**Methylophaga nitratireducenticrescens** sp. nov. and **Methylophaga frappieri** sp. nov., isolated from the biofilm of the methanol-fed denitrification system treating the seawater at the Montreal Biodome

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Two bacterial strains, designated JAM1<sup>T</sup> and JAM7<sup>T</sup>, were isolated from a methanol-fed denitrification system treating seawater at the Montreal Biodome, Canada. They were affiliated within the genus *Methylophaga* of the Gammaproteobacteria by analysis of the 16S rRNA gene sequences. Strain JAM1<sup>T</sup> had the capacity to grow under denitrifying conditions by reducing nitrate into nitrite which is unique among the species of the genus *Methylophaga*. Major fatty acids were C<sub>16:1ω7c</sub> or ω6c, C<sub>16:0</sub> and C<sub>18:1ω7c</sub> or ω6c. The major ubiquinone was Q<sub>8</sub>. Both strains required vitamin B<sub>12</sub> and Na<sup>+</sup> ions for growth. The genomes of strains JAM1<sup>T</sup> and JAM7<sup>T</sup> have been completely sequenced and showed a DNA G+C content of 44.7 mol% and 47.8 mol%, respectively. Growth occurred at pH 6–11 and at 0.5–8% NaCl. Both genomes contained predicted ORFs encoding the key enzymes of the ribulose monophosphate pathway. Also, operons encoding two nitrate reductases (Nar), two nitric oxide reductases (Nor), one nitrous oxide reductase (Nos) and one truncated nitrite reductase (NirK) were clustered in a 67 kb chromosomal region in strain JAM1<sup>T</sup>. No such operons were found in strain JAM7<sup>T</sup>. These results supported the affiliation of the two strains as novel species within the genus *Methylophaga*. The names *Methylophaga nitratireducenticrescens* sp. nov. for type strain JAM1<sup>T</sup>(=DSM 25689<sup>T</sup>=ATCC BAA-2433<sup>T</sup>) and *Methylophaga frappieri* sp. nov. for type strain JAM7<sup>T</sup>(=DSM 25690<sup>T</sup>=ATCC BAA-2434<sup>T</sup>) are proposed.

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**Abbreviations:** RAST, Rapid Annotation using Subsystem Technology; RuMP, ribulose-monophosphate.

A supplementary figure is available with the online version of this paper.

The genus *Methylophaga* was first described by Janvier *et al.* (1985) and at the time of writing has eight members with validly published names: *Methylophaga alcalica* (Doronina *et al.*, 2003b), *Methylophaga thiooxydans* (Boden *et al.*, 2010), *Methylophaga aminisulfidovorans* (Kim *et al.*, 2007), *Methylophaga marina* (Janvier *et al.*, 1985), *Methylophaga muralis* (Doronina *et al.*, 2005) *Methylophaga sulfidovorans* (de Zwart *et al.*, 1996), *Methylophaga thalassica* (Janvier *et al.*, 1985) and *Methylophaga lonarensis* (Antony *et al.*, 2012). Members of this genus are Gram-negative, aerobic and methylotrophic. Phylogenetic analyses based on the 16S rRNA gene sequences indicate that the eight species can be divided into two clades (Boden, 2012). Some species of the genus *Methylophaga* are auxotrophic for vitamin B<sub>12</sub>. They are typically isolated from marine environments or brackish waters. A strict requirement for Na<sup>+</sup> and a low DNA G+C content (42–50 mol%) are characteristics that distinguish species of the genus *Methylophaga* from the other ribulose-monophosphate (RuMP) pathway methylobacteria (Boden *et al.*, 2011). In previous work, we isolated two bacterial strains (designated JAM1<sup>T</sup> and JAM7<sup>T</sup>) from the denitrifying biofilm of the methanol-fed denitrification system treating the seawater of the St. Lawrence mesocosm at the Montreal Biodome, Canada (Auclair *et al.*, 2010). Analysis of the 16S rRNA gene sequences indicated that they were affiliated with the genus *Methylophaga*. Here we report the taxonomic analysis of the two strains.

