Methylophaga aminisulfidivorans sp. nov., a restricted facultatively methylotrophic marine bacterium

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A novel restricted facultatively methylotrophic marine strain, MP^T, possessing the ribulose monophosphate pathway of C₁-carbon compound assimilation was isolated from a seawater sample obtained from Mokpo, South Korea. The novel isolate is aerobic, Gram-negative, asporogenous and a non-motile short rod. It grows well on methanol, methylated amines, dimethylsulfide and DMSO. Optimal growth occurs with 3 % NaCl at 30 °C and pH 7.0. Fructose is utilized as a multicarbon source. Growth factors are not required and vitamin B₁₂ does not stimulate growth. The cellular fatty acid profile of the novel strain consists primarily of straight-chain saturated C_{16:0} and unsaturated C_{16:1} acids. The major ubiquinone is Q-8. The dominant phospholipids are phosphatidylethanolamine and phosphatidylglycerol. The DNA G + C content is 44.9 mol% (T_m). Based on 16S rRNA gene sequence analysis and DNA–DNA relatedness (25–41 %) with the type strains of marine methylotrophs belonging to the genus *Methylophaga*, it is suggested that isolate MP^T represents a novel species, *Methylophaga aminisulfidivorans* sp. nov. (type strain MP^T=KCTC 12909^T=VKM B-2441^T=JCM 14647^T).

Bacteria belonging to the genus *Methylophaga* are a unique group of aerobic, halophilic, non-methane-utilizing methylotrophs. These organisms have been isolated from various marine sediments and soda lakes. They use the ribulose monophosphate (RuMP) pathway of C₁-carbon compound assimilation and some grow on a single multicarbon compound, fructose. Three neutrophilic species, *Methylophaga marina*, *Methylophaga thalassica* and *Methylophaga sulfidovorans*, have been described (Janvier *et al.*, 1985; De Zwart *et al.*, 1996). More recently, *Methylophaga alcalica* and '*Methylophaga natronica*' – moderately haloalcalophilic obligately and restricted facultatively methylotrophic bacteria were described by Doronina *et al.* (2003a, b). Species of the genus *Methylophaga* are distinguished from methylotrophs of the other RuMP pathway by their requirement for Na⁺, Mg²⁺ and vitamin B₁₂, tolerance of NaCl and the low G + C content (38.0–49.0 mol%) of their DNA. On the other hand, two strains, *Methylophaga marina* KM3 and KM5, isolated recently from Red Sea algae were not found to require vitamin B₁₂ (Li *et al.*, 2007).

Here, we report the taxonomic characterization of a neutrophilic, moderately halophilic, vitamin B₁₂-independent, RuMP pathway restricted facultative methylotroph belonging to the genus *Methylophaga*. The name *Methylophaga aminisulfidivorans* sp. nov. is proposed for this new isolate.

The novel strain was isolated from a seawater sample collected from Mokpo, South Korea. A 1 ml sample was used to inoculate a 500 ml flask containing 50 ml mineral salts medium [MSM; 2 g KH₂PO₄ l⁻¹; 2 g (NH₄)₂SO₄ l⁻¹; 0.2 g MgSO₄·7H₂O l⁻¹; 30 g NaCl l⁻¹; 0.002 g FeSO₄·7H₂O l⁻¹; pH 7.0] supplemented with 1 % methanol (v/v) and the flask was then incubated at 150 r.p.m. for 2 days at 30 °C. An aliquot of 0.5 ml of the turbid suspension was then transferred to fresh medium with 1 % (v/v) methanol and incubated as before. After serial transfers, a small amount of the suspension was spread on an agar-containing methanol plate and incubated at 30 °C for 3 days. From the plate, fast-growing colonies were selected and transferred to fresh plates. After several purification steps, a novel fast-growing strain, MP^T, was obtained. The culture purity of the isolated methylotrophic bacteria was tested by examining colonies and cell suspensions with light and electron microscopes.

