Published in final edited form as:
J Microbiol Methods. 2008 March ; 72(3): 257–262.

A trap for *in situ* cultivation of filamentous actinobacteria

Ekaterina Gavrish, **Annette Bollmann**, **Slava Epstein**, and **Kim Lewis***
Department of Biology and Antimicrobial Discovery Center, Northeastern University, Boston, Massachusetts 02115

Abstract

The approach of growing microorganisms *in situ*, or in a simulated natural environment is appealing, and different versions of it have been described by several groups. The major difficulties with these approaches are that they are not selective for actinomycetes – a group of gram-positive bacteria well known as a rich source of antibiotics. In order to efficiently access actinomycetes, a trap for specifically capturing and cultivating these microorganisms *in situ* has been developed, based on the ability of these bacteria to form hyphae and penetrate solid environments. The trap is formed by two semi-permeable membranes (0.2 – 0.6 μm pore-size bottom membrane and 0.03 μm pore-size top membrane) glued to a plastic washer with sterile agar or gellan gum inside. The trap is placed on top of soil, and filamentous microorganisms selectively penetrate into the device and form colonies. Decreasing the size of the pores of the lower membrane to 0.2 μm restricted penetration of fungi. The trap produced more filamentous actinobacteria, and a higher variety of them, as compared to a conventional Petri dish cultivation from the same soil sample. Importantly, the trap cultivation resulted in the isolation of unusual and rare actinomycetes.

1. Introduction

Traditionally, the main source for antibiotics has been natural products derived from bacteria and fungi (Demain and Fang, 2000). However, this source has been steadily drying up, which is likely due to repeated isolation and screening of the same microorganisms. Indeed, the rediscovery of common antibiotics from cultivable species is a major obstacle for antibiotic discovery (Baltz, 2006). Such over-mining is not surprising considering the limited number of culturable species, which make up only a fraction of the total diversity in the environment (Osburne et al., 2000). The majority of known antibiotics come from Actinomycetes (Hopwood, 2006) and developing methods to access new representatives of this group of microorganisms is of particular importance.

We previously introduced a method of *in situ* cultivation that bypassed the difficulties of replicating the natural environment inherent in traditional Petri dish-based approaches (Kaeberlein et al., 2002). That method is based on placing bacteria in a diffusion chamber that is then introduced back into the environment from which the sample originated. The diffusion chamber is a mix of agar and diluted environmental sample sandwiched between two semi-permeable membranes glued onto a washer. The chamber allows for a free diffusion of chemicals while restricting the movement of cells, and allowed for the cultivation of up to 40% of bacterial cells from a marine sediment environment, as compared to 0.05% that grew on a Petri dish (Kaeberlein et al., 2002). We found that the diffusion chambers can be used to grow

* Corresponding author. Mailing address: Department of Biology, 134 Mugar Hall, 360 Huntington Avenue, Boston, MA 02115. Phone: (617) 373-8238. Fax: (617) 373-3724. E-mail: k.lewis@neu.edu.

soil microorganisms as well, but only a small portion of the bacteria that grow in the chamber are actinomycetes.

Here we describe a novel method for targeted isolation of Actinomycetes. This method employs a diffusion chamber in a very different way: as a trap for filamentous microorganisms. While in the original approach (Kaeberlein et al., 2002), the diffusion chamber is inoculated with target organisms, and is then incubated in the natural environment from which these organisms originate, the trap is filled with sterile agar and placed back in the environment. The expectation is that filamentous actinomycetes would penetrate through the pores of the membrane (Hirsch, Christensen, 1983; Polsinelli, Mazza, 1984) and grow in the unoccupied space within the trap. The conditions inside the trap will closely mimick those outside of the trap, leading to growth of filamentous species. This study describes the successful application of the trap method to cultivate novel Actinomycetes.

2. Materials and Methods

2.1. Soil sampling

Garden soil samples were collected in Verrill Farm (Massachusetts, USA) in October 2005 and Pasadena (California, USA) in February 2006. The pine soil sample was collected in Dover (Massachusetts, USA) in September 2006. Samples were transported to the laboratory in Boston (Massachusetts, USA) and stored at room temperature before use.

