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ORIGINAL ARTICLE

Dissimilatory reduction of nitrate in seawater by a *Methylophaga* strain containing two highly divergent *narG* sequences

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Methylophaga **spp. are methylotrophs commonly associated with marine environments and have been defined as strict aerobic methylotrophs. They have been shown previously to represent 50–70% of the bacterial population in the biofilm of the methanol-fed denitrification reactor treating a large seawater aquarium at the Montreal Biodome. It was therefore surprising to find such a high concentration of** ***Methylophaga*** **spp. in anoxic conditions. In this study, we showed by cultivation-independent and -dependent approaches that one** ***Methylophaga*** **strain present in the anoxic biofilm is involved in the denitrification process. DNA stable-isotope probing (SIP) experiments in which the biofilm was cultured under denitrifying conditions with ^{13}C-methanol have revealed the enrichment of one particular taxon. By screening a 16S ribosomal RNA gene library derived from a ^{13}C-DNA fraction of the SIP gradients, 62% of the library was composed of one sequence affiliated with the genus** ***Methylophaga*****. One strain, named JAM1, representing this** ***Methylophaga*** **species was isolated. It grows aerobically but also under denitrifying conditions by reducing nitrate into nitrite. The nitrate-reducing activity was correlated with the presence and the expression of two highly divergent** ***narG*** **genes (*narG*1 and *narG*2).** ***narG*****1 showed a high percentage of identity with the corresponding part of** ***narG*** **found in** ***Thiobacillus denitrificans,*** **which suggests a recent acquisition of** ***narG*** **in strain JAM1 by horizontal gene transfer. This study provides the first direct evidence of the adaptation of a** ***Methylophaga*** **species to an oxygen-limited environment.**
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Introduction

Public aquariums and aquaculture tanks that are operated in closed circuit require water treatment facilities such as provision of sand filters or biological filtration systems for removing organic matter and other waste (Parent and Morin, 2000; Hamlin *et al.*, 2008a). However, highly soluble molecules such as nitrate, which is generated from animal-derived nitrogenous compounds (that is urea), could prove to be toxic for the fish and invertebrates (Grguric *et al.*, 2000; Parent and Morin, 2000; Hamlin *et al.*, 2008b). For instance, in marine environments, the nitrate concentration should not exceed 20 mg NO_3^--N/L to protect aquatic animals (Camargo *et al.*, 2005). On the other hand, the option of replacing the water for decreasing the nitrate concentration is expensive, especially in artificial seawater aquarium, as well as stressful to the fauna. Biological denitrification is a valuable alternative for removing nitrate from seawater (Balderston and Sieburth, 1976; Grguric *et al.*, 2000; Tal *et al.*, 2003). Denitrification is a process that generally occurs in the absence of oxygen and in which heterotrophic bacteria reduce nitrate into N_2 (Zumft, 1997).

In 1998, the Montreal Biodome established a methanol-fed, fluidized denitrification reactor for controlling the concentration of nitrate in its three million liter seawater aquarium wherein the concentration had reached a critical level of 200 mg NO_3^--N/L (Parent and Morin, 2000). Owing to the low organic charge of the seawater, methanol was used as an exogenous carbon source to sustain denitrification (Mateju *et al.*, 1992). The bacterial biota of the denitrifying biofilm in the denitrification system was estimated to contain between 15 and 20 species, among which two methanol-utilizing denitrifying bacteria (methylotrophs), *Hyphomicrobium zavarzinii* and *Paracoccus denitrificans,* were isolated (Labbé *et al.*, 2003). However, it was estimated by fluorescence *in situ* hybridization

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(FISH) that more than 50% of the biofilm was composed of bacteria that belonged to the genus *Methylophaga* (Labbé *et al.*, 2007).

Methylophaga spp. are halophilic, methylotrophic bacteria that were isolated from diverse marine environments (Janvier *et al.*, 1985; Doronina *et al.*, 2003a, b). They have been shown to be involved in methanol assimilation in marine environments (Neufeld *et al.*, 2007) or associated with phytoplankton that produce methanol (Janvier *et al.*, 1985; Neufeld *et al.*, 2008a). Thus, the presence of *Methylophaga* spp. in the methanol-fed marine reactor at the Biodome was no surprise. However, their high proportion in the denitrifying biofilm was not expected as they have been defined as strictly aerobic (Janvier *et al.*, 1985; Doronina *et al.*, 2003a, b; Kim *et al.*, 2007). Their presence suggested that *Methylophaga* spp. were involved in the denitrification process or were involved in cross-feeding with other denitrifying bacteria.

Some *Methylophaga* species have been shown to reduce nitrate into nitrite; however, very limited information is available from these reports regarding this activity (Doronina *et al.*, 2003b; Kim *et al.*, 2007). Osaka *et al.* (2008) found 16S ribosomal RNA (rRNA) gene sequences associated with the genus *Methylophaga* when a wastewater sludge was acclimated with 4% NaCl in a methanol-fed denitrifying reactor. However, direct evidence of the denitrifying activity of *Methylophaga* spp. *in situ* has not been shown.

