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

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ABSTRACT

Aims: A syntrophically growing actinomycete strain, designated Act-7, was isolated from Gunung Halimun 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 Halimun 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
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).

**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 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.

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

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Strain Act-7</th>
<th>Strain D-2</th>
</tr>
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<tbody>
<tr>
<td>13:0</td>
<td>0.4</td>
<td>-</td>
</tr>
<tr>
<td>iso-14:0</td>
<td>3.9</td>
<td>7.2</td>
</tr>
<tr>
<td>14:0</td>
<td>2.1</td>
<td>0.5</td>
</tr>
<tr>
<td>iso-15:0</td>
<td>7.1</td>
<td>25.7</td>
</tr>
<tr>
<td>anteiso-15:0</td>
<td>1.0</td>
<td>5.4</td>
</tr>
<tr>
<td>15:0</td>
<td>5.6</td>
<td>1.9</td>
</tr>
<tr>
<td>iso-16:1 H</td>
<td>1.5</td>
<td>-</td>
</tr>
<tr>
<td>16:1 ω 7c alcohol</td>
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</tr>
<tr>
<td>iso-16:0</td>
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<tr>
<td>Sum In Feature 3</td>
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<td>-</td>
</tr>
<tr>
<td>16:1 ω 11c</td>
<td>-</td>
<td>2.4</td>
</tr>
<tr>
<td>16:0</td>
<td>6.8</td>
<td>1.0</td>
</tr>
<tr>
<td>15:0 2OH</td>
<td>0.8</td>
<td>-</td>
</tr>
<tr>
<td>10 Me 16:0</td>
<td>4.3</td>
<td>-</td>
</tr>
<tr>
<td>iso-17:1 ω 10c</td>
<td>-</td>
<td>1.7</td>
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<td>anteiso-17:1</td>
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<td>iso-17:0</td>
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<tr>
<td>17:1 ω 8c</td>
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<tr>
<td>16:0 2OH</td>
<td>0.9</td>
<td>-</td>
</tr>
<tr>
<td>10Me 17:0</td>
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<td>-</td>
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<tr>
<td>iso-18:0</td>
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<td>-</td>
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<tr>
<td>18:1 ω 9c</td>
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<tr>
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<td>-</td>
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<tr>
<td>10Me18:0</td>
<td>0.9</td>
<td>-</td>
</tr>
</tbody>
</table>

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-spor 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*
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 16:1o7c 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 tabaci 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
actinomycete is a good example for the discovery of uncultured or not-yet-cultured microorganisms.

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