Micromonospora polyrhachis sp. nov., an actinomycete isolated from edible Chinese black ant (Polyrhachis vicina Roger)

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A novel actinomycete, designated strain NEAU-ycm2T, was isolated from edible Chinese black ants (Polyrhachis vicina Roger) and characterized using a polyphasic approach. The organism was found to have morphological and chemotaxonomic characteristics typical of the genus Micromonospora. The 16S rRNA gene sequence of strain NEAU-ycm2T showed highest similarity to those of Micromonospora sonneratiae 274745T (99.12 %), Micromonospora pattaloongensis TJ2-2T (98.85 %), Micromonospora pisi GUI 15T (98.76 %), Polymorphospora rubra TT 97-42T (98.42 %) and Micromonospora eburnea LK2-10T (98.21 %). Phylogenetic analysis based on the 16S rRNA gene and gyrB gene demonstrated that strain NEAU-ycm2T is a member of the genus Micromonospora and supported the close phylogenetic relationship to M. sonneratiae 274745T, M. pattaloongensis JCM 12833T and M. pisi GUI 15T. Furthermore, a combination of DNA–DNA hybridization and some physiological and biochemical properties indicated that the novel strain could be readily distinguished from its closest phylogenetic relatives. Therefore, it is proposed that NEAU-ycm2T represents a novel species of the genus Micromonospora, for which the name Micromonospora polyrhachis sp. nov. is proposed. The type strain is NEAU-ycm2T (=CGMCC 4.7100T=DSM 45886T).

Insects are the most abundant and diverse animal class on earth (May, 1988), and they are associated with an amazing variety of symbiotic micro-organisms (Buchner, 1965). Among symbiotic associations, insect–actinobacteria symbioses may be of particular interest in natural product discovery. Recently, a range of novel compounds with antifungal activity have been obtained from insect-symbiotic actinomycetes (Scott et al., 2008; Oh et al., 2009; Kroiss et al., 2010; Poulsen et al., 2011). Species of the genus Micromonospora are best known for producing antibiotics, especially aminoglycosides, enediyne and oligosaccharide antibiotics. Thus, their effects on medicine are considerable. As part of a programme to discover actinomycetes with novel antibiotic production properties from Chinese black ant (Polyrhachis vicina Roger), an aerobic actinomycete strain, NEAU-ycm2T, was isolated. In this study, we performed polyphasic taxonomy on this strain and propose that strain NEAU-ycm2T represents a novel species of the genus Micromonospora.

Strain NEAU-ycm2T was isolated from Chinese black ant (Polyrhachis vicina Roger) collected at North-east Agriculture University (Harbin, Heilongjiang, China). Five individual of Chinese black ants were caught on the lawn adjacent to formicary in June 2012. Each individual was surface-disinfected in 70% ethanol for 60 s and then washed three times in sterile distilled water. Surface-disinfected ants were dissected separately, followed by immersion in 500 μl sterile water and shaking on a rotary shaker at 180 r.p.m. at 28 °C for 30 min. A 200 μl sample of the suspension was spread on a plate of humic acid–vitamin agar (HV) (Hayakawa & Nonomura, 1987) supplemented with cycloheximide (50 mg l⁻¹) and nalidixic acid (20 mg l⁻¹). After 21 days of aerobic incubation at 28 °C, colonies were transferred and purified on oatmeal agar (ISP medium 3) (Shirling & Gottlieb, 1966) and incubated at 28 °C for 2–3 weeks.