Strains JAM1<sup>T</sup> and JAM7<sup>T</sup> grew well on the American Type Culture Collection (ATCC) *Methylophaga* medium 1403 [per 990 ml: 24 g NaCl, 3 g MgCl<sub>2</sub>, 6 H<sub>2</sub>O, 2 g MgSO<sub>4</sub>, 7 H<sub>2</sub>O, 0.5 g KCl, 1 g CaCl<sub>2</sub>, 0.5 g Bistris, 3 ml methanol (sterilized by passage through a 0.2 µm filter), 20 ml solution T (per 100 ml: 0.7 g KH<sub>2</sub>P<sub>4</sub>, 10 g NH<sub>4</sub>Cl, 10 g Bistris, 0.3 g Ferric ammonium citrate, pH 8), 1 ml vitamin B<sub>12</sub> 0.1 mg ml<sup>−1</sup>, 10 ml Wolf's mineral solution (ATCC), pH 8.0]. This medium was used to determine the
optimal growth conditions and to perform all other growth assays. Colonies on medium 1403 agar (1.5 % agar) were beige, 2–3 mm in diameter, smooth, circular and convex. Catalase was detected in both strains by adding 3 % peroxide on a colony on medium 1403 agar. Oxidase was detected in both strains by swapping colonies from medium 1403 agar and adding oxidase reagent (BBL, Becton Dickinson).

The fatty acid profiles and the respiratory quinones were determined from cells that were grown in liquid medium 1403 for 2 days (stationary phase) at 30 °C for strain JAM1T and at 34 °C for strain JAM7T. Fatty acid analysis was carried out in accordance with the standard protocol of the Microbial Identification System at Keystone laboratories (Edmonton, AB, Canada). The fatty acid methyl esters mixtures were separated using the Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID). Peaks were automatically integrated and fatty acid names and percentages calculated by the MIS Standard Software (Microbial ID). Analysis of respiratory quinones was carried out by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany following their standard operating protocol (http://www.dsmz.de/services/services-microorganisms/identification/analysis-of-respiratory-quinones.html). Briefly, respiratory lipoquinones were separated into their different classes (menaquinones, ubiquinones, etc.) by TLC on silica gel using hexane:tert-butylmethyl ether (9:1 v/v) as a solvent. UV absorbing bands were separated into their different quinone classes (e.g. menaquinones or ubiquinones) were removed from the plate and further analysed by HPLC, using methanol:heptane 9:1 (v/v) as the eluant. The major cellular fatty acids were C16:1ω7c or C16:1ω6c (38.6 %), C16:0 (32.0 %) and C18:1ω7c or C18:1ω6c (9.9 %) for strain JAM1T and C16:1ω7c or C16:0ω6c (38.5 %), C16:0 (29.4 %) and C18:1ω7c or C18:1ω6c (13.9 %) for strain JAM7T. These fatty acids were also found in M. alcalica M39T, M. murius Kr3T and M. lonarensis MPL1T, and two (C16:0 and C16:1ω7c) were found in M. aminisulfidivorans MP7T (Table 1). The respiratory lipoquinone is ubiquinone Q-8 (100 %) in both cases, which was found in all the other species for which information on quinones was available (Table 1).

Optimal aerobic growth conditions were determined by following turbidity of liquid cultures over time in medium 1403 using a Bioscreen C MBR apparatus (Oy Growth Curves). Growth assays were performed at temperatures of 15, 20, 25, 30 and 37 °C, at pH 5, 6, 7, 8, 9, 10 and 11 and at NaCl concentrations of 0, 0.5, 1, 3, 5, 8 and 10 % (w/v) (Table 1). Growth inhibition by selected antibiotics was tested in liquid cultures with antibiotic concentrations ranging from 15 to 500 μg ml⁻¹ in medium 1403. Growth of strain JAM1T was inhibited by 50 μg ml⁻¹ gentamicin, 50 μg ml⁻¹ kanamycin, 50 μg ml⁻¹ streptomycin, 100 μg ml⁻¹ tetracycline and 50 μg ml⁻¹ trimethoprim. No growth inhibition of strain JAM1T was detected for ampicillin, chloramphenicol and tetracycline at the concentrations tested.

