Phytohabitans suffuscus gen. nov., sp. nov., an actinomycete of the family *Micromonosporaceae* isolated from plant roots

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An actinomycete strain, K07-0523^T, was isolated from the roots of an orchid collected in Okinawa prefecture, Japan. 16S rRNA gene sequence analysis indicated that the new strain belonged to the family *Micromonosporaceae* and the similarity values between strain K07-0523^T and the type species of 24 genera in the family *Micromonosporaceae* were 93.3–97.7 %. Strain K07-0523^T contained D-glutamic acid, glycine, D-alanine, meso-diaminopimelic acid, hydroxydiaminopimelic acid and L-lysine in the cell wall. The major menaquinones were MK-9(H₆), MK-10(H₄) and MK-10(H₆). Galactose, glucose, mannose, ribose and xylose were present in the whole-cell sugars. The acyl type of the peptidoglycan was glycolyl. Major fatty acids were anteiso-C_{17:0}, iso-C_{17:0}, iso-C_{16:0} and iso-C_{15:0}. Phosphatidylethanolamine was detected as the major phospholipid and corresponded to phospholipid type II. The G + C content of the genomic DNA was 73 mol%. On the basis of phylogenetic and chemotaxonomic data, the new strain represents a member of a new genus and novel species, namely *Phytohabitans suffuscus* gen. nov., sp. nov., in the family *Micromonosporaceae*. The type strain of the type species is K07-0523^T (=DSM 45306^T=NBRC 105367^T).

It is well known that the majority of actinomycete strains isolated from soil samples belong to the genus *Streptomyces* (Lechevalier & Lechevalier, 1967). Okazaki *et al.* (1995) and Matsumoto *et al.* (1998) reported that the microbial flora in plant matter was different from that in soil samples and that the majority of species in such samples were rare actinomycetes. We tried to isolate actinomycete strains from plants in order to discover new microbial resources for screening for potential novel natural products. As part of these studies, we have discovered many rare or novel actinomycete strains. One of these strains, K07-0523^T, was identified as being a member of the family *Micromonosporaceae*. The family *Micromonosporaceae* was first described by 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* comprised 24 genera: *Micromonospora* (Ørskov, 1923), *Actinoplanes* (Couch, 1950), *Pilimelia* (Kane, 1966), *Dactylosporangium* (Thiemann *et al.*, 1967), *Catellatospora* (Asano & Kawamoto, 1986), *Catenuloplanes* (Yokota *et al.*, 1993), *Couchioplanes* (Tamura *et al.*, 1994), *Spirilliplanes* (Tamura *et al.*, 1997), *Verrucosisporea* (Rheims *et al.*, 1998), *Virgisporeangium* (Tamura *et al.*, 2001), *Asanoa* (Lee & Hah, 2002), *Longisporea* (Matsumoto *et al.*, 2003), *Salinispora* (Maldonado *et al.*, 2005), *Actinocatenisporea* (Thawai *et al.*, 2006), *Polymorphosporea* (Tamura *et al.*, 2006), *Luedemannella* (Ara & Kudo, 2007a), *Krasilnikovia* (Ara & Kudo, 2007b), *Planosporeangium* (Wiese *et al.*, 2008), *Pseudosporeangium* (Ara *et al.*, 2008a), *Catelliglobosisporea* (Ara *et al.*, 2008b), *Hamadaea* (Ara *et al.*, 2008b), *Plantactinospora* (Qin *et al.*, 2009), *Rugosimonosporea* (Monciardini *et al.*, 2009) and *Actinaurisporea* (Thawai *et al.*, 2010). The characterization and classification of strain K07-0523^T is described in this paper.

Strain K07-0523^T was isolated from the roots of a variety of orchid collected in Okinawa prefecture, Japan, in March

Abbreviation: SEM, scanning electron microscope.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain K07-0523^T is AB490769.

Additional phylogenetic trees based on maximum-likelihood and maximum-parsimony analyses of 16S rRNA gene sequences are available with the online version of this paper.

