Rhizocola hellebori gen. nov., sp. nov., an actinomycete of the family Micromonosporaceae containing 3,4-dihydroxydiaminopimelic acid in the cell-wall peptidoglycan

Atsuko Matsumoto,1,2 Yoko Kawaguchi,1 Takuji Nakashima,3 Masato Iwatsuki,1,2 Satoshi Ōmura2 and Yōko Takahashi1,2

1Graduate School of Infection Control Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan
2Kitasato Institute for Life Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan
3Research Organization for Infection Control Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan

An actinomycete strain, K12-0602T, was isolated from the root of a Helleborus orientalis plant in Japan. The 16S rRNA gene sequence of strain K12-0602T showed that it had a close relationship with members of the family Micromonosporaceae and the 16S rRNA gene sequence similarity values between strain K12-0602T and type strains of type species of 27 genera belonging to the family Micromonosporaceae were below 96.2 %. MK-9 (H4) and MK-9 (H6) were detected as major menaquinones, and galactose, xylose, mannose and ribose were present in the whole-cell hydrolysate. The acyl type of the peptidoglycan was glycolyl. Major fatty acids were iso-C15 : 0, iso-C16 : 0, C17 : 1v9c and anteiso-C17 : 0. Phosphatidylethanolamine was detected as the phospholipid corresponding to phospholipid type II. The G+C content of the genomic DNA was 67 mol%. Analyses of the cell-wall peptidoglycan by TLC and LC/MS showed that it was composed of alanine, glycine, hydroxylglutamic acid and an unknown amino acid, which was subsequently determined to be 3,4-dihydroxydiaminopimelic acid using instrumental analyses, including NMR and mass spectrometry. On the basis of the phylogenetic analysis and chemotaxonomic characteristics, strain K12-0602T represents a novel species of a new genus in the family Micromonosporaceae, for which the name Rhizocola hellebori gen. nov., sp. nov. is proposed. The type strain of the type species is K12-0602T (=NBRC 109834T=DSM 45988T). This is the first report, to our knowledge, of 3,4-dihydroxydiaminopimelic acid being found as a diamino acid in bacterial cell-wall peptidoglycan.

In the course of the isolation of actinomycete strains from plants for new microbial resources to discover novel natural products, we have discovered many rare actinomycete strains including representatives of a novel genus, Phytohabitans (Inahashi et al., 2010) and seven novel species belonging to the genera Streptosporangium, Actinomallomurus and Phytohabitans (Inahashi et al., 2011a, 2012; Koyama et al., 2012; Matsumoto et al., 2012). Notably, Streptosporangium oxazolinicum K07-0460T produces the novel bioactive compounds, spoxazomicins (Inahashi et al., 2011b), which exhibit antitrypanosomal activity. Furthermore, Polymorphospora rubra K07-0510 produces novel photo-oxidative haemolysis inhibitors, the trehangelins (Nakashima et al., 2013). Therefore, we focused one line of our search for rare actinomycete (non-Streptomyces) strains in plant roots. One of the isolates, strain K12-0602T was phylogenetically identified as being a member of the family Micromonosporaceae ([Krasil’nikov, 1938], emended by Koch et al. (1996) on the basis of chemotaxonomic data and further emended by Stackebrandt et al. (1997) and Zhi et al. (2009) on the basis of 16S rRNA gene sequence analysis). At the time of writing, the family Micromonosporaceae comprises 27 genera with validly published names. It is known that diamino acids in cell-wall peptidoglycans of micro-organisms

Abbreviations: d, doublet; dd, doublet of doublets; odd, doublet of doublet of doublets; δ, hydrogen shift; J, coupling constant; m, multiplet; m/z, mass to charge ratio.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain K12-0602T is AB848707.

Five supplementary figures are available with the online version of this paper.
belonging to these genera are meso-diaminopimelic acid, 3-hydroxydiaminopimelic acid and/or lysine. An unknown amino acid was detected in the cell-wall composition of strain K12-0602^T. In this study we describe the cell-wall amino acid composition and the classification of strain K12-0602^T.