Physiological and biochemical characteristics were further investigated using the API 50CH (acid production and carbon assimilation), API 20NE (biochemical reactions and carbon assimilation) and API ZYM strips (extracellular enzyme activity) (bioMérieux). The three API systems were used as described by the manufacturer, with some modifications. The API 50CH and API 20NE strips were inoculated with strain JAM1T or strain JAM7T in medium 1403 without methanol, while the API ZYM strips were inoculated with 2 day cultures washed and dispersed in 3 % saline. Strips were incubated at 30 °C for strain JAM1T and at 34 °C for strain JAM7T for 12 h (API ZYM) or 48 h (API 50CH and API 20NE). Both strains were negative in all tests in API 50CH and API 20NE strips, with the exception of the reduction of nitrate into nitrite, which was positive for strain JAM1T only. With API ZYM strips, both strains were positive for esterase (C4), leucine arylamidase and naphthol-AS-BI-phosphohydrolase activities. Strain JAM1T was also positive for alkaline phosphatase. Fructose, methylamine, acetate, methylsulfate, glycerol and methanol (as a control) were further tested as carbon sources in liquid medium 1403 (containing no methanol); both strains grew exclusively with methanol as a carbon source. The genomes from both strains have been sequenced and closed (GenBank accession numbers CP003390.1 and CP003380.1) (Villeneuve et al., 2012). The genome length of strain JAM1T is 3137192 bp, and that for strain JAM7T is 2697465 bp. Strain JAM7T also contains a 48.8 kb plasmid (CP003381.1). The DNA G+C content of both genomes was calculated from the genome sequence (Table 1). Strains JAM1T and JAM7T contain three 16S rRNA gene copies which differ by few nucleotides. Pairwise comparisons of the three copies between strains JAM1T and JAM7T showed sequence similarity ranged from 96.9 % to 97.3 %. Both genomes were aligned at the Rapid Annotations using Subsystems Technology (RAST) Server (http://rast.nmpdr.org; June 2012) (Aziz et al., 2008) with either strain JAM1T or strain JAM7T as a reference sequence. On average, 2178 orthologues were found between the two strains with 64.5 % overall identity (deduced amino acid sequences). Nucleic acid sequence of the two genomes were aligned with mVista using the LAGAN alignment program (http://genome.lbl.gov/vista/index.shtml; June 2012) (Brudno et al., 2003; Frazer et al., 2004) using strain JAM1T as a reference. On average, 84.5 % of both genomes could be aligned with 49.6 % identity. Finally, we determined the percentage of conserved DNA of strain JAM7T in the genome of strain JAM1T as described by Goris et al. (2007). Briefly, the JAM1T genome was cut in silico into 1000 nt pieces (3138 fragments) which were compared with the whole JAM7T genome (BLASTN, word size 11, E set at 10⁻⁶). Only fragments with >90 % identity were kept. The lengths of the aligned regions of these fragments were summed and the sum was divided by the total length of the genomic
Table 1. Physiological, chemotaxonomic and molecular properties of species of the genus *Methylophaga*

<table>
<thead>
<tr>
<th>Taxa</th>
<th>1</th>
<th>2</th>
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<th>4</th>
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<th>7</th>
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<td>47.8</td>
<td>48.3</td>
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<td>43.0</td>
<td>44.6</td>
<td>42.4</td>
<td>44.0</td>
<td>45.9</td>
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<tr>
<td>Cell morphology and size (μm)</td>
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<td>Rods 0.7 × 1.5</td>
<td>Rods 0.6–0.8 × 1.4–2.8</td>
<td>Rods 0.2–0.4 × 1.0–1.2</td>
<td>Rods 1.2–2.0 × 0.2</td>
<td>Rods 0.2 × 1.0</td>
<td>Rods 0.7 × 1.7–2.0</td>
<td>Rods 0.2 × 0.9</td>
<td>Rods 0.2 × 1.0</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
<td>–/+</td>
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<td>–</td>
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<td>+</td>
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<td>C_{16:0}, C_{16:1}</td>
<td>C_{16:0}, C_{16:1}</td>
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<td>UQ-8</td>
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<td>ND</td>
<td>6–10</td>
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<td>+</td>
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<td>+</td>
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*Nitrated reduction into nitrite was positive with *M. marina* strains KM3 and KM5 (Li et al., 2007), but not with the type strain 222T (ATCC 35842T). From *M. marina* strains KM3 and KM5 (Li et al., 2007).
DNA of strain JAM1\textsuperscript{T}. Based on this analysis, only 1.34\% of the genome of the strain JAM7\textsuperscript{T} genome is conserved (>90\%) in strain JAM1\textsuperscript{T}. Goris et al. (2007) showed that bacterial strains with DNA–DNA hybridization values of more than 70\% have a percentage of conserved DNA of over 69\%. All these results strongly suggest that strains JAM1\textsuperscript{T} and JAM7\textsuperscript{T} are different species of the genus Methylophaga.