2007. The roots were soaked in 70 % ethanol for 30 s and then soaked in 1 % sodium hypochlorite for 30 s. The roots were then rinsed in sterilized water. A 0.5 g root sample was dried in a chamber with silica gel and then ground with a mortar and pestle in 5.0 ml extraction solution [0.38 % K_2HPO_4 , 0.12 % KH_2PO_4 , 0.51 % $MgSO_4 \cdot 7H_2O$, 0.25 % NaCl, 0.005 % $Fe_2(SO_4)_3 \cdot nH_2O$ (Wako Pure Chemical Industries, Ltd.), 0.005 % $MnSO_4 \cdot 5H_2O$]. A 0.2 ml aliquot of the resultant liquid was mixed into cellobiose asparagine agar (1.0 % cellobiose, 0.1 % L-asparagine, 0.1 % K_2HPO_4 , 0.0001 % $FeSO_4 \cdot 7H_2O$, 0.0001 % $MnCl_2 \cdot 4H_2O$, 0.0001 % $ZnSO_4 \cdot 7H_2O$, 1.5 % agar, pH 7.0) and colonies were picked up after incubation for 5 weeks at 27 °C. After culturing on ISP 3 (Nihon Pharmaceutical) and nutrient agar (Difco) for 2–4 weeks at 27 °C, morphology was observed using a scanning electron microscope (SEM; model JSM-5600, JEOL). For SEM inspection, the cultures were fixed with 4 % osmium tetroxide vapour *in situ* for 16 h at room temperature and then dried at room temperature. Cultural characteristics were examined on ISP 3, ISP 4 (Difco), ISP 5 (Nihon Pharmaceutical), nutrient agar and HV agar (Hayakawa & Nonomura, 1987). For the spore motility test, spores of the isolate cultured on nutrient agar for 2 weeks at 27 °C were suspended and mixed with 0.1 ml sterile tap water or nutrient broth (Difco). After incubation for 1 h at 27 °C, the spore suspension was observed under a light microscope.

The temperature range, pH range and the NaCl tolerance for growth were determined on nutrient agar. Utilization of carbohydrates as sole carbon sources was tested using ISP 9 (Nihon Pharmaceutical) as the basal medium according to the method of Shirling & Gottlieb (1966). Production of acid was evaluated by using the media of Gordon *et al.* (1974). ISP 4 (Difco) was used for starch hydrolysis tests, ISP 8 (0.5 % peptone, 0.3 % beef extract, 0.1 % KNO_3 , pH 7.0) was used for nitrate reduction, glucose-peptone-gelatin medium (2.0 % glucose, 0.5 % peptone, 20 % gelatin, pH 7.0) was used to test for gelatin liquefaction, 10 % skimmed milk (Difco) was used for coagulation and peptonization of milk, ISP 6 (Nihon Pharmaceutical) was used for H_2S production and skimmed milk agar (Gordon *et al.*, 1974) was used to test for casein hydrolysis.

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 3 weeks at 27 °C. After extraction, as described by Collins *et al.* (1977), isoprenoid quinones were analysed by HPLC with 802-SC chromatography (JASCO) using a CAPCELL PAK C18 column (Shiseido) (Tamaoka *et al.*, 1983). Isomers of diaminopimelic acid were determined by TLC using whole-cell hydrolysis (Becker *et al.*, 1965). Purified cell walls were prepared according to the method of Kawamoto *et al.* (1981). Cell-wall amino acids were identified by TLC (Hasegawa *et al.*, 1983) and HPLC (Nozawa *et al.*, 2007). The N-acyl types of muramic acid were determined by the method of Uchida & Aida (1977). Phospholipids in cells were extracted and identified

according to the method of Minnikin *et al.* (1977). The presence of mycolic acids was examined by TLC following Tomiyasu (1982). Whole-cell sugar composition was analysed according to the methods of Becker *et al.* (1965). Methyl esters of cellular fatty acids were prepared by direct transmethylation with methanolic hydrochloride using cells grown in nutrient broth (Oxoid) for 10 days at 27 °C and analysed on a GLC system (HP 6890; Hewlett Packard). Identification and quantification of the fatty acid methyl esters, as well as the numerical analysis of the fatty acid profiles, were performed according to the instructions for the Microbial Identification System (MIDI).

Chromosomal DNA was prepared following the procedure of Saito & Miura (1963) and the DNA G+C content was determined by HPLC according to Tamaoka & Komagata (1984). The 16S rRNA gene was amplified using the primers described by Takahashi *et al.* (2002). Amplifications were performed in a TaKaRa thermal cycler (Takara) with an initial incubation of 1 min at 95 °C followed by 30 cycles of 1 min at 95 °C, 1 min at 50 °C and 1.5 min at 72 °C, followed by 2 min final extension at 72 °C. The PCR products were sequenced on a DNA sequencer (Applied Biosystems 3130 Genetic Analyzer) using a BigDye Terminator v3.1 cycle Sequencing kit (Applied Biosystems), according to the manufacturer's instructions. The CLUSTAL_X program version 1.83 (Thompson *et al.*, 1997) was used for multiple alignments with selected sequences for calculating evolutionary distances (Kimura, 1980). Alignments were manually verified and a phylogenetic tree was constructed based on the neighbour-joining method (Saitou & Nei, 1987). Data were resampled with 1000 bootstrap replications (Felsenstein, 1985). For the creation of phylogenetic trees by the maximum-likelihood (Felsenstein, 1981) and the maximum-parsimony methods (Kluge & Farris, 1969), SeaView version 4.2 was used and data were resampled with 100 bootstrap replications. The values for sequence similarities among the most closely related strains were determined using the EzTaxon server (Chun *et al.*, 2007).

