Actinoallomurus radicium sp. nov., isolated from the roots of two plant species

Atsuko Matsumoto, Asami Fukuda, Yuki Inahashi, Satoshi Ōmura and Yōko Takahashi

Kitasato Institute for Life Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan

Correspondence
Atsuko Matsumoto
amatsu@lisci.kitasato-u.ac.jp

Three strains, K08-0182^T, K08-0178 and K08-0195 were isolated from the paste of ground plant roots collected in Kanagawa Prefecture, Japan. These strains contained *meso*-diaminopimelic acid and lysine as the diamino acids in cell-wall peptidoglycan, and MK-9(H_6) and MK-9(H_8) as the predominant menaquinones. The G+C contents of the DNA were 72–73 mol%. Taken together, these characteristics combined with 16S rRNA gene sequence analyses revealed that the isolated strains belong to the genus *Actinoallomurus*. DNA–DNA hybridization values showed that the three strains belonged to a novel species of the genus *Actinoallomurus*. Therefore strains K08-0182^T, K08-0178 and K08-0195 are proposed as representatives of a novel species, *Actinoallomurus radicium* sp. nov. The type strain is K08-0182^T (=DSM 45523^T =NBRC 107678^T =JCM 17294^T).

Numerous attempts have been made to isolate many actinomycete strains from various samples for the purpose of finding new microbial resources in screening for novel natural products. In many trials we have used plant roots to isolate rare or novel actinomycete strains, and we reported the novel genus and species *Phytohabitans suffuscus*, which was isolated from an orchid variety (Inahashi *et al.*, 2010), and *Actinoallomurus acaciae* (Thamchaipenet *et al.*, 2010) and *Actinoallomurus oryzae* (Indananda *et al.*, 2011) that were isolated from separate plants in Thailand. The genus *Actinoallomurus*, the type species of which is *Actinoallomurus spadix*, was transferred from the genus *Actinomadura* based on a proposal by Tamura *et al.* (2009). Here we report the taxonomic characteristics of another novel species in the genus *Actinoallomurus*.

Strains K08-0182^T and K08-0178, and strain K08-0195 were isolated from the roots of a Mondo grass and a Chusan Palm collected in Kanagawa Prefecture in March 2008, respectively. The roots were washed with sterilized water and dried in a chamber with silica gel for several days. After soaking the roots in 70 % ethanol for 30 s and then in 1 % sodium hypochlorite for 30 s, the samples were rinsed in sterilized water before being ground in extraction solution (0.38 % K_2HPO_4, 0.12 % KH_2PO_4, 0.51 % $MgSO_4 . H_2O$, 0.25 % NaCl, 0.005 % $Fe_2(SO_4)_3 . nH_2O$, 0.005 % $MnSO_4 . 5H_2O$) and plated on cellobiose-asparagine agar (1.0 % cellobiose, 0.1 % L-asparagine, 0.1 % K_2HPO_4, 0.0001 % $FeSO_4 . 7H_2O$, 0.0001 % $MnCl_2 . 4H_2O$, 0.0001 % $ZnSO_4 . 7H_2O$, 1.5 % agar, pH 7.0). Colonies were picked up after incubation for 6 weeks at 27 °C.

Morphological characteristics of the aerial mycelium were observed by scanning electron microscopy (JSM-5600, JEOL) after incubation on ISP medium 4 (Difco) for 1 month at 27 °C and fixation with 4 % osmium tetroxide vapour. Cultural and physiological characteristics were observed after incubation for 3 weeks at 27 °C. Cultural characteristics were examined on ISP medium 2 (Difco), ISP medium 3 (DAIGO, Nihon Pharmaceutical), ISP medium 4 and ISP medium 7 (DAIGO). ISP medium 4 was used to examine starch hydrolysis, ISP medium 8 (0.5 % peptone, 0.3 % beef extract, 0.5 % KNO_3, pH 7.0) for nitrate reduction, gelatin medium (2.0 % glucose, 0.5 % peptone, 20 % gelatin, pH 7.0) for gelatin liquefaction, 10 % skim milk (Difco) for coagulation and peptonization of milk, ISP medium 6 (DAIGO) for H_2S production, and skim milk agar (Gordon *et al.*, 1974) for casein hydrolysis. NaCl tolerance, and pH and temperature ranges for growth were determined on ISP medium 2. Utilization of sole carbon sources at 1 % (w/v) was tested using ISP medium 9 (DAIGO) as basal medium according to the method of Pridham & Gottlieb (1948). The API ZYM system (bioMérieux) was used to examine biochemical characteristics of the strains according to the manufacturer's instructions for 24 h at 37 °C.