Phylogenetic analysis of the 16S rRNA gene sequences of the type strains of species of the genus Methylophaga was performed along with those of strains JAM1\textsuperscript{T} and JAM7\textsuperscript{T} (Fig. 1). Sequences were aligned by CLUSTAL W 1.4 (Thompson et al., 1994). Phylogenetic analyses were carried out with software applications from the PHYLIP package, version 3.65 (Felsenstein, 1989) with the SEQBOOT (bootstrap), DNADIST (distance matrix), FITCH (calculate a tree) and CONSENSE (derive the consensus tree) programs. Strains JAM1\textsuperscript{T} and JAM7\textsuperscript{T} branched with the alkaliphilic clade, which includes M. alcalica M39\textsuperscript{T}, M. lonarensis MPL\textsuperscript{T} and M. muralis Kr\textsuperscript{3T}. This result correlates with the pH range (between 7 and 10), at which both strains grow very well. No growth was observed at pH 5 and 6. Above pH 10, the mineral medium precipitated. Sequence similarity in the 16S rRNA gene sequences between strain JAM1\textsuperscript{T} and the three alkaliphilic species ranged from 94.1\% to 96.3\%, and for strain JAM7\textsuperscript{T} from 94.2\% to 96.1\%.

Both genomes have predicted open reading frames encoding 3-hexulose-6-phosphate synthase (HPS) and 6-phospho-3-hexuloseisomerase (PHI), the two key enzymes for the ribulose monophosphate (RuMP) pathway according to annotation done with the Integrated Microbial Genomes Expert Review (IMG/ER) (January to March 2012; https://img.jgi.doe.gov/cgi-bin/er/main.cgi). This pathway is the one used by the other species of the genus Methylophaga to assimilate one-carbon substrates. They also have the gene encoding the 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase, which indicates that the Entner–Doudoroff variant of the RuMP pathway is used. Strain JAM1\textsuperscript{T} has the gene coding for one specific enzyme of the serine cycle for one-carbon assimilation (the hydroxypyruvate reductase) and for the ribulose-biphosphate carboxylase (ribulose-biphosphate pathway). However, strain JAM1\textsuperscript{T} does not have genes coding for the other specific enzymes of the serine cycle for one-carbon assimilation like the serine glyoxylate transaminase or the isocitrate lyase. Strain JAM7\textsuperscript{T} does not have any genes coding for specific enzymes of the serine cycle for one-carbon assimilation or the ribulose-biphosphate pathway.

Strain JAM1\textsuperscript{T} was, to our knowledge, the first strain of a species of the genus Methylophaga to be reported to grow under denitrifying conditions by reducing nitrate into nitrite. No growth was observed for strain JAM7\textsuperscript{T} under these conditions (Auclair et al., 2010). Most species of the genus Methylophaga have been reported to reduce nitrate into nitrite, except M. thalassica ATCC 33146\textsuperscript{T}, M. sulfidovorans RB-1\textsuperscript{T} and M. marina 222\textsuperscript{T} (Antony et al., 2012; Boden et al., 2010; de Zwart et al., 1996; Doronina et al., 2003a; Doronina et al., 2003b; Doronina et al., 1997; Doronina et al., 2005; Janvier et al., 1985; Kim et al., 2007; Li et al., 2007). None of these species have been shown or reported to grow with nitrate as an electron acceptor (denitrifying conditions). We cultured M. alcalica M39\textsuperscript{T} (=ATCC BAA-297\textsuperscript{T}; NMS medium) and M. marina 222\textsuperscript{T} (=ATCC 35842\textsuperscript{T}; medium 1403) under denitrifying conditions (0.3\% methanol, 4800–8500 µg NaNO\textsubscript{3} ml\textsuperscript{-1}). Both cultures showed no sign of growth, and nitrate was not reduced under these conditions.