In this paper, we used cultivation-independent and -dependent approaches to identify the denitrifying methylotrophic bacteria present in the denitrification reactor of the Montreal Biodome. DNA stable-isotope probing (DNA-SIP) was first performed with the denitrifying biofilm in artificial seawater containing nitrate and ^{13}C-methanol. Our results indicated a specific enrichment of *Methylophaga* spp., which strongly suggested their involvement in denitrification. Then, one *Methylophaga* strain, named JAM1, representing the most dominant bacteria enriched in the SIP assay was isolated from the biofilm. It has the capacity of growing in the presence of methanol in denitrifying conditions by reducing nitrate into nitrite. This was correlated with the presence and the expression in strain JAM1 of two putative nitrate reductase genes (*narG*). This study is the first to reveal the capacity of a *Methylophaga* strain to reduce nitrate into nitrite under denitrifying conditions.

Materials and methods

Determination of the concentration of nitrate, nitrite and ammonium

The nitrate concentration was measured by spectrophotometry or high-performance liquid chromatography (HPLC). The NO_3^- concentration was determined by spectrophotometry using the standard method (Clesceri *et al.*, 1998) with $NaNO_3$ as the standard. The nitrate and nitrite concentrations were measured using a Dionex DX 500 HPLC (Dionex Canada Ltd, Oakville, ON, Canada) equipped with a IonPac AS20 column (250 mm, 4 mm ID) and coupled to an ASRS 300 4 mm suppressor operating in recycle mode and an ED-40 electrochemical detector as recommended by the manufacturer's instructions using a gradient of sodium hydroxide. Ammonium concentration was measured by means of the colorimetric method as described by Mulvaney (1996).

DNA-SIP

The denitrification system at the Montreal Biodome was described in Labbé *et al.* (2007). The denitrifying biofilm was carefully scraped from the Bioflow 9-mm carriers (1.020 density; Rauschert, Steinwiessen, Germany) taken from the reactor and rinsed three times with artificial seawater (Labbé *et al.*, 2007) to remove residual methanol. Incubation was carried out in 120 ml serum vials in which 1.5 g wet weight of biofilm and 30 ml of artificial seawater supplemented with 300 mg NO_3^--N/L (21.4 mM final concentration) were added. CO_2 traps (1 ml of 1 M KOH) were used to prevent bacterial uptake of $^{13}CO_2$ derived from mineralization (Labbé *et al.*, 2007). The vials were purged with nitrogen, then sealed and preincubated for 1 h before adding either ^{13}C- or ^{12}C-methanol (37.5 mM final concentration) in a 1.5 C/N ratio to remove residual oxygen. The 1.5 C/N ratio was determined earlier in our laboratory and was shown to support optimal denitrifying activity (data not shown). The vials were incubated for 7 days at room temperature. Each day, the cultures were shaken to disperse the biomass, and subsequently a 1-ml volume was taken and centrifuged. The residual nitrate concentration was measured daily in the supernatant by spectrophotometry. On the basis of the nitrate removal, methanol and nitrate were added in a 1.5 C/N ratio to maintain the denitrifying activity. Subsequently, the nitrite concentration was determined by HPLC.

The biomass from the daily samples was extracted for the total DNA. DNA extraction was carried out as described in Labbé *et al.* (2007). A portion of the extracted DNA was used for ^{13}C incorporation measurement in thymine by HPLC coupled with mass spectrometry (HPLC-MS) in both ^{12}C- and ^{13}C-methanol amended cultures. DNA (2.5 μg) was hydrolyzed with 0.5 ml of 88% formic acid at 140 °C for 45 min. The sample was flushed with nitrogen to remove the acid, and the hydrolyzed DNA was suspended in 60 μl of water containing 1% acetic acid. The separation of nitrogenous bases was achieved using an HP 1100 HPLC (Agilent Technologies Canada, Mississauga, ON, Canada) equipped with a Zorbax Eclipse XDB-C8, 5 μm, 4.6 × 150-mm column (Agilent Technologies Canada) by isocratic elution with 1% acetic acid–water

solution at a flow rate of 0.4 ml min^{-1}. The HPLC was coupled to a Quattro II triple-quadrupole mass spectrometer (Waters, Mississauga, ON, Canada) that was operated in positive electrospray ionization mode. The analyses were performed in selected ion monitoring mode using the ions appearing at *m*/*z* 127, 128, 129, 130, 131, 132 at the retention time of thymine. The isotope incorporation percentages were calculated as the sum of the intensities of the isotopic ion peaks' area divided by the intensity of the pseudomolecular ion (*m*/*z* 127). The HPLC-MS measurements were carried out in triplicate on each DNA sample. It was found that the ^{13}C incorporation measurement in cytosine generated similar results (data not shown).

