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Isolation of a syntrophic actinomycete strain whose growth depend on co-culture with a *Lysinibacillus* strain

Akira Yokota[1, 2*], Wellyzar Sjamsuridzal[1, 2], Ariyanti Oetari[1, 2], Iman Santoso[1, 2]

[1]Center of Excellence for Indigenous Biological Resources-Genome Studies, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Kampus UI, Depok 16424, Indonesia.
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Kampus UI, Depok 16424, Indonesia.
Email: uayoko@gmail.com

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ABSTRACT

Aims: A syntrophically growing actinomycete strain, designated Act-7, was isolated from Gunung Halimum mountain soil, West Java, Indonesia, and taxonomically characterized.
Methodology and results: The strain Act-7 was isolated by the standard dilution technique using ISP 2 agar. It was identified and characterized based on the phenotypic, chemotaxonomic and genotypic methods. The phylogenetic analysis using 16S rRNA gene sequences suggested strain Act-7 belong to the genus *Microtetraspora*. The actinomycete strain required a diffusible compound for its growth from a spore-forming bacterial strain, designated D-2, which was identified as a member of the genus *Lysinibacillus*.
Conclusion, significance and impact study: Isolation of syntrophic rare actinomycete is a good example for the discovery of uncultured or not-yet-cultured microorganisms by syntrophic growth response.

Keywords: Syntrophic growth, *Microtetraspora*, rare actinomycetes, co-culture, *Lysinibacillus*

INTRODUCTION

The majority of microorganisms distributed in nature are not cultivable by conventional technique. One of the primary reasons for such uncultivability might be caused by syntrophic growth between microorganisms, since some bacterial strains cannot grow on artificial media alone have been cultured in the presence of other bacteria (Ohno *et al*., 2000; Rhee *et al*., 2000; Kaeberlein *et al*., 2002; Tanaka *et al*., 2004; Morris *et al*., 2013). Presence of many not-yet-cultured actinomycetes strains are also known. To obtain such microorganisms¸ they must be screened with growth factors supplied by other microorganisms.

In this study, we obtained an actinomycete strain as a mixed culture with other microorganisms, and then the strain was purified in the presence of a diffusible compound from other microorganisms, and was subjected to taxonomic studies.

MATERIALS AND METHODS

Isolation methods

The isolate was obtained by the following procedure. Soil samples collected from Gunung Halimum mountain, West Java, Indonesia, was dried for one day at a room temperature. An aliquot of soil sample was diluted to 10^{-4} with sterile water and inoculated on ISP 2 agar plate (International *Streptomyces* Project medium No. 2 containing yeast extract 4 g, malt extract 10 g, glucose 4 g, water 1 L, pH 7.3). After incubation at 30 °C for 7 days, actinomycete colonies that emerged the agar plate were picked and streaked on a new agar plate.

Identification and characterization methods

Morphological characterization

The strain Act-7 was grown at 30 °C for 7 days on a conditioned medium of ISP 2, which was prepared by culturing strain D-2 in ISP 2 broth at 30 °C for 4 days, centrifuging and filter-sterilizing. The cell morphology was observed with light microscope.

Chemotaxonomic characterization

For chemotaxonomy, freeze-dried cells were prepared by collecting from 7 day-old cultures grown in a conditioned medium of ISP 2 broth at 30 °C. The cell-wall

*Corresponding author

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peptidoglycan was prepared by the method of Schleifer and Kandler (1972). Amino acids in the cell wall were determined by the thin-layer chromatography (TLC) method of Staneck and Roberts (1974). Isoprenoid quinones were extracted by the method of Collins *et al*. (1977) and then analyzed by HPLC equipped with a Cosmosil 5C18 column (4.6 by 150 mm, Nacalai Tesque, Kyoto Japan). Methylesters of cellular fatty acids were prepared from cells grown at 30 °C for 7 days on culture filtrate of ISP 2 broth as described above, and identified by gas liquid chromatography (GLC) according to the Microbial Identification System (MIDI) (Sasser, 1990; Kämpfer and Kroppenstedt, 1996).

Genotypic characterization

Chromosomal DNA was extracted from cells grown in conditioned medium of ISP 2. The G+C content of the DNA was determined by HPLC (Mesbah *et al.*, 1989). Sequences of 16S rRNA gene was determined by using primers 9F (5`GAGTTTGATCCTGGCTCG) and 1510R (5`GGCTACCTTGTACGACTT) as described by Nurkanto *et al*. (2012). The 16S rRNA gene sequences was multialigned with selected sequences obtained from the GenBank/EMBL/DDBJ databases by the CLUSTAL_W (Thompson *et al*., 1997). The aligned sequences were manually edited before constructing the phylogenetic tree by the neighbour-joining (Saitou and Nei, 1987). The branches confidence values of the phylogenetic tree were examined by bootstrap analysis (Felsenstein, 1985) based on 1,000 resamplings. Gaps and ambiguous nucleotides were deleted manually before analyzing the similarity values by CLUSTAL_W (Thompson *et al*., 1997).

