Award Lecture

Studies on the Isolation and Distribution of Rare Actinomycetes in Soil

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INTRODUCTION

The search for novel antibiotics and other bioactive microbial metabolites for potential agricultural, pharmaceutical and industrial applications has been, and still is, important (Cross, 1982; Williams & Wellington, 1982; Okami & Hotta, 1988; Goodfellow & O'Donnel, 1989; Labeda & Shearer, 1990; Takahashi & Omura, 2003; Berdy, 2005). Members of the actinomycete genus *Streptomyces* have long been recognized as prolific producers of useful bioactive compounds (Tanaka & Omura, 1990), providing more than half of the naturally occurring antibiotics discovered to date and continuing to be a major source of new bioactive metabolites (Miyadoh, 1993). However, the rate of discovery of new metabolites from these common and ubiquitous actinomycetes has declined (Kurtböke *et al.*, 1992). In modern natural-product screening strategies, the adoption of improved methodologies for isolating the uncommon and less studied rare actinomycetes is required to avoid re-isolating strains that produce known bioactive metabolites and to improve the quality of the natural products screened (Lazzarini *et al.*, 2001; Takahashi & Omura, 2003; Berdy, 2005).

We have developed various methods for isolating desirable rare actinomycete genera from natural habitats (Nonomura & Hayakawa, 1988; Hayakawa, 1990, 1994, 2003; Hayakawa & Nonomura, 1993). These methods include a variety of pretreatment techniques in combination with enrichment techniques that appropriately supplement agar media with selective antimicrobial agents. The present paper reviews a range of selective isolation methods that we have developed for the rare actinomycete genera *Actinoplanes*, *Actinokineospora*, *Acrocarpospora*, *Actinosynnema*, *Actinomadura*, *Amycolatopsis*, *Catenuloplanes*, *Cryptosporangium*, *Dactylosporangium*, *Kineospora*, *Kutzneria*, *Microbispora*, *Micromonospora*, *Microtetraspora*, *Nocardia*, *Nonomuraea*, *Thermomonospora*, *Pseudonocardia*, *Thermobifida*, *Saccharomonospora*, *Spirilliplanes*, *Streptosporangium*, and *Virgosporangium*. Results of investigating the occurrence and distribution of these rare actinomycetes in Japanese soils are also reviewed.

NEW ISOLATION MEDIA

Because actinomycetes are at a competitive disadvantage when grown on agar media in association with other soil-

inhabiting microbes, isolation media must be designed to reduce the development of competing microbes without adversely affecting actinomycete propagules (Cross, 1982; Williams *et al.*, 1982; Goodfellow & O'Donnel, 1989). Hayakawa and Nonomura (1987a,b) formulated humic acid-vitamin (HV) agar, a new medium containing soil humic acid as the sole carbon and nitrogen source. Humic acids are extremely heterogeneous cross-linked polymers (Kumada, 1975) and generally are resistant to biological decomposition. However, actinomycetes have been shown to utilize humic acids (Federov & Ilyina, 1963; Yanze & Blondeau, 1991; Dari *et al.*, 1995). Hayakawa and Nonomura (1987a,b) also demonstrated that HV agar was useful for efficient recovery and adequate growth of streptomycetes and various rare actinomycetes, while restricting growth of non-filamentous bacteria colonies. The HV agar also supported good sporulation for these actinomycetes, which offers a considerable advantage for rapid morphological identification. To successfully eliminate bacterial contaminants, HV agar was supplemented with synthetic antibacterial agents, nalidixic acid and trimethoprim (Hayakawa *et al.*, 1996a). In addition, another medium, LSV-SE agar containing a Kraft lignin as the carbon and nitrogen source, was formulated (Hayakawa *et al.*, 1996b) to facilitate an increased recovery of *Microtetraspora* spp.

SELECTIVE ISOLATION METHODS

In natural soil habitats, streptomycetes are common and are usually a major component of the total actinomycetes population. Therefore, to selectively isolate a particularly rare genus of actinomycetes, techniques that enhance the desirable actinomycetes in natural habitat samples (enrichment) or eliminate undesirable streptomycetes and other contaminants from the isolation plate media (pretreatment) must be employed. We have developed various enrichment and pretreatment techniques in our laboratory to selectively isolate rare actinomycetes, as well as to successfully increase the selectivity of the isolation media for desirable rare actinomycetes with the supplementation of several antibiotics (summarized in Table 1). Photographs of specific chambers and plates used for the enrichment of rare actinomycetes of natural habitat (soils and root fragments) are shown in Figure 1 and preparations of actinomycetes on HV isolation plates after pretreatment or enrichment are

Table 1. Summary of methods developed for the selective isolation of rare actinomycete from soil (1987–2007)

