

Sphingomonas oligophenolica sp. nov., a halo- and organo-sensitive oligotrophic bacterium from paddy soil that degrades phenolic acids at low concentrations

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The taxonomic position of a halo- and organo-sensitive, oligotrophic soil bacterium, strain S213^T, was investigated. Cells were Gram-negative, non-motile, strictly aerobic, yellow-pigmented rods of short to medium length on diluted nutrient broth. When 0.1–0.4% (w/v) NaCl was added to diluted media composed of peptone and meat extract, growth was inhibited with increasing NaCl concentration and the cells became long aberrant forms. When 6 mM CaCl₂ was added, the cells grew quite normally and aberrant cells were no longer found at 0.1–0.5% (w/v) NaCl. Chemotaxonomically, strain S213^T contains chemical markers that indicate its assignment to the *Sphingomonadaceae*: the presence of ubiquinone Q-10 as the predominant respiratory quinone, C_{18:1} and C_{16:0} as major fatty acids, C_{14:0} 2-OH as the major 2-hydroxy fatty acid and sphingoglycolipids. 16S rRNA gene sequence analysis indicated that strain S213^T belongs to the genus *Sphingomonas*, exhibiting high sequence similarity to the 16S rRNA gene sequences of *Sphingomonas mali* IFO 15500^T (98.3%), *Sphingomonas pruni* IFO 15498^T (98.0%), *Sphingomonas asaccharolytica* IFO 15499^T (97.9%) and *Sphingomonas echinoides* DSM 1805^T (97.8%). The results of DNA–DNA hybridization experiments and its phenotypic characteristics clearly distinguished the strain from its nearest neighbours and demonstrate that strain S213^T represents a novel *Sphingomonas* species, for which the name *Sphingomonas oligophenolica* sp. nov. is proposed. The type strain is S213^T (=JCM 12082^T = CIP 107926^T).

In very early studies on soil bacterial populations, soil microbiologists noted that the best results of plate counting were obtained on media containing low levels of organic nutrients (e.g. Conn, 1914). The low organic content of the media undoubtedly holds in check certain rapidly growing organisms that would prevent the growth of the more numerous but more slowly growing bacteria. In the 1970s and 1980s, a series of studies by Hattori and co-workers demonstrated that a number of soil bacteria isolated on low-nutrient media were not only slow growing but also

highly sensitive to mineral salts and organic compounds (Hattori, 1976; Hattori & Hattori, 1980; Ohta & Hattori, 1980; Suwa & Hattori, 1984; Whang & Hattori, 1988). Such low-nutrient bacteria were classified as members of oligotrophic groups by their ability to grow on media containing trace amounts of organic nutrients (Ohta & Hattori, 1983a; Suwa & Hattori, 1984). Although members of the genus *Arthrobacter* (class *Actinobacteria*) are widespread in soil and are assumed to be examples of soil oligotrophic bacteria (Williams, 1985), a majority of oligotrophic isolates from paddy field soils in Japan belonged to the ‘*Alphaproteobacteria*’, ‘*Betaproteobacteria*’ or *Cytophaga–Flexibacter–Bacteroides* group (Mitsui *et al.*, 1997a).

A soil bacterium, strain S213^T, was isolated on a low-nutrient medium from Kashimadai paddy soil near Sendai

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Sphingomonas oligophenolica* S213^T is AB018439.

Micrographs of the fine structures of strain S213^T are available as supplementary material in IJSEM Online.

in Japan (Hattori, 1976), and its growth was severely suppressed by full-strength nutrient broth (Hattori & Hattori, 1980). Strain S213^T was tentatively identified as a Gram-negative aerobic bacterium close to *Pseudomonas paucimobilis* (now *Sphingomonas paucimobilis*) (Ohta, 1982). Strain S213^T is able to grow rapidly with low concentrations (<1 mM) of lignin-related ferulic acid, but growth is inhibited at concentrations above this (Ohta, 2001). Because ferulic acid concentrations in normal paddy soils are low (Shindo & Kuwatsuka, 1977), it seems likely that strain S213^T-related organisms play an important role in the process of lignin degradation. With respect to the salt sensitivity of soil oligotrophs, it was reported that a drastic change in the outer membrane structure of a soil bacterium, strain S34, related to the genus *Deinococcus* was induced by 0.2–0.4% (w/v) NaCl, and that this change in structure was relieved by 6 mM CaCl₂ and induced by 1 mM EGTA (Mitsui *et al.*, 1997b). The aim of the present study was to determine the taxonomic relationships between strain S213^T and related species in the *Sphingomonas* group, using phenotypic, chemotaxonomic and genomic analyses. In addition, we examined the effect of NaCl and CaCl₂ on the cell morphology and ultrastructure of strain S213^T to show its dependence on calcium.