Strain K12-0602^T was isolated from the root of Helleborus orientalis collected at the Medicinal Plant Garden of Kitasato University in Kanagawa Prefecture, Japan, in July 2011. The roots were washed with sterilized water and dried in a chamber with silica gel. The sample was ground with a pestle and mortar in extraction solution (0.38 % K2HPO4, 0.12 % KH2PO4, 0.51 % MgSO4·H2O, 0.25 % NaCl, 0.005 % Fe2(SO4)3·nH2O, 0.005 % MnSO4·5H2O) and plated in water proline agar (1.0 % proline, 1.2 % agar, tap water). Colonies were picked up after incubation for 8 weeks at 27 °C. Morphology was observed using a scanning electron microscope (model JSM-5600; JEOL) using previously described methods (Matsumoto et al., 2012). Cultural characteristics were examined on ISP media 2, 3, 4, 5 and 7 (Difco or Nihon Pharmaceutical) and YS agar (0.2 % yeast extract, 1 % soluble starch, 1.5 % agar, pH 7.0) and water agar (1.5 % agar, tap water). For the spore motility test, cells cultured on ISP medium 2 for 4 weeks at 27 °C were suspended in 0.1 ml sterile tap water or casamino acid solution (0.1 % casamino acid, 0.1 % CaCO3, 0.01 % Tween 80). After incubation for 1 h at 27 °C, the spore suspension was observed under a light microscope. The temperature range, obtained using a gradient incubator, (5–50 °C), the pH range (pH 4–10, interval of 1 pH unit) and the NaCl tolerance (0, 1, 2 and 3 %) for growth were determined on ISP medium 2. Utilization of carbohydrates as sole carbon sources was tested using ISP medium 9 (Nihon Pharmaceutical) as basal medium according to the method of Pridham & Gottlieb (1948). ISP medium 4 (Difco) for starch hydrolysis, ISP medium 8 (0.5 % peptone, 0.3 % beef extract, 0.1 % KNO3, pH 7.0) for nitrate reduction, glucose-peptone–gelatin medium (2.0 % glucose, 0.5 % peptone, 20 % gelatin, pH 7.0) for gelatin liquefaction, 10 % skimmed milk (Difco) for coagulation and peptonization of milk and skimmed milk agar for casein hydrolysis were used. Biomass for the molecular systematics and the chemotaxonomic studies was obtained after cultivation in yeast extract–glucose broth (1.0 % yeast extract, 1.0 % glucose, pH 7.0) for 2 weeks at 27 °C. Menaquinones, which were extracted and purified by the method of Collins et al. (1977), were subsequently analysed by LC/MS (JMS-T100LP; JEOL) with ODS column (Capcellpak C18, 150 mm × 2 mm, Shiseido) using methanol/2-propanol (7 : 3). Purified cell wall was prepared according to the method of Kawamoto et al. (1981). Cell-wall amino acids were identified by TLC (Hasegawa et al., 1983) and LC/MS using advanced Marfey’s method (Nozawa et al., 2007). The derivative amino acids were separated by chromatography with an Inertsil ODS-4 (150 mm × 3 mm, GL Sciences), using a linear gradient from 15 % to 85 % of acetonitrile containing 0.1 % formic acid. ESI-MS spectra were measured on a QSTAR Elite Hybrid MS/MS spectrometer (AB Sciex). An unknown amino acid was characterized by instrumental analyses including LC/MS and NMR. The amino acid was eluted with 5 % aqueous methanol (flow rate, 1.0 ml min⁻¹) by HPLC (Capcellpak CR, 250 mm × 4.6 mm, Shiseido). MS/MS spectra were collected using collision-induced dissociation with collision energy 20 V. NMR spectra were measured using an Agilent Technologies NMR System 400 with ¹H NMR at 400 MHz in D2O. The N-acyl types of muramic acid were determined by the method of Uchida & Aida (1977). Phospholipids in cells were extracted and identified by the method of Minnikin et al. (1984). The presence of mycolic acids was examined by TLC after the method of Tomiyasu (1982). Whole-cell sugar composition was analysed according to the methods of Becker et al. (1965). After methyl esters of cellular fatty acids were prepared by direct transmethylation with methanolic hydrochloride, fatty acid analyses were performed according to the procedures for the Sherlock Microbial Identification System (Microbial ID) using the ACTIN6 method. Genomic DNA was prepared following the procedure of Saito & Miura (1963) and the G+C content of genomic DNA was determined by HPLC according to the method of Tamaoka & Komagata (1984). PCR amplification and sequencing of the 16S rRNA genes followed described methods (Matsumoto et al., 2012). Identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity values were achieved using the EzTaxon-e server (Kim et al., 2012). The CLUSTALW2 program was used for multiple alignments with selected sequences and SeaView version 4.2 (Gouy et al., 2010) was used for evolutionary distances (Kimura, 1980). The phylogenetic trees were reconstructed based on the neighbour-joining method (Saitou & Nei, 1987), maximum-likelihood method (Felsenstein, 1981) and the maximum-parsimony method (Fitch, 1971). Data were resampled with 1000 bootstrap replications (Felsenstein, 1985).

