COVER SHEET

Rhizomicrobium palustre gen. nov., sp. nov., a facultatively anaerobic, fermentative stalked bacterium in the class *Alphaproteobacteria* isolated from rice plant roots

Full paper

Running head: *Rhizomicrobium palustre* gen. nov., sp. nov.

Names of authors, institutions and addresses:

Atsuko Ueki[1], Yumiko Kodama[2], Nobuo Kaku[1], Takuya Shiromura[1], Atsuya Satoh[1], Kazuya Watanabe[2] and Katsuji Ueki[1]

[1]Faculty of Agriculture, Yamagata University, Wakaba-machi 1-23, Tsuruoka 997-8555, Japan

[2]Exploratory Research for Advanced Technology (ERATO), JST, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

Correspondent footnote: Atsuko Ueki. Mailing address: Faculty of Agriculture, Yamagata University, Wakaba-machi 1-23, Tsuruoka 997-8555, Japan

Phone: 0235-28-2866. Fax: 0235-28-2846.

E-mail: uatsuko@tds1.tr.yamagata-u.ac.jp

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Summary

Facultatively anaerobic bacterial strains (A48^T, RR25 and RR54) were isolated from roots of living rice plants in irrigated rice-field in Japan. The three strains had the identical 16S rRNA gene sequences and showed almost the same phenotypic properties examined. Cells of the strains were Gram-negative, non-spore-forming rods. Reproduction of cells was by binary fission as well as by budding. Cells occurred singly or in pairs arranged angular. Some of cells, including dividing cells, were motile with a single polar flagellum. Cells developed a polar prostheca (stalk) with a holdfast-like structure and the cell with the stalk budded a daughter cell. The strains were chemoorganotrophs and utilized various sugars as growth substrates. The strains fermentatively produced acetate and lactate as well as small amounts of ethanol and H_2 from the substrates. Growth temperature and pH ranges for growth were 15-40^{o}C and pH 5.5-7.3 with optimum growth at 30-35^{o}C and pH 6.8. NaCl concentration range for growth was 0-1.0% (wt/vol) with an optimum at 0% (wt/vol). Catalase and oxidase activities were not detected. The strains reduced Fe(III) to Fe(II) in the presence of glucose, while did not reduce nitrate, fumarate, malate and sulfate. The major cellular fatty acids of the strains were $C_{18:1}\omega7$, anteiso-$C_{15:0}$, iso-$C_{15:0}$, $C_{16:0}$ and $C_{18:0}$. Ubiquinone Q-10 was the major respiratory quinone and the genomic DNA G + C contents were 53.4-55.4 mol%. Phylogenetic analysis based on 16S rRNA gene sequences placed the strains in the class *Alphaproteobacteria* and the strains formed a novel deep branch in the phylogenetic trees constructed. Based on the differences in 16S rRNA gene sequences and phenotypic properties of the novel strains from those of the relatives, we proposed that the strains should be assigned in the novel genus and species as *Rhizomicrobium palustre* gen. nov., sp. nov. The type strain of the novel species is strain A48^T (= JCM 14971^T = DSM 19867^T).

Key words: *Alphaproteobacteria*; anaerobic Gram-negative rods; Fe(III)-reduction, *Rhizomicrobium*; stalked bacterium

Introduction

In Japan, rice is widely cultivated as the principal food using irrigated fields, which develop a highly reduced condition in soil during the flooding period (Takai, 1970; Wassmann et al., 2000). The submerged, anoxic rice-field soil harbors anaerobic microbial communities composed of diverse species of microbes including fermentative and anaerobically-respiratory (nitrate-, Fe(III)- or sulfate-reducing) bacteria as well as methanogenic archaea (Boone, 2000). In anoxic rice-field soil, plant residue such as rice straw ploughed into the soil provides major amounts of growth substrates for microbes and dense microbial populations usually develop on it (Kaku et al., 2000). Roots of living rice plants also supply substrates for microbes in rhizosphere by secreting various compounds such as saccharides, amino acids and organic acids or by peeling-off the root epidermis and cortex. Substrates derived from these sources stimulate the microbial activity in the rice-field soil and thereby increase production and emission of methane from the fields as one of greenhouse gases (Boone, 2000; Kaku et al., 2000; Khalil, 2000; Seiler et al., 1984; Takai, 1970; Wassmann et al., 2000).

We have isolated various fermentative anaerobes from different samples collected from an irrigated rice-field in Japan and already described some novel species derived from plant residue as well as living rice roots (Akasaka et al., 2003a, b; Satoh et al. 2002; Ueki et al., 2006, 2007). In this study, we have characterized three strains isolated from living rice roots, which were assigned to the class *Alphaproteobacteria* and phylogenetically distant from any recognized species.

Materials and Methods

Isolation of strains. Strains A48^T (= JCM 14971^T = DSM 19867^T), RR25 (= JCM 14973 = DSM 19866) and RR54 (= JCM 14972 = DSM 19865) were isolated from roots of living rice plants (*Oryza sativa* cv. Haenuki, type japonica) in August of 1993 (strain A48^T) and 2000 (strains RR25 and RR54) collected from the same irrigated rice field soil in the Shonai Branch of the Yamagata Agricultural Research Center (Tsuruoka, Yamagata, Japan) during the intermittent irrigation period (Satoh et al., 2002). Cultivation practices for rice plants and other conditions of the fields were described previously (Ueki et al., 2000). The rice plants were taken by digging out the roots together with the surrounding soil layer using a trowel, and then soil adhering to the roots was removed by washing several times with a sterilized, anaerobic dilution solution (Satoh et al., 2002; Akasaka et al., 2003a). The root samples were cut to about 1-cm pieces and homogenized with the dilution solution using a Waring blender (10000 rpm, 10 min) under N_2. The homogenized root samples were diluted by the one-tenth serial dilution under O_2-free N_2 gas flow and inoculated to the medium described below by the anaerobic roll tube method (Holdeman et al., 1977; Hungate, 1966). Colonies formed on the roll tube agar after incubation for about one month were picked and the strains were obtained after purification using the anaerobic roll tube method. The three strains used were derived from the roll tubes inoculated with 10^{-5} diluted samples of the homogenized root samples.