Strain S213^T was maintained as a stab culture in a 100-fold dilution (10⁻² NB) of the nutrient broth (NB) containing 0.4% (w/v) agar at room temperature. NB comprised 1% (w/v) meat extract (Kyokuto Seiyaku), 1% (w/v) polypeptone (Nihon Seiyaku) and 0.5% (w/v) NaCl. pH was adjusted to 7.0 with 1 M NaOH. Cellular morphologies were examined after growth in the late-exponential phase at 27 °C by phase-contrast microscopy and electron microscopy (Mitsui *et al.*, 1997b). The following liquid media were used: (1) 10⁻² PM (peptone and meat extract), which comprised 0.01% (w/v) each of peptone (Kyokuto Seiyaku) and meat extract; (2) 10⁻² PM supplemented with 0.1, 0.2, 0.3, 0.4 or 0.5% (w/v) NaCl; and (3) 10⁻¹ PM, which comprised 0.1% (w/v) each of peptone and meat extract. CaCl₂ was added to media 2 and 3 to a final concentration of 6 mM to determine the effect of calcium ions.

Heat resistance was examined with 28-day-old cultures on 10⁻² NB semi-solid agar medium by testing viability after standing for 10 min at 80 °C. Cultures surviving the heat treatment were regarded as spore-formers. To determine the ability of cells to grow anaerobically, cultures of 10⁻² NB or 10⁻² NB with 0.2% (w/v) glucose both in the presence and in the absence of 0.1% (w/v) NaNO₃ were incubated in an atmosphere containing 80% (v/v) N₂, 10% (v/v) H₂ and 10% (v/v) CO₂.

Substrate utilization profile was tested in a 1000-fold diluted NB (10⁻³ NB) liquid medium supplemented with L-arabinose, D-xylose, D-glucose, D-galactose, D-mannose, D-fructose, cellobiose, maltose, lactose, raffinose, acetic acid, DL-lactic acid, gluconic acid, pyruvic acid, 2-oxoglutaric acid, citric acid, succinic acid, L-malic acid or methanol. All compounds were sterilized by filtration and were added to

autoclaved 10⁻³ NB medium. Sugars were added at a concentration of 0.1% (w/v), organic acids at 0.03% (w/v) and methanol at 50 mM. Growth was measured over a 2-week period and utilization was assessed by comparing the growth both in the presence and in the absence of an added compound. To test the degradation profile of aromatic compounds, strain S213^T was grown at 30 °C in a 10-fold dilution of Difco nutrient broth supplemented with one of the following compounds (1 mM): benzoic acid, ferulic acid, *o*-, *m*- and *p*-hydroxybenzoic acid, *o*-, *m*- and *p*-toluic acid, *o*- and *p*-anisic acid, *o*-, *m*- and *p*-cresol, cinnamic acid, *m*- and *p*-coumaric acid, phenylacetic acid, phenol, vanillic acid or caffeic acid. Cultures were left for 10 days and degradation of a compound was judged by a change in the UV absorption spectrum. Organic acids including aromatic acids were prepared as sodium salts. Assay for hydrolysis of starch, gelatin and casein and tests for oxidase, catalase and nitrate reduction were carried out as described by Ohta & Hattori (1983b).

Analyses of cellular fatty acids and isoprenoid quinones were performed as described by Komagata & Suzuki (1987). For detection of hydroxy fatty acids, TLC (Kieselgel F-254, Merck) was employed with a solvent system of n-hexane and diethyl ether (1:1, v/v). Fatty acid methyl ester analysis by GLC was carried out on a glass column (5 m) packed with 10% diethyleneglycol succinate (DEGS) using standard fatty acid methyl esters, as described by Ohta & Hattori (1983b). Cellular lipids were analysed by the method of Hirai *et al.* (1995) using *Sphingomonas paucimobilis* JCM 7516^T as the reference strain. For detection of alkaline-stable glycolipids, the total extractable lipids were incubated in 1 M KCl and methanol (2:1, v/v) for 2 h at 37 °C and analysed by TLC with a solvent system composed of chloroform, methanol and water (70:30:5, by volume).