2.2 Media used for isolation of bacteria

For all traps 1% agar or 1.2% gellan gum supplemented with a 1% vitamin supplement (ATCC) was used as a cultivation medium. For pine soil traps the pH of the medium was adjusted to 4 by adding a few drops of HCl.

For standard plating isolation CN agar medium (0.1% Casamino acids, 0.1% Nutrient broth (Difco), 1% Bacto agar), CN gellan gum medium (CN medium with 1.2% gellan gum) and Actinomycete Isolation (AI) Agar (Difco) were used. For isolation from the pine soil sample the pH of the media was adjusted to 4 by adding a few drops of HCl. Nystatin (50 μg/ml) and cycloheximide (100 μg/ml) were added to the media in order to prevent the growth of fungi.

2.3. The trap design and in situ cultivation

To form the trap, a 0.2- to 0.6- μm pore-size polycarbonate membrane (47 mm diameter, Isopore, Millipore, USA) was glued to the bottom of a nylon washer (56 mm outer diameter, 35 mm inner diameter, 3 mm thickness, # SPS-2209-1375-0125, from WashersUSA.com), and 3 ml of sterile 1% agar or 1.2% gellan gum with vitamin supplement was poured onto the filter. After the medium solidified, a top polycarbonate membrane (0.03-μm pore-size, 47 mm in diameter, Osmonics Inc., USA) was glued to the washer sealing the trap (Fig. 1). The membranes and washer were autoclaved prior to use. A diffusion chamber formed by two 0.03-μm pore-size filters with sterile medium inside was used as a negative control. All manipulations were made aseptically to prevent contamination. After the glue dried, the traps were placed on top of moist soil, insuring that the bottom filter was in good contact with soil.

Large Petri dishes (15 cm diameter) were used as a soil reservoir. The plates were sealed with parafilm to prevent evaporation and were incubated for 14–21 days at room temperature in the dark.

2.4. Isolation of bacteria with traps

After incubation the traps were opened, the solid agar or gellan content was removed in one piece, inverted and placed into a sterile Petri dish. The solid disks were examined under a

stereomicroscope at 20–100x magnification (Discovery V12, Zeiss, Germany). Visible microcolonies were picked with sterile needles and streaked out on plates with CN agar or CN gellan gum medium. Subcultivation was repeated to obtain pure cultures.

An additional incubation of the medium from the trap for 5–7 days allowed for some actinomycetes to form an aerial mycelium, which made it easier to pick and subcultivate microcolonies. To prevent drying several drops of water were added to the Petri dish that contained trap medium.

2.3. Isolation of bacteria by a standard isolation method

One gram of air-dry soil was mixed with 9 ml sterilized water and vortexed. After the soil particles settled, serial dilutions were made and a 100 μl aliquot from each dilution was plated on CN agar, CN gellan gum, and Actinomycetes agar plates. After two weeks of incubation individual colonies were randomly picked and streaked out to obtain pure cultures.

2.4. Sequencing of 16S rRNA genes and phylogenetic analysis

For identification purposes, single colonies were suspended in PCR grade water with approximately 0.05 g of zirconium beads (0.1 mm, BioSpec Products, Inc. USA) and vortexed for 5 minutes at maximum speed. The supernatants were used for PCR amplification with actinobacteria-specific primers Act-235F (5′ CGCGGCCTATCAGCTTGTTG) and Act-878R (5′ CCGTACTCCCCAGGCGGG) (modified after (Stach et al., 2003) and GoTaq Green Master Mix (Promega, USA) with an annealing temperature of 60° C running for 30 cycles. The PCR products were purified and sequenced commercially (Seqwright, Houston Texas, USA) using Fluorescent dye terminator. The sequences were edited using 4Peaks software (http://mekentosj.com/4peaks/), aligned to the ARB database (Ludwig et al., 2004), and added to the tree in the ARB database with the parsimony addition tool. Nucleotide sequences obtained in this study have been deposited in the GenBank database under accession numbers EF599956 – EF600030, EF601722, EF601723