Media and cultivation method. The strains were isolated by using peptone/yeast extract (PY) medium or 1/10 PY medium (the concentrations of both peptone and yeast extract were decreased to one tenth of those in PY medium) as basal medium with oxygen-free, 95% N_2/ 5% CO_2 mixed gas as the headspace as described by Satoh et al., (2002). PY or 1/10PY medium supplemented with (L^{-1}) 0.25 g each of glucose,

cellobiose, maltose and soluble starch as well as 15 g agar (Difco) were designated PY4S or 1/10 PY4S agar and used for isolation. The strains were anaerobically maintained in agar slants of PY4S medium. Cells for physiological and chemotaxonomic examinations were usually cultivated in PY medium supplemented with 10 g L^{-1} glucose (PYG). The composition of the B-vitamin mixture was as described previously (Akasaka 2003b). The strains were cultivated at 30ºC under the anaerobic condition unless otherwise stated. Growth in liquid medium was monitored by changes in OD_{660}.

Characterization of the strains. Growth of the strains under aerobic conditions was examined by plate culture on nutrient agar (Nissui Pharmacy) as well as R2A agar (Satoh et al., 2002). PY4S and 1/10 PY4S agar modified to exclude Na_2CO_3, L-cysteine·HCl·H_2O and sodium resazurin were also used. Cell morphology was assessed by observation of cells by phase-contrast microscopy in addition to observation of Gram-stained cells by light-microscopy. Cell morphology was also observed using transmission electron microscopy to confirm the presence of flagella and stalks (Kodama and Watanabe, 2004). Growth of cells exposed to 80ºC for 10 min was examined to check the presence of heat-tolerant cells. Oxidase and catalase activities were determined according to the methods described by Akasaka et al. (2003b). Utilization of carbon sources was tested in PY liquid medium with each substrate added at 10 g L^{-1} (for sugars and sugar alcohols) or 30 mM (organic acids, amino acids and alcohols). Fermentation products (fatty acids, alcohols and H_2) were analyzed by gas-chromatography (GC) as described previously (Akasaka et al., 2003a; Satoh et al., 2002; Ueki et al., 1986). Nitrate-reducing activity was determined in PY liquid medium supplemented with 2 g L^{-1} $NaNO_3$ using glucose (2 g L^{-1}), acetate, lactate, methylamine, methanol or ethanol (20 mM each) as a possible electron donor. The Fe(III)-reducing ability was examined with ferric ammonium citrate (20 mM each) as an Fe(III) source using glucose (2 g L^{-1}), acetate, pyruvate, lactate methanol, ethanol or

L-serine (20 mM each) as a possible electron donor. The concentrations of Fe(II) formed in the medium were determined with the ferrozine method (Stookey, 1970). Malate-, fumarate- and sulfate-reducing activities were determined according to the methods described by Satoh et al. (2002). Other characterizations were performed according to the methods described by Holdeman et al. (1977) and Ueki et al. (2006).

Chemotaxonomical analyses. Whole-cell fatty acids (CFAs) were converted to methyl esters (Miller, 1982) and analyzed by GC (Hp6890; Hewlett-Packard or G-3000; Hitachi) equipped with a HP Ultra 2 column. CFAs were identified by equivalent chain-length (ECL) (Miyagawa et al., 1979) according to the protocol of TechnoSuruga Co., Ltd (Shimidu, Japan) based on the MIDI microbial identification system (Microbial ID) of MOORE (Moore et al., 1994). Isoprenoid quinone was extracted as described by Komagata and Suzuki (1987) and analyzed by using a mass spectrometer (JMS-SX102A; JEOL). G + C content of genomic DNA was measured by HPLC (HITACHI L-7400) equipped with a μBondapak C18 column (3.9 × 300 mm; Waters) (Suzuki et al., 2009).

Phylogenetic analysis. Almost full-length of 16S rRNA gene was PCR amplified using the primer set of 8f and 1546r. The PCR-amplified 16S rRNA gene was sequenced by using an ABI Prism BigDye Terminator Cycle Sequencing ready reaction kit and ABI Prism 3730 automatic DNA sequencer (Applied Biosystems). Multiple alignments of the sequences with references in GenBank were performed with the BLAST program (Altschul et al., 1997) as well as the FASTA alignment tool (Pearson and Lipman, 1988). A phylogenetic tree was constructed with the neighbor-joining method (Saitou and Nei, 1987) by using the CLUSTAL W program (Thompson et al., 1994). All gaps and unidentified base positions in the alignments were excluded before sequence assembly.

Results

Various phenotypic characteristics were determined for all three strains (A48^T, RR25 and RR54) cultivated under the anaerobic conditions unless otherwise stated, and they showed similar properties for all characteristics examined. Out of the characteristics of the strains shown below, detailed descriptions such as amounts of fermentation products and the composition of CFAs will be mainly based on the data from strain A48^T as a representative strain.