Isolation of DNA (Saito & Miura, 1963) and determination of the DNA G+C content by the thermal denaturation method (Marmur & Doty, 1962) followed standard procedures. DNA–DNA relatedness was estimated by a chemiluminescence DNA–DNA hybridization method with photobiotin-labelled probes in microplate wells, as described by Ezaki *et al.* (1989). For enzymic development, alkaline phosphatase–streptavidin conjugate (Vector) was used with CDP-Star (Tropix) as the substrate and chemiluminescence was measured on a Wallac 1420 ARVOsx multilabel counter.

The 16S rRNA gene sequence of strain S213^T was obtained by PCR amplification of genomic DNA using a universal primer set, and the nucleotide sequence (nucleotide positions 28–1390; *Escherichia coli* numbering) was determined as described by Hiraishi (1992). The DNA sequence was aligned with reference sequences of representative species of *Sphingomonas sensu stricto*, *Novosphingobium*, *Sphingobium* and *Sphingopyxis*. Multiple alignments, calculation of nucleotide substitution rates (*K*_{nuc} values) as described by Kimura (1980) and construction of a phylogenetic tree by the neighbour-joining method (Saitou

& Nei, 1987) were performed by using the CLUSTAL W program (Thompson *et al.*, 1994). The robustness of tree topology was evaluated by a bootstrap analysis (1000 replications).

Strain S213^T showed a range of phenotypic properties typical of members of the genus *Sphingomonas* (Yabuuchi *et al.*, 1990; Takeuchi *et al.*, 2001). Cells are strictly aerobic, Gram-negative, non-sporulating and catalase-positive rods (0.4–0.6 × 1.0–1.5 μm). Colonies are pale yellow on 10⁻² NB agar and yellow on a 10-fold diluted NB agar medium. When ferulate-limited chemostat-cultured cells (Ohta, 2001) were used for analysis of acetone-soluble pigments (70 mg wet cells with 400 μl acetone), the spectrum of the acetone extract had peaks at 454 and 482 nm and a shoulder at 431 nm. Strain S213^T grew at 4, 10, 25, 30 and 37 °C but not at 42 °C in 10⁻² NB liquid cultures.

When grown in 10⁻² PM, strain S213^T clearly shows the typical cell wall structure of Gram-negative bacteria, as recognized from the presence of the outer membrane and the peptidoglycan layer (see Fig. A available as supplementary material in IJSEM Online). When 20 phase-contrast microscopic fields, each containing 50–70 cells, were observed, the ratios of aberrant to normal cells in 10⁻² PM supplemented with 0, 0.1, 0.2 and 0.3 % (w/v) NaCl were 0 (Fig. 1A), 0.23, 0.64 and 0.83, respectively. With increasing NaCl concentration, cells became longer and, at 0.4 % (w/v) NaCl, all cells resembled long filaments and seemed to be aberrant in form (Fig. 1B). At 0.5 % (w/v) NaCl, faint ghost-like filaments were observed exclusively. When cells were grown in 10⁻¹ PM, they became longer and aberrant but less remarkably so (data not shown). Calcium significantly relieved this effect of NaCl as in the case of strain S34, a highly halo-sensitive soil bacterium (Mitsui *et al.*, 1997b). When 6 mM CaCl₂ was added, cells of strain S213^T grew quite normally and the ratio of aberrant to normal cells was 0 at every NaCl concentration tested (0.1–0.5 %, w/v) in 10⁻² PM or in

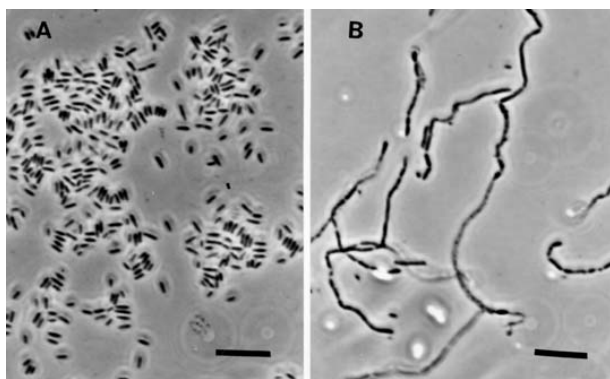


Fig. 1. Phase-contrast micrographs showing the effect of NaCl on cell morphology. (a) Cells grown in 10⁻² PM; (b) cells grown in 10⁻² PM supplemented with 0.4 % (w/v) NaCl. Bars, 5 μm.