3. Results and Discussion

Mycelia of filamentous Actinobacteria can grow through solid substrates, and have been shown to penetrate pores as small as 0.22 μm (Hirsch and Christensen (1983) and Polsinelli and Mazza (1984). This ability was used for selective isolation of actinomycetes from mixed populations (soil, water, vegetable materials) on the surface of cellulose membranes placed onto nutrient agar (Hirsch and Christensen, 1983; Polsinelli and Mazza, 1984). During the incubation, actinomycetes mycelia penetrated through the filter pores to the underlying agar medium and formed colonies, whereas other bacteria and fungi were restricted from the agar by the membranes. This procedure is simple and suitable for actinomycetes isolation but requires cultivation in Petri plates and does not simulate the natural environment. We reasoned that we could similarly select for actinobacteria and enable growth of species that do not grow in Petri dishes by a reverse use of the diffusion chamber (Kaeberlein et al., 2002). In this way, a diffusion chamber is not inoculated with cells, but rather placed into the environmental sample empty, and then traps filamentous microorganisms that penetrate its membrane. Traps were initially incubated on top of garden soil (collected in California and transported to the home lab). After 14 days of incubation the traps were opened, and the solid agar or gellan content was removed and examined. A great majority of colonies formed well-developed vegetative mycelia that were easily detected under the stereomicroscope. This showed that the trap could indeed selectively capture filamentous organisms that penetrated the pores of the filters and settled in the unoccupied space of the chamber. The trap with 0.4 μm and larger pore size filters contained both bacteria and fungi (Fig. 2A). The fungi grew more rapidly than the actinomycetes and filled the entire trap with fungal hyphae, effectively spreading throughout

the trap, making isolation of actinomycetes impractical. Actinomycetes form thinner hyphae compared to fungi, and it seemed possible to selectively trap them by using a membrane with smaller pores that would exclude fungi. Decreasing the pore diameter from 0.4 to 0.2 μm indeed excluded fungi, and the trap was found to primarily contain colonies of filamentous actinomycetes (Fig. 2B).

The traps with 0.2 μm pore-size bottom filters were used for further experiments. The solid media slabs from traps were incubated for an additional 5–7 days, after which well-separated aerial mycelia were apparent in a number of cases (Fig. 3). Colonies were picked with sterile needles and purified by streaking them out on agar plates with CN medium. The majority of isolates showed good growth after approximately one week of cultivation.

In order to survey the actinomycetes obtained by conventional Petri dish plating, an inoculum was prepared from the same garden soil sample collected in California that served for *in situ* cultivation. Approximately 90 colonies were picked randomly from the traps and the Petri dishes containing either agar or gellan gum.

Of the ~90 randomly picked colonies, 69 isolates recovered from the agar trap and 81 isolates from the gellan gum traps were actinobacteria (Table 1). The diversity of isolates obtained from the gellan gum trap was higher compared to the agar trap. Representatives from 11 actinobacterial genera were isolated from gellan, and most of the isolates formed mycelia. Strains of *Streptomyces*, together with *Agromyces*, *Cellulomonas* and *Cellulosimicrobium* represented the dominant groups among the isolates. Based on 16S rRNA gene sequence data, these isolates are closely related to at least eight streptomycetes species, two different *Agromyces* and three different *Cellulomonas* species (Table 2). All isolates from *Cellulosimicrobium* were closely related to the same species - *C. cellulans* with 98.8– 100% 16S rRNA gene sequence identity. Additionally, isolates from 8 other genera were obtained, including relatives of *Actinoplanes*, *Kribbella, Nocardioides* and *Promicromonospora* (Table 1 and 2).

Among the isolates from the agar trap, the majority of the isolates belong to the genus *Streptomyces* (Table 1). In addition, nine *Kitasatospora* strains, two different *Actinoplanes*, and *Dactylosporangium* closely related to *D. aurantiacum* were isolated (Table 2). The overlap between the two different traps (agar and gellan gum) was small; only relatives of *Actinoplanes italicus* and several *Streptomycetes* strains were shared between the traps (Table 2), indicating that agar and gellan gum may select for different actinobacterial strains.