The pure culture of strain MP^T was stored in liquid MSM for 10 days, on agar slants at 4 °C for 2 weeks or freeze-dried

Abbreviations: H₄MPT, tetrahydromethanopterin; PMS, phenazine methosulfate; PQQ, pyrroloquinoline quinone.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MP^T is DQ463161.

with a protectant (skimmed milk) for over a year. Cell morphology was examined using batch cultures in the late-exponential growth phase. An aliquot of cell suspension was mounted on a Formvar-coated copper grid and stained with 0.2 % (w/v) phosphotungstic acid (pH 7.2). For thin sectioning, cells were collected by centrifugation and pre-fixed with 1.5 % (w/v) glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) and washed three times with 1 % (w/v) OsO_4 in the same buffer for 3 h at 20 °C. After dehydration in a series of alcohols, the cells were embedded in Spurr epoxy resin and sectioned with a microtome (LKB 2128 Ultratome; LKB). Ultrathin sections were mounted on copper grids and double-stained with uranyl acetate and lead citrate (Reynolds, 1963). Negatively stained preparations and thin sections were viewed with a transmission electron microscope (JEM-100B; JEOL) at operating voltages of 60 kV and 80 kV, respectively.

The methods, reagents and media used for phenotypic characterization were as described by Doronina *et al.* (1995). Utilization of a wide range of growth substrates was determined in MSM after cultivation for 2 weeks with methanol replaced by the other carbon compounds (more than 60 were tested). Organic acids, amino acids and methylated amines were added at concentrations of 0.2 % (w/v), while carbohydrates and alcohols were added at concentrations of 0.2–0.5 % (w/v or v/v). To test alternative nitrogen sources, $(\text{NH}_4)_2\text{SO}_4$ was replaced by other nitrogen compounds. Methane utilization was tested in an atmosphere containing methane and air (1 : 1, v/v) in 700 ml conical flasks containing 100 ml MSM and fitted with rubber stoppers. Hydrogen utilization was tested by the same procedure but under an atmosphere consisting of $\text{H}_2/\text{O}_2/\text{CO}_2$ (7 : 2 : 1, v/v).

Fatty acids were extracted from cell biomass dried by lyophilization. A 200 µl aliquot of a 5.4 M solution of anhydrous HCl in methanol was added to 30 mg of dry biomass and the mixture was heated at 70 °C for 2 h. The methyl esters of fatty acids and aldehyde derivatives obtained were extracted twice with 100 µl hexane. The extract was dried and silylated in 20 µl N,O-bis-(trimethylsilyl)trifluoroacetamide for 15 min at 65 °C. A 1 µl portion of the reaction mixture was analysed with a GC-MS system (model HP-5985B; Hewlett Packard) equipped with a capillary column (25 × 0.25 mm) consisting of fused quartz containing an Ultra-1 nonpolar methylsilicone phase. The temperature program was run from 150 °C (2 min isotherm) to 250 °C at 5 °C min⁻¹ and then from 250 to 300 °C at 10 °C min⁻¹. Data processing was carried out with a computer (HP-1000; Hewlett Packard) by using the standard programs of the GC-MS system (Hewlett Packard). Phospholipid composition of the cells was determined according to previously described methods (Govorukhina & Trotsenko, 1989). Ubiquinones were extracted and purified according to Collins (1985). Analysis of ubiquinones was carried out using a mass spectrometer (MX-1310; Finnigan). Enzyme assays were

performed as described previously (Trotsenko *et al.*, 1986; Doronina *et al.*, 1995).

DNA was isolated and purified according to Marmur (1961). The DNA G + C content was determined by using the thermal denaturation (T_m) method with a spectrophotometer (DU-8B; Beckman) at a heating rate of 0.5 °C min⁻¹. DNA G + C content was calculated according to Owen & Lapage (1976) using the equation: $\text{mol G + C} = (T_m \times 2.08) - 106.4$. The DNA of *Escherichia coli* K-12 was used as the standard. DNA–DNA relatedness was defined by DNA reassociation (Johnson, 1985). *M. marina* ATCC 35842^T, *M. thalassica* ATCC 33146^T and *M. sulfidovorans* LMD 95.210^T were used as the reference strains.