10⁻¹ PM. In the presence of 0.1 % (w/v) NaCl, deposition of electron-dense particles between the outer and inner membranes became apparent (supplementary Figs B and C in IJSEM Online). With the addition of 0.4 % (w/v) NaCl, three types of structural change were observed: (1) membrane-deposited particles were excluded from the cells (supplementary Fig. D), (2) membrane structure remained normal but division was inhibited (supplementary Fig. E), and (3) cell lysis occurred (supplementary Fig. C). These detrimental effects of NaCl on the cellular structure were not detected in the presence of CaCl₂ (supplementary Fig. F).

Strain S213^T utilized some pentoses, hexoses, oligosaccharides and organic acids. Methanol was not utilized. The organism was able to degrade four phenolic acids (ferulic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid and vanillic acid) of 20 aromatic compounds tested. The strain reduced nitrate to nitrite but denitrification was not detected. Other details relating to biochemical characteristics are included below in the species description. Diagnostic characteristics are shown in Table 1.

Chemotaxonomically, strain S213^T contains chemical markers that support its assignment to the *Sphingomonas sensu lato* group. Cells contain ubiquinone Q-10 as the predominant respiratory quinone and have C_{18:1} (56 %, mean of independent duplicate determinations), C_{16:0} (10 %) and C_{16:1} (6 %) as major fatty acids and C_{14:0} 2-OH (19 %) as the major 2-hydroxy fatty acid. Other fatty acids detected were C_{14:0} (3 %), C_{17:1} (1 %) and C_{12:0} (<0.5 %); the summed amount of unknown acids was 5 %. No visible spot of 3-hydroxy fatty acids was detected in TLC of the fatty acid methyl esters. When the total extractable lipid pattern of strain S213^T on TLC was compared with that of *Sphingomonas paucimobilis* JCM 7516^T, three major spots of polar lipids of R_F values 0.41, 0.57 and 0.78 were found in common. Based on the results of polar lipid analysis by Yabuuchi *et al.* (1990), the major polar lipids of R_F values 0.57 and 0.78 corresponded to phosphatidyl ethanolamine and cardiolipin, respectively. In our preparation of polar lipids, the spot of phosphatidyl glycerol (PG) was not clearly detected in strain S213^T. Mild alkaline hydrolysis of the total extractable lipids and subsequent TLC analysis revealed that the major lipid of R_F value 0.41 was an alkaline-stable glycolipid, probably glucuronosyl ceramide reported with *Sphingomonas paucimobilis* JCM 7516^T by Yabuuchi *et al.* (1990). A minor spot of alkaline-stable glycolipid of R_F value 0.25 was also detected in strain S213^T but its relation to the glycolipids GX₁, GX₂, GX₃ and GL' of the *Sphingomonas* group (Yabuuchi *et al.*, 1990) is currently unknown.

The 16S rRNA gene sequence (1306 bp) of strain S213^T was used for searches in the GenBank, EMBL and DDBJ databases by the FASTA program (Pearson & Lipman, 1988). Strain S213^T is a member of the *Sphingomonadaceae*, most closely related to the genus *Sphingomonas*. Sequence similarity calculations indicated that the nearest relatives of the strain are *Sphingomonas mali* IFO 15500^T (98.3 %),

Table 1. Phenotypic characteristics of strain S213^T and the type strains of nine phylogenetically related *Sphingomonas* species

Strains: 1, strain S213^T (this study); 2, *S. echinoides* DSM 1805^T (Denner *et al.*, 1999); 3, *S. asaccharolytica* IFO 15499^T (Takeuchi *et al.*, 1995); 4, *S. mali* IFO 15500^T (Takeuchi *et al.*, 1995); 5, *S. pruni* IFO 15498^T (Takeuchi *et al.*, 1995); 6, *S. koreensis* JSS26^T (Lee *et al.*, 2001); 7, *S. melonis* DAPP-PG 224^T (Buonaurio *et al.*, 2002); 8, *S. aquatilis* JSS7^T (Lee *et al.*, 2001); 9, *S. adhaesiva* GIFU 11458^T (Yabuuchi *et al.*, 1990); 10, *S. paucimobilis* GIFU 2395^T (type strain for the type species of the genus, Yabuuchi *et al.*, 1990). ND, No data; +, positive reaction; −, negative reaction; w, weakly positive reaction. All strains are positive for utilization of glucose and maltose and contain C_{14:0} 2-OH as the major 2-hydroxy fatty acid.