Cell morphology and colony

The three strains grew under the anaerobic conditions by using PY medium as basal medium. Colonies on PY4S agar were translucent and thin with smooth surface. Reduction of concentrations of peptone and yeast extract in PYG medium (1/10 PYG) did not affect the anaerobic growth of the strains. Cells of the strains were Gram-negative rods, 0.3-0.5 μm in width and 1.0-1.5 μm in length. Cells after 2-4 days of cultivation (in PY4S slant cultures or PYG liquid medium) were straight rods with tapered ends as observed by phase-contrast microscopy, and showed snapping-like divisions to produce angular arrangement of two cells (Fig. 1A). Only a small number of cells, even in relatively younger cultures, were motile singly or in pairs under the phase-contrast microscope, and the number of motile cells decreased furthermore in older cultures. Transmission electron microscopy confirmed that cells, as a single cell or one of cells in pairs, had a polar flagellum (up to 4 μm long) (Fig. 2A, B). Cells in pairs are connected by a constriction, showing sharply-pointed ends after division (Fig. 2C). It was confirmed by electron microscopy that about 30% of cells in the 4-days culture had prosthecae on one poles of the cells. The length of the prosthecae ranged

from 0.2 to about 1.5 μm with constant diameter of 0.1-0.15 μm (Fig. 2C-H). Some prosthecae swelled at the ends to develop holdfast-like structures (Fig. 2F). The sizes of cells in pairs on the prosthecae were usually unequal, indicating that the cells without the prosthecae were budded from the cells having the prostheca (Fig. 2E-H). The daughter cells became fusiform and were connected to the mother cells with slender constrictions before separation (Fig. 2H). Based on the observations, the prosthecae were considered to be stalks, but not hyphae. The presence of stalks on the cells could not be certainly confirmed by phase-contrast microscopy (Fig. 1A, B). When Gram-stained cells were observed by light-microscopy (Fig. 1D-F), however, the stalk-like structures of the cells could be confirmed for the 5-days culture (Fig. 1E). The number of stalked-cells seemed to decrease in the older cultures (Fig. 1F).

Although the strains grew in air weakly either on PYG or nutrient agar, the aerobic growth was not improved in other several media examined including 1/10 PY4S agar, R2A and the diluted nutrient agar. Cells cultivated in the aerobic condition had the same morphological features as described above for the cells cultivated in the anaerobic conditions, although the aerobically-grown cells seemed to be slightly more slender than the anaerobically-grown cells (Fig. 1C). Cultivation under the light did not affect the color of the colonies in both conditions. Spores were not observed, and the strains did not produce heat-tolerant cells. Growth of the strains was not affected by the presence of B-vitamin mixture in PYG medium.

Physiological characteristics and substrate spectra

Catalase activity was not detected in the cells grown under the anaerobic condition, while very slight bubble formation was observed by the addition of 3% (vol/vol) H_2O_2 solution to cells cultivated in the aerobic condition. Oxidase activity was not detected. The strains grew fermentatively and utilized arabinose,

xylose, fructose, galactose, glucose, rhamnose, cellobiose, lactose, maltose, sucrose, soluble starch and xylan as growth substrates. The strains did not use ribose, mannose, sorbose, trehalose, raffinose, cellulose, glycogen, erythritol, glycerol, inositol, mannitol, sorbitol, citrate, fumarate, lactate, malate, pyruvate, succinate, L-alanine, L-aspartate, L-glutamate, glycine, L-serine, aesculin, methanol, ethanol and propanol. Fermentation products in PYG medium were acetate (6.2 mmol L^{-1}), lactate (10.4 mmol L^{-1}), ethanol (6.7 mmol L^{-1}) and H_2 (0.19 mmol L^{-1}), and final pH grown in PYG medium was 4.8. The strains produced the same fermentation products from all substrates used, although the amounts of each product were slightly different according to the substrates. The strains reduced Fe(III) (ferric ammonium citrate) to Fe (II) rather actively in the presence of glucose. Other possible electron donors examined (acetate, lactate, pyruvate, methanol, ethanol and serine) were not used for Fe(III)-reduction. Nitrate was not reduced when examined with glucose, acetate, lactate methylamine, methanol or ethanol as a possible electron donor. The strains did not reduce malate, fumarate and sulfate. The strains were negative for the production of urease, hydrogen sulfide and indole. Gelatin was not hydrolysed.

Growth conditions

The strains grew at 15-40°C and pH 5.5-7.3 with optimum growth at 30-35°C and pH 6.8. Addition of NaCl to PYG medium significantly decreased the growth rates of the strains, and the concentration range of NaCl for growth was 0-1.0% (wt/vol).

Chemotaxonomic characteristics

The three strains had almost the same profiles of the cellular fatty acid composition. For strain A48^T, the

major CFAs were $C_{18:1}\omega7$ (29.4%), anteiso-$C_{15:0}$ (16.2%), $C_{16:0}$ (13.2%), iso-$C_{15:0}$ (11.2%) and $C_{18:0}$ (9.1%). CFAs such as $C_{14:0}$ (2.4%), 3-OH $C_{14:0}$ (1.4%), 3-OH iso-$C_{15:0}$ (1.9%), 3-OH $C_{16:0}$ (2.6%), iso-$C_{17:0}$ (1.3%), 2-OH $C_{17:0}$ (1.2%) and $C_{18:1}\omega9$ (1.1%) were detected as minor components. The predominant respiratory quinone of the strains was ubiquinone Q-10 (H_0). The G + C contents of genomic DNA were 55.4 mol% (strain A48^T) and 53.4 mol% (strain RR54). For strain RR25, the respiratory quinone composition and the G + C content were not determined.