The 16S rRNA gene of the novel strain was amplified and sequenced (Lane, 1991). The 16S rRNA gene sequences determined were aligned against the sequences of representative methylobacteria by using the CLUSTAL program. The position of sequence uncertainties was omitted; in total, 1415 nucleotides were used in the analysis. The phylogenetic relationships were determined by the neighbour-joining method and programs from the TREECON software package (Van de Peer & DeWachter, 1994), by maximum-likelihood using the PUZZLE program (Strimmer & von Haeseler, 1996) and by maximum-parsimony with the DNAPARS program from the PHYLIP package (Felsenstein, 1989) with bootstrap analysis of 100 trees. The sequences were compared with representatives of the class *Gamma-proteobacteria*, including recognized methylotrophic species. In preliminary trials, a total of 98 sequences were used and several phylogenetic trees were generated. Phylogenetic analyses that employed different algorithms showed similar results.

Cells of the novel isolate were Gram-negative, non-motile, non-spore-forming rods and 0.2–0.4 × 0.8–1.0 µm in size. Reproduction occurred by binary fission. No internal complex membrane or capsules were observed. When grown on methanol agar medium, colonies were 0.5–1.5 mm in diameter after 3 days at 30 °C and were opaque, milky and raised with a smooth surface with a round edge.

The novel isolate utilized C₁-compounds: methanol, methylamine, dimethylamine, trimethylamine, dimethylsulfide, DMSO and dimethylformamide (Table 1). Fructose was utilized as a multicarbon source. Strain MP^T could not utilize other sugars, organic acids, amino acids, C₂–C₆ alcohols, nutrient or peptone broth supplemented with 3 % (w/v) NaCl at pH 7.0. It did not grow under gas mixtures of $\text{CO}_2/\text{H}_2/\text{O}_2$ or CH_4/O_2 . Nitrate was reduced to nitrite. Hydrolysis of starch was not observed. No cell aggregation and pigmentation occurred in MSM. The novel isolate was catalase- and oxidase-positive. The isolate grew in the presence of 9 % NaCl (optimum 3 % NaCl), over the pH range of 6.0 to 8.0 and over the temperature range of 25 to 32 °C (optimum pH 7.0, optimum temperature 30 °C). No growth occurred at 45 °C. Strain MP^T did not require additional growth factors when grown on methanol and

Table 1. Characteristics of the type strains of species of the genus *Methylophaga*

Taxa: 1, strain MP^T; 2, *M. marina* ATCC 35842^T; 3, *M. thalassica* ATCC 33146^T; 4, *M. sulfidovorans* LMD 95.210^T; 5, *M. alcalica* ATCC BAA-297^T; 6, '*M. natronica*' VKM B-2288. All taxa are positive for growth on methanol and methylamine. All taxa are negative in tests for utilization of glucose and sucrose. +, Positive; –, negative; ND, not determined.

Characteristic	1	2	3	4	5	6
Requirement for vitamin B ₁₂	–	+	+	+	+	+
Motility	–	+	+	+	+	+
Nitrate reduction	+	–	–	–	+	+
Growth at:						
37 °C	+	+	+	–	–	+
42 °C	–	–	+	–	–	–
Optimal pH	6.8–7.0	7.0–7.5	7.0–7.5	7.4–7.8	9.0–9.5	ND
Tolerance for NaCl (%)	9	12	12	12	10	10
Utilization of:						
Fructose	+	+	+	–	–	+
Dimethylamine	+	–	–	+	–	–
Trimethylamine	+	–	–	–	–	–
Dimethylsulfide	+	–	–	+	–	–
DMSO	+	ND	ND	ND	ND	ND
Dimethylformamide	+	ND	ND	ND	ND	ND
Major ubiquinone	Q-8	Q-8	Q-8	Q-8	Q-8	Q-8
DNA G + C content (mol%)	44.9	43.0	44.0	42.4	48.3	45.0

the addition of vitamin B₁₂ or yeast extract did not stimulate growth.

