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\textsuperscript{T} and JAM7\textsuperscript{T}, 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\textsuperscript{T} 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\textsubscript{16:1}ω7c or ω6c, C\textsubscript{16:0} and C\textsubscript{18:1}ω7c or ω6c. The major ubiquinone was Q\textsubscript{8}. Both strains required vitamin B\textsubscript{12} and Na\textsuperscript{+} ions for growth. The genomes of strains JAM1\textsuperscript{T} and JAM7\textsuperscript{T} 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\textsuperscript{T}. No such operons were found in strain JAM7\textsuperscript{T}. 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\textsuperscript{T} (=DSM 25689\textsuperscript{T} =ATCC BAA-2433\textsuperscript{T}) and Methylophaga frappieri sp. nov. for type strain JAM7\textsuperscript{T} (=DSM 25690\textsuperscript{T} =ATCC BAA-2434\textsuperscript{T}) are proposed.

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 alcalka (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\textsubscript{12}. They are typically isolated from marine environments or brackish waters. A strict requirement for Na\textsuperscript{+} 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\textsuperscript{T} and JAM7\textsuperscript{T}) 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\textsuperscript{T} and JAM7\textsuperscript{T} grew well on the American Type Culture Collection (ATCC) Methylophaga medium 1403 [per 990 ml: 24 g NaCl, 3 g MgCl\textsubscript{2}, 6 H\textsubscript{2}O, 2 g MgSO\textsubscript{4}, 7 H\textsubscript{2}O, 0.5 g KCl, 1 g CaCl\textsubscript{2}, 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\textsubscript{2}PO\textsubscript{4}, 10 g NH\textsubscript{4}Cl, 10 g Bistris, 0.3 g Ferric ammonium citrate, pH 8), 1 ml vitamin B\textsubscript{12} 0.1 mg ml\textsuperscript{-1}, 10 ml Wolf’s mineral solution (ATCC), pH 8.0]. This medium was used to determine the

Abbreviations: RAST, Rapid Annotation using Subsystem Technology; RuMP, ribulose-monophosphate.
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-butylmethylether (9:1 v/v) as a solvent. UV absorbing bands corresponding to the 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 a solvent. UV absorbing bands corresponding to the different quinone classes (e.g. menaquinones or ubiquinones) 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

| Taxa: 1, strain JAM1<sup>T</sup> (data from this study); 2, strain JAM7<sup>T</sup> (this study); 3, M. alcalica M39<sup>T</sup> (Doronina et al., 2003b); 4, M. aminisulfidivorans MP<sup>T</sup> (Kim et al., 2007); 5, M. lonarensis MPL<sup>T</sup> (Antony et al., 2012); 6, M. marina ATCC 35842<sup>T</sup> (Janvier et al., 1985; Li et al., 2007); 7, M. muralis Kr3<sup>T</sup> (Doronina et al., 2005); 8, M. sulfidovorans RB-1<sup>T</sup> (de Zwart et al., 1996); 9, M. thalassica ATCC 33146<sup>T</sup> (Janvier et al., 1985); 10, M. thiooxydans DSM010<sup>T</sup> (Boden et al., 2010). Motility: swimming was observed by microscopy and flagella were detected by electron microscopy for strains JAM1<sup>T</sup> and JAM7<sup>T</sup>. +, Positive; −, negative; MMA, monomethylamine; UQ, ubiquinone; ND, not determined.

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| Predominant cellular fatty acids | C<sub>16:0</sub>, C<sub>16:1ω3</sub> | C<sub>16:0</sub>, C<sub>16:1ω7c</sub> | C<sub>16:0</sub>, C<sub>16:1ω9c</sub> | C<sub>16:0</sub>, C<sub>16:1ω6c</sub> | ND | C<sub>16:0</sub>, C<sub>16:1ω3</sub> | ND | ND | ND |
| Quinone                  | UQ-8 | UQ-8 | UQ-8 | UQ-8 | UQ-8 | UQ-8 | UQ-8 | ND | ND | UQ-8 |
| Range of growth pH       | 6–11 | 6–11 | 6–11 | 6–8 | 7–10 | 6–11 | 6–11 | 0–42 | ND | ND |
| Range of growth temperatures (°C) | 15–37 | 15–37 | 4–35 | ND | 20–37 | 10–40 | 10–40 | ND | ND |

Growth on: Methanol + + + + + + + + + + + + + + Fructose + + + + + + + + + + + + + + MMA + + + + + + + + + + + + + +

