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Isolation of Novel Bacteria and Actinomycetes Using Soil-Extract Agar Medium

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Novel bacteria were discovered using an isolation technique consisting of (i) selection of microorganisms that grew on soil-extract agar medium, but not on conventional media, and (ii) detection of small microbial colonies with a microscope. Three bacterial strains thus isolated were provisionally designated Shinshu-th1, -th2, -th3, and five actinomycete strains, Shinshu-MS-01, -02, -03, -04, -05, respectively. Sequence analysis of their 16S rDNA showed that th1 had 95–96% homology with three unculturable bacteria, and th2 had 96% similarity to *Bradyrhizobium* sp., one unculturable and one unidentified bacterial strain. A phylogenetic study indicated that both strains were α-Proteobacteria belonging to the order *Rhizobiales* and the family *Bradyrhizobiaceae*. Since they had low homology (96%) with their close relatives, it is possible that th1 and th2 belong to a new genus. The actinomycetes Shinshu-MS-02 and -03 had 95–96% homology with four strains of *Actinomadura*, -04 had 95–96% similarity to *Streptosporangium* and *Microbispora*, and -05 had 97–98% homology with three strains of *Acrocarpospora*, *Herbidospora* and *Planotetraspora*. According to the phylogenetic study, both 02 and 03 are possibly new species of *Actinomadura*, -04 of *Streptosporangium*, and -05 of *Acrocarpospora*. Shinshu-th3 and -MS-01 were identified as *Mycobacterium cookii* and *Frankia* sp., respectively, having 99% homology with these species.

[**Key words:** micro-colony-forming bacteria, soil-extract agar medium, *Bradyrhizobiaceae* of new genus, new species of Actinomycetes]

It has become clear that less than 1% of microorganisms in the natural environment can be cultivated using conventional culture techniques (1): culture media, culture conditions and detection of microbial colonies with the naked eye. Thus, from the viewpoint of applied microbiology, it is necessary to develop new methodologies that allow us to cultivate and isolate unculturable microorganisms, leading to the exploitation of their new functions.

In the field of investigational search for new biologically active substances, where more than 10,000 secondary metabolites of microbial origin have been discovered, the possibility of finding a new compound with practical significance using conventional methodologies of microbial isolation and assay is remote. A new collection of novel microorganisms is needed. Particularly, actinomycetes are important microorganisms since more than 90% of practical antibiotics originate from them, and two thirds of 10,000 biologically active substances of microbial origin are produced by them.

Thus, we have studied the cultivation and isolation of microorganisms that nature can cultivate but researchers cannot (2).

This paper deals with the discovery of several bacterial and actinomycete strains that grow on soil-extract agar medium but not on conventional media.

MATERIALS AND METHODS

Microbial source Soil samples were collected in a forest of Japanese *larch* belonging to the campus of Shinshu University located at Minamiminowa-mura, Nagano, Japan.

Culture media Culture media used for bacterial growth were as follows. Luria–Bertani medium (LB) consisted of 10 g/*l* peptone, 5 g/*l* yeast extract and 5 g/*l* NaCl. Nutrient broth medium (NB) consisted of 10 g/*l* Polypepton, 5 g/*l* meat extract and 5 g/*l* NaCl. Yeast starch medium (YS) contained 2 g/*l* yeast extract and 10 g/*l* soluble starch. R medium (R) consisted of 10 g/*l* Polypepton, 5 g/*l* yeast extract, 5 g/*l* malt extract, 5 g/*l* casamino acids, 2 g/*l* meat extract, 2 g/*l* glycerol, 50 mg/*l* Tween 80 and 1 g/*l* $MgSO_4 \cdot 7H_2O$. Schaffer's medium contained 8 g/*l* nutrient broth (Becton, Sparks,

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MD, USA), 1 g/*l* KCl, 0.25 g/*l* $MgSO_4 \cdot 7H_2O$, 10^{-3} M $Ca(NO_3)_2 \cdot 4H_2O$, 10^{-5} M $MnCl_2 \cdot 4H_2O$ and 10^{-6} M $FeSO_4 \cdot 7H_2O$. Yeast mannitol medium (YM) consisted of 10 g/*l* mannitol, 0.4 g/*l* yeast extract, 0.5 g/*l* K_2HPO_4, 0.2 g/*l* $MgSO_4 \cdot 7H_2O$ and 0.1 g/*l* NaCl. Complete yeast medium (CYM) contained 20 g/*l* glucose, 2 g/*l* Polypepton, 2 g/*l* yeast extract, 1 g/*l* K_2HPO_4 and 0.46 g/*l* KH_2PO_4. Casein yeast medium (CY) contained 3 g/*l* casamino acids, 1 g/*l* yeast extract and 1.36 g/*l* $CaCl_2 \cdot 2H_2O$. In order to prepare a solid medium, agar (Nacalai Tesque, Kyoto) was added at a concentration of 1.5%. Agar medium (AG) contained only 15 g/*l* agar powder. LB was diluted 10–10^6 times to prepare 1/10–1/10^6 LB medium, and named 1/10 LB or 1/10^6 LB. The media were sterilized at 121°C for 20 min.