RESULTS AND DISCUSSION

Isolation of syntrophic actinomycete strain

Among them, bacterial mixed culture but separated on agar plate was selected. Consequently, we isolated one actinomycete strain, Act-7, of which growth was stimulated by another bacterial strain, designated strain D-2. Strain Act-7 did not grow on ISP 2 agar or ISP 2 broth, however, addition of supernatant prepared from ISP 2 broth culture of strain D-2 caused the growth of actinomycete strain Act-7.

An actinomycete strain, Act-7, was isolated from soil, but the strain grew on ISP 2 medium only in the presence of another bacterial strain, strain D-2, which were separately isolated (Figure 1). Growth of strain Act-7 was stimulated by a diffusible component from strain D-2. This could be considered as syntrophic growth (Morris *et al*., 2013).

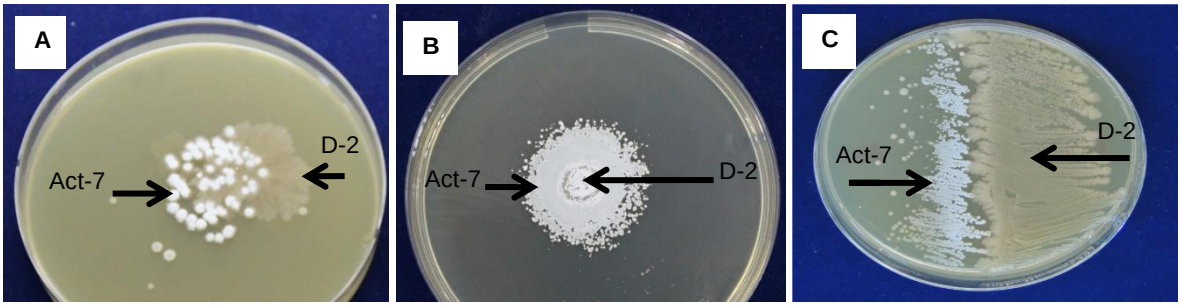

Figure 1: Syntrophic growth of strain Act-7 with strain D-2 on ISP-2 agar medium. Spore suspension of strain Act-7 were spread on the agar plate, and cells of strain D-2 were streaked in the center of agar plate, and incubated at 30 °C for 7 days. White-colored colonies; strain Act-7, dark-colored colonies, strain D-2.

Identification and characterization of the isolates

Strain Act-7 is Gram-stain positive, non-motile, branched mycelium- and aerial mycelium-forming actinomycete (Figure 2). No soluble pigment is produced. Straight chains of four spores are formed on sporophores branching from aerial mycelia. Spores are oval to short cylindrical. The 16S rRNA gene of strain Act-7 was partially sequenced (928 bases, corresponding to positions 439-1383 of *Escherichia coli* 16S rRNA gene), and the sequence obtained showed 99.9% similarity to that of *Microtetraspora malaysiensis* (Nakajima *et al.*, 2003). Phylogenetic tree of strain Act-7 is shown in Figure 3. Based on phylogenetic comparison, the strain was considered a member of genus *Microtetraspora*, most closely related to *Microtetraspora malaysiensis*.

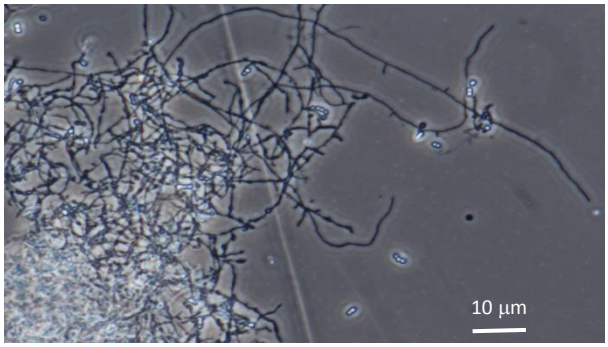

Figure 2: Photomicrograph of vegetative branched mycelium cells of strain Act-7, viewed by phase-contrast microscopy.