Biomass for chemotaxonomy was prepared by cultivation in YD (1 % yeast extract, 1 % glucose, pH 7.0) at 27 °C. *N*-Acyl types of muramic acid were determined by the method of Uchida & Aida (1977). Purified cell wall was

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains K08-0182^T, K08-0178 and K08-0195 are AB578855, AB578856 and AB578857, respectively.

Two supplementary tables and a supplementary figure are available with the online version of this paper.

obtained by using the method of Kawamoto *et al.* (1981), and the amino acid composition of hydrolysed cell walls was determined by TLC. Whole-cell sugars were analysed after Becker *et al.* (1965) and the presence of mycolic acids was examined by TLC following Tomiyasu (1982). Phospholipids were extracted and identified following the method of Minnikin *et al.* (1977). Menaquinones, which were extracted and purified by the method of Collins *et al.* (1977), were subsequently analysed by LC/MS (JMS-T100LP, JEOL) with a PEGASIL ODS column (20 × 50 mm) using methanol/2-propanol (7 : 3). After methyl esters of cellular fatty acids were prepared by direct transmethylation with methanolic hydrochloride, fatty acid analysis was performed according to the procedures for the Sherlock Microbial Identification System (Microbial ID).

Chromosomal DNA was isolated using a slight modification of the method described by Saito & Miura (1963). DNA base composition was estimated by the HPLC method of Tamaoka & Komagata (1984) and DNA–DNA hybridization experiments were performed as described by Ezaki *et al.* (1989). PCR amplification and sequencing of the 16S rRNA genes were performed using previously described methods (Matsumoto *et al.*, 2008). CLUSTAL X version 1.83 (Thompson *et al.*, 1994) was used for multiple alignments with selected sequences for calculating evolutionary distances (Kimura, 1980). A phylogenetic tree was reconstructed based on the neighbour-joining method (Saitou & Nei, 1987). Data were resampled with 1000 bootstrap replications (Felsenstein, 1985). PHYLIP version 3.67 was used for generating a phylogenetic tree by the maximum-likelihood method (Felsenstein, 1981). Sequence similarity values were determined by visual comparison and manual calculation.

Cell-wall peptidoglycans of strains K08-0182^T, K08-0178 and K08-0195 contained *meso*-diaminopimelic acid and lysine as diamino acids, as well as alanine and glutamic acid. Madurose and galactose were detected from whole-cell hydrolysates, and the acyl type of the peptidoglycan was acetyl. Phosphatidylglycerol and diphosphatidylglycerol were detected but phosphatidylethanolamine and phosphatidylcholine were not detected. The major menaquinones were MK-9(H_6) and MK-9(H_8) and the predominant fatty acids were iso-$C_{16:0}$ and 10-methyl $C_{17:0}$ (Supplementary Table S1, available in IJSEM Online). Mycolic acids were not detected. The G + C contents of genomic DNA ranged from 72 to 73 mol%.