As seen from Table 2, the predominant cellular fatty acids of the novel isolate were straight-chain saturated C_{16:0} and unsaturated C_{16:1ω7c} acids. The presence of 3-hydroxy fatty acids was observed, but 2-hydroxy fatty acids were not

observed. The novel isolate had a cellular fatty acid composition of type A and was similar to group 11 of the marine methylobacteria (Urakami & Komagata, 1987). Analysis of the cellular phospholipids revealed the presence of phosphatidylethanolamine and phosphatidylglycerol as the dominant phospholipids and phosphatidic acid, phosphatidylserine and cardiolipin (diphosphatidylglycerol) as minor components. The major isoprenoid ubiquinone is Q-8.

The enzyme profiles of methanol- or methylamine-grown cells indicated that the novel isolate oxidized methanol to formaldehyde by an inducible pyrroloquinoline quinone (PQQ)-linked methanol dehydrogenase (Table 3). Methylamine dehydrogenase PQQ was present in methylamine-grown cells. Formaldehyde and formate dehydrogenases were active with phenazine methosulfate (PMS) while NAD⁺-dependent activities were absent. Formaldehyde assimilation occurred via the RuMP cycle (Entner–Doudoroff variant), as confirmed by the presence of 3-hexulosephosphate synthase and 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase. Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenases were active with both NAD⁺ and NADP⁺. Rather high levels of these enzymes indicated the preferential oxidation of formaldehyde to CO₂ via the dissimilatory hexulosephosphate cycle which provided the methylotroph with the reduced equivalents and energy for biosynthesis. However, we could not rule out the same potential role of the tetrahydromethanopterin (H₄MPT)-dependent oxidation pathway in formaldehyde dissimilation in the novel isolate because high activities of methenyl H₄MPT cyclohydrolase

Table 2. Cellular fatty acid content of strain MP^T grown on methanol

Fatty acid	Total fatty acids (%)
Straight-chain acids:	
C _{12:0}	2.20
C _{14:0}	6.51
C _{14:1ω5}	1.01
C _{15:0}	1.00
C _{16:0}	42.91
C _{16:1ω7c}	35.94
C _{16:1ω5c}	0.15
C _{17:0}	0.09
C _{18:0}	0.18
C _{18:1ω7}	2.38
Cyclopropane acids:	
C _{17:0} cyclo	7.39
C _{19:0} cyclo	–
Hydroxy acids:	
3-OH C _{10:0}	0.12
3-OH C _{15:0}	0.14

Table 3. Enzymic activities in cell extracts of strain MP^T grown with methanol or methylamine

GSH, Reduced glutathione; PMS, phenazine methosulfate; KDPG, 2-keto-3-deoxy-6-phosphogluconate.

Enzyme (co-factors)	Enzyme activity [nmol min ⁻¹ (mg protein) ⁻¹]	
	Methanol	Methylamine
Methanol dehydrogenase (PMS)	61	0
Methylamine dehydrogenase (PMS)	0	47
Formaldehyde dehydrogenase (PMS)	25	19
Formaldehyde dehydrogenase (NAD ⁺ , GSH)	0	0
Formate dehydrogenase (PMS)	64	57
Formate dehydrogenase (NAD ⁺)	0	0
3-Hexulosephosphate synthase	194	171
Hydroxypyruvate reductase (NADH, NADPH)	0	0
Serine glyoxylate aminotransferase (NADH, NADPH)	0	0
Ribulose-1,5-bisphosphate carboxylase	0	0
Glucose-6-phosphate dehydrogenase (NAD ⁺)	163	125
Glucose-6-phosphate dehydrogenase (NADP ⁺)	632	583
6-Phosphogluconate dehydrogenase (NAD ⁺)	23	21
6-Phosphogluconate dehydrogenase (NADP ⁺)	25	24
Fructose-1,6-bisphosphate aldolase	0	0
KDPG aldolase	137	119
α -Ketoglutarate dehydrogenase	0	0
Isocitrate dehydrogenase (NAD ⁺)	134	95
Isocitrate dehydrogenase (NADP ⁺)	38	33
Isocitrate lyase	0	0
Malate synthase	0	0
Glutamate dehydrogenase (NADH)	44	39
Glutamate dehydrogenase (NADPH)	23	20
Glutamate synthase (NADPH)	57	51
Glutamine synthetase (ATP, Mn)	110	102

and NAD(P)-dependent methylene H₄MPT dehydrogenases are found in *Methylobacillus flagellatus* KT^T (Vorholt *et al.*, 1999).

