mycobacterium that was identified in the cultured broth of one of these *Actinobacteria* strains.

Hawksworth (1991) reported that the number of

Table 6. PCR product profile and DNA G+C content of Gram-positive strains isolated from soil samples.

Strain	PCR product		G+C content (mol%)
	270 bp	380 bp	
K99B-0001	—	+	64.3
K99B-0002	—	+	68.2
K99B-0003	—	+	61.7
K99B-0004	—	+	65.1
K99B-0005	+	+	61.6
K99B-0006	+	+	50.4
K99B-0008	+	+	68.4
K99B-0010	+	+	68.0
K99B-0012	—	+	67.7
K99B-0013	+	—	36.8
K99B-0017	—	+	68.7
K99B-0019	+	—	38.7
K99B-0020	—	+	55.2
K99B-0021	+	—	37.0
K99B-0022	—	+	61.1
K99B-0023	+	—	44.3
K99B-0024	+	—	41.8
K99B-0025	+	+	70.5
K99B-0026	—	+	71.5
K99B-0027	—	+	66.4
K99B-0028	—	+	62.3
K99B-0029	+	+	52.3
K99B-0030	—	+	65.2
K99B-0031	+	+	63.6
K99B-0032	+	—	37.5
K99B-0034	—	+	60.3
K99B-0035	—	+	68.1
K99B-0036	+	—	46.3
K99B-0037	—	+	60.9
K99B-0038	—	+	63.2
K99B-0039	—	+	65.8
K99B-0041	—	+	71.3

known bacteria is not more than 10% of the estimated total species in the world. This indicates that new microorganisms will be isolated by devising isolation methods.

In our preliminary experiment, we observed that colony appearances were different depending on preservation conditions of the soil samples after collection (unpublished results). The highest number and most diverse actinomycete strains were observed when isolation was conducted immediately after collection. Although certain strains were able to be isolated after preservation at room temperature for 1

week, we were not able to isolate actinomycetes from the sample preserved at 5°C. After preservation for 4 weeks, no actinomycete strain was isolated from either the sample stored at room temperature or at 5°C, be-

cause the surfaces of the isolation plates were covered with bacterial colonies (Fig. 8). It seems likely that bacterial cell populations increased during preservation of the soil samples. Dilution of the samples was

Table 7. New bioactive compounds from actinomycetes isolated under unusual conditions.

Environment (Habitat)	Isolation method	Producer	Compound	Activity
Rice field (Soil)		<i>Micromonospora echinospora</i> subsp. <i>armeniaca</i> KMR-593 ^T	Clostomicin ^a	Antibacterial
Marine (Sea water)		<i>Nocardiopsis</i> sp. MM-380	New α -pyrone ^a	Differentiation inducer
Desert (Sand)		<i>Streptomyces pseudoverticillus</i> OM-5186	Panosialin ^a	α -Mannosidase inhibitor
Desert (Sand)		<i>Streptomyces</i> sp. OH-5093	4-Chlorothreonine ^a	Herbicidal
Plant (Pine needle)		<i>Streptomyces</i> sp. K96-5794	Diketopiperazine	Antitumor
Pond (Mud)	Chemotactic	<i>Actinoplanes capillaceus</i> K95-5561 ^T	2-Hydroxyethyl-3-methyl-1,4-naphthoquinone ^a	Antibacterial
Pond (Mud)	Chemotactic	<i>Actinoplanes</i> sp. K95-5076	K95-5076 ^a	Antibacterial
Field (Soil)	Novobiocin	<i>Kitasatospora cineracea</i> SK-3255 ^T	None	
Wood (Soil)	Novobiocin	<i>Kitasatospora griseola</i> AM-9660 ^T	Setamycin ^a	Nematocidal
Field (Soil)	Novobiocin	<i>Kitasatospora niigatensis</i> SK-3406 ^T	None	
Field (Soil)	Novobiocin	<i>Kitasatospora phosalacinea</i> KA-338 ^T	Phosalacine ^a	Herbicidal
Park (Soil)	Novobiocin	<i>Kitasatospora setae</i> KM-6054 ^T	Setamycin ^a	Nematocidal
Garden (Soil)	Gellum gum	<i>Longispora albida</i> K97-0003 ^T	Actinohibin ^a	Anti HIV

^a New compound.

Fig. 9. Structures of new bioactive compounds from actinomycetes isolated under unusual conditions.

needed, moreover, to isolate actinomycete strains. This means that low population strains will be not isolated. The data shown above would suggest that we can isolate new microorganisms by devising appropriate preservation methods for soil samples.

6. Concluding Remarks

We were able to isolate new actinomycete strains using various approaches, including particular habitats and unusual isolation methods, and were able to identify novel bioactive compounds possessing different activities from culture broths of these strains (Table 7). The new compounds and their structures are shown in Fig. 9. Furthermore, we proposed new taxa, including two new genera (*Kitasatospora setae* gen. nov., sp. nov. and *Longispora albida* gen. nov., sp. nov.), five new species (*Actinoplanes capillaceus*, *K. cineracea*, *K. griseola*, *K. niigatensis*, *K. phosalacinea*) and one new subspecies (*Micromonospora echinospora* subsp. *armeniaca*) (Table 7). And we showed further possibilities to explore new microbial resources.

Our results strongly indicate that we may be able to identify novel bioactive compounds by isolating new microorganisms using different approaches.

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