Almost complete 16S rRNA gene sequences (1487 bp) were determined for strains K08-0182^T, K08-0178 and K08-0195. The sequence similarity among the three strains was 100 %. Phylogenetic analysis using the neighbour-joining and maximum-likelihood methods showed that these strains are closely related to the genus *Actinoallomurus* (Fig. 1). It is clear that the strains belong to the genus *Actinoallomurus* based on the phylogenetic analyses and chemotaxonomic data. The highest similarity value between the 16S rRNA gene sequences of the isolates and species of the genus *Actinoallomurus* was 99.0 % (*Actinoallomurus iriomotensis* TT02-47^T), which was shown as the nearest species in the phylogenetic tree (Fig. 1). Similarity values between the three strains and other species were below 98.8 %.

Strains K08-0182^T, K08-0178 and K08-0195 grew on ISP media 2, 3, 4 and 7, but aerial mycelium was produced slightly only on ISP medium 4 (Table 1). No soluble pigment was produced. Spores produced on the tips of aerial mycelium were oval (0.7 × 1.0 μm, approximately) with smooth surfaces (Supplementary Fig. S1).

All isolated strains exhibited similar phenotypic characteristics. The isolates grew at temperatures ranging from 12 to 36 °C and at pH 5–7, but not on 2 % NaCl medium. Liquefaction of gelatin was positive but hydrolysis of starch, urea and casein were negative. The physiological characteristics of the three strains are listed in the species description below, and the physiological differences among strains K08-0182^T, K08-0178, K08-0195 and *A. iriomotensis* NBRC 103685^T are shown in Table 2. All three isolated strains differed from *A. iriomotensis* NBRC 103685^T in some characteristics; utilization of *myo*-inositol, maltose

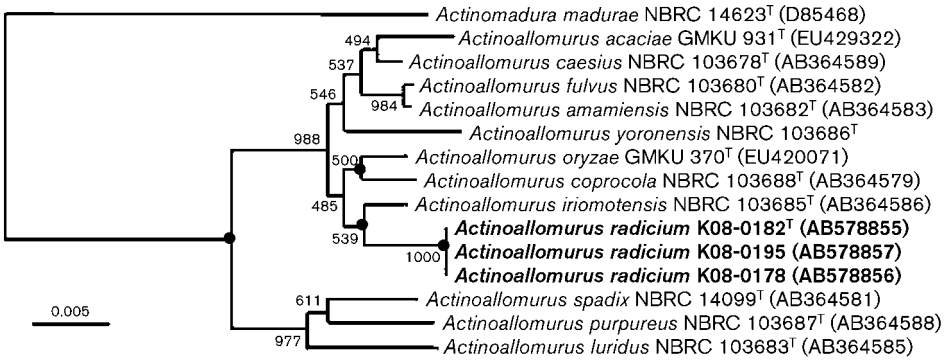

Fig. 1. Phylogenetic tree, derived from 16S rRNA gene sequences, recreated using the neighbour-joining method. Only bootstrap values above 40 % (from 1000 replications) are indicated. Solid circles indicate that the corresponding nodes were also recovered in the maximum-likelihood tree. Bar, 0.005 substitutions per nucleotide position.

Table 1. Cultural characteristics of strains K08-0182^T, K08-0178, K08-0195 and *Actinoallomurus iriomotensis* NBRC 103685^T

All strains showed good growth, pale yellow colonies and no aerial mycelium on ISP 2 medium.

Characteristic	K08-0182^T	K08-0178	K08-0195	*A. iriomotensis* NBRC 103685^T
Growth on ISP 3				
Growth	Good	Good	Moderate	Good
Colony colour	Brownish white	Pale yellow	Brownish white	Brownish white
Aerial mycelium	None	None	None	None
Growth on ISP 4				
Growth	Good	Good	Good	Good
Colony colour	Brownish white	Brownish grey	Brownish white	Brownish white
Aerial mycelium/colour	Poor/White	Poor/White	None	None
Growth on ISP 7				
Growth	Good	Moderate	Good	Moderate
Colony colour	Dark brown	Brownish grey	Dark brown	Brownish white
Aerial mycelium	None	None	None	None

and sucrose, enzyme activities of lipase (C4), trypsin, chymotrypsin, α-galactosidase and β-galactosidase, hydrolysis of starch and casein, and growth with sodium chloride

Table 2. Differential physiological characteristics of strains K08-0182^T, K08-0178, K08-0195 and *A. iriomotensis* NBRC 103685^T

Strains: 1, K08-0182^T; 2, K08-0178; 3, K08-0195; 4, *A. iriomotensis* NBRC 103685^T. +, Positive; −, negative; +^{w}, doubtful positive.