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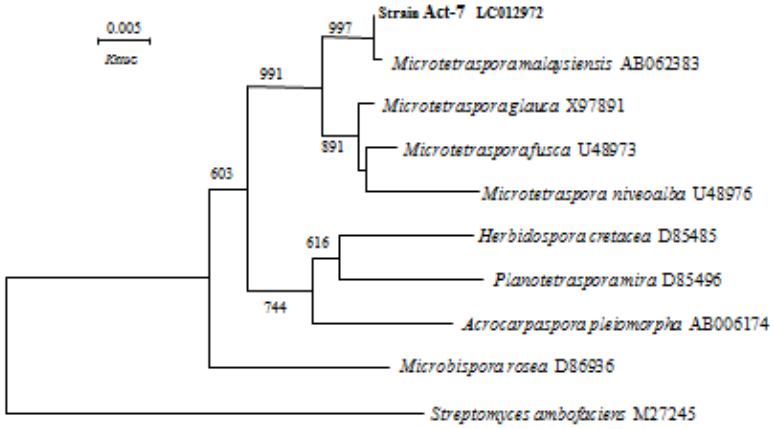

Figure 3: Neighbour-joining tree based on partial 16S rRNA gene sequences (1000 positions were taken into account). Bootstrap values above 60% (based on 1000 replications) are represented at the branch points. The 16S rRNA gene sequence of *Streptomyces ambofaciens* (M27245) was included as an out group. Bar, 0.005 substitutions per nucleotide position.

Table 1: Fatty acid composition of strains Act-7 and D-2.

Fatty acid	Strain Act-7	Strain D-2
13:0	0.4	-
iso-14:0	3.9	7.2
14:0	2.1	0.5
iso-15:0	7.1	25.7
anteiso-15:0	1.0	5.4
15:0	5.6	1.9
iso-16:1 H	1.5	-
16:1 ω 7c alcohol	-	21.2
iso-16:0	37.7	26.6
Sum In Feature 3	1.8	-
16:1 ω 11c	-	2.4
16:0	6.8	1.0
15:0 2OH	0.8	-
10 Me 16:0	4.3	-
iso-17:1 ω 10c	-	1.7
anteiso-17:1	-	1.2
iso-17:0	0.7	3.1
anteiso-17:0	0.6	2.3
17:1 ω 8c	3.0	-
17:1 ω 6c	6.1	-
17:0	2.3	-
16:0 2OH	0.9	-
10Me 17:0	9.7	-
iso-18:0	0.5	-
18:1 ω 9c	0.5	-
18:1 ω 7c	0.4	-
18:0	1.0	-
10Me18:0	0.9	-

Sum In Feature 3 contains iso-15:0 2OH and/or 16:1 ω 7c.

Strain Act-7 contained iso-16:0,10Me 17:0, iso-15:0, 16:0, and 17:1ω6c as major cellular fatty acids (Table 1). Cell wall peptidoglycan of Act-7 contained glutamic acid, alanine and *meso*-diaminopimelic acid. These characteristics agree with those of species of the genus *Microtetraspora*. Therefore, the strain Act-7 was identified as *Microtetraspora* sp.

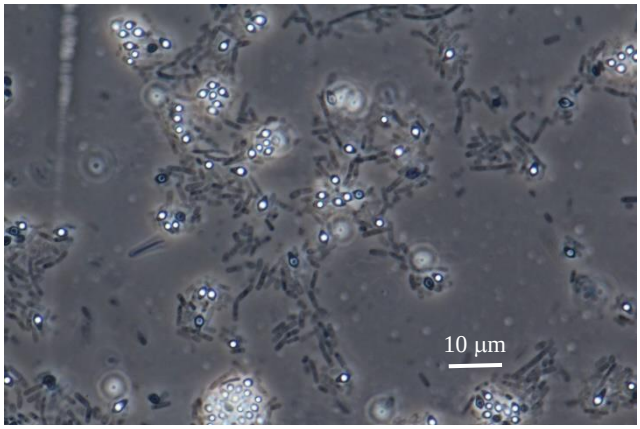

Figure 4: Photomicrograph of sporangia and vegetative cells of strain D-2, viewed by phase-contrast microscopy; ellipsoidal spores lie terminally in the sporangia that are swollen.

Strain D-2 is Gram positive, terminal sphaerical-spore forming, straight and club-shaped rods with swollen sporangium (Figure 4). Young cells are motile. The 16S rRNA gene of strain D-2 was partially sequenced (971 bases, corresponding to positions 24-1021 of *Escherichia*