Strain K12-0602^T grew well on ISP medium 2, moderately on ISP media 3, 4 and 5, nutrient agar and YS agar and...
poorly on water agar following cultivation for 4 weeks. The colony colour was yellow. No aerial mycelia were produced on all these media. However, scant aerial mycelia were observed on only ISP medium 7, though the growth was weak. Spore chains comprising more than 10 spores developed on short sporophores on the substrate mycelia and were flexible. The surface of the spore was smooth and 0.8 × 1.0 μm in size (Fig. 1 and Fig. S1 available in the

Fig. 2. Phylogenetic tree, derived from 16S rRNA gene sequences, created using the neighbour-joining method. Only bootstrap values above 40% (percentages of 1000 replications) are indicated. Filled circles and asterisks indicate that the corresponding nodes were also recovered in the maximum-parsimony and maximum-likelihood trees, respectively. Bar, 0.01 substitutions per nucleotide position.
online Supplementary Material). Sporangia and motile spores were not observed. The temperature and pH range for growth were 8–36 °C and pH 6–8. Optimum growth occurred at 28–32 °C and pH 7. Strain K12-0602T did not grow on ISP medium 2 with 1 % NaCl though it grew with 0 % NaCl. Other physiological characteristics are given in the species description.

The almost complete 16S rRNA gene sequence (1451 bp) determined for strain K12-0602T showed a close relationship with those of members of the family Micromonosporaceae. 16S rRNA gene sequence similarity values between strain K12-0602T and type strains of type species of all genera belonging to the family Micromonosporaceae with validly published names were 92.2–96.2 %. The recognized species showing the highest similarity value to strain K12-0602T was Catelliglobosispora koreensis DSM 44566T (96.2 %), followed by Catellatospora bangladeshensis 2-70(23)T (94.7 %), Catellatospora methionotrophica IMSNU 22006T (94.7 %) and Hamadacea tsunoiensis IMSNU 22005T (94.6 %) (Ara & Kudo, 2006; Ara et al., 2008). The phylogenetic analyses based on the 16S rRNA gene sequences also indicated that the isolate fell within the lineage of the family Micromonosporaceae and formed a branch with the genus Catelliglobosispora (Ara et al., 2008) (Fig. 2).

The N-acyl type of muramic acid of strain K12-0602T was glycolyl. Galactose, xylose, mannose and ribose were detected as whole-cell sugars. Phosphatidylethanolamine and unidentified phospholipids were detected but phospholipids containing glucosamine, phosphatidylcholine and phosphatidylglycerol were absent, corresponding to phospholipid type II as described by Lechevalier and Lechevalier (1977) (Fig. S2). Mycolic acids were not detected. The predominant menaquinones were MK-9 (H₄) (65 %) and MK-9 (H₆) (20 %) and the minor menaquinones were MK-10 (H₄) (7 %), MK-10 (H₆) (4 %), MK-9 (H₈) (3 %) and MK-10 (H₈) (1 %). The major cellular fatty acids of strain K12-0602T were accounted for by saturated fatty acids, namely iso-C₁₅:0 (18.0 %), iso-C₁₆:0 (14.5 %), anteiso-C₁₇:0 (10.6 %), 10-methyl-C₁₇:0 (7.8 %), C₁₇:0 (7.7 %), anteiso-C₁₅:0 (7.1 %), C₁₅:0 (5.1 %), iso-C₁₄:0 (2.9 %), 9-methyl-C₁₆:0 (1.7 %) and iso-C₁₇:0 (1.7 %), together with a few unsaturated fatty acids; C₁₇:0 9c (12.2 %), C₁₆:1 2-OH (1.6 %) and anteiso-C₁₇:1 (1.5 %). The G + C content of the genomic DNA was 67 mol%. The analysis of the cell-wall peptidoglycan by TLC (Fig. S3) and LC/MS showed that the strain contains alanine, glycine, hydroxyglutamic acid and an unknown amino acid, which was apparently more highly polar than 3-hydroxydiaminopimelic acid, as indicated by a lower Rate of flow (Rf) value in TLC. Therefore, we purified this unknown amino acid to characterize it. The unknown amino acid was estimated to be a diamino acid with a molecular weight of 222, because two derivatives labelled with one and two 1-fluoro-2,4-dinitrophenylleucinamide (FDLA) (molecular weights of 516 and 810) were detected as peak 1 and peak 2 by LC/MS, respectively (Fig. S4a–e). The molecular formula was deduced to be C₁₁H₁₄O₆N₂ by HRESI-MS (high resolution electrospray ionization-mass spectrometry) [M+H]⁺ m/z 223.0922 (calculated for C₁₁H₁₅O₆N₂ 223.0930) (Fig. S5a) and, a major fragment peak at m/z 74 observed in MS/MS analysis also supported the presence of an α-amino acid moiety (Fig. S5b). Consequently, it was proved to be 3,4- or 3,5-dihydroxydiaminopimelic acid. The 1H NMR spectrum in D₂O revealed the presence of two oxymethines at δH 4.01 (J =2.4 and 4.4 Hz, H-3) and 3.76 (m, H-4, two nitrogenated methines at δH 3.82 (dd, J =2.4 and 6.0 Hz, H-2) and 3.78 (m, H-6) and one unequivocal methylene at δH 2.03 (ddd, J =4.0, 5.6 and 14.8 Hz, H-5a and δH 2.14 (ddd, J =8.0, 10.0 and 14.8 Hz, H-5b). These data indicated that the unknown amino acid is 3,4-dihydroxy-2,6-diaminopimelic acid (Fig. 3), since it was found to be an asymmetrical compound via NMR analysis. This is a novel amino acid. On the basis of these results, it is clear that the cell-wall peptidoglycan of this strain is composed of alanine, glycine, hydroxyglutamic acid and 3,4-dihydroxydiaminopimelic acid. Neither diaminopimelic acid nor monohydroxydiaminopimelic acid were detected by TLC and LC/MS. This is the first report, to our knowledge, that a diamino acid of peptidoglycan in bacterial cell-wall is 3,4-dihydroxydiaminopimelic acid.