The remaining DNA samples were separated by CsCl density-gradient ultracentrifugation as described in Ginige *et al*. (2004), with 10 μg of DNA per centrifuge tubes (3.9 ml). Ethidium bromide was included in the gradient to visualize the efficiency of the separation. Each CsCl gradient was fractionated from the bottom of the tube to the top into 13 fractions of 275 μl and the fractions were extracted with isopropanol saturated with NaCl to remove ethidium bromide, and diluted four times; the DNA was precipitated with 2-volume ethanol and dissolved in water.

PCR-denaturing gradient gel electrophoresis and 16S rRNA gene library

The PCR protocols, the denaturing gradient gel electrophoresis (DGGE), the extraction of DGGE bands and their reamplification, and the construction of the 16S rRNA gene library were carried out as described by Lafortune *et al*. (2009) using previously published primers (Table 1). The 16S clones were screened by PCR-DGGE, and grouped according to their migration profile. At least one representative clone of each group was sequenced. For comigration experiments, representative clones were run side by side with the heavy fractions of the ^{13}C-methanol cultures on DGGE.

16S rRNA gene sequence analyses

The 16S rRNA gene sequences were examined for chimeras with BELLEROPHON (Huber *et al*., 2004). No chimeric sequences were detected. Using the ARB program and the ARB databases (SSURef_100_SILVA_20_03_09), sequences related to *Methylophaga* spp. were aligned with the lineage of Thiotrichales using Pt_servers for this lineage, and Fastaligner from ARB with 10 neighbors (Ludwig *et al*., 2004; Pruesse *et al*., 2007). The resulting alignments were refined by hand and then used for positioning the sequences in the ARB tree by the fast parsimony method using the *pos_var_Bacteria* filter. From the consensus positions in the tree, representative sequences were selected for deriving a multiple alignment sequence with the *pos_var_Bacteria* filter. These alignments were refined using BIOEDIT 7.0.4.1 (Hall, 1999). Phylogenetic analyses were performed using DNADIST (F84 distance method) and FITCH (Fitch Margoliash method) and SEQBOOT/CONSENSE (bootstrap analysis) programs in PHYLIP 3.65 software package (Felsenstein, 1989).

Isolation of Methylophaga strains

The denitrifying biofilm was carefully scraped from carriers, homogenized and diluted in a 10-fold series (10^{-1} to 10^{-3}), and spread on the following agar media: R2A (Becton Dickinson Co., Cockeysville, MD, USA), ASW (Janvier *et al*., 1985), MSM (Kim *et al*., 2007), ATCC medium 1090 (marine methanol) and on artificial seawater. Artificial seawater was prepared as described above and was supplemented to obtain a final concentration of 0.3% methanol, 0.1 μg ml^{-1} B12 vitamin and 1.5% agar. The culture media were incubated in the presence of oxygen at 30 °C for 1 week to promote the growth of *Methylophaga* species. Unique isolated colonies were picked and grown in liquid medium according to their isolation medium. DNA was extracted from 1.5 ml centrifuged cultures as described earlier (Labbé *et al*., 2007). To identify the strains, 25 ng of DNA was used as template to amplify the nearly full-length 16S rRNA gene with the 27f-YM and 1541r primers (Table 1), and the PCR product was sequenced.

In addition to cultured isolates, *M. alcalica* ATCC BAA-297 was cultured with the NMS medium (Doronina *et al*., 2003b) whereas *M. marina* ATCC 35842 was cultured with the ASW medium (Janvier *et al*., 1985). These species were obtained from the American Type Culture Collection (Manassas, VA, USA).

Detection of denitrification genes in the isolated strains

Deduced amino-acid sequences of the denitrification genes *narG, napA, nirK* and *cnorB* taken from type-strain bacteria were collected from protein databases (National Center for Biotechnology Information (NCBI), January 2006, http://www.ncbi.nlm.nih.gov/) and aligned with ClustalW (Thompson *et al*., 1994). Degenerated oligonucleotides were designed based on the amino-acid consensus sequences, and used as primers for PCR (Table 1). For *nirS* and *nosZ*, we used previously published primers (Table 1). All these primers showed positive PCR amplifications for the respective genes with total DNA extracted from the biofilm.

Expression of narG in Methylophaga sp. strain JAM1

The *Methylophaga* sp. strain JAM1 was incubated in 25 ml ASW liquid medium supplemented with 200 mg NO_3^--N/L in a 125 ml serum vials that were flushed previously for 10 min with nitrogen gas to