Pretreatment	Culture media	Genera selected	Reference
<u>Physical:</u>			
None	HV agar with or without nalidixic acid and trimethoprim	<i>Streptomyces</i> and other genera	Hayakawa & Nonomura (1987a,b), Hayakawa <i>et al.</i> (1996a)
Dry heat at 120 °C for 1 h	HV agar with nalidixic acid	Many rare actinomycete genera including <i>Spirilliplanes</i> **	Hayakawa <i>et al.</i> (1991a) Tamura <i>et al.</i> (1997)
<u>Chemical:</u>			
SDS 0.05% and Yeast extract 5%	HV agar with nalidixic acid	<i>Streptomyces</i> and other genera	Hayakawa & Nonomura (1989)
Phenol 1.5%	HV agar with nalidixic acid and tunicamycin	<i>Micromonospora</i>	Hayakawa <i>et al.</i> (1991a)
Phenol 1.5%	HV agar	<i>Streptomyces violaceusniger</i> cluster	Hayakawa <i>et al.</i> (2004)
Chloramine-T	HV agar with nalidixic acid	<i>Herbidospora</i> , <i>Microbispora</i> , <i>Microtetraspora</i> , <i>Nonomuraea</i> and <i>Streptosporangium</i>	Hayakawa <i>et al.</i> (1997)
<u>Physical and chemical:</u>			
Dry heat at 110 °C for 1 h and phenol 1.0%	HV agar with kanamycin, josamycin, lysozyme and nalidixic acid	<i>Actinomadura viridis</i>	Hayakawa <i>et al.</i> (1995a)
Dry heat at 120 °C for 1 h and phenol 1.5%-CG 0.01%	HV agar with nalidixic acid	<i>Microbispora</i>	Hayakawa <i>et al.</i> (1991a)
Dry heat at 120 °C for 1 h and BC 0.01% (or 0.03%)	HV agar with nalidixic acid and leucomycin (or tunicamycin)	<i>Streptosporangium</i> or <i>Dactylosporangium</i>	Hayakawa <i>et al.</i> (1991b)
Dry heat at 110 °C for 1 h and BC 0.05%	LSV-SE agar with kanamycin, nalidixic acid and norfloxacin	<i>Microtetraspora</i>	Hayakawa <i>et al.</i> (1996b)
<u>Enrichment:</u>			
Chemotaxis (γ -collidine, vanillin)	HV agar with nalidixic acid	<i>Actinoplanes</i> , <i>Catenuloplanes</i> , <i>Dactylosporangium</i> , <i>Virgosporangium</i> **	Hayakawa <i>et al.</i> (1991c, 1995b) Tamura <i>et al.</i> (2001)
<u>Enrichment and physical:</u>			
Pollen-baiting and drying	HV agar with nalidixic acid	<i>Actinoplanes</i>	Hayakawa <i>et al.</i> (1991d), Nonomura <i>et al.</i> (1979)
Rehydration (30 °C, 90 min) and centrifugation (1,500 × g, 20 min)	HV agar with nalidixic acid and trimethoprim	<i>Actinoplanes</i> , <i>Actinokineospora</i> , <i>Actinosynnema</i> , <i>Catenuloplanes</i> , <i>Cryptosporangium</i> **, <i>Dactylosporangium</i> , <i>Geodermatophilus</i> , <i>Kineospora</i> , <i>Sporichthya</i>	Hayakawa <i>et al.</i> (2000) Tamura <i>et al.</i> (1995, 1998, 1999)
CaCO ₃ , rehydration and centrifugation	HV agar with fradiomycin, kanamycin, nalidixic acid and trimethoprim	<i>Actinokineospora</i>	Otoguro <i>et al.</i> (2001a,b)
Sucrose-gradient centrifugation (240 × g, 30 min)	HV agar with nalidixic acid and chlortetracycline	<i>Nocardia</i>	Yamamura <i>et al.</i> (2003a,) b, 2004, 2005)
Moist incubation and drying	HV agar with nalidixic acid and trimethoprim	<i>Streptomyces</i> and other genera	Matsukawa <i>et al.</i> (2007a,b)

*Both HV agar and LSV-SE agar contain the antifungal antibiotic cycloheximide.

**Proposed as new genera.

Fig. 1. Enrichment of actinomycetes to soil samples before plating. (a) A Lucite plate and a capillary filled with chemotaxis buffer containing an attractant for motile actinomycetes, which has been placed in the channel (Hayakawa *et al.*, 1991c); (b) *Pinus* pollen grains floating on a soil suspension as useful baits for motile actinomycetes (Hayakawa *et al.*, 1991d); (c) A soil sample incubated with calcium carbonate to multiply the population of *Actinokineospora* spp. (Otoguro *et al.*, 2001a); (d) Growth of actinomycetes on root fragments of *Perilla frutescens* placed on HV agar (Matsukawa *et al.*, 2007a,b).

shown in Figure 2. Scanning electron micrographs of a diverse range of rare actinomycetes from pretreated and HV-plated samples are shown in Figure 3 to 8. Representative isolation methods are described in detail below.