Within the strains isolated from direct plating the majority were not Actinobacteria. Among the actinobacterial isolates most of the strains belong to *Arthrobacter, Microbacterium* and *Streptomycetes*, with several additional strains present as single isolates (Table 1). The isolates from the plates appeared to contain relatively more non-filamentous actinobacterial species as compared to the traps, for example *Arthrobacter, Microbacterium, Microsphaera, Subtercola, Terrabacter, Williamsia* etc. (Tables 1 and 2). Some filamentous actinobacteria such as *Streptosporangium, Micromonospora* and *Nonomuraea* strains were only found on plates but not in the traps. The overlap between the plates and the traps was small. Only relatives of *Agromyces, Microbacterium* and several *Streptomycetes* strains were shared between the plates and traps (Table 2).

We also tested the ability of the trap to capture various actinomycetes from two additional soil samples. We used a garden soil sample collected in Massachusetts and stored at room temperature for more than one year, and a pine soil sample also collected in Massachusetts (the sample had pH 3.9–4 and very high fungal diversity). The traps were made and maintained the same way as described above. Only traps with 0.2 μm pore-size bottom filter were used in the experiment. The vast majority of the microorganisms captured in the trap were mycelium-

forming actinobacteria with a very small number of other non-actinobacterial isolates. Different species of *Streptomyces* were dominant among the isolates. In addition several rare and unusual actinobacterial strains were isolated, including Streptacidophilus, Catellatospora, Lentzea and the recently described new genus *Catenulispora* (Busti et al., 2007), that were not captured in the parallel experiment by Petri plate cultivation (Table 3).

From this first evaluation of the trap for *in situ* cultivation, we conclude that:

- The trap allows for specific capture of filamentous actinobacteria;
- Decreasing the filter pore size to 0.2 μm effectively excludes fungi and allows for specific capture of actinomycetes, even in the absence of antifungal agents;
- The majority of the organisms captured in the trap are actinomycetes, including representatives of unusual and rare groups such as *Dactilosporangium*, *Catellatospora*, *Catenulispora*, *Lentzea*, *and Streptacidiphilus*;
- Gellan gum favors captured of more diverse actinobacterial groups compared to agar;
- The trap favors filamentous actinobacteria compared to the Petri dish, which produces more non-filamentous isolates.

Soil is rich in actinomycetes species, and any method, including the trap, probably captures the most abundant ones from a given environment. At the same time, the trap has the potential of selectively enriching actinomycetes, and could probably do this in an environment relatively poor in these organisms, where rare species are likely to be found. Our data show that the trap for *in situ* actinomycetes cultivation is a promising technique for gaining access to interesting isolates that are not recovered by conventional Petri dish cultivation.

Acknowledgements

We are grateful to Dr. Eric Stewart for assistance with the manuscript. This work was supported by grants NIH AI059489-01 and DOE DE-FG02-04ER63782.

References

Baltz RH. Marcel Faber Roundtable: is our antibiotic pipeline unproductive because of starvation, constipation or lack of inspiration? J Ind Microbiol Biotechnol 2006;33:507–513. [PubMed: 16418869]

Busti EL, Cavaletti P, Monciardini P, Schumann M, Rohde M, Sosio S, Donadio. *Catenulispora acidiphila* gen. nov., sp. nov., a novel mycelium-forming actinomycete and proposal of *Catenulisporaceae* fam. nov. Int J Syst Evol Microbiol 2006;56:1741–1746. [PubMed: 16902001]

Demain, AL.; Fang, A. The natural functions of secondary metabolites. In: Fiechter, IA., editor. History of Modern Biotechnology. Springer; Berlin: 2000. p. 1-39.

Hirsch CF, Christensen DL. Novel method for selective isolation of actinomycetes. Appl Environ Microbiol 1983;46:925–929. [PubMed: 6639036]

Hopwood DA. Soil to genomics: the Streptomyces chromosome. Annu Rev Genet 2006;40:1–23. [PubMed: 16761950]