*Nitrate reduction into nitrite was positive with M. marina strains KM3 and KM5 (Li et al., 2007), but not with the type strain 222<sup>T</sup> (ATCC 35842<sup>T</sup>).
†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 (\textgreater 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. lonarenensis MPL\textsuperscript{T} and M. muralis Kr3\textsuperscript{T}. 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-hexulose isomerase (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., 1992; 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 \textmu M 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
region of strain JAM1<sup>T</sup> where operons related to the denitrification pathway were found. Besides the two expected nar operons, it was surprising to find also two nitric oxide reductase (nor) operons and one nitrous oxide reductase (nos) operon. A truncated nitrite reductase (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<sup>T</sup>. The nar1 and nor2 gene sequences and gene arrangements are similar to those found in some Betaproteobacteria (e.g., *Thiobacillus denitrificans*), suggesting horizontal gene transfer for this specific region as suggested by Auclair 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 (moaA) were found. The nitrate reductases are molybdopterin proteins. Finally, the nnrS gene that is involved in nitric oxide response is present. No such operons were present in strain JAM7<sup>T</sup>.

We clearly showed that strain JAM1<sup>T</sup> and strain JAM7<sup>T</sup> 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<sup>T</sup> cannot be achieved by strain JAM7<sup>T</sup>, *M. alcalica* M39<sup>T</sup> and *M. lonarensis* MPL<sup>T</sup>. Also strain JAM1<sup>T</sup> differs from strain JAM7<sup>T</sup> by the presence of alkaline phosphatase activity. Strains JAM1<sup>T</sup> and JAM7<sup>T</sup> and *M. lonarensis* MPL<sup>T</sup> cannot grow on monomethylamine as does *M. alcalica* M39<sup>T</sup>. A difference of more than 5% in the DNA G+C content separates strain JAM1<sup>T</sup> from *M. lonarensis* MPL<sup>T</sup>. Strain JAM1<sup>T</sup> and *M. alcalica* M39<sup>T</sup> share 96.3% sequence similarity in their 16S rRNA gene sequences, and there is 94.7% sequence similarity between strain JAM1<sup>T</sup> and *M. lonarensis* MPL<sup>T</sup>. All these differences support the suggestion that strain JAM1<sup>T</sup> is a different species from *M. alcalica* M39<sup>T</sup> and *M. lonarensis* MPL<sup>T</sup>. Besides the differences in the monomethylamine, strain JAM7<sup>T</sup> and *M. alcalica* M39<sup>T</sup> share 95.9% sequence similarity in their 16S rRNA gene sequences. The only discrimination factor between strain JAM7<sup>T</sup> and *M. lonarensis* MPL<sup>T</sup> is the 94.9% sequence similarity in their 16S rRNA gene sequence. The fact that genome sequences of strains JAM1<sup>T</sup> and JAM7<sup>T</sup> are significantly different despite a 97% sequence similarity in their 16S rRNA gene sequences, strongly suggests that strain JAM7<sup>T</sup> is a different species from *M. alcalica* M39<sup>T</sup> and *M. lonarensis* MPL<sup>T</sup>.

**Description of *Methylophaga nitratireducenticrescens* sp. nov.**

*Methylophaga nitratireducenticrescens* (ni.tr.a.ti.re.du.cen. ti.cres.cens. N.L. n. nitrati-atis nitrate; L. part. adj. reducens

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**Fig. 2.** Chromosomal arrangement of genes related to the denitrification pathway in strain JAM1<sup>T</sup>. The chromosomal region of strain JAM1<sup>T</sup> from positions 434688 to 501331 (GenBank accession no. CP003390) is illustrated. Only genes that showed affiliation to the denitrification pathway are depicted. The genome was analysed at the Rapid Annotation using Subsystem Technology (RAST) website (http://rast.nmpdr.org; January to March 2012) (Aziz et al., 2008) for comparison with the most related genomes. **nor**: nitric oxide reductase; **nos**: nitrous oxide reductase; **nirK**: copper-containing nitrite reductase (truncated); **nar**: nitrate reductase; **nnrS**: protein involved in response to nitric oxide; **N-trp**: nitrate/nitrite transporter; **N-sensor**: nitrate/nitrite sensor protein; **N-reg**: nitrate/nitrite response regulator protein; **mogA**: molybdopterin biosynthesis Mog protein, molybdochelatase; **moaA**: Molybdenum cofactor biosynthesis protein; **IS982 tnp**: transposase; **ISSod6 tnp**: transposase. Regions marked Betaproteobacteria are regions where the nucleic sequence and the gene arrangement are more closely similar to those found in Betaproteobacteria than those found in Gammaproteobacteria based on RAST analysis.
-entis converting to a different state; L. part. adj. crescens growing; N.L. part. adj. nitratireducercentis crescens 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 or absence of oxygen. Grows in the absence of oxygen with nitrate. Grows at temperatures between 15 and 37 °C (optimum, 30 °C). 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 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 T (=DSM 25689 T =ATCC BAA-2433 T) 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 47.8 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 °C (optimum, 34 °C). 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 JAM7 T (=DSM 25690 T =ATCC BAA-2434 T) 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 47.7 mol%.

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References