Characteristic	1	2	3	4	5	6	7	8	9	10
Motility	−	+	+	+	+	+	−	+	+	+
Reduction of nitrate	+	+*	+*	+*	+*	−	−	−	−	+
Hydrolysis of gelatin	+	−	−	−	+	−	−	−	−	+
Acid production from glucose	−	−	w	−	w	w	+	+	−	+
Assimilation of:										
Acetate	+	−	−	−	w	ND	ND	ND	−	ND
L-Arabinose	+	+	w	w	+	−	+	+	−	+
Cellobiose	+	w	−	+	+	ND	+	ND	+	+
D-Fructose	−	−	−	w	+	ND	+	ND	+	+
D-Galactose	+	+	−	+	+	ND	+	ND	−	+
Gluconate	+	+	−	+	+	−	−	−	−	−
DL-Lactate	+	−	−	−	−	ND	+	ND	−	−
Lactose	+	ND	w	+	+	ND	+	ND	+	+
L-Malate	−	+	−	−	w	+	+	−	+	+
D-Mannose	+	−	w	+	+	−	+	−	+	+
Raffinose	+	ND	−	+	−	ND	−	ND	−	+
D-Xylose	+	−	+	+	+	ND	+	ND	−	+
Major 2-hydroxy fatty acid:										
C _{15:0} 2-OH	−	−	+	+	+	+	−	−	−	−
C _{16:0} 2-OH	−	−	−	−	−	+	−	−	−	−

*Data from Takeuchi *et al.* (2001).

Sphingomonas pruni IFO 15498^T (98.0%), *Sphingomonas asaccharolytica* IFO 15499^T (97.9%) and *Sphingomonas echinoides* DSM 1805^T (97.8%). Similarities of 95 to 97% were found to the 16S rRNA gene sequences of *Sphingomonas melonis* DAPP-PG 224^T (96.9%), *Sphingomonas adhaesiva* GIFU 11458^T (96.1%), *Sphingomonas sanguinis* IFO 13937^T (95.4%) and *Sphingomonas parapaucimobilis* JCM 7510^T (95.6%). Construction of a 16S rRNA gene sequence-based phylogenetic tree indicated that strain S213^T branched with *Sphingomonas echinoides* DSM 1805^T with high bootstrap support (Fig. 2).

The DNA G+C content of strain S213^T was 64.2 mol% and fell within the range (62–68 mol%) reported for the genus *Sphingomonas* (Takeuchi *et al.*, 2001). DNA–DNA relatedness between strain S213^T and strains of phylogenetically related *Sphingomonas* species was analysed. Strain S213^T showed very low DNA–DNA relatedness values to its closest phylogenetic neighbours, *Sphingomonas echinoides* NBRC 15742^T (=DSM 1805^T) (10%), *Sphingomonas asaccharolytica* IFO 15499^T (10%), *Sphingomonas mali* IFO 15500^T (12%) and *Sphingomonas pruni* IFO 15498^T (6%), whereas relatedness values of 22, 17 and 16% were found between *Sphingomonas echinoides* DSM 1805^T

and the type strains of *Sphingomonas pruni*, *Sphingomonas asaccharolytica* and *Sphingomonas mali*, respectively.

Takeuchi *et al.* (2001) have shown that polyamine patterns and nitrate reduction provide good diagnostic markers for differentiation of *Sphingomonas*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis* species. Although polyamine was not analysed in this study, the assignment of strain S213^T to the genus *Sphingomonas* is supported by its phylogenetic position and nitrate reduction data. As shown in Table 1, strain S213^T can be clearly differentiated from other phylogenetically related *Sphingomonas* species on the basis of major 2-hydroxy fatty acids and several phenotypic characteristics. The genomic and phenotypic evidence presented here clearly indicate that strain S213^T is representative of a novel species of the genus *Sphingomonas*, for which the name *Sphingomonas oligophenolica* sp. nov. is proposed.

Description of *Sphingomonas oligophenolica* sp. nov.

Sphingomonas oligophenolica (o.li.go.phe.no'li.ca. Gr. adj. *oligos* little, scanty; N.L. n. *phenol* phenol; N.L. fem. adj.