Morphological characteristics were observed by light microscopy (ECLIPSE E200; Nikon) and electron microscopy (S-3400N; Hitachi) using cultures grown on ISP3 agar at 28 °C for 14 days. Cultural characteristics were...
determined after 2 weeks at 28 °C by methods using International Streptomyces Project (ISP) media 2–7 (Shirling & Gottlieb, 1966), tap-water agar (Gordon et al., 1974), nutrient agar (Waksman, 1961) and Czapek’s agar (Waksman, 1967). The Inter-Society Color Council–National Bureau of Standards (ISCC–NBS) colour charts were used to determine the designations of colony colours (Kelly, 1964). Growth at different temperatures (4, 16, 22, 28, 37 and 45 °C) was determined on ISP3 medium after incubation for 14 days. Growth tests for pH range were carried out using media adjusted to pH 2–12 with 4 M HCl or 5 M KOH after sterilization, and NaCl tolerance was determined in tryptic soy broth (TSB) supplemented with 0–7 % NaCl (w/v) at 28 °C for 14 days on a rotary shaker. Production of catalase, esterase and urease were tested as described by Smibert & Krieg (1994). The utilization of sole carbon and nitrogen sources, decomposition of cellulose, hydrolysis of starch and aesculin, reduction of nitrate, peptonization of milk, liquefaction of gelatin and production of H2S were examined as described previously (Gordon et al., 1974; Yokota et al., 1993).

Biomass for chemical studies was prepared by growing the strain in TSB at 28 °C for 7 days. Cells were harvested by centrifugation, washed with distilled water and freeze-dried. The isomers of diaminopimelic acid (DAP) in the whole cell hydrolysates were derivatized according to the protocol of McKerrow et al. (2000) and analysed by HPLC using a TC-C18 column (250 × 4.6 mm internal diameter 5 μm; Agilent) with a mobile phase consisting of acetonitrile/0.05 mol l−1 phosphate buffer pH 7.2 (15 : 85) at a flow rate of 0.5 ml min−1. The peak detection used a G1321A fluorescence detector (Agilent) with a 365 nm excitation and 455 nm long-pass emission filters. The whole-cell sugars were analysed according to the procedures developed by Lechevalier & Lechevalier (1980). The phospholipids were examined by two-dimensional TLC and identified using the method of Minnikin et al. (1984). Menaquinones were extracted from freeze-dried biomass and purified according to the protocol of Collins (1985). Extracts were analysed by the HPLC-UV method using an Extend-C18 column (150 × 4.6 mm, internal diameter 5 μm; Agilent), typically at 270 nm. The mobile phase was acetonitrile/propyl alcohol (60 : 40, v/v) and the flow rate was set to 1.0 ml min−1 and the run time was 60 min. The injection volume was 20 μl, and the chromatographic column was controlled at 40 °C (Wu et al., 1989). Mycolic acids were checked by the acid methanolysis method as described previously (Minnikin et al., 1980). Fatty acids were analysed by GC-MS using the method of Xiang et al. (2011).

Extraction of chromosomal DNA, PCR-mediated amplification of the 16S rRNA gene and direct sequencing of the purified products were carried out using a standard procedure (Kim et al., 2000). The PCR product was purified and cloned into the vector pMD19-T (Takara) and sequenced by using a DNA sequencer (model 3730XL; Applied Biosystems) and software provide by the manufacturer. Almost full-length 16S rRNA gene sequence (1510 nt) was obtained and aligned with multiple sequences obtained from the GenBank/EMBL/DDBJ databases using the CLUSTAL_X 1.83 software. Phylogenetic trees were generated with the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) algorithms using the MEGA software version 5.05 (Tamura et al., 2011). The stability of the clades in the trees was appraised using a bootstrap value with 1000 repeats (Felsenstein, 1985). A distance matrix was generated using the Kimura’s two-parameter model (Kimura, 1980). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). 16S rRNA gene sequence similarities between strains were calculated on the basis of pairwise alignment using the EzTaxon-e database (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012). PCR amplification of the gyrB gene was carried out using primers GFY1 and GYB3 (Garcia et al., 2010) and the PCR program for the 16S rRNA gene. Sequencing and phylogenetic analysis was performed as described above. Actinoplanes regularis IFO 12514T was used as an outgroup. The G+C content of the genomic DNA was determined using the thermal denaturation (Tm) method (Mandel & Marmur, 1968) with Escherichia coli JM109 as the control. DNA–DNA relatedness between isolate NEAU-ycz2T and Micromonospora sonneratiae JCM 12745T, Micromonospora pattaloogensis JCM 12833T and Micromonospora pisi GUI 151 was tested as described by De Ley et al. (1970) with consideration of the modifications described by Huß et al. (1983), using a model Cary 100 Bio UV/VIS spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multieell changer and a temperature controller with in-situ temperature probe (Varian).