Reduction of nitrate into nitrite by strain JAM1\textsuperscript{T} was correlated with the presence of two narG genes (Auclair et al., 2010). Fig. 2 illustrates the 67 kbp chromosomal

![Fig. 1. Phylogenetic analysis of 16S rRNA gene sequences of species of the genus Methylophaga. Evolutionary distance among 16S rRNA gene sequences of strains JAM1\textsuperscript{T} and JAM7\textsuperscript{T} and all species of the genus Methylophaga with validly published names is illustrated by an unrooted phylogenetic tree. The tree was inferred from a matrix of pairwise distance using aligned sequences containing 1443 positions. The numbers at the branches indicate the percentage of 1000 bootstrap resamplings; only values above 70\% are shown. The numbers in parentheses are GenBank accession numbers and the second numbers for strains JAM1\textsuperscript{T} and JAM7\textsuperscript{T} are the locus tags. 16S rRNA sequences from Cycloclastus pugetii PS-1\textsuperscript{T} and Thiomicrospira arctica SVALE\textsuperscript{E} were used as the outgroup species. The scale bar indicates nucleotide substitutions per position.](http://ijs.sgmjournals.org/2219)
region of strain JAM1\textsuperscript{T} where operons related to the denitrification pathway were found. Besides the two expected \textit{nar} operons, it was surprising to find also two nitric oxide reductase (\textit{nor}) operons and one nitrous oxide reductase (\textit{nos}) operon. A truncated nitrite reductase (\textit{nirK}) sequence encoding an 82 amino acid protein was found, which could explain why only the reduction of nitrate into nitrite occurs in strain JAM1\textsuperscript{T}. The \textit{nar1} and \textit{nor2} gene sequences and gene arrangements are similar to those found in some Betaproteobacteria (e.g., \textit{Thiobacillus denitrificans}), suggesting horizontal gene transfer for this specific region as suggested by Auclair \textit{et al.} (2010). The remaining sequences and arrangements are more closely related to those found in Gammaproteobacteria. Two genes encoding the molybdenum cofactor biosynthesis protein MogA and the molybdenum cofactor biosynthesis protein A (\textit{moaA}) were found. The nitrate reductases are molybdopterin proteins. Finally, the \textit{nnrS} gene that is involved in nitric oxide response is present. No such operons were present in strain JAM7\textsuperscript{T}.

We clearly showed that strain JAM1\textsuperscript{T} and strain JAM7\textsuperscript{T} have significant differences in their genomes, which supports the hypotheses that they represent two different species. The growth under denitrification conditions displayed by strain JAM1\textsuperscript{T} cannot be achieved by strain JAM7\textsuperscript{T}, \textit{M. alcalica} M39\textsuperscript{T} and \textit{M. lonarensis} MPL\textsuperscript{T}. Also strain JAM1\textsuperscript{T} differs from strain JAM7\textsuperscript{T} by the presence of alkaline phosphatase activity. Strains JAM1\textsuperscript{T} and JAM7\textsuperscript{T} and \textit{M. lonarensis} MPL\textsuperscript{T} cannot grow on monomethylamine as does \textit{M. alcalica} M39\textsuperscript{T}. A difference of more than 5\% in the DNA G+C content separates strain JAM1\textsuperscript{T} from \textit{M. lonarensis} MPL\textsuperscript{T}. Strain JAM1\textsuperscript{T} and \textit{M. alcalica} M39\textsuperscript{T} share 96.3\% sequence similarity in their 16S rRNA gene sequences, and there is 94.7\% sequence similarity between strain JAM1\textsuperscript{T} and \textit{M. lonarensis} MPL\textsuperscript{T}. All these differences support the suggestion that strain JAM1\textsuperscript{T} is a different species from \textit{M. alcalica} M39\textsuperscript{T} and \textit{M. lonarensis} MPL\textsuperscript{T}. Besides the differences in the monomethylamine, strain JAM7\textsuperscript{T} and \textit{M. alcalica} M39\textsuperscript{T} share 95.9\% sequence similarity in their 16S rRNA gene sequences. The only discrimination factor between strain JAM1\textsuperscript{T} and \textit{M. lonarensis} MPL\textsuperscript{T} is the 94.9\% sequence similarity in their 16S rRNA gene sequences. The fact that genome sequences of strains JAM1\textsuperscript{T} and JAM7\textsuperscript{T} are significantly different despite a 97\% sequence similarity in their 16S rRNA gene sequences, strongly suggests that strain JAM7\textsuperscript{T} is a different species from \textit{M. alcalica} M39\textsuperscript{T} and \textit{M. lonarensis} MPL\textsuperscript{T}.