The absence of the serine pathway-specific enzymes (hydroxypyruvate reductase and serine glyoxylate transaminase) and the ribulose biphosphate pathway enzymes (ribulose biphosphate carboxylase) enabled these pathways to be discounted from operating in the methylotroph studied.

The tricarboxylic acid cycle was deficient in α -ketoglutarate dehydrogenase activity. The absence of isocitrate lyase and malate synthase activities indicated a non-functional glyoxylate shunt in the novel isolate. Primary ammonia assimilation occurred by reductive amination of α -ketoglutarate to glutamate and via the glutamate cycle (GS/GOGAT system).

The DNA G+C content of strain MP^T was estimated via T_m as 44.9 mol% (Table 1). The level of DNA–DNA relatedness between the novel isolate and reference strains of the genus *Methylophaga* (*M. marina* ATCC 35842^T, *M. thalassica* ATCC 33146^T and *M. sulfidovorans* LMD 95.210^T) did not exceed 25–42 %, consistent with the assignment of the novel strain to a separate species of the

genus *Methylophaga*. According to 16S rRNA gene sequence analysis, strain MP^T had gene sequence similarity of 96–98 % with *M. marina*, *M. thalassica* and *M. sulfidovorans*.

In the phylogenetic tree derived from 16S rRNA gene sequences (Fig. 1), strain MP^T consistently branched together with the cluster of species within the genus *Methylophaga* within the class *Gammaproteobacteria*. The relatively high level of 16S rRNA gene sequence identity (94–98 %) between strain MP^T and members of the genus *Methylophaga* indicated their close relationship. Thus, phenotypic and genotypic data allow the separation of strain MP^T as a distinct genospecies of the genus *Methylophaga*. Consequently, strain MP^T is classified as the type strain of a novel species of the genus *Methylophaga*, *Methylophaga aminisulfidivorans* sp. nov.

Description of *Methylophaga aminisulfidivorans* sp. nov.

Methylophaga aminisulfidivorans (am.i.ni'sul.fi.di'vo.rans. N.L. n. *aminum* the amine group; N.L. n. *sulfidum* sulfide; L. v. *voro* to devour; N.L. part. adj. *aminisulfidivorans* amine-sulfide-devouring, amine-sulfide-utilizing).

Fig. 1. 16S rRNA gene sequence-based dendrogram showing the phylogenetic position of *Methylophaga aminisulfidovorans* sp. nov. MP^T among methylobacteria of the class *Gammaproteobacteria*. Numbers at the branch points are bootstrap values from 100 replicates. Bar, 10 nucleotide substitutions per 100 nucleotides.

Cells are Gram-negative rods, 0.8–1.2 µm in length and 0.2–0.4 µm in diameter. Colonies on mineral salts/methanol agar are white to slightly cream and 1–2 mm in diameter. Non-motile, strictly aerobic and does not require vitamin B₁₂. Moderately halophilic. Able to grow at 20–37 °C, at pH 6.0–8.0 on MSM with 1.5–9% (w/v) NaCl. Optimal growth takes place at 30 °C, pH 6.8–7.0 and 3% NaCl. Catalase- and oxidase-positive. Reduces nitrate to nitrite. Restricted facultative methylobacter that utilizes methanol, monomethylamine, dimethylamine, trimethylamine, dimethylsulfide, DMSO, dimethylformamide and fructose. Utilizes C₁-compounds via the RuMP pathway. Does not grow on peptone-yeast extract medium with or without NaCl. The prevailing cellular fatty acids are C_{16:0} and C_{16:1}. The major ubiquinone is Q-8. DNA G+C content is 44.9 mol%.

The type strain, MP^T (=KCTC 12909^T=VKM B-2441^T=JCM 14647^T), was isolated from a seawater sample collected from Mokpo, South Korea.

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