Strain K07-0523^T showed moderate growth and produced long chains of more than 10 spores on ISP 3, ISP 4, ISP 5,

Fig. 1. Scanning electron micrograph of cells of strain K07-0523^T grown on ISP 3 for 4 weeks at 27 °C. Bar, 1 μm.

nutrient agar and HV agar. Vegetative mycelia were branched, but not fragmented. The surface of the spores was smooth and the spores were $0.8 \times 1.0 \mu\text{m}$ in size (Fig. 1). Spores were non-motile. Sporangia were not observed. The temperature and pH range for growth were 22–37 °C and pH 7–11, respectively. Good growth occurred at 26–35 °C and pH 7–10. Strain K07-0523^T did not grow on 3% (w/v) NaCl medium. Additional physiological characteristics are given in the species description.

The cell-wall peptidoglycan contained D-glutamic acid, glycine, D-alanine, *meso*-diaminopimelic acid, hydroxydiaminopimelic acid and L-lysine. Galactose, glucose, mannose, ribose and xylose were detected as whole-cell sugars. The *N*-acyl type of the muramic acid was glycolyl. Phosphatidylethanolamine and five unknown phospholipids were detected but phospholipids containing glucosamine, phosphatidylcholine and phosphatidylglycerol were absent. This polar lipid profile corresponded to phospholipid type II of Lechevalier

Fig. 2. Neighbour-joining tree based on 16S rRNA gene sequences showing relationships between strain K07-0523^T and members of family *Micromonosporaceae*. Only bootstrap values >50% (1000 replications) are indicated. The solid circles and asterisks indicate that the corresponding nodes were also recovered in the maximum-likelihood tree and maximum-parsimony trees, respectively. Bar, 0.01 nucleotide substitutions per site.

Table 1. Morphological features and chemotaxonomic characteristics of the genus *Phytohabitans* gen. nov. and other genera in the family *Micromonosporaceae*