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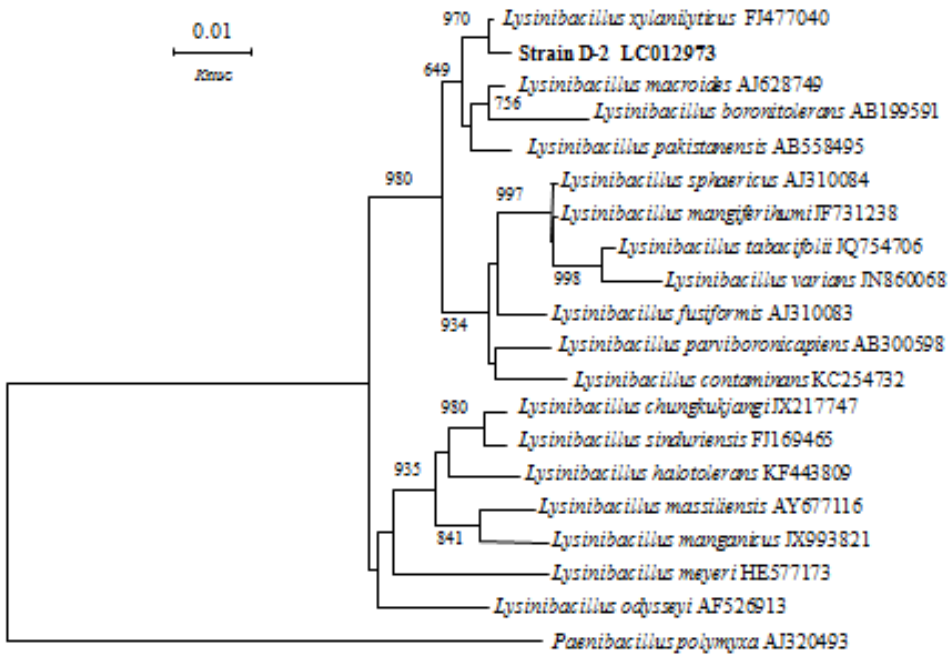

Figure 5: Neighbour-joining tree based on partial 16S rRNA gene sequences (1000 positions were taken into account). Bootstrap values above 60% (based on 1000 replications) are represented at the branch points. The 16S rRNA gene sequence of *Paenibacillus polymyxa* (AJ320493) was included as an out group. Bar, 0.005 substitutions per nucleotide position.

coli 16S rRNA gene), and the sequence obtained showed 99.3% similarity to that of *Lysinibacillus xylanilyticus* (Lee *et al*., 2010). Phylogenetic tree of strain Act-7 is shown in Figure 5. Based on phylogenetic comparison, the strain D-2 was considered as a member of genus *Lysinibacillus.*

Strain D-2 contained MK-7 as predominant isoprenoid quinone, and iso-16:0, iso-15:0, and 16:1ω7c alcohol as major cellular fatty acids (Table 1). Cell wall peptidoglycan of D-2 contained glutamic acid, alanine, aspartic acid and lysine. The peptidoglycan type was very similar to that found in species of the genus *Lysinibacillus* (Shaw and Keddie, 1983; Ahmed *et al*., 2007; Krishnamurthi *et al*., 2010). Therefore, the strain D-2 was identified as *Lysinibacillus* sp.

Growth of strain Act-7

Growth of the strain Act-7 was investigated on ISP 2 agar plate. It could grow in the presence of *Lysinibacillus* strain D-2. When the strain D-2 was replaced to other bacterial strains, the growth of strain Act-7 occurred in *Lysinibacillus tabacifolii* KCTC 33042, *L. odysseyi* NBRC 100172, *L. sphaericus* NBRC 15095, *L. fusiformis* NBRC 15717, *L. boronitolerans* NBRC 103108, *L. chungkukjangi* NBRC 108498, *L. massiliensis* KCTC 13178, *L. pakistanensis* KCTC 13795, *L. sinduriensis* JCM 15800. Growth, however, did not occur in *Bacillus subtilis* NBRC 13719, *Staphylococcus aureus* NBRC 100910, *Kocuria rhizophila* NBRC 12708, and *Escherichia coli* NBRC 3301. The results suggest diffusible substance necessary for the growth of strain Act-7 is produced specifically by *Lysinibacillus* strains.

CONCLUSION

A syntrophically growing actinomycete was isolated from mountain soil and taxonomically characterized. The strain was isolated by the standard dilution technique using ISP 2 agar. It was identified based on the phenotypic, chemotaxonomic and genotypic characteristics. The phylogenetic analysis using 16S rRNA gene sequences suggested it belong to the genus *Microtetraspora*. The actinomycete strain required a diffusible compound for its growth from a spore-forming bacterial strain, which was identified as a strain of the genus *Lysinibacillus*.

Strains of the genus *Microtetraspora* belong to rare actinomycetes (non-streptomycete actinomycetes). The rare actinomycetes are usually regarded as strains of actinomycetes whose isolation frequency by conventional methods is much lesser than that of streptomycete strains (Seong *et al.*, 2001). Isolation of a syntrophic rare

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actinomycete is a good example for the discovery of uncultured or not-yet-cultured microorganisms.

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