Characteristic	1	2	3	4
Utilization of:				
D-Fructose	+	+	−	−
D-Galactose	+	+	−	−
myo-Inositol	−	−	−	+
D-Mannitol	+	+	−	−
Maltose	−	−	−	+
D-Rhamnose	+	+	−	−
Sucrose	−	−	−	+
API ZYM test				
Lipase (C4)	−	−	−	+^{w}
Trypsin	−	−	−	+^{w}
Chymotrypsin	−	−	−	+
α-Galactosidase	−	−	−	+^{w}
β-Galactosidase	−	−	−	+^{w}
α-Glucosidase	+^{w}	+^{w}	+	+
N-Acetyl-β-glucosaminidase	+^{w}	+^{w}	+^{w}	+
Hydrolysis of:				
Starch	−	−	−	+
Casein	−	−	−	+
Gelatin	+	+	+	−
Temperature range (°C) for growth	16–34	12–36	12–34	20–37*
Tolerance of NaCl (%)	1	<1	<1	5
pH range for growth	5–7	5–7	5–7	5–8

*Data taken from Tamura *et al.* (2009).

greater than 1 % were negative, but hydrolysis of gelatin was positive. Though the dominant cellular fatty acid of the three strains was i-$C_{16:0}$ like *A. iriomotensis* NBRC 103685^T, 10-methyl $C_{17:0}$ was detected in greater proportion (Supplementary Table S1).

DNA–DNA hybridization values were 77–100 % among the three isolates and 3–12 % between the isolates and *A. iriomotensis* TT02-47^T (Supplementary Table S2). The latter values are considered to be sufficiently below the 70 % threshold required for assigning strains to the same species (Wayne *et al.*, 1987). Therefore, we propose to assign strains K08-0182^T, K08-0178 and K08-0195 to a novel species of genus *Actinoallomurus*, *Actinoallomurus radicium* sp. nov.

Description of *Actinoallomurus radicium* sp. nov.

Actinoallomurus radicium (ra.di′ci.um. L. gen. pl. n. *radicium* of roots, isolated from a plant root).

Colonies are yellow to brown. Aerial mycelium is white. The tips of the aerial mycelium divide into oval to rod-shaped spores at maturity. Spore surface is smooth. Spore chains form spirals. Aerobic. Temperature range for growth is 12–36 °C. Growth occurs at pH 5–7 and not with 2 % NaCl. Hydrolysis of gelatin is positive, but starch, urea and casein hydrolysis is negative. Coagulation and peptonization of milk and nitrate reduction are negative. D-Glucose and D-xylose are utilized, D-arabinose, dulcitol, *myo*-inositol, maltose, D-sorbitol and sucrose are not utilized as sole carbon source, and D-fructose, D-galactose, D-mannitol, raffinose and D-rhamnose utilization is variable. Acid phosphatase, alkaline phosphatase, leucine arylamidase and naphthol-AS-BI-phosphohydrolase are present, but chymotrypsin, α- and β-galactosidase, β-glucosidase, lipase (C4), α-fucosidase, α-mannosidase and trypsin are absent by API ZYM. The predominant cellular fatty acids are i-$C_{16:0}$, 10-methyl $C_{17:0}$ and ai-$C_{17:0}$. The G+C content of DNA is 72–73 mol%.

The type strain is K08-0182^T (=DSM 45523^T =NBRC 107678^T =JCM 17294^T), isolated from plant roots collected in Kanagawa Prefecture, Japan.