**Description of \textit{Methylophaga nitratireducenticrescens} sp. nov.**

\textit{Methylophaga nitratireducenticrescens} (\textit{ni.trat.ri.re.du.cen}.ti.cres.cens. N.L. \textit{n}. \textit{nitras} -atis nitrate; L. part. adj. \textit{reducens}}
-ents converting to a different state; L. part. adj. crescens growing; N.L. part. adj. nitratireductocrescens growing with the reduction of nitrate).

Gram-negative. Cells are short, straight rods 0.6 × 1.5 μm (Fig. S1, available in IJSEM Online). Colonies on ATCC *Methylophaga* medium 1403 agar are beige, 2–3 mm in diameter, smooth, circular and convex. Catalase- and oxidase-positive. Reduces nitrate into nitrite in the presence of oxygen. Grows in the absence of oxygen with nitrate. Grows at temperatures between 15 and 37 ºC (optimum, 30 ºC). Optimum pH is 8.0 but grows between pH 6 and 11. Optimum NaCl concentration is 3%; grows between 0.5 and 8% NaCl. Sensitive to gentamicin, streptomycin, tetracycline and trimethoprim. Resistant to chloramphenicol and ampicillin up to 500 μg ml⁻¹. Vitamin B₁₂ and Na⁺ are required for growth. Positive for esterase (C₄), leucine arylamidase, naphthol-AS-BI-phosphohydrolase and alkaline phosphatase activities. The major cellular fatty acids are C₁₆ : 1ω7c or ω6c, C₁₆ : 0 and C₁₈ : 1ω7c or ω6c. The major ubiquinone is Q₈.

The type strain JAM1 ( =DSM 25689T =ATCC BAA-2433T) was isolated from a methanol-fed denitrification system treating seawater at the Montreal Biodome, Canada. The DNA G+C content of the type strain is 44.7 mol%.

**Description of Methylophaga frappieri sp. nov.**

*Methylophaga frappieri* ([frap.pi.e’ri. N.L. gen. masc. n. frappieri of Frappier, in honour of Armand Frappier, a renowned Canadian microbiologist].

Gram-negative. Cells are short straight rods 0.7 × 1.5 μm. Colonies on ATCC *Methylophaga* medium 1403 agar are beige, 2–3 mm in diameter, smooth, circular and convex. Catalase- and oxidase-positive. Does not reduce nitrate into nitrite. Grows between 15 and 37 ºC (optimum, 34 ºC). Optimum pH is 8.0, but grows between pH 6 and 11. Optimum NaCl concentration is 3%; grows between 0.5 and 8% NaCl. Sensitive to gentamicin, kanamycin, streptomycin, tetracycline and trimethoprim. Resistant to chloramphenicol, ampicillin and tetracycline up to 500 μg ml⁻¹. Vitamin B₁₂ and Na⁺ are required for growth. Does not grow in the absence of oxygen with nitrate. Positive for esterase (C₄), leucine arylamidase and naphthol-AS-BI-phosphohydrolase activities. The major cellular fatty acids are C₁₆ : 1ω7c or ω6c, C₁₆ : 0 and C₁₈ : 1ω7c or ω6c. The major ubiquinone is Q₈.

The type strain JAM1T (=DSM 25690T =ATCC BAA-2434T) was isolated from the methanol-fed denitrification system treating seawater at the Montreal Biodome, Canada. The DNA G+C content of the type strain is 47.8 mol%.

**Acknowledgements**

This work was supported by funding from the Natural Sciences and Engineering Research Council of Canada to R.V. and by Le Fonds de Recherche du Québec – Nature et Technologies to C.M. We thank Karla Vazquez for excellent technical support.

**References**