Culture media used for the growth of actinomycetes were tryptone yeast medium (ISP1), yeast malt-extract medium (ISP2), starch mineral medium (ISP4), glycerol Asn medium (ISP5), peptone yeast iron medium (ISP6), tyrosine medium (ISP7), nitrate medium (ISP8), Pridham and Gottlieb medium (ISP9) (3, 4), and modified Bennett's medium (MB) which contained 1 g/*l* meat extract, 1 g/*l* yeast extract, 2 g/*l* casamino acids, 5 g/*l* glucose and 5 g/*l* malt extract.

In order to prepare the soil extract, 1000 g of soil was mixed with 2 *l* of 50 mM NaOH and incubated overnight at room temperature. The mixture was filtrated and then centrifuged for 60 min at 18,000 rpm. The supernatant was sterilized through a 0.2-μm membrane filter. Soil-extract agar medium contained 500 ml/*l* soil extract and 15 g/*l* agar. For the isolation of actinomycetes, 50 mg/*l* of cycloheximide was added to preclude the growth of fungi.

Culture conditions For solid culture, microorganisms were incubated at 30°C unless otherwise noted. In order to perform bacterial incubation under extremely low oxygen tension, an anaerobic pack (Mitsubishi Gas Chemical, Tokyo) was employed. Bacterial growth was detected under microscope (×40), and mycelia were observed under a microscope (×200).

16S rDNA sequencing and phylogenetic analysis 16S rDNA fragments from the crude lysate were amplified by PCR with a universal set of primers (5). PCR products were sequenced with a SequiTherm cycle sequencing kit (Epicentre Technologies, Madison, WI, USA); this was followed by detection with a Pharmacia laser fluorescent DNA sequencer (6). Sequence determination was carried out in cooperation with Tomomi Koide (NCIMB Japan, Shimizu). The 16S rDNA sequences of strains Shinshu-th1 and -th2 were deposited in the DDBJ under accession nos. AB121772 and AB121773, respectively, and -MS-02, -MS-03, -MS-04, and -MS-05 under nos. AB193569, AB193570, AB193571, and AB193572, respectively.

The sequences determined were compared with those retrieved from the DDBJ/EMBL/GenBank nucleotide sequence databases. A distance matrix tree was constructed by the neighbor-joining method (7), and the topology of the phylogenetic tree was built by bootstrap analysis (8), using the CLUSTAL W program (9).

RESULTS

Microorganisms grown on soil-extract agar medium but not on conventional media After a two-week incubation on soil-extract agar medium, bacterial colonies were isolated. A total of 1261 colonies was then transplanted to several media including LB, NB and Schaffer's, which are conventionally employed for bacterial growth. Among them, three colonies that could not grow on these media were selected. They were designated Shinshu-th1, -th2 and -th3.

After observation under the microscope (×200), 217 colonies assumed to be actinomycetes were selected from soil-extract agar medium containing cycloheximide. Microscopic observation was effective to distinguish actinomycete colonies from bacterial ones. Then all of the actinomycete colonies were plated on the media conventionally employed for the growth of actinomycetes, such as ISP2, ISP4, ISP5, ISP9 and MB media. Among the strains, five could not grow on the media but could grow on the soil-extract agar medium with cycloheximide. They were designated Shinshu-MS-01, -02, -03, -04 and -05.

TABLE 1. Growth of three bacterial strains isolated on several types of solid media

Medium	Shinshu-th1	Shinshu-th2	Shinshu-th3
AG	−	−	+
LB	−	−	−
1/10 LB	−	−	+
1/10^2 LB	+	−	+
1/10^3 LB	+	−	+
1/10^4 LB	−	+	+
1/10^5 LB	−	+	+
1/10^6 LB	−	+	+
NB	−	−	−
R	−	−	−
YM	−	+	−
YS	−	+	−
Shaffer's	−	−	−
CYM	−	−	−
CY	−	+	+
Soil-extract agar	+	+	+

Bacterial culture was performed at 30°C for two weeks.

Growth of isolated strains on other media The isolated microorganisms were grown on several other conventional media and nutritionally poor media to elucidate their growth characteristics.

The three newly isolated bacterial strains, Shinshu-th1, -th2 and -th3 required nutritionally poor medium although the extent of the requirement was different for each strain. The requirement for poor medium of th1 was the strictest: it grew well on 1/10^2 LB and 1/10^3 LB but not on 1/10 LB, 1/10^4 LB (Table 1) and other media conventionally used (Table 1). The relatively nutritionally poor media YM, YS and CY could support the growth of th2, while media containing Polypepton and/or meat extract such as LB, NB, R, Schaffer's and CYM could not. Strain th2 could grow on 1/10^4, 1/10^5 and 1/10^6 LB. Growth of th3 was possible on nutritionally poor medium including CY and diluted LB medium ranging from 1/10–10^6, however, the growth was much better on soil-extract agar medium. Strain th3 could grow even on AG without degrading agar, indicating that residual organic compounds in the agar were sufficient for the formation of small colonies (Table 1). Common characteristics of these bacteria were as follows. Their growth was extremely slow. After growing for two weeks at 30°C, they formed barely visible small colonies. The diameter of the colonies was less than 0.1 mm and so they were detected only under the microscope (×40). All three bacterial strains could not grow under anaerobic conditions.

Although five actinomycete strains growing on soil-extract agar medium but not on ISP2, ISP4, ISP5, ISP9 and MB were isolated, the growth characteristics of three of them, MS-02, MS-04 and MS-05, changed during mainte-