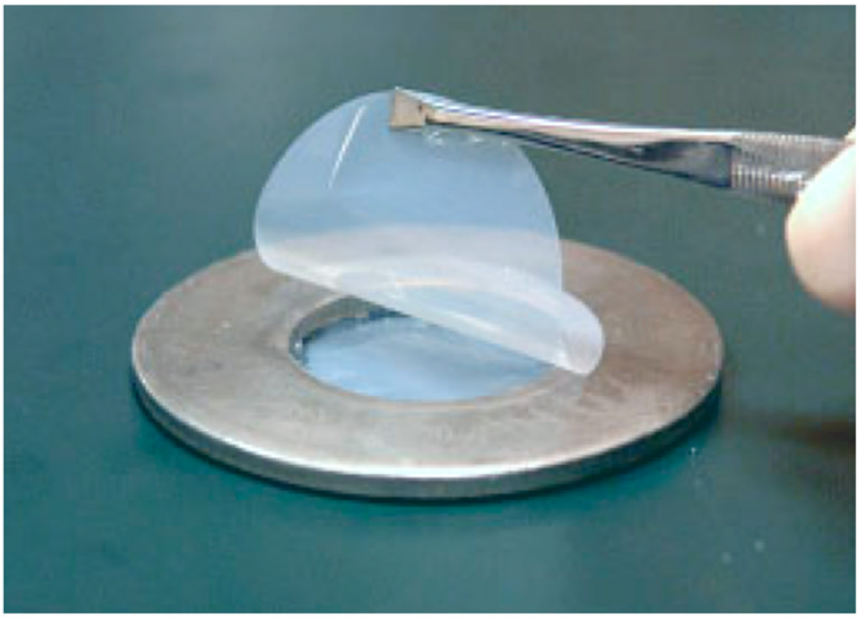
Kaeberlein T, Lewis K, Epstein SS. Isolating “uncultivable” microorganisms in pure culture in a simulated natural environment. Science 2002;296:1127–1129. [PubMed: 12004133]

Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar, Buchner A, Lai T, Steppi S, Jobb G, Forster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, Konig A, Liss T, Lussmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A, Schleifer KH. ARB: a software environment for sequence data. Nucleic Acids Res 2004;32:1363–1371. [PubMed: 14985472]

Osburne MS, Grossman TH, August PR, MacNeil IA. Tapping into microbial diversity for natural products drug discovery. ASM News 2000;66:411–417.

Polsinelli M, Mazza PG. Use of membrane filters for selective isolation of actinomycetes from soil. FEMS Microbiol Lett 1984;22:79–83.

Stach JE, Maldonado LA, Ward AC, Goodfellow M, Bull AT. New primers for the class Actinobacteria: application to marine and terrestrial environments. Environ Microbiol 2003;5:828–841. [PubMed: 14510836]



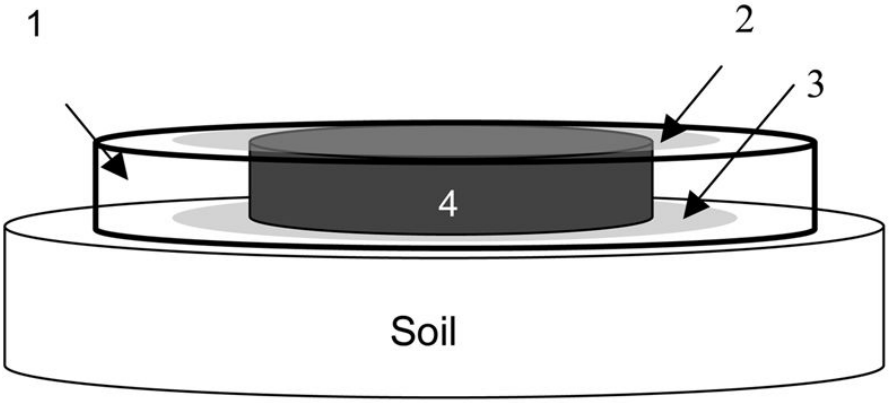


Fig. 1. Image and diagram of the trap

1, plastic or metal washer; 2, top membrane filter (0.03-μm pore-size); 3, bottom membrane filter (0.2- to 0.6-μm pore-size); 4, agar or gellan gum.