Morphological observation of a 14 day-old culture of strain NEAU-ycz2T grown on ISP3 agar revealed that it formed extensively branched substrate mycelia without fragmentation. Aerial hyphae and sporangia were not present. Non-motile spores (0.73–1.26 × 0.50–0.99 μm) were borne singly on the substrate mycelium and the spore surface was smooth (Fig. 1). Cultural characteristics of strain NEAU-ycz2T are shown in Table 1. Good growth was observed on ISP2, ISP3, ISP4, ISP6, ISP7 and nutrient agar; poor growth was observed on Czapek’s agar; no growth was observed on ISP5 and tap water agar. The colour of substrate hyphae varied from strong orange–yellow to pale yellow (Table 1). Diffusible pigments were not formed on any of the tested media. Melanin was not observed on ISP6 or ISP7 medium. Strain NEAU-ycz2T was observed to grow well at pH 7–11, with an optimum pH of 7.0. The range of growth temperatures was determined to be 16–37 °C, with an optimum growth temperature of 28 °C. Strain NEAU-ycz2T was observed to grow in the presence of 0–2 % NaCl (w/v). Detailed physiological characteristics are presented in the species description.

The cell wall of strain NEAU-ycz2T was observed to contain meso-diaminopimelic acid and glycine, indicating that strain NEAU-ycz2T was of chemotype II (Lechevalier et al., 1970).
The almost-complete 16S rRNA gene sequence (1510 nt) of strain NEAU-ycm2T showed a close relationship with members of family Micromonosporaceae. Based on EzTaxon analysis, the species most closely related to the novel isolate were *M. sonneratiae* 274745T, *M. pattaloongensis* TJ2-2T, *M. pisi* GUI 15T and *P. rubra* TT 97-42T. The differences between NEAU-ycm2T and *M. sonneratiae* 274745T, *M. pattaloongensis* TJ2-2T, *M. pisi* GUI 15T and *P. rubra* TT 97-42T were 90.76 %, 97.56 %, 98.84 % and 88.41 %, respectively. Strain NEAU-ycm2T was dispersive with *P. rubra* TT 97-42T, *M. pisi* GUI 15T and *M. eburnea* LK2-10T, but formed a monophyletic clade with *M. sonneratiae* 274745T and *M. pattaloongensis* TJ2-2T that was supported by a bootstrap value of 64 % in the phylogenetic tree based on 16S rRNA gene sequences of strain NEAU-ycm2T and the type strains of other members of the genus *Micromonospora* and one member of the family *Micromonosporaceae* (Fig. S3). These results demonstrated that strain NEAU-ycm2T is a member of the genus *Micromonospora* and supported the close phylogenetic relationship to *M. sonneratiae* 274745T, *M. pattaloongensis* JCM 12833T and *M. pisi* GUI 15T. DNA–DNA hybridization was employed to further clarify the relatedness between NEAU-ycm2T and *M. sonneratiae* 274745T, *M. pattaloongensis* JCM 12833T and *M. pisi* GUI 15T; the levels of DNA–DNA relatedness between them were 55.21 ± 0.76, 44.08 ± 0.82 and 52.04 ± 1.02 %, respectively. These values were below the threshold value of 70 % recommended by Wayne et al. (1987) for assigning strains to the same species. A comparative study between strain NEAU-ycm2T and *M. sonneratiae* 274745T, *M. pattaloongensis* JCM 12833T and *M. pisi* GUI 15T also revealed that it differed from them in physiological and biochemical characteristics as summarized in Table 2.

In conclusion, it is evident from the genotypic, chemotaxonomic and phenotypic data that strain NEAU-ycm2T represents a novel species of the genus *Micromonospora*, for which the name *Micromonospora polyrhachis* sp. nov. is proposed.