Phylogenetic affiliation

The three strains had the identical 16S rRNA gene sequences and the strains were assigned to the class *Alphaproteobacteria* (Garrity et al., 2005). The closest relative on the database was strain Mfc52 isolated from a biofilm attaching onto anode graphite of a microbial fuel cell (Kodama & Watanabe, 2008) with sequence similarity of 97.5%. The next closest relative was a clone RB521 (AB240224) with sequence similarity of 96.8% derived from rhizosphere biofilm of reed bed reactor.

The three strains were placed at almost equal distances from many described species in the different families of the *Rhizobiales* as follows: *Hyphomicrobium hollandicum* ATCC 27498^T (sequence similarity based on the FASTA search, 88.2%), *Blastochloris sulfoviridis* DSM 729^T (88.0%) and *Rhodoplanes serenus* DSM 18633^T (88.0%) in the *Hyphomicrobiaceae*; *Methylosinus trichosporium* NCIMB 11131^T (88.0%) and *Methylocystis parvus* NCIMB 11129^T (87.9%) in the *Methylocystaceae*; *Afipia birgiae* CIP 106344^T (88.2%) in the *Bradyrhizobiaceae*; *Pseudoxanthobacter soli* DSM 19599^T (88.2%) in the *Xanthobacteraceae*. The closest relatives of the isolates in the *Sphingomonadales* were *Sphingomonas melonis* DSM 14444^T and *Sphingomonas aquatilis* KCTC 2881^T (87.3% for both) in the

Sphingomonadaceae (Arun et al., 2008; Garrity et al., 2005; Okamura et al., 2009).

Discussion

The novel three strains were isolated from rice plant roots of the same irrigated rice-filed at the two different times with an interval of seven years. The submerged rice-field soil is anoxic and the highly reduced condition usually develops in it. Although the rice roots may remain relatively oxic with oxygen transported through aerenchyma of plants, it was shown that many anaerobes including methanogens were present on rice roots in submerged soil (Kaku et al., 2000; Satoh et al., 2002). Fe(III)-reduction usually proceeds actively in submerged rice-filed soil and Fe(II) accumulated in the soil acts as a reducing agent in the soil to maintain the strictly anaerobic, methanogenic environment (Ueki et al., 2000). It is known that Fe(III)-reduction also occurs on rice plant roots in submerged soil and a layer of Fe(II)-deposition develops on rice roots. The bacterial group relating to the three strains should live by fermentation using various saccharides supplied from roots or by Fe(III)-reduction, and these abilities of the three strains should be important to understand the bacterial ecology on rice roots. The closest strain (Mfc52) was derived from biofilm on the anode of microbial fuel cell operated with rice field soil as a source of microorganisms. The cells of strain Mfc52 also possessed prosthecae-like appendages, which were considered to play an important role in the generation of electricity (Kodama and Watanabe, 2008). The closest clone (RB521) was also derived from rhizosphere biofilm of reed in the laboratory. These facts suggest that bacterial groups relating to the three novel strains should be common and important members of microbial communities in biofilm on solid surface under submerged anoxic conditions.

Some characteristics of the novel three strains were compared with those of some related genera in the

Rhizobiales as well as the genus *Sphingomonas* in the *Sphingomonadales* (Table 1). Based on the observations of cells by transmission electron microscopy, we confirmed that reproduction of our strains is by binary fission as well as by budding from mother cells. Furthermore, we concluded that the polar prosthecae produced by the cells are stalks, but not hyphae, since the daughter cells budded directly from the mother cells having the prosthecae. The *Hyphomicrobium* species, one of the closest relatives of the three strains, are aerobic, and the cell produces a polar hypha developing a daughter cell on the tip (Garrity et al., 2005). *Blastochloris* species in the *Hyphomicrobiaceae* are photoheterotrophic under anoxic conditions in the light, and the mother cell of *Blastochloris viridis* also forms a daughter cell at the end of a slender prostheca (Hiraishi, 1997). *Rhodoplanes* species are also photoheterotrophic and multiply by budding and asymmetric cell division, and the presence of a division tube between the mother and daughter cells has been confirmed for *Rhodoplanes serenus* (Okamura et al., 2009). Thus, the novel strains have a common feature to produce polar prosthecae with some relatives in the *Hyphomicrobiaceae*, however, the function of the prosthecae of the novel strains seems to be different from those of the related species. Cells of other relatives in the *Rhizobiales* and the genus *Sphingomonas* in the *Sphingomonadales* do not produce any prostheca-like structure. In addition, all these organisms are aerobic and other physiological properties are basically different from those of the three strains.

The novel strains had $C_{18:1}$ as the dominant CFA, which is usually present in most of the species in the class *Alphaproteobacteria* as a major CFA, while the profiles of CFAs containing branched-$C_{15:0}$ as one of major CFAs were distinct in those of the relatives. The G + C contents of the strains were significantly lower than those of the related organisms (Garrity et al., 2005).

The class *Alphaproteobacteria* includes some species other than the *Hyphomicrobium* species that form

polar prosthecae (stalks or hyphae) on the cells. The known species producing polar prosthecae are currently assigned to the different orders, that is, *Rhizobiales*, *Rhodobacterales* and *Caulobacterales* (Garrity et al., 2005) or *Rhizobiales* and *Caulobacterales* (Lee et al., 2005). The morphological features of the novel three strains with polar stalks on the cells resemble to those of the species in the genera *Caulobacter* (Garrity et al., 2005), *Maricaulis* (Abraham et al., 1999, 2002), *Oceanicaulis* (Strömpl et al., 2003) and *Woodsholea* (Abraham et al., 2004). The three strains, however, are also only remotely related to any of the members of these stalked bacteria; similarities of 16S rRNA gene sequences between A48^T and *Caulobacter vibrioides* DSM 9893^T, *Maricaulis maris* ATCC 15268^T, *Oceanicaulis alexandrii* DSM 11625^T and *Woodsholea maritime* LMG 21817^T are 85.0, 86.1, 83.3 and 84.4%, respectively.