The 16S rRNA gene analyses indicated that strain K12-0602T belongs to the family Micromonosporaceae. Moreover, this strain has N-glycolated muramic acid, one of the distinctive chemotaxonomic features of the genera of the family Micromonosporaceae. Therefore strain K12-0602T should be classified as a member of the family Micromonosporaceae. The cell-wall peptidoglycan of strain K12-0602T contains a unique diamino acid; 3,4-dihydroxydiaminopimelic acid, which has never been reported, not only from the family Micromonosporaceae but also from other bacteria. Furthermore, strain K12-0602T containing MK-9 (H₄) as the major menaquinone is also distinguished from the most closely related member of the genus Catelliglobosispora, which has MK-10 (H₄).

Based on phenotypic and phylogenetic evidence, strain K12-0602T represents a novel species of a new genus in the family Micromonosporaceae, for which the name Rhizocola hellebori is proposed. The phenotypic characteristics of this genus are listed in Table 1.
Aerobic, Gram-stain-positive and mesophilic actinomycetes. Cells are non-motile. Vegetative mycelia are branched and not fragmented. Sporangia are not observed. Aerial mycelium is produced, and long spore chains develop on short sporophores on the substrate mycelia. Cell walls contain 3,4-dihydroxydiaminopimelic acid as a diamino acid, along with alanine, glycine and hydroxylglutamic acid. Galactose and xylose are detected as diagnostic whole-cell sugars. The acyl type of the peptidoglycan is glycolyl. Phospholipid type is PII. Mycolic acids are not detected. Predominant menaquinones are MK-9 (H4) and MK-9 (H6).

The type species is *Rhizocola hellebori*.

### Description of *Rhizocola hellebori* sp. nov.

*Rhizocola hellebori* (hel.le.bo’ri. N.L. fem. n. hellebori of Helleborus, a plant genus name).

General morphological and chemotaxonomic characteristics are as given in the genus description. The colony colour is yellow to orange. Spore chains comprising more than 10 spores developed on short sporophores at maturity. Aerobic. The surface of the oval spores is smooth. Spore chains are flexible. The temperature range for growth is 8–36 °C. Growth occurs at initial pH 6–8 and does not occur with 1 % NaCl. Hydrolysis of starch and casein are positive but that of gelatin is negative. Coagulation and peptonization of milk and nitrate reduction are negative. D-Arabinose, D-fructose, D-glucose, D-rhamnose, D-mannitol and maltose are utilized, but cellulose, myo-inositol, melibiose, raffinose, D-xylose and sucrose are not utilized as sole carbon sources. The predominant components of the cellular fatty acid are iso-C15:0, iso-C16:0, C17:1ω9c and anteiso-C17:0.

The type strain, K12-0602 T (=NBRC 109834 T =DSM 45988 T) was isolated from the root of *Helleborus orientalis* in Japan. The G+C content of the genomic DNA of the type strain is 67 mol%.

### Acknowledgements

We thank Dr Tatsuo Fukuda (Kitasato University) for provision of plant samples. We also thank Professor Bernhard Schink (University of Konstanz) for his help with the nomenclature. Part of this work was financially supported by the Institute for Fermentation, Osaka (IFO), Japan.

### References


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### Table 1. Phenotypic characteristics of *Rhizocola* gen. nov. and other closely related genera

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*3,4-OH-DAP, 3,4-dihydroxy-2,6-diaminopimelic acid; 3-OH-DAP, 3-hydroxydiaminopimelic acid; meso-DAP, meso-diaminopimelic acid; Ala, alanine; Gly, glycine; OH-Glu, hydroxylglutamic acid.

†According to the classification of Lechevalier et al. (1977).


+ Present, – absent; ND, not determined. Spore motility is of all genera is negative.