Taxa: 1, *Phytohabitans* gen. nov.; 2, *Actinaurispora*; 3, *Actinocatenispora*; 4, *Actinoplanes*; 5, *Asanoa*; 6, *Catellatospora*; 7, *Catelliglobospora*; 8, *Catenuloplanes*; 9, *Couchioplanes*; 10, *Dactylosporangium*; 11, *Hamadaea*; 12, *Krasilnikovia*; 13, *Longispora*; 14, *Luedemannella*; 15, *Micromonospora*; 16, *Pilimelia*; 17, *Planosporangium*; 18, *Plantactinospora*; 19, *Polymorphospora*; 20, *Pseudosporangium*; 21, *Rugosimonospora*; 22, *Salinispora*; 23, *Spirilliplanes*; 24, *Verrucosisporea*; 25, *Virgisporangium*. Data were taken from Thawai *et al.* (2006, 2010), Couch (1950), Lee & Hah (2002), Asano & Kawamoto (1986), Ara *et al.* (2008a, b), Ara & Kudo (2006, 2007a, b), Yokota *et al.* (1993), Kudo *et al.* (1999), Tamura *et al.* (1994, 1997, 2001, 2006), Thiemann *et al.* (1967), Matsumoto *et al.* (2003), Ørskov (1923), Kane (1966), Wiese *et al.* (2008), Qin *et al.* (2009), Monciardini *et al.* (2009), Maldonado *et al.* (2005) and Rheims *et al.* (1998). Fatty acid type is according to the classification of Kroppenstedt (1985). Phospholipid type is according to the classification of Lechevalier *et al.* (1977). +, Positive; –, negative; ND, not determined; *m*-DAP, *meso*-diaminopimelic acid; 3-OH-DAP, 3-hydroxydiaminopimelic acid; L-Lys, L-lysine; Ara, arabinose; Gal, galactose; Glc, glucose; Man, mannose; Rha, rhamnose; Rib, ribose; Xyl, xylose.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Spore motility	–	–	–	+	–	–	–	+	+	+	–	–	–	–	–	+	+/-	–	–	–	–	–	+	–	–
Sporangium	–	–	–	+	–	–	–	–	–	+	–	–	–	+	–	+	+	–	–	–	–	–	–	–	+
Diamino acid(s)	<i>m</i> -DAP, L-Lys	<i>m</i> -DAP	<i>m</i> -DAP	<i>m</i> -DAP	<i>m</i> -DAP, 3-OH-DAP	<i>m</i> -DAP, 3-OH-DAP	<i>m</i> -DAP	L-Lys	L-Lys	<i>m</i> -DAP	<i>m</i> -DAP, 3-OH-DAP	<i>m</i> -DAP	<i>m</i> -DAP	<i>m</i> -DAP	<i>m</i> -DAP and/or 3-OH-DAP	<i>m</i> -DAP	<i>m</i> -DAP	<i>m</i> -DAP	<i>m</i> -DAP	<i>m</i> -DAP	3-OH-DAP, 3-OH-DAP	<i>m</i> -DAP	<i>m</i> -DAP	3-OH-DAP	
Whole-cell sugars	Gal, Glc, Man, Rib, Xyl	Gal, Man, Xyl	Ara, Gal, Xyl	Ara, Xyl	Ara, Gal, Xyl	Ara, Gal, Man, Rha, Rib, Xyl or only Xyl	Gal, Glc, Man, Rha, Rib, Xyl	Man, Rib, Xyl	Ara, Gal, Xyl	Ara, Xyl	Ara, Gal, Glc, Man, Rha, Rib, Xyl	Ara, Gal, Man, Rib, Xyl	Ara, Gal, Xyl	Ara, Gal, Man, Rha, Rib, Xyl	Ara, Xyl	Ara, Xyl	Ara, Xyl	Ara, Gal, Xyl	Xyl	Ara, Gal, Glc, Man, Rib, Xyl	Ara, Gal, Xyl	Ara, Gal, Xyl	Gal, Man, Xyl	Man, Xyl	Ara, Gal, Xyl
Fatty acid type	2d	3b	3b	2c	2d	3b	3b	2c	2c	2d	3b	2d	2d	2d	3b	2b	3b	2d	2a	2d	2d	3a	2d	2b	2d
Major menaquinones	9(H ₆), 10(H _{4,6})	9(H ₆), 10(H ₆)	9(H _{4,6}), 10(H ₄)	9(H ₄), 10(H ₄)	10(H _{6,8}), 10(H ₄)	9(H _{4,6}), 10(H ₄) or 10(H ₄)	10(H ₄)	10(H ₄), 11(H ₄)	9(H ₄)	9(H _{4,6,8})	9(H ₆), 10(H _{4,6,8})	10(H _{4,6})	9(H _{4,6})	9(H _{4,6}), 10(H _{4,6})	9(H _{2,4})	10(H ₄)	10(H _{4,6,8})	9(H _{4,6}), 10(H _{4,6})	9(H ₆), 10(H _{4,6})	9(H ₈)	9(H ₄)	10(H ₄)	9(H ₄)	10(H _{4,6,8})	
Phospholipid type	II	II	II	II	II	II	II	III	II	II	II	II	II	II	II	II	II	II	II	II	II	II	II	II	II
DNA G+C content (mol%)	73	72	72	72–73	71–72	70–72	70	70–73	70–72	72–73	70	71	70	71	71	ND	71	70	71	73	72–73	70–73	69	71	71

et al. (1977). Mycolic acids were not detected. The predominant menaquinones were MK-9(H₆) (16%), MK-10(H₄) (21%) and MK-10(H₆) (33%) and the minor menaquinones were MK-9(H₄) (6%), MK-9(H₈) (11%) and MK-10(H₈) (13%). The cellular fatty acids representing >1% of total fatty acids were anteiso-C_{17:0} (28.6%), iso-C_{17:0} (19.5%), iso-C_{16:0} (12.4%), iso-C_{15:0} (10.2%), 9-cis-C_{18:1} (5.0%), 2-OH-C_{16:1} (4.9%), C_{18:0} (4.5%), 9-cis-C_{17:1} (2.6%), anteiso-C_{15:0} (2.2%), 10-methyl-C_{16:0} (2.2%), anteiso-C_{17:1} (1.6%), C_{17:0} (1.5%), C_{16:0} (1.3%), 9-cis-C_{16:1} (1.3%) and iso-C_{18:0} (1.2%). This profile corresponded to fatty acid type 2d of Kroppenstedt (1985). The G + C content of the genomic DNA was 73 mol%.