Except for anoxic, phototrophic bacteria, most of the species in the class *Alphaproteobacteria* are aerobic and have a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. Only restricted species in the genera such as *Gemmobacter* and *Ketogulonicigenium* in the order *Rhodobacterales*, *Zymomonas* in the order *Sphingomonadales* and *Ancalomicrobium* in the family *Hyphomicrobiaceae* are described to be facultatively anaerobic and fermentative. *Gemmiger formicilis* in the family *Hyphomicrobiaceae* seems to be only one strictly anaerobic, fermentative species in the class *Alphaproteobacteria*. Although the novel three strains grew under the aerobic conditions, the growth was much weaker than that in the anaerobic conditions and the cells of the strains did not have a distinct catalase activity irrespective of the cultivation conditions. Many species in the class *Alphaproteobacteria* have ability of nitrate reduction, while our strains did not reduce nitrate. The facultatively anaerobic, fermentative and Fe(III)-reducing properties of the three strains are rather unique in all species in the class *Alphaproteobacteria* including the phylogenetically and phenotypically related species (Garrity et al.,

2005).

Classification of species belonging to the class *Alphaproteobacteria* is fundamentally based on the 16S rRNA gene sequences and the class is currently divided into nine orders (Cho and Giovannoni, 2003; Garrity et al., 2005; Kurahashi et al., 2008; Kwon et al., 2005), most of which consisted of morphologically and physiologically heterogeneous species. Phylogenetic trees including the three strains and the type species of representative genera in the nine orders in the class *Alphaproteobacteria* (Garrity et al., 2005; Lee et al., 2005) were constructed with the neighbor-joining method (Saitou and Nei, 1987) (Fig. 3). Although the topology of the trees slightly varied depending on the species selected, the novel three strains always formed a deep branch together with strain Mfc52 and clone RB521 diverged from a major branch including the families in the order *Sphingomonadales*.

Several significant phylogenic analyses on the class *Alphaproteobacteria* have been carried out recently based on different standpoints and various data have shown that further reconstruction of the higher taxonomic groups in the class must be established. Especially, Lee et al., (2005) proposed that the order *Rhodobacterales* (Garrity et al., 2005) should be eliminated and the family '*Rhodobacteraceae*' must be included in the order *Caulobacterales* based on the comprehensive analysis of 16S rRNA gene sequences in the class. Furthermore, Badger et al. (2005) indicated that although 16S rRNA gene sequence analysis supported the affiliation of *Hyphomonas neptunium* with the order *Rhodobacterales*, phylogenies based on the sequences of the 23S rRNA gene and three proteins supported the grouping of the genus *Hyphomonas* as a member of the *Caulobacterales*. Badger et al. (2006) further indicated based on the genome sequence of *H. neptunium* that the species shared more genes with *Caulobacter crescentus* than it did with the closer relative according to the 16S rRNA phylogeny. Williams et al. (2007) got the same conclusion according to

the multiprotein tree analysis and also suggested that the order *Caulobacterales* should include the family *Parvularculaceae* with the abandonment of the order *Parvularculales*, which was designated mainly on the 16R rRNA sequence (Cho and Giovannonl, 2003). Gupta and Mok (2007) have also presented just the same conclusion based on the comprehensive phylogenic analyses of genome sequences for many species in the class *Alphaproteobacteria*. These reports especially suggested that the species producing prostheca in the class *Alphaproteobacteria* should be reclassified using more diverse sequence data other than 16S rRNA gene.

Based on the differences in 16S rRNA gene sequences and phenotypic properties of the three strains from those of phylogenetically and phenotypically related species, we considered that the three strains were not validly assigned to any of the families or orders currently recognized in the class *Alphaproteobacteria*. Taking into consideration for the above-mentioned various reports for the phylogeny of the species in the class of *Alphaproteobacteria*, however, we propose here that the strains should be assigned in the novel genus and species as *Rhizomicrobium palustre* gen. nov., sp. nov. without definition of the higher taxonomic affiliation in the class *Alphaproteobacteria*. The type strain of the novel species is A48^{T} (= JCM 14971^{T} = DSM 19867^{T}) isolated from rice plant roots in irrigated rice field soil in Japan. Strain RR25 (= JCM 14973 = DSM 19866) and RR54 (= JCM 14972 = DSM 19865) as reference strains were also derived from rice roots of living rice plants obtained from the same field in different year. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains A48^{T}, RR25 and RR54 are AB081581, AB174809 and AB174822, respectively.

Description of *Rhizomicrobium* gen. nov.

Rhizomicrobium (Rhi.zo.mi.cro'bi.um.. Gr. n. *rhiza* a root; N.L. neut. n. *microbium* a microbe; N.L. neut. n. *Rhizomicrobium* microbe living in a root or rhizosphere).

Cells are Gram-negative, non-spore-forming, motile rods. Cells divide by binary fission as well as by budding and have polar stalks. The major respiratory quinone is ubiquinone Q-10. The type species is *Rhizomicrobium palustre*.

Description of *Rhizomicrobium palustre* sp. nov.