Table 1 Oligonucleotides used as PCR primers

Name	*Sequence (5′–3′)*	*Hybridization temperature (°C)*	*Primer concentration (nM)*	*References*
Bacteria[a]				
27f-YM	AGAGTTTGATYMTGGCTCAG	55	200	Frank *et al.* (2008)
1541r	AAGGAGGTGATCCARCCGCA			
Bacteria[b]				
341f	CCTACGGGAGGCAGCAG	55	200	Muyzer *et al.* (1993)
534r	ATTACCGCGGCTGCTGG			
narG[c]				
narG-A241f	TTYTAYGAYTGGTAYGCNGA	46	500	This study
narG-C241f	TTYTAYGAYTGGTAYTGYGA		500	
narG-631r	TTYTCYTGNCCNACRTARTG		1000	
narG1-180f[d]	CCCTGTACGCTCGATACCA	56	200	This study
narG1-384r	GCCAGTACGCAAGGTTAAGC			
narG2-175f[d]	TCACGCGCAATTTGAACCAC	57	200	This study
narG2-397r	TCCAACAGCGAAAAGTACCG			
napA[c]				
napA-206f	GAYCCNAAYGCNMGNCAYTGYATGGC		1000	This study
napA-F457r	TGYTGRTTRAANCCCATNGTCCA	51	500	
napA-M457r	TGYTGRTTNATNCCCATNGTCCA		500	
nirS				
primer F1acd	TAYCACCCSGARCCGC	57	1000	Hallin and Lindgren (1999)
primer R4cd	CGTTGAACTTRCCGGTSGG			
nirK				
nirK-203f	TTYGTNTAYCAYTGYGCNCC	56	8000	This study
nirK-326r	TCNCCRTGNCCNCCDATNARRTGNGG			
cnorB[c]				
cnorB-V212f	TGGGTNGARGGNGTNTGGGA	51	500	This study
cnorB-T212f	TGGGTNGARGGNACNTGGGA		500	
cnorB-355r	TANGCNCCRWARAANGC		1000	
nosZ				
Nos661f	CGGCTGGGGGCTGACCAA	56	400	Scala and Kerkhof (1998)
Nos1773r	ATRTCGATCARCTGBTCGTT			

[a]The 27f-YM and 1541r primers were used to derive the 16S rRNA gene library.
[b]The 341f and 534r primers were used for re-amplifying DNA fragments extracted from denaturing gradient gel electrophoresis (DGGE). 341F-GC had the same sequence as 341f except for the following sequence upstream: CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G, and were used with 534r for PCR-DGGE.
[c]The forward primers narG-A241f/narG-C241f were used with the reverse primer narG-631r; the forward primer napA-206f with the reverse primers napA-F457r/napA-M457r; and the forward primers cnorB-V212f/cnorB-T212f with the reverse primer cnorB-355r.
[d]The narG1-180f/narG1-384r and the narG2-175f/narG2-397r primer pairs were used for RT–PCR experiments.

remove oxygen. The vials were then sealed and incubated at 30 °C with agitation (250 r.p.m.). In addition, the strains were cultured under aerobic conditions (25 ml ASW medium, 30 °C, 250 r.p.m.) either with 200 mg NO_3^--N/L or in the absence of nitrate. In all, 10 ml of culture was centrifuged for 8 min at 3650 *g* and 4 °C, and RNA was extracted from the pellet using the RiboPure-Bacteria kit (Ambion Inc., Austin, TX, USA) according to the manufacturer's specifications. The total RNA (40 μg) was treated with 20 U DNase I in 40 mM Tris–HCl (pH 7.9), 10 mM NaCl, 6 mM $MgCl_2$ and 1 mM $CaCl_2$ at 37 °C for 1 h in a 60-μl volume. Another 20 U of DNase I was added after 1 h of incubation and the reaction mix was incubated for an additional 30 min at 37 °C. DNase I was inactivated by the DNase Inactivation Reagent supplied with the RiboPure-Bacteria kit. The treated RNA (1 μg) was used for reverse transcription (RT) with 2 μM of the narG1-384r or narG2-397r primer (Table 1) as described earlier (Villemur *et al.*, 2007). In all, 5 μl of the RT reaction was used for PCR amplification with the narG1-180f/narG1-384r or narG2-175f/narG2-397r primer pairs (Table 1) as described earlier (Villemur *et al.*, 2007). The amplifications were performed at 94 °C for 5 min, at 56 or 57 °C for 5 min, followed by 30 cycles at 72 °C for 60 s, at 94 °C for 60 s, at 56 or 57 °C for 60 s and finally an extension period of 10 min at 72 °C.

The deduced amino-acid sequences of *narG1* and *narG2* were compared against the protein databases with BLASTP (NCBI). The most related sequences of

type-strain organisms were selected. The sequences were aligned with ClustalW (Thompson *et al.*, 1994). The alignments were manually refined in BIOEDIT (Hall, 1999). Phylogenic analysis was performed using the PROTDIST (Jones—Taylor–Thornton distance method), FITCH (Fitch Margoliash method) and SEQBOOT/CONSENSE (bootstrap analysis) programs in PHYLIP 3.65 software package (Felsenstein, 1989).

Results

Enrichment of denitrifying methylotrophic bacteria by DNA-SIP

The biofilm taken from the denitrification reactor of the Montreal Biodome was incubated under denitrifying conditions in artificial seawater supplemented with nitrate and ^{13}C-methanol for 7 days. A control experiment was carried out with ^{12}C-methanol. The biofilm sustained denitrifying activity in both cultures (Figure 1a). Ammonium was not detected in the cultures (data not shown).