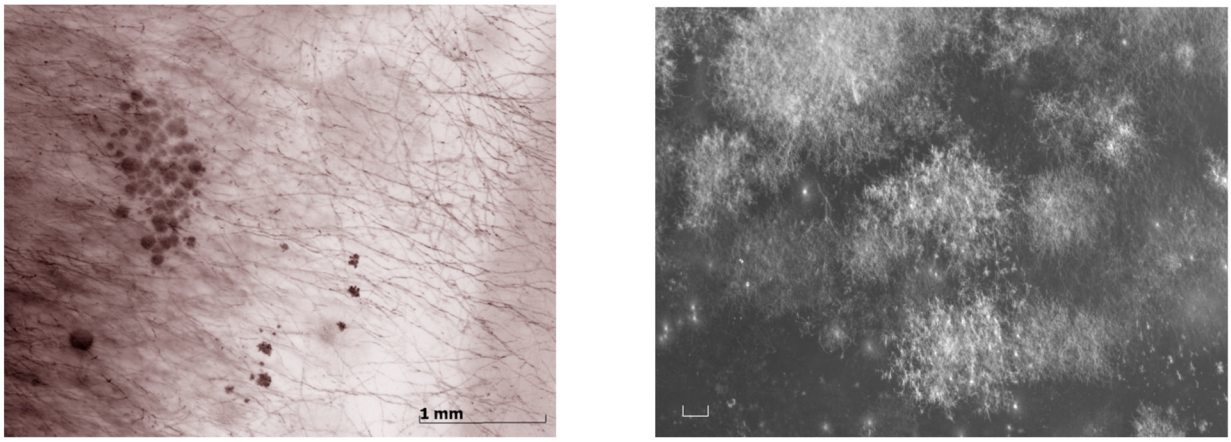


Fig. 2. Microorganisms growing in the trap

The traps were opened after 2 weeks of cultivation. a. Bacterial colonies and microfungal hyphae, agar-base trap with 0.4-μm pore-size bottom membrane. Bar, 1 mm.
b. Actinomycetes-like microcolonies, gellan gum trap with 0.2-μm pore-size bottom membrane. Bar, 0.1 mm.

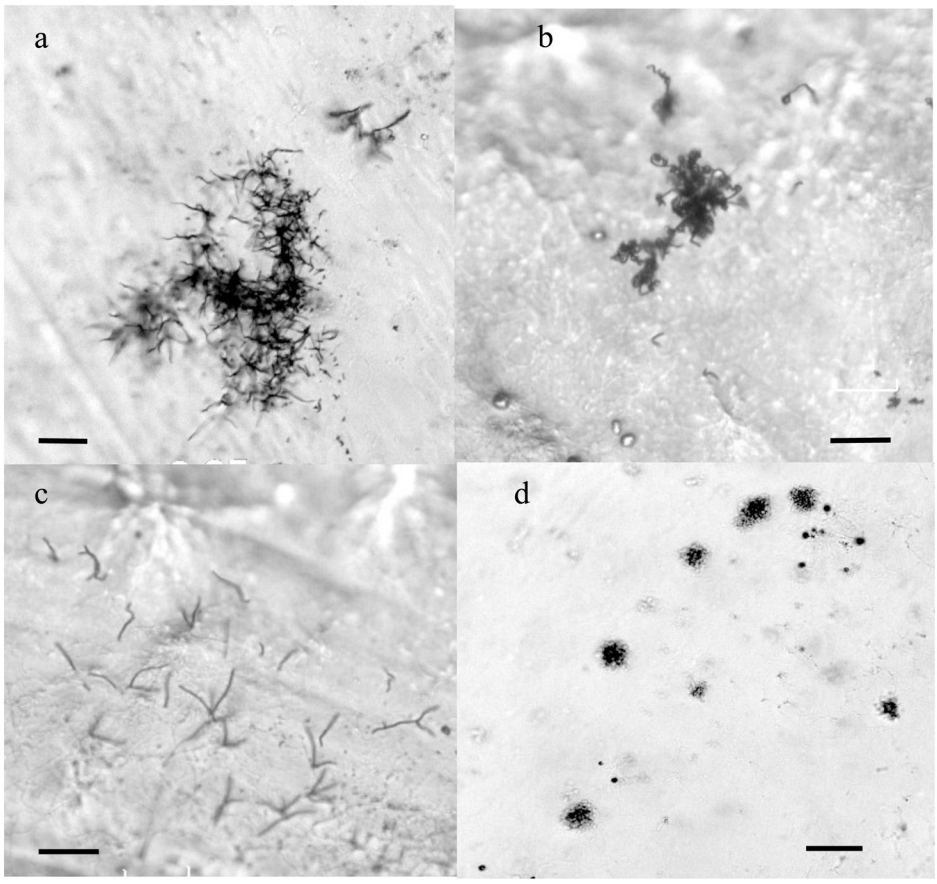


Fig. 3. Microcolonies of Actinobacteria from the trap

The trap was incubated for an additional week at room temperature after it was opened. Actinobacterial aerial (a, b, c) and substrate (d) mycelia. Gellan gum trap with 0.2-μm pore-size bottom membrane. Bar, 0.05 mm

Table 1

Number of actinobacterial strains isolated from garden soil sample.