Rhizomicrobium palustre (pa.lu.st're. L. neut. adj. *palustre* living in swamps)

Has the following characteristics in addition to those described for the genus. Cells are Gram-negative rods, 0.3-0.5 µm in width and 1.0-1.5 µm in length. Cells occur singly or in pairs arranged angular, some of which are motile with a single polar flagellum. Some cells develop a polar stalk with a holdfast structure. The cells with stalks bud daughter cells. Chemoorganotroph. Facultatively anaerobic. Have a fermentative metabolism. Does not require B-vitamins. Utilizes arabinose, xylose, fructose, galactose, glucose, rhamnose, cellobiose, lactose, maltose, saccharose, starch and xylan as fermentative substrates and produces acetate and lactate as well as small amounts of ethanol and H_2 from the substrates. Does not use ribose, mannose, sorbose, trehalose, raffinose, cellulose, glycogen, erythritol, glycerol, inositol, mannitol, sorbitol, citrate, fumarate, lactate, malate, pyruvate, succinate, alanine, aspartate, glutamate, glycine, serine, aesculin, methanol, ethanol and propanol. Growth temperature range is 15-40^{o}C with optimum at 30-35^{o}C. pH range for growth is pH 5.5-7.3; optimum at 6.8. NaCl concentration range for growth is 0-1.0% (wt/vol). Does not have catalase and oxidase activities. Reduces Fe(III) to Fe(II) in the presence of glucose. Does not reduce nitrate, fumarate, malate and sulfate. Does not produce indole, hydrogen sulfide and urease. The major

cellular fatty acids are $C_{18:1}\omega7$, anteiso-$C_{15:0}$, iso-$C_{15:0}$, $C_{16:0}$ and $C_{18:0}$. The genomic DNA G + C contents are 53.4-55.4 mol%. The type strain of the novel species is A48^T (= JCM 14971^T =DSM 19867^T) isolated from rice plant roots in flooded rice field soil. Strain RR25 (= JCM 14973 = DSM 19866) and strain RR54 (= JCM 14972 = DSM 19865) as reference strains are derived from roots of living rice plants of the same field collected in different years.

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Legends for figures

Fig. 1. Photomicrographs of cells of strain A48^T: A-C, cells observed by phase-contrast microscopy; anaerobically cultivated cells for 4 days (A) and 7 days (B), and aerobically cultivated cells for 7 days (C). D-F, Gram-stained cells; cultivated anaerobically for 2 days (D), 5 days (E) and 7 days (F). Bar, 5 μm.

Fig. 2. Transmission electron microscopy of cells cultivated anaerobically for 4 days, showing a polar flagellum (A and B), binary fission (B and C), development of a polar stalk (C-H), budding (E-H), holdfast (F) and constriction connecting dividing cells (C and H). A-C, strain A48^T; D-G, strain RR54; H, strain RR25. Bar, 0.5 μm.

Fig. 3. Neighbor-joining tree based on 16S rRNA gene sequences including the three novel strains (A48^T, RR54 and RR25) and the type species of representative genera in the nine orders within the class *Alphaproteobacteria* (Cho & Giovannoni, 2003; Garrity et al., 2005; Kurahashi et al. 2008; Kwon et al., 2005; Lee et al., 2005). Bootstrap values above 500 based on analysis of 1000 replicates are shown at branch nodes. *Escherichia coli* ATCC 11775^T was used as the outgroup. Bar, estimated difference of 2% in nucleotide sequence positions.

Fig.1.

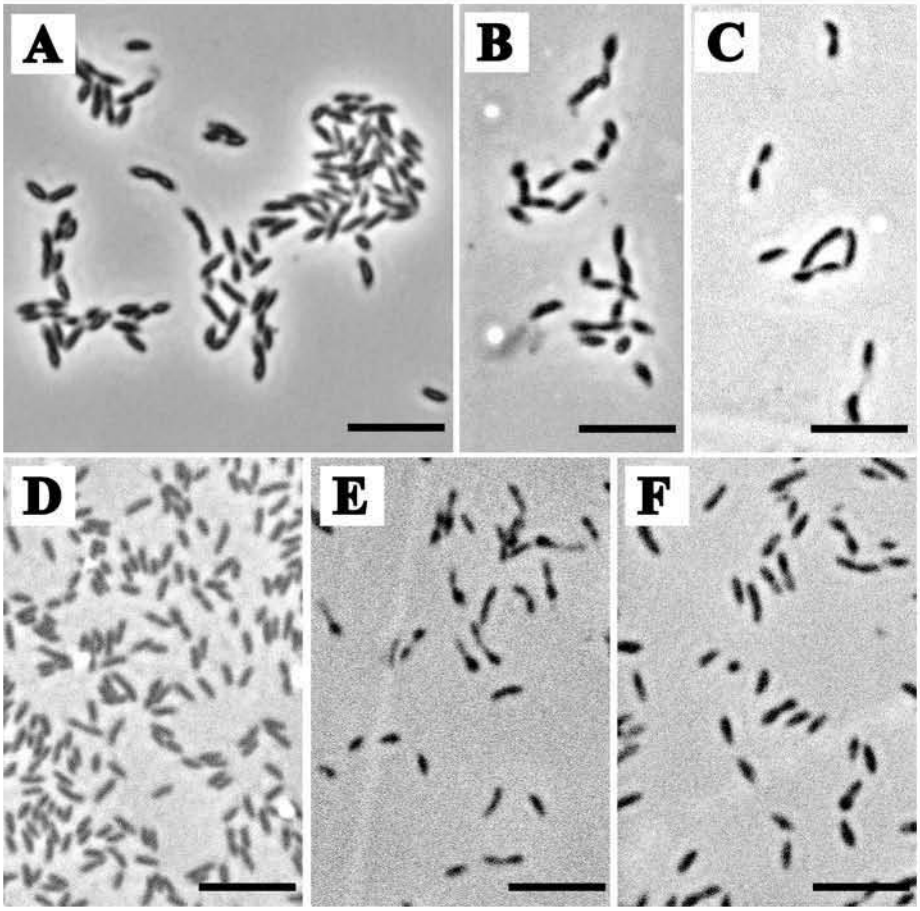

Fig.2.