Acknowledgements

This work was financially supported by the Institute for Fermentation, Osaka (IFO), Japan.

References

Becker, B., Lechevalier, M. P. & Lechevalier, H. A. (1965). Chemical position of cell-wall preparations from strains of various form-genera of aerobic actinomycetes. *Appl Microbiol* **13**, 236–243.

Collins, M. D., Pirouz, T., Goodfellow, M. & Minnikin, D. E. (1977). Distribution of menaquinones in actinomycetes and corynebacteria. *J Gen Microbiol* **100**, 221–230.

Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.

Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**, 368–376.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.

Gordon, R. E., Barnett, D. A., Handerhan, J. E. & Pang, C. H.-N. (1974). *Nocardia coeliaca*, *Nocardia autotrophica*, and the Nocardin Strain. *Int J Syst Bacteriol* **24**, 54–63.

Inahashi, Y., Matsumoto, A., Danbara, H., Ōmura, S. & Takahashi, Y. (2010). *Phytohabitans suffuscus* gen. nov., sp. nov., an actinomycete of the family *Micromonosporaceae* isolated from plant roots. *Int J Syst Evol Microbiol* **60**, 2652–2658.

Indananda, C., Thamchaipenet, A., Matsumoto, A., Inahashi, Y., Duangmal, K. & Takahashi, Y. (2011). *Actinoallomurus oryzae* sp. nov., an endophytic actinomycete isolated from roots of a Thai jasmine rice plant. *Int J Syst Evol Microbiol* **61**, 737–741.

Kawamoto, I., Oka, T. & Nara, T. (1981). Cell wall composition of *Micromonospora olivoasterospora*, *Micromonospora sagamiensis*, and related organisms. *J Bacteriol* **146**, 527–534.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

Matsumoto, A., Yamada, M., Ōmura, S. & Takahashi, Y. (2008). *Microterricola viridarii* gen. nov., sp. nov., a new member of the family *Microbacteriaceae*. *Int J Syst Evol Microbiol* **58**, 1019–1023.

Minnikin, D. E., Patel, P. V., Alshamaony, L. & Goodfellow, M. (1977). Polar lipid composition in the classification of *Nocardia* and related bacteria. *Int J Syst Bacteriol* **27**, 104–117.

Pridham, T. G. & Gottlieb, D. (1948). The utilization of carbon compounds by some *Actinomycetales* as an aid for species determination. *J Bacteriol* **56**, 107–114.

Saito, H. & Miura, K. I. (1963). Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochim Biophys Acta* **72**, 619–629.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.

Tamura, T., Ishida, Y., Nozawa, Y., Otoguro, M. & Suzuki, K. (2009). Transfer of *Actinomadura spadix* Nonomura and Ohara 1971 to *Actinoallomurus spadix* gen. nov., comb. nov., and description of *Actinoallomurus amamiensis* sp. nov., *Actinoallomurus caesius* sp. nov., *Actinoallomurus coprocola* sp. nov., *Actinoallomurus fulvus* sp. nov., *Actinoallomurus iriomotensis* sp. nov., *Actinoallomurus luridus* sp. nov., *Actinoallomurus purpureus* sp. nov. and *Actinoallomurus yoronensis* sp. nov. *Int J Syst Evol Microbiol* **59**, 1867–1874.

Thamchaipenet, A., Indananda, C., Bunyoo, C., Duangmal, K., Matsumoto, A. & Takahashi, Y. (2010). *Actinoallomurus acaciae* sp. nov., an endophytic actinomycete isolated from *Acacia auriculiformis* A. Cunn. ex Benth. *Int J Syst Evol Microbiol* **60**, 554–559.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Tomiyasu, I. (1982). Mycolic acid composition and thermally adaptative changes in *Nocardia asteroides*. *J Bacteriol* **151**, 828–837.

Uchida, K. & Aida, K. (1977). Acyl type of bacterial cell wall: its simple identification by colorimetric method. *J Gen Appl Microbiol* **23**, 249–260.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.