The ^{13}C incorporation percentages in ^{13}C-methanol cultures increased during the experiment (Figure 1b) and reached almost 50% after 7 days. On the basis of the minimal isotopic enrichment of 20% proposed by Radajewski *et al.* (2000) to obtain a clear separation of ^{13}C-DNA and ^{12}C-DNA, and to minimize the bias generated by bacterial cross-feeding, the biomass taken at day 3, for which there was 28% of ^{13}C incorporation, was chosen to extract the DNA and then to perform the CsCl density-gradient ultracentrifugations.

A bacterial diversity profile was derived for the 'light' and 'heavy' DNA fractions from the SIP CsCl gradients (Figure 2). There was little difference in the profiles between the heavy and the light fractions for the ^{12}C-methanol cultures, with both profiles being very similar to the one derived from the original biofilm. However, there was a clear difference in the number of distinct DGGE bands in the profile of the heavy fraction of the ^{13}C-methanol culture, reflecting ^{13}C incorporation into methylotrophic bacteria. One intense DNA band was found to dominate this profile. Sequencing of this band showed affiliation with *Methylophaga* spp. To determine the non-methylotrophic bacteria that were present in the biofilm, sequencing of some bands from the light fraction was also performed, and the bands for which readable sequences were successfully obtained showed affiliation with Oceanospirillales, *Vibrio* spp., and Rhizobiales (Figure 2). The band related to *Methylophaga* spp. was still present in the light fraction, at a lower intensity than the heavy fraction. This is probably because of carryover from the heavy fraction as the ^{13}C-*Methylophaga* DNA band in the heavy fraction represented a high proportion of the total DNA.

The DNA recovered from the heavy fractions of ^{13}C-methanol cultures at day 3 was used to derive a 16S rRNA gene library. The screening of 93 clones by PCR-DGGE showed seven different migration profiles. Phylogenetic analyses showed that two groups of clones (IAFJAsip1 and IAFJAsip4), which composed 69% of the screened clones, were affiliated with the genus *Methylophaga* (Table 2; Figure 3). The most abundant clones (62%; representative sequence IAFJAsip1) were closely related (96%) to *M. alcalica* (Table 2). PCR-DGGE experiments with the heavy fraction (fraction 4) and IAFJAsip1 showed comigration between the most

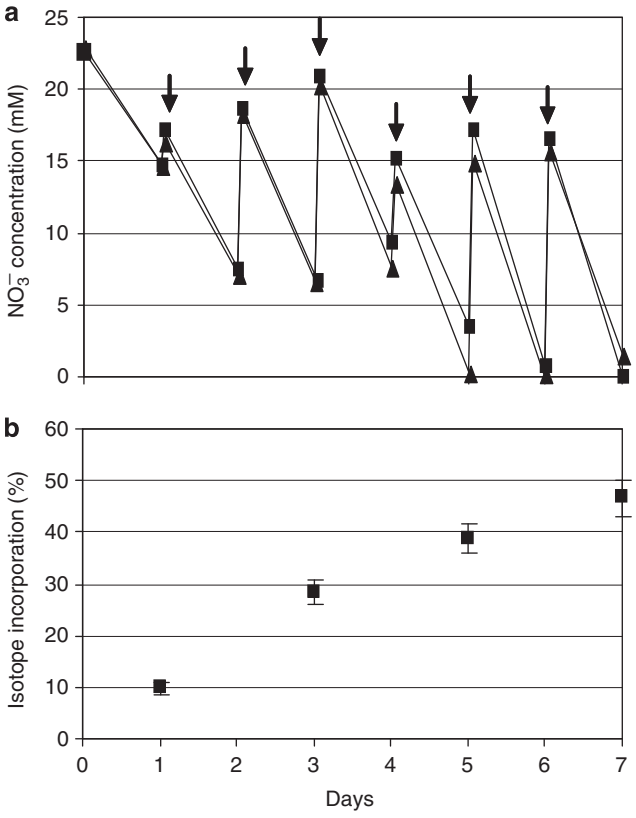

Figure 1 NO_x removal (**a**) and ^{13}C incorporation in DNA (**b**) during SIP cultures. (**a**) The biofilm taken from the denitrification reactor of the Montreal Biodome was cultured under denitrifying conditions in artificial seawater supplemented with 300 mg NO_3^--N/L and ^{13}C-methanol (C/N ratio of 1.5) for 7 days. Nitrate and methanol at the same C/N ratio were added on a daily basis (arrows) for maintaining the biofilm under denitrifying conditions. Total residual NO_x concentration was measured by spectrophotometry. The nitrite concentration was afterwards determined by HPLC. Nitrite accounted for the residual NO_x concentration for the first 4 days. ^{13}C-methanol (triangle) and ^{12}C-methanol (square) cultures. (**b**) The biomass from ^{12}C- and ^{13}C-methanol cultures was sampled on days 0, 1, 3, 5 and 7, and the total DNA was extracted. This DNA was acid hydrolyzed and thymine was analyzed by HPLC-MS for ^{13}C incorporation. Each value was subtracted with the ^{13}C incorporation percentages in thymine in the day 0 samples (before adding ^{13}C-methanol), which represented the natural abundance ^{13}C incorporation in thymine. The ^{13}C incorporation percentage for the ^{12}C-methanol cultures remained stable throughout the experiment with a percentage similar to the theoretical isotopic distribution of thymine because of the natural 1% abundance of ^{13}C (data not shown).