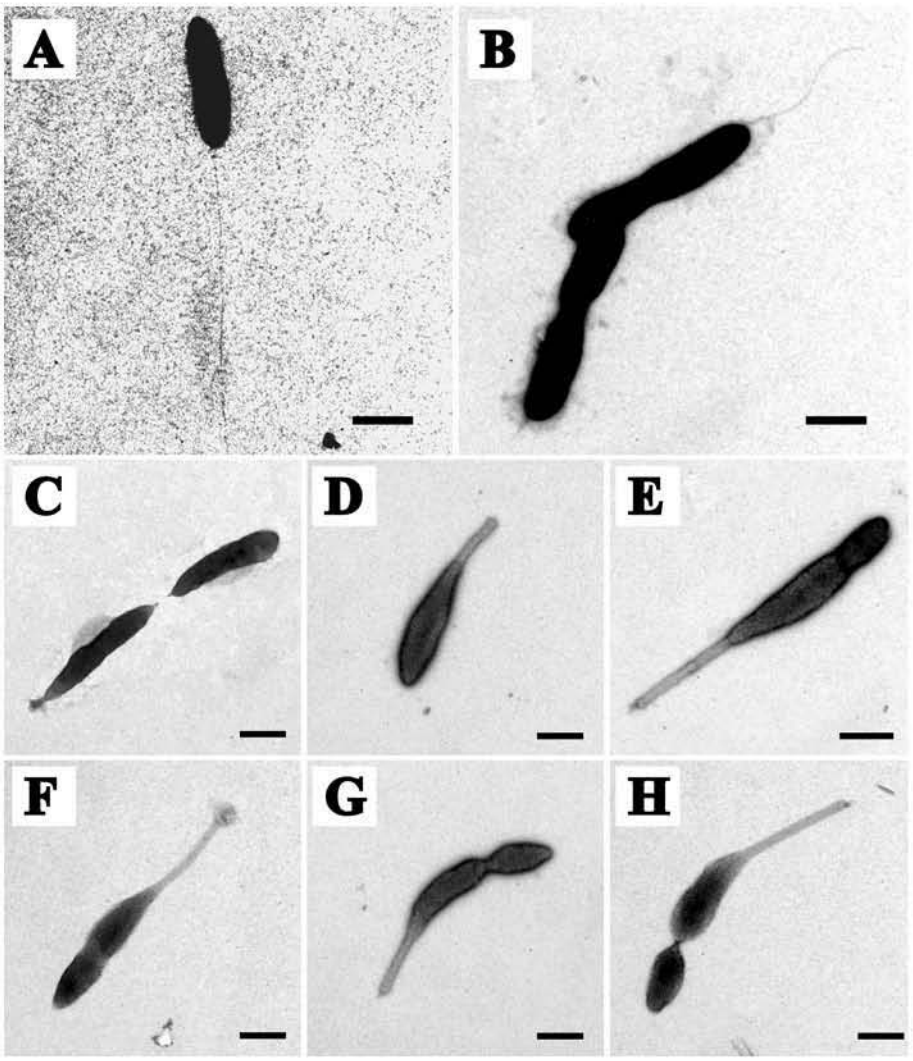

Fig. 3

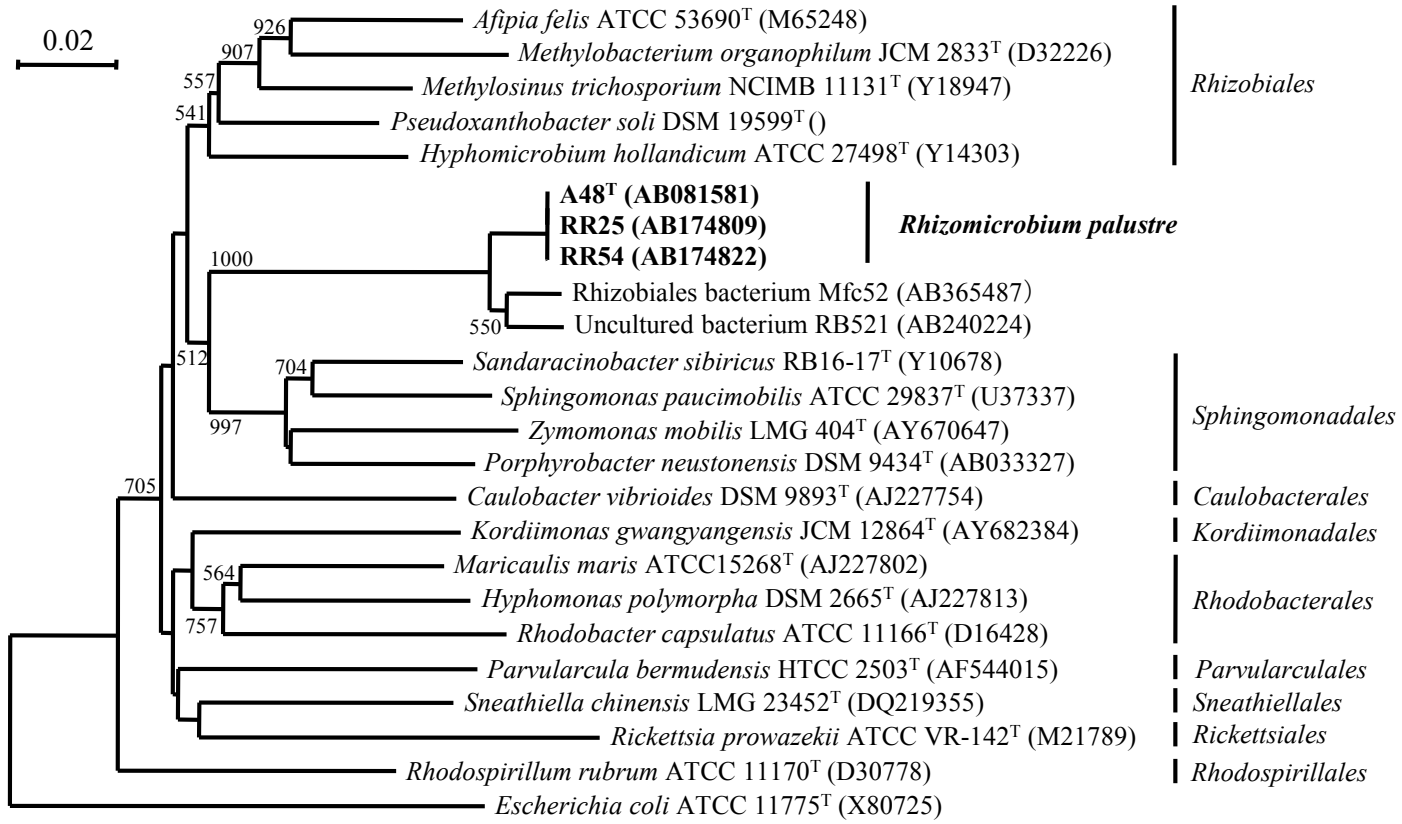
0.02
926
907
557
541
1000
550
512
704
997
705
564
757
Afipia felis ATCC 53690T (M65248)
Methylobacterium organophilum JCM 2833T (D32226)
Methylosinus trichosporium NCIMB 11131T (Y18947)
Pseudoxanthobacter soli DSM 19599T ()
Hyphomicrobium hollandicum ATCC 27498T (Y14303)
A48T (AB081581)
RR25 (AB174809)
RR54 (AB174822)
Rhizomicrobium palustre
Rhizobiales bacterium Mfc52 (AB365487)
Uncultured bacterium RB521 (AB240224)
Sandaracinobacter sibiricus RB16-17T (Y10678)
Sphingomonas paucimobilis ATCC 29837T (U37337)
Zymomonas mobilis LMG 404T (AY670647)
Porphyrobacter neustonensis DSM 9434T (AB033327)
Caulobacter vibrioides DSM 9893T (AJ227754)
Kordiimonas gwangyangensis JCM 12864T (AY682384)
Maricaulis maris ATCC15268T (AJ227802)
Hyphomonas polymorpha DSM 2665T (AJ227813)
Rhodobacter capsulatus ATCC 11166T (D16428)
Parvularcula bermudensis HTCC 2503T (AF544015)
Sneathiella chinensis LMG 23452T (DQ219355)
Rickettsia prowazekii ATCC VR-142T (M21789)
Rhodospirillum rubrum ATCC 11170T (D30778)
Escherichia coli ATCC 11775T (X80725)
Rhizobiales
Sphingomonadales
Caulobacterales
Kordiimonadales
Rhodobacterales
Parvularculales
Sneathiellales
Rickettsiales
Rhodospirillales

Table 1. Some characteristics that differentiate the three strains (A48^T, RR25 and RR54) from other related genera in the class *Alphaproteobacteria* .