Genus	Agar trap	Gellan gum trap	Actinomycetes agar	CN gellan gum medium	CN agar medium
Actinoplanes	3	3			
Agromyces		11	3		
Arthrobacter			4	5	11
Cellulomonas		19			
Cellulosimicrobium		14			
Dactylosporangium	1				
Frigoribacterium		1			
Kineosporia					1
Kitasatospora	9				
Kribbella		4			
Microbacterium		2	3	4	4
Micromonospora				2	
Microsphaera				2	
Mycobacterium			1		
Nocardioides		1		1	
Nonomuraea			1		
Oerskovia		4			
Promicromonospora		4			
Rhodococcus			1	7	
Streptomyces	56	18	9	20	8
Streptosporangium			1		
Subtercola			1		
Terrabacter					1
Williamsia			1		
Total number	69	81	60	87	63
Non-actinobacterial strains			35	46	38
Actinobacterial strains	69	81	25	41	25

Table 2

List of the strains isolated from traps and plates and their closest relatives based on 16S rRNA gene partial sequences.

Closest match	Accession number	% similarity	Agar trap	gellan gum trap	AI agar	CN gellan	CN agar	Sequenced strain	Accession number
Actinoplanes derwentensis	AB036999	99.5		1				CATR-197	EF600021
Actinoplanes italicus	AB037005	99.8–100	1	2				CATR-14	EF600022
Actinoplanes pallidoaurantiacus	AJ277578	99.7	2					CATR-7	EF600020
Agromyces sp.	AJ252586	99.7–100		10	3			Act-77	EF600006
Agromyces ulmi	AY427830	98.6		1				CATR-150	EF600007
Arthrobacter bergeri	AJ609632	98.6–98.8			1	1	2	SM-42	EF601722
Arthrobacter globiformis	AY167856	99.5–99.7			3	2	7	SM-8	EF599994
Arthrobacter histidinolovorans	AF501358	99.8–100				2	1	SM-31	EF601723
Arthrobacter sp	AY238502	99.8					1	SM-63	EF6000??
Cellulomonas fimi	X83803	99.5–99.8		5				CATR-124	EF600010
Cellulomonas humilata	X82449	99.5–100		13				CATR-118	EF600009
Cellulomonas sp	AF060791	99.3		1				CATR-192	EF599996
Cellulosimicrobium cellulans	AB116667	98.8–100		14				CATR-146	EF599997
Dactylosporangium aurantiacum	D85480	99.8	1					CATR-61	EF600019
Frigoribacterium sp.	AJ297441	96.3		1				CATR-116	EF600008
Kineosporia rhizophila	AB003933	99.5					1	SM-48	EF600013
Kitasatospora nipponensis	AY442263	99.0–100	9					CATR-80	EF599993
Kribbella fulva	AF005017	99.8		1				CATR-139	EF600026
Kribbella sp.	AY253866	99.8		3				CATR-178	EF600025
Microbacterium imperiale	AB042081	99.3–99.5		1		1		G-78	EF599999
Microbacterium sp.	AY040877	99.5					3	SM-55	EF600001
Microbacterium sp.	AB042083	100				1		G-47	EF600000
Microbacterium sp.	AB042070	99.7		1	1	1	1	SM-47	EF600002
Microbacterium terregens	AB004721	100			1	1		Act-60	EF600003
Microbacterium testaceum	X77445	100			1			Act-103	EF599998
Micromonospora lacustris	X92622	100				1		G-71	EF600018
Micromonospora sp.	AY221486	99.8				1		G-74	EF600017
Microsphaera multipartita	Y08541	99.8				2		G-96	EF600014
Mycobacterium madagascariense	X55600	98.3			1			Act-64	EF599976
Nocardioides plantarum	X69973	99.8				1		G-51	EF600024
Nocardioides sp.	AY423719	96.5		1				CATR-114	EF600023
Nonomuraea turkmeniaca	AF277201	99.8			1			Act-73	EF600016
Oerskovia paurometabola	AJ314851	100		4				CATR-180	EF600011
Promicromonospora sukumoe	AJ272024	99.3–99.5		4				CATR-188	EF599995
Rhodococcus opacus	AF095715	100			1	6		G-50	EF599977