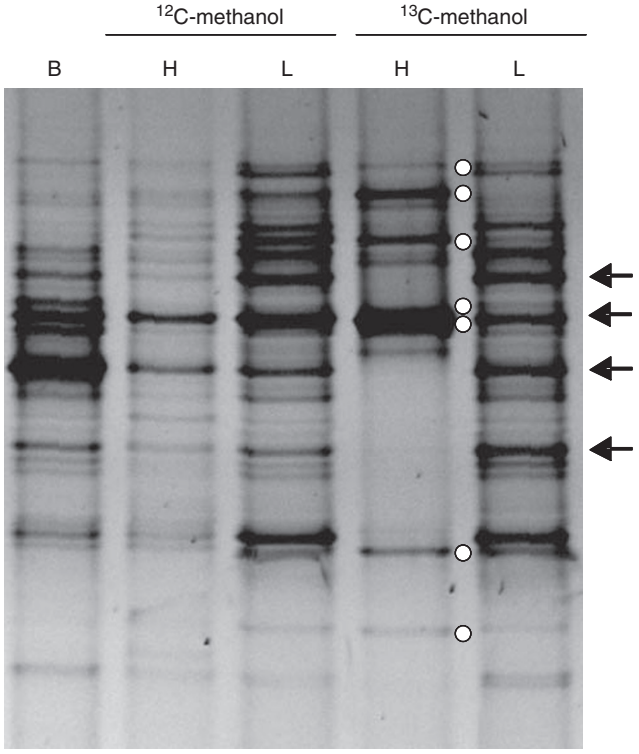

Figure 2 Denaturing gradient gel electrophoresis (DGGE) profiles for the 'light' and 'heavy' DNA from the SIP CsCl gradients. DNA extracted from day 3 of the ^{12}C- and ^{13}C-methanol cultures was subjected to CsCl gradient ultracentrifugations, and fractionated. PCR was performed on each fraction for part of the 16S rRNA gene sequences, and the resulting amplicons were separated by DGGE. DGGE migration profiles from representatives of the heavy (fraction 4) and light (fraction 7) fractions are shown. Migrating profile of DNA extracted from the original biofilm before the beginning of the experiment is included for comparison. Dots: correspondence between the 16S rRNA clones and the heavy DNA fraction of the ^{13}C-methanol culture as determined by PCR-DGGE comigration experiments. From top to bottom: IAFJAsip25, IAFJAsip3, IAFJAsip28, IAFJAsip4, IAFJAsip1, IAFJAsip30 and IAFJAsip2 (see Table 2). Arrows: these DNA bands were extracted from gel, reamplified and sequenced. From the top to the bottom, bands were affiliated with Oceanospirillales, *Methylophaga* spp., *Vibrio* spp. and Rhizobiales, respectively. H: heavy fraction; L: light fraction; B: biofilm.

prominent band and the IAFJAsip1-derived band (data not shown).

IAFJAsip3, representing about 20% of the screened clones, showed a low-level affiliation with *M. thalassica* (93%) (Table 2). Phylogenetic analysis showed that IAFJAsip3 rather grouped with uncultured bacteria retrieved from different marine habitats (Figure 3). The other clones (10/93) were all affiliated with Proteobacteria (Table 2).

Isolation of Methylophaga strains from the biofilm

Five different strains were isolated when the biofilm was spread on different solid media in aerobic conditions to isolate *Methylophaga* colonies. The analyses of 16S rRNA gene sequences showed that strains JAM1 and JAM7 were related (96%) to *M. alcalica* and *M. murata,* respectively (Figure 3). These two strains were both isolated from the ASW and artificial seawater media. The three other strains were affiliated with the genera *Bacillus* (99%), *Halomonas* (99%) and *Vibrio* (98%). The 16S rRNA gene sequence of strain JAM1 has only one nucleotide difference with IAFJAsip1. This result suggests that the *Methylophaga* sp. strain JAM1 represents the most abundant bacterial species in the biofilm.

The five strains were tested for their denitrification capacity. No significant growth or nitrate reduction occurred under denitrifying conditions with the *Bacillus*, *Halomonas* and *Vibrio* strains or with the *Methylophaga* sp. strain JAM7 (data not shown). However, nitrate was completely depleted by strain JAM1 in 10 h (Figure 4a), with equimolar production of nitrite. Moreover, nitrate reduction was observed when strain JAM1 was cultured in the presence of nitrate and oxygen (Figure 4a). Reduction of nitrate into nitrite and growth were not observed when *M. alcalica* or *M. marina* was cultured under denitrifying conditions (data not shown).