Physical or chemical pretreatment methods

Nonomura and Ohara (1969) showed that heating air-dried soil samples (120 °C or 100 °C) reduced the numbers of filamentous bacteria and streptomycetes on isolation plates, resulting in the selective isolation of various rare actinomycetes genera. Hayakawa *et al.* (1991a,b) also used dry-heating in combination with chemical pretreatment for highly selective isolation. Thus, to isolate *Streptosporangium* spp., an air-dried soil sample is first subjected to dry heat treatment (120 °C, 1 h); a water suspension of the heated sample is then treated with 0.01% benzethonium chloride (BC), diluted and then cultured on HV agar supplemented with nalidixic acid and leucomycin. The dry-heating and BC treatment drastically eliminate unicellular bacteria and unwanted actinomycetes, including *Streptomyces* spp. from the isolation plates, thereby facilitating the selective isolation of *Streptosporangium* spp. The use of leucomycin in the HV agar increases the selectivity for *Streptosporangium* spp. For isolations from various soils, this method achieved 20% selective isolation of *Streptosporangium* spp. (Hayakawa *et al.*, 1991b). On the other hand, the combined use of dry-heating (120 °C, 1 h) and treatment with a solution of 1.5% phenol and 0.03% chlorhexidine gluconate resulted in a 90% selective isolation of *Microbispora* spp. (Hayakawa *et al.*, 1991a).

Enrichment Methods

An improved chemotactic method developed by Hayakawa *et al.* (1991c) utilizes the strong chemotactic response of actinomycete zoospores to γ -collidine and shows increased recovery of *Actinoplanes* spp. and *Dactyl-*

Fig. 2. Selective isolation plates (HV agar) of different genera of actinomycetes. (a) common actinomycetes; SDS-YE method (Hayakawa & Nonomura, 1989); (b) *Micromonospora*; phenol-tunicamycin method (Hayakawa *et al.*, 1991a); (c) *Microbispora*; dry heat-phenol chlorhexidine gluconate method (Hayakawa *et al.*, 1991a); (d) *Streptosporangium*; dry heat-0.01% benzethonium chloride-leucomycin method (Hayakawa *et al.*, 1991b); (e) motile genera including *Actinoplanes*, *Catenuloplanes*, *Dactylosporangium* and *Kineosporia*; RC method (Hayakawa *et al.*, 2000); (f) *Actinokineospora*; CaCO₃-RC method (Otoguro *et al.*, 2001a).

Fig. 3. Scanning electron micrographs of isolates of monosporic, non-motile actinomycetes (Hayakawa *et al.*, 1991a). (a) *Micro-monospora* sp.; (b) *Saccharomonospora* sp.; (c–e) *Thermobifida* spp.; (f) *Microbispora* sp. Bar intervals: (a–e), 10 µm; and (f), 1 µm.

Fig. 4. Scanning electron micrographs of isolates of oligosporic, non-motile actinomycetes (Hayakawa *et al.*, 1991a,b, 1995a, 1996b, 1997, Yamamura *et al.*, 2003a). (a) *Microbispora* sp.; (b, d, g) *Actinomadura* spp.; (c) *Microtetrastroma* sp.; (e, f) *Nonomuraea* sp.; (h) *Herbidospira* sp.; (i) *Pseudonocardia* sp. Bar intervals: (a–i), 10 µm.

osporangium spp. from various soil samples. Hayakawa *et al.* (1995b) also found that vanillin is successful as a chemoattractant for isolating *Catenuloplanes* spp. from soil; the use of vanillin also enables the isolation of members of the recently discovered genus *Virgosporangium* (Tamura *et al.*, 2001).

Another simple technique using *Pinus* pollen grain baiting was employed to isolate *Actinoplanes* spp. from soil (Hayakawa *et al.*, 1991d). The bait bearing the sporangia in the dried soil particles is desiccated (30 °C, 2 h) with the aid of silica gel and the spores are liberated

upon immersion in water. A portion of the enriched zoospore sample is plated on HV agar containing nalidixic acid. The desiccation stage almost completely eliminates contamination of bacteria from the colonized bait and resulted in 83% selective isolation of *Actinoplanes* spp. More recently, Matsukawa *et al.* (2007a,b) also successfully used desiccation for the selective isolation of *Streptomyces* spp. with anti-phytopathogenic activity from root fragments of healthy herbaceous plants.