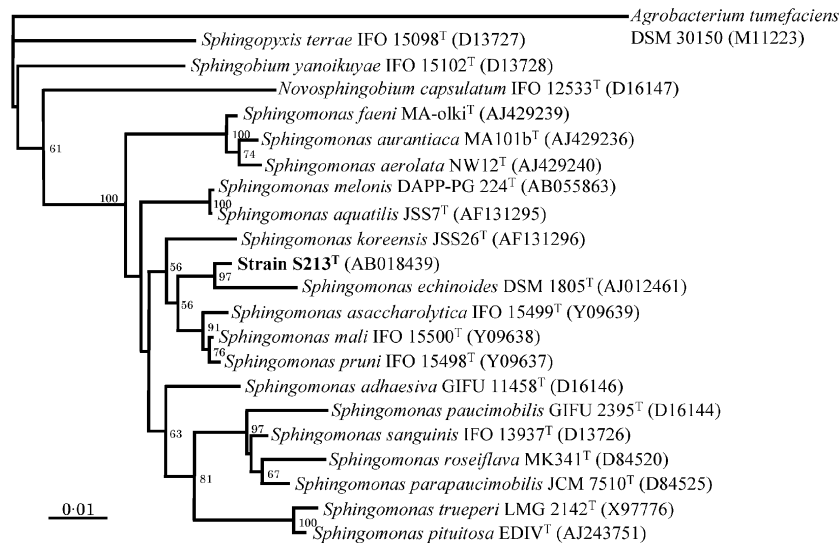


Fig. 2. Unrooted tree showing the phylogenetic relationships of strain S213^T and representative species of the genera *Sphingomonas*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*. The tree, constructed using the neighbour-joining method, was based on a comparison of a region corresponding to *Escherichia coli* positions 28–1390. Bootstrap values, expressed as a percentage of 1000 replications, are given at branching points. Bar, 0.01 nucleotide substitution rate (K_{nuc}) unit.

oligophenolica relating to small amounts of phenolic compounds).

Cells are Gram-negative, non-motile, non-sporulating rods (0.4–0.6 μm wide and 1.0–1.5 μm long). Colonies are circular and convex with entire margins, pale yellow and pinpoint after 3 days incubation on a 100-fold diluted nutrient broth medium and yellow and 0.6–0.8 mm in diameter after 3 days incubation on a 10-fold diluted nutrient broth medium. Growth is suppressed slightly in the presence of 0.1% (w/v) NaCl; this suppression is enhanced with increasing NaCl concentration in cultures. In the presence of 0.5% (w/v) NaCl cells become faint ghost-like filaments. NaCl-induced aberrant cell morphologies are significantly reduced by the addition of calcium (6 mM CaCl_2). Able to grow at 4–37 $^{\circ}\text{C}$ but not at 42 $^{\circ}\text{C}$. Strictly aerobic, catalase-positive and oxidase-positive. Nitrate is reduced to nitrite but not to N_2 . Hydrolyses gelatin, but not starch or casein. Acid is not produced from glucose. Assimilates L-arabinose, D-xylose, D-glucose, D-galactose, D-mannose, cellobiose, maltose, lactose, raffinose, acetic acid, DL-lactic acid, gluconic acid, pyruvic acid, 2-oxoglutaric acid, ferulic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid and vanillic acid. The following compounds are not utilized: D-fructose, citric acid, succinic acid, L-malic acid, benzoic acid, *o*- and *m*-hydroxybenzoic acid, *o*-, *m*- and *p*-toluic acid, *o*- and *p*-anisic acid, *o*-, *m*- and *p*-cresol, cinnamic acid, *m*-coumaric acid, phenylacetic acid, phenol, caffeic acid and methanol. The major non-polar fatty acids are $\text{C}_{18:1}$ and $\text{C}_{16:0}$, and the major 2-hydroxy fatty acid is $\text{C}_{14:0}$ 2-OH. No 3-OH fatty acids are present. The major isoprenoid quinone is ubiquinone Q-10. Sphingoglycolipid is present. Acetone-soluble pigment is characterized by λ_{max} at 454 and 482 nm. The DNA G+C content is 64.2 mol%.

The type strain, S213^T (= JCM 12082^T = CIP 107926^T), was isolated from paddy soil near Sendai in Japan.

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