Growth was observed when strain JAM1 was cultured with methanol and nitrate in the presence or the absence of oxygen (Figure 4b). Microbial flocs and attachment of the biomass to glass surface were observed during the growth. Aerobic conditions resulted in an increased growth yield, especially when nitrate was not present (Figure 4b). This difference could be explained by the low energetic yield of nitrate respiration when compared with oxygen respiration and by the accumulation of the toxic nitrite. There was a fourfold decrease in biomass when strain JAM1 was cultured aerobically in presence of 0.36 mM (5 mg l^{-1}) nitrite at the beginning of the culture, and no growth occurred in the presence of 0.71 mM (10 mg l^{-1}) nitrite (Figure 4c).

Detection of denitrification genes in Methylophaga strains and their expression

Detection of the different reductases involved in denitrification such as *narG, napA, nirS, nirK, cnorB* and *nosZ* sequences in the *Methylophaga* strains was assessed with degenerated PCR primers (Table 1). PCR amplifications occurred with the *narG* primers on DNA extracted from strain JAM1. Subsequent cloning of the PCR product showed that two sequences related to *narG* were present in strain JAM1. Using specific pairs of primers for each of these *narG*, PCR amplifications were still occurring, confirming the presence of these two genes in the JAM1 genome. No PCR amplification occurred with primers targeting sequences related to *napA*, *nirS*, *nirK*, *cnorB* and *nosZ* in strain JAM1. There was no PCR amplification with primers targeting all these genes, including *narG,* with the *Methylophaga* sp. strain JAM7, *M. alcalica* and *M. marina*.

Table 2 Closest affiliation of the screened clones from the 16S rRNA gene library derived from the heavy fraction (fraction 4) of the ^{13}C-methanol cultures.

Affiliation	*Number of clones*	*Representative clone*	*%*	*Accession identity number*
Gamma-Proteobacteria, Thiotrichales, Piscirickettsiaceae (83/93)				
Methylophaga alcalica	58/93	IAFJAsip1	96	GU567807
Methylophaga thalassica	19/93	IAFJAsip3	93	GU567809
Methylophaga marina	6/93	IAFJAsip4	95	GU567810
Gamma-Proteobacteria, unclassified Gamma-Proteobacteria				
Sedimenticola selenatireducens	1/93	IAFJAsip25	98	GU567811
Delta-Proteobacteria, Bdellovibrionales, Bacteriovoracaceae				
Bacteriovorax sp.	4/93	IAFJAsip28	98	GU567812
Delta-Proteobacteria	3/93	IAFJAsip2	<90	GU567808
Alpha-Proteobacteria, Rhodobacterales, Rhodobacteraceae				
Rhodovulum sp.	2/93	IAFJAsip30	96	GU567813

DGGE, denaturing gradient gel electrophoresis.
Ninety-three clones were screened by PCR-DGGE and classified based on their migration profile. One to three representative clones were sequenced.

The *narG*1 and *narG*2 cloned sequences contain each a continuous coding frame of 359 and 370 codons, respectively. They share 61% identity in their nucleic sequence and 55% identity in the deduced amino-acid sequence (65% similarity). Phylogenetic analyses showed that NarG1 has a high percentage of identity (88%; 93% similarity) to the corresponding sequence of NarG in *Thiobacillus denitrificans* (Figure 5). The phylogenetic analyses also showed the NarG2 affiliation with the Gamma-Proteobacteria.

The expression of *narG*1 and *narG*2 was monitored by RT–PCR in *Methylophaga* sp. strain JAM1 (Figure 6). *narG*1 and *narG*2 were expressed under denitrifying conditions but also in the presence of both oxygen and nitrate. Finally, *narG*1 and *narG*2 were expressed under aerobic conditions in the absence of nitrate.

Discussion

By using cultivation-independent and -dependent approaches, we clearly showed the involvement of the dissimilatory reduction of nitrate by one *Methylophaga* strain present in the biofilm of the denitrification reactor at the Montreal Biodome. The DNA-SIP experiments and the subsequent screening of the 16S rRNA gene library showed a clear enrichment of one bacterial species in the ^{13}C fractions that was affiliated with the genus *Methylophaga*. Cultivation approach allowed the isolation of this *Methylophaga* strain, JAM1, from the biofilm, which had the capacity to reduce nitrate into nitrite, but not further. The 16S rRNA gene sequence of strain JAM1 is almost identical to clone IAFDn39 (1-nt difference), the most numerous clones present in the 16S rRNA gene library of the biofilm reported by Labbé *et al*. (2003). This suggests that JAM1 represents the major bacteria in the biofilm of the denitrification reactor of the Montreal Biodome.