The rehydration and centrifugation (RC) method is a simple enrichment method incorporating differential

Fig. 5. Scanning electron micrographs of isolates of sporangiate, non-motile actinomycetes (Hayakawa *et al.*, 1991b, 1997). (a-d) *Streptosporangium* spp.; (e, f) *Kutzneria* spp. Bar intervals: (a-c), 100 µm; and (d-f), 10 µm.

Fig. 6. Scanning electron micrographs of isolates of sporangiate, motile actinomycetes (Hayakawa *et al.*, 1991c,d, 2000). (a-c) *Actinoplanes* sp.; (d, e) *Dactylosporangium* spp.; (f, g) *Virgosporangium* spp.; (h, i) *Cryptosporangium* spp. Bar intervals: (a, d, f), 100 µm; and (b, c, e, g, h, i), 10 µm.

centrifugation for the isolation of motile actinomycetes (Hayakawa *et al.*, 2000). RC consists of immersing air-dried natural habitat samples in 10 mM phosphate buffer containing 10% soil extract, allowing the preparation to stand at 30 °C for 90 min, followed by centrifugation at $1,500 \times g$ for 20 min. The supernatant enriched with actinomycete zoospores is plated on HV agar containing nalidixic acid and trimethoprim. The phosphate buffer-soil extract solution liberates motile zoospores and centrifugation eliminates streptomycetes and other non-motile actinomycetes from the supernatant, thereby facilitating selective

growth of motile actinomycetes on the isolation plates subsequent to inoculation. The RC method consistently achieved 37 to 86% selective isolation of motile actinomycetes from various soil and leaf-compost samples. The most frequently isolated motile actinomycetes were *Actinoplanes* and *Dactylosporangium*; strains of *Actinokineospora*, *Actinosynnema*, *Catenuloplanes*, *Couchioplanes*, *Geodermatophilus*, *Kineospora* and *Sporichthya* were also recovered. In other studies, Otoguro *et al.* (2001a,b) found that the combined use of calcium carbonate treatment with the RC method resulted in the selective isolation of

Fig. 7. Scanning electron micrographs of isolates of oligosporic, motile actinomycetes (Hayakawa *et al.*, 2000, Tamura *et al.*, 1999). (a–c) *Catenuloplanes* spp.; (d–f) *Sporichthya* spp. Bar intervals: (a–d), 10 μ m; (e, f), 1 μ m.

Fig. 8. Scanning electron micrographs of isolates of oligosporic or monosporic, motile actinomycetes (Hayakawa *et al.*, 2000, Otaguro *et al.*, 2001a). (a–c) *Actinokineospora* spp.; (d–f) *Actinosynnema* spp.; (g–i) *Kineosporia* spp. Bar intervals: (h, i), 100 μ m; (a, c–g), 10 μ m; (b), 1 μ m.

Actinokineospora spp. on HV agar supplemented with fradiomycin, kanamycin, nalidixic acid and trimethoprim; Yamamura *et al.* (2003a,b, 2004, 2005) applied sucrose-gradient centrifugation for selective isolation and ecological study of *Nocardia* spp. in soil and sediment samples.

DISTRIBUTION OF ACTINOMYCETES

Hayakawa *et al.* (1988) investigated the distribution of rare actinomycetes in Japanese natural soil habitats using HV agar. Their distribution seemed to be associated with

environmental factors such as soil type, pH, humus content, and the characteristics of the humic acid. They found that *Microbispora* and *Streptosporangium* were most abundant in humus-rich acidic (pH 5.0–7.5) soils with low humic acid $\Delta \log K$ values (black colored humic acid); conversely, *Saccharomonospora* spp. were most abundant in relatively humus-poor alkaline (pH 7.0–7.5) soils with higher $\Delta \log K$ values (brown humic acid). Species of *Dactylosporangium* and *Microtetraspora*, *Saccharomonospora*, and *Micromonospora* were frequently isolated from mountainous forest soils, level-land forest soils, and pasture soils, respectively.

Hayakawa *et al.* (1995c) demonstrated that the incidence of antibiotic producers in *Microbispora* spp. increased with soil pH. In addition, Matsukawa *et al.* (2007b) reported that a significantly higher percentage of organisms with activity against plant pathogens are found among streptomycetes isolated from root samples than for those isolated from soil samples. The mercury volatilization activity of *Streptomyces* spp. distributed in heavy metal-polluted soils was also reported (Hayakawa *et al.*, 1982; Hayakawa & Nonomura, 1983).

CONCLUSIONS

By combining pretreatment with suitable media supplemented with specific antibiotics, diverse rare actinomycete genera that previously were only recovered incidentally by conventional dilution-plating techniques can now be successfully isolated. The range of isolation methods shown in Table 1 is a valuable tool for discovering novel bioactive secondary metabolites. These methods will contribute to improving our knowledge concerning the occurrence, distribution, ecology, taxonomy and evolution of actinomycetes.

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