### Table 1. Cultural characteristics of strain NEAU-ycm2T

<table>
<thead>
<tr>
<th>Agar medium</th>
<th>Growth</th>
<th>Colony colour</th>
</tr>
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<tbody>
<tr>
<td>Yeast extract–malt extract agar (ISP2)</td>
<td>Good</td>
<td>Strong orange–yellow</td>
</tr>
<tr>
<td>Oatmeal agar (ISP3)</td>
<td>Good</td>
<td>Brilliant orange</td>
</tr>
<tr>
<td>Inorganic salts–starch agar (ISP4)</td>
<td>Good</td>
<td>Pale orange–yellow</td>
</tr>
<tr>
<td>Peptone–yeast extract–iron agar (ISP6)</td>
<td>Good</td>
<td>Pale yellow</td>
</tr>
<tr>
<td>Tyrosine agar (ISP7)</td>
<td>Good</td>
<td>Brilliant orange–yellow</td>
</tr>
<tr>
<td>Nutrient agar</td>
<td>Good</td>
<td>Yellowish white</td>
</tr>
<tr>
<td>Czapek’s agar</td>
<td>Poor</td>
<td></td>
</tr>
</tbody>
</table>

*Fig. 1.* Scanning electron micrograph of strain NEAU-ycm2T grown on ISP3 medium for 21 days at 28 °C. Bar, 2 μm.
Description of *Micromonospora polyrhachis* sp. nov.

*Micromonospora polyrhachis* (po. ly. rha’chis. N.L. gen. n. polyrhachis of Polyrhachis, referring to the insect *Polyrhachis vicina* Roger from which the organism was isolated).

Gram-strain-positive and aerobic. Grows well on ISP2, ISP3, ISP4, ISP6, ISP7 and nutrient agar; poorly on Czapek’s agar; no growth is observed on ISP5 and tap water agar. Diffusible pigments are not formed on any of the tested media. Melanin is not observed on ISP6 or ISP7 medium. Cells form extensively branched substrate mycelia, which carry singly, smooth–surfaced non-motile spores (0.73–1.26 x 0.50–0.99 μm). Positive for liquefaction of gelatin, hydrolysis of aesculin, peptonization of milk and reduction of nitrate, and negative for hydrolysis of starch, decomposition of cellulose and urea and production of H₂S. Inositol, D-galactose, D-glucose, raffinose, sucrose, D-mannose, D-mannitol, D-sorbitol, maltose and D-ribose are utilized as sole carbon sources but L-arabinose, D-fructose, D-xylose, D-ribose and L-rhamnose are not. L-Glutamic acid, L-threonine, L-aspartic acid, L-asparagine, L-tyrosine, L-arginine and L-creatine are utilized as sole nitrogen sources but L-alanine, L-serine, L-glutamine and L-glycine are not. Tolerates up to 2 % NaCl and grows at temperatures of between 16 and 37 °C, with an optimum temperature of 28 °C. Growth occurs at initial pH values between 7 and 11, the optimum being pH 7.0. The cell wall is chemotype II and the whole-cell sugars are xylose and...
Table 2. Differential characteristics of strain NEAU-ycm2\(^T\) and the most closely related species of the genus Micromonospora

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tbody>
<tr>
<td>Soluble pigment on ISP2/3</td>
<td>None</td>
<td>None</td>
<td>Yellow</td>
<td>None</td>
</tr>
<tr>
<td>Utilization of</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-Fructose</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>l-Rhamnose</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>d-Xylose</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Raffinose</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Cellulose decomposition</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Maximum NaCl tolerance (%)</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Growth at pH 11</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

glucose. The major menaquinones are MK-10(H\(_4\)), MK-10(H\(_3\)), MK-10(H\(_2\)), MK-9(H\(_6\)) and MK-9(H\(_8\)). The phospholipid profile comprises diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidyl-

The type strain is NEAU-ycm2\(^T\) (=CGMCC 4,7100\(^T\) = DSM 45886\(^T\)), isolated from Chinese black ants collected in North-east Agriculture University (Harbin, Heilongjiang, China). The DNA G+C content of the DNA of the type strain is 76 mol%.

Acknowledgements

This work was supported in part by grants from the National Key Project for Basic Research (no. 2010CB126102) and the National Natural Science Foundation of China (nos 30971937 and 30771427), the National Outstanding Youth Foundation (no. 31225024), the Special Foundation for Scientific and Technological Innovation Research of Harbin (no. 2011RFXXN038) and the Natural Science Foundation of Heilongjiang Province (no. C201029).

References