Closest match	Accession number	% similarity	Agar trap	gellan gum trap	AI agar	CN gellan	CN agar	Sequenced strain	Accession number
Rhodococcus sp.	AB023374	99.3				1		G-81	EF599978
Streptomyces clavuligerus	AB045869	99.7					2	SM-23	EF599990
Streptomyces coelicolor	AL356612	100	2	1				CATR-54	EF599981
Streptomyces laceyi	AY094367	98.8–99.0	5	1	4			Act-100	EF599986
Streptomyces lateritius	AY277559	99.1–100		2				CATR-149	EF599992
Streptomyces neyagawaensis	AJ399493	99.5–100	14		3	16	1	G-22	EF599988
Streptomyces sacchari	AF306660	99.7	1	1				CATR-9	EF599985
Streptomyces sp.	AY237556	99.5–99.7	14	1		1		CATR-60	EF599987
Streptomyces sp.	AF101414	99.7–100	3	1	1		3	CATR-4	EF599989
Streptomyces sp.	AY114179	99.8					1	SM-10	EF599991
Streptomyces sp.	AJ001433	99.8	1					CATR-40	EF599979
Streptomyces sp.	AF060793	99.8			1			Act-99	EF599980
Streptomyces sp.	AF112169	98.5–99.1	7	9				CATR-13	EF599982
Streptomyces sp.	AY465202	98.6	1					CATR-41	EF599984
Streptomyces sp.	AY465216	99.8–100	8	2		3	1	CATR-86	EF599983
Streptosporangium brasiliense	X89937	99.8			1			Act-98	EF600015
Subtercola pratensis	AJ310412	100			1			Act-78	EF600005
Terrabacter tumescens	AF005023	98.6					1	SM-59	EF600012
Williamsia murale	Y17384	99.3			1			Act-84	EF599975
Other non-actinobacterial strains					35	46	38		

Table 3

List of non-streptomycetes mycelium-forming actinomycetes, isolated by traps from soil samples collected in Massachusetts, USA.

Strain	accession number	Closest relative	accession number	% similarity	soil sample
MS-2 (2)*	EF599956	*Actinoplanes utahensis*	AJ277574	99–100	Garden soil
MS-8 (3)	EF599960	*Actinoplanes italicus*	AB037005	98–100	Garden soil
MS-G2	EF599974	*Actinoplanes roseosporangius*	AJ277583	99	Garden soil
MS-13	EF599961	*Amycolatopsis keratiniphila*	AJ508238	96	Garden soil
MS-E4	EF599972	*Catellatospora coxensis*	AB200232	100	Garden soil
MS-18	EF599965	*Kribbella koreensis*	AY253865	99	Garden soil
MS-15	EF599963	*Lentzea albidocapillata*	X84321	100	Garden soil
MS-6 (2)	EF599958	*Lentzea flaviverrucosa*	AF183957	98–99	Garden soil
MS-5	EF599957	*Lentzea sp.*	DQ008601	100	Garden soil
MS-17	EF599964	*Nocardioides albus*	AF005004	100	Garden soil
MS-10 (4)	EF599962	*Nocardioides luteus*	AF005007	100	Garden soil
PS–1 (3)	EF600027	*Catenulispora* sp. Neo15	AJ865860	100	Pine soil
PS-3-2 (5)	EF600030	*Kitasatospora kifunense*	U93322	99–100	Pine soil
PS–37 (4)	EF600029	*Streptacidiphilus sp.* Aac-32	AB180775	99–100	Pine soil

* The numbers in parentheses are the numbers of sequenced strains.