Family		*Hyphomicrobiaceae*	*Hyphomicrobiaceae*	*Hyphomicrobiaceae*	*Methylocystaceae*	*Methylocystaceae*	*Bradyrhizobiaceae*	*Xanthobacteraceae*	*Sphingomonadaceae*
Strain or genus	A48^T, RR25, RR54	*Hyphomicrobium*	*Blastochloris*	*Rhodoplanes*	*Methylosinus*	*Methylocystis*	*Afipia*	*Pseudoxanthobacter*	*Sphingomonas*
Cell division	Budding, binary divisor	Polar growth, budding	Polar growth, budding	Budding	Binary, budding	Binary divison	Binary division	Binary divisiom	Binary division
Prostheca	Stalk	Hypha	Hypha	(Division tube)	-	-	-	-	-
Growth	Facultatively anaer-obic, fermentative, Fe(III) reduction	Aerobic, nitrate reduction	Phototrophic (anoxic), chemotrophic (micro-oxic)	Phototrophic (anoxic) chemotrophic (oxic), nitrate reduction	Aerobic, obligately methanotrophic	Aerobic, obligately methanotrophic	Aerobic	Aerobic	Aerobic
Utilization of C_1 compounc	-	+	-	-	+	+	-	-	-
Quinone	Q-10	Q-9	Q-8/9/10, MK-7/8/9	Q-10, RQ-10	Q-8	Q-8	Q-10	Q-10	Q-10
Major CFAs	$C_{18:1}$, anteiso-$C_{15:0}$ iso-$C_{15:0}$, $C_{16:0}$, $C_{18:0}$	$C_{18:1}$	$C_{18:1}$	$C_{18:1}$, $C_{16:0}$	$C_{18:1}$	$C_{18:1}$	$C_{18:1}$, branched-$C_{19:}$ $C_{17:0\ cyclo}$	$C_{18:1}$, $C_{19:0\ cyclo}$, $C_{16:0}$	$C_{18:1}$, $C_{14:0\ 2\text{-}OH}$, $C_{16:0}$
G+C (%) of genome DNA	53-55	59-65	66-71	67-70	62-67	61-67	61.5-69		59-68

+, Positive; -, negative.

Data except for the genus *Pseudoxanthobacter* were taken from Garrity et al., (2005), and data for the genus *Pseudoxanthobacte* r were from Arun et al. (2008).