The 16S rRNA gene sequence of strain K07-0523^T showed a close relationship with members of family *Micromonosporaceae*. Similarity values between strain K07-0523^T and the type species of all genera belonging to the family *Micromonosporaceae* were 93.3–97.7%. The species showing the highest similarity values to strain K07-0523^T were *Micromonospora pattaloongensis* (97.7%), *Pseudosporangium ferrugineum* (97.7%), *Micromonospora auratinigra* (97.6%) and *Catenuloplanes crispus* (97.6%). The phylogenetic analysis based on the 16S rRNA gene sequences also indicated that the new isolate fell within the lineage of the family *Micromonosporaceae* and formed a cluster within the genera *Catenuloplanes* and *Asanoa* (Fig. 2) (see Supplementary Figures S1 and S2 in IJSEM Online). Signature nucleotides of the 16S rRNA gene sequence of strain K07-0523^T were shared in all positions with genera of the family *Micromonosporaceae* (Zhi *et al.*, 2009).

Strain K07-0523^T could be classified as a member of the family *Micromonosporaceae* on the basis of both the phylogenetic analysis and the fact that the *N*-acyl type of muramic acid was glycolyl. However, strain K07-0523^T could be distinguished from all genera of the family *Micromonosporaceae* by the unique characteristic that the cell-wall peptidoglycan contained both *meso*-diaminopimelic acid and L-lysine as the diamino acids (Table 1). Furthermore, strain K07-0523^T was clearly different from members of the genera *Micromonospora*, *Pseudosporangium* and *Catenuloplanes*, with which it showed high 16S rRNA gene sequence similarity values, on the basis of their morphology, as these genera form only single spores, pseudosporangia or motile spores, respectively. The genera *Catenuloplanes* and *Asanoa*, which clustered with strain K07-0523^T on the phylogenetic tree, could be distinguished from strain K07-0523^T by the major menaquinones, the characteristic whole-cell sugars, the fatty acid profile and the phospholipid profile (Table 1). On the basis of the above results, it is proposed that strain K07-0523^T represents a novel genus and species in the family *Micromonosporaceae*, namely *Phytohabitans suffuscus* gen. nov., sp. nov.

Description of *Phytohabitans* gen. nov.

Phytohabitans (Phy.to.ha'bi.tans. Gr. n. *phyton* plant; L. part. adj. *habitans* inhabiting; N. L. part. adj. used as a

masc. n. *Phytohabitans* plant-inhabiting, isolated from plants).

Aerobic, Gram-positive and mesophilic actinomycetes. Vegetative mycelia are branched and not fragmented. Spores are non-motile. Cell wall contains D-glutamic acid, glycine, D-alanine, *meso*-diaminopimelic acid and L-lysine. Whole-cell sugars are galactose, glucose, mannose, ribose and xylose. The acyl type of the peptidoglycan is glycolyl. Phosphatidylethanolamine is detected, corresponding to phospholipid type II. Mycolic acids are not detected. Predominant menaquinones are MK-9(H₆), MK-10(H₄) and MK-10(H₆). The G + C content of the genomic DNA is 73 mol%. The type species is *Phytohabitans suffuscus*.

Description of *Phytohabitans suffuscus* sp. nov.

Phytohabitans suffuscus (suff.us'cus. L. masc. adj. *suffuscus* brownish, named after the brownish colour of the vegetative mycelium).

Morphological, chemotaxonomic and cultural characteristics are as given in the genus description above. Good growth occurs at 26–35 °C and at pH 7–10. No growth on 3% (w/v) NaCl. Exhibits moderate growth and forms long chains, of more than 10 spores, on ISP 3, ISP 4, ISP 5, nutrient agar and HV agar. The surface of the spores is smooth and spore size is 0.8 × 1.0 µm. The vegetative mycelia are pale orange to pale brown in colour. Soluble pigment is not produced. Major fatty acids are anteiso-C_{17:0}, iso-C_{17:0}, iso-C_{16:0}, iso-C_{15:0}. Degrades casein and weakly hydrolyses gelatin. Does not hydrolyse starch. Peptonizes and coagulates milk. Acid is produced from fructose, arabinose, mannitol, rhamnose, galactose and glucose. No acid is produced from raffinose. Nitrate is not reduced to nitrite. H₂S is not produced. Utilizes D-glucose, D-mannitol, sucrose, D-fructose, D-galactose, L-rhamnose, D-xylose, D-mannose, raffinose, L-arabinose. Does not utilize maltose, dulcitol, D-sorbitol, methyl α-D-glucopyranoside, β-lactose, trehalose, adonitol, melibiose, *myo*-inositol or *meso*-erythritol.

The type strain, K07-0523^T (=DSM 45306^T=NBRC 105367^T), was isolated from the roots of an orchid.

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