These results are in contradiction with the previously obtained results by microautoradiography coupled with FISH (MAR-FISH) (Labbé *et al*., 2007). In the MAR-FISH experiments, the denitrifying biofilm was incubated for 17 h with ^{14}C-methanol under denitrifying conditions. No positive MAR response was obtained for *Methylophaga* spp. suggesting that *Methylophaga* spp. was not involved in methanol consumption under these conditions. One possible explanation is that the incubation time of 17 h was insufficient for *Methylophaga* spp. to assimilate enough ^{14}C-methanol under denitrifying conditions and to be detected by MAR-FISH. In fact, Figure 1 shows that there was <10% ^{13}C incorporation in the biofilm after 17 h under denitrifying conditions.

16S rRNA gene sequences affiliated with the genus *Methylophaga* were observed elsewhere in anoxic environments. About 10% of the population of a methanol-fed denitrification reactor for the treatment of industrial saline wastewater was reported to be affiliated with this genus (Osaka *et al*., 2008). In another study involving an offshore oil field, deoxygenated seawater was injected into the underground oil reservoir to increase pressure and oil recovery. To avoid souring, nitrate was added to the deoxygenated seawater to favor denitrifying conditions to prevent the growth of sulfate-reducing bacteria. The deoxygenation system used methanol in the presence of a catalyst to consume O_2. Schwermer *et al*. (2008) studied the changes in the bacterial biota in the biofilm that developed in the pipes when nitrate was added. They observed an enrichment of 16S rRNA gene sequences related to *Methylophaga* spp., which

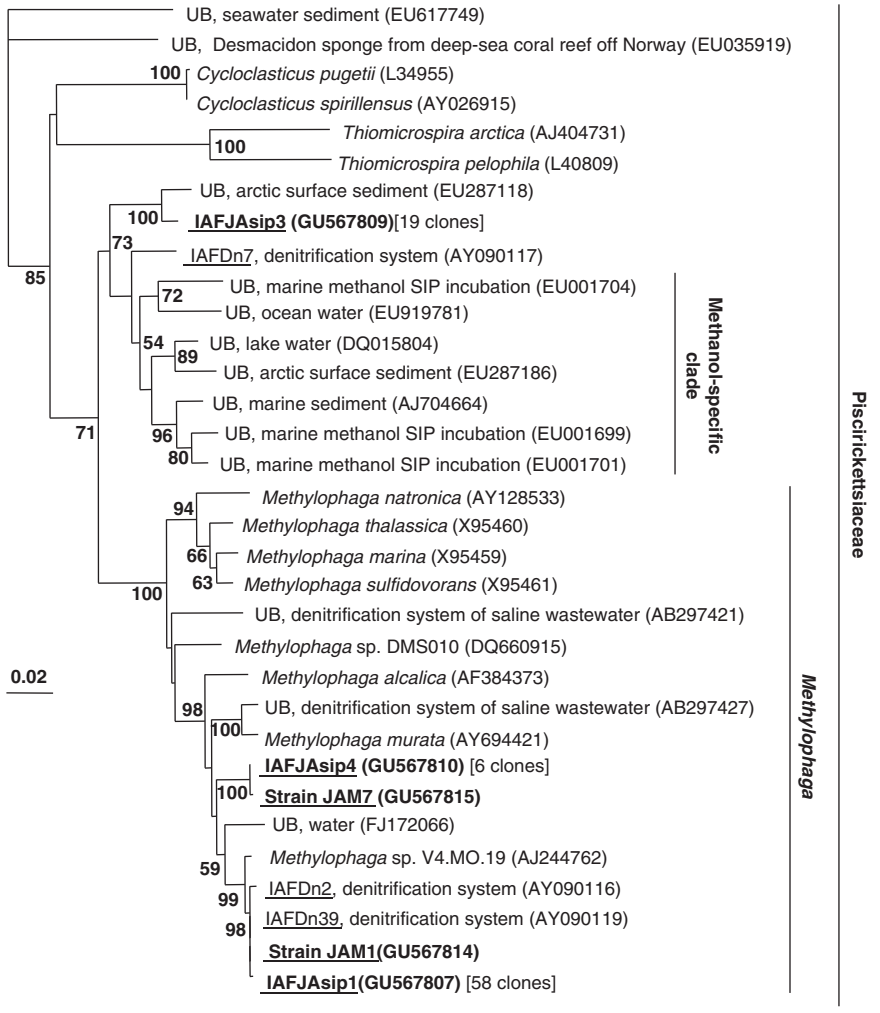

Figure 3 Phylogenetic analysis of 16S rRNA gene sequences affiliated with the genus *Methylophaga*. Unrooted phylogenetic trees showed the evolutionary relationship among 16S rRNA gene sequences from clones (IAFJAsip1, IAFJAsip3 and IAFJAsip4) and strains (JAM1 and JAM7), and from representative members of the genus *Methylophaga* and closely related Piscirickettsiaceae. Trees were inferred from a matrix of pairwise distance using aligned sequences containing 789 positions. The scale bar represents nucleotide substitutions per position. Bootstrap values above 50% are indicated at the branches. GenBank accession numbers are in parentheses beside each species name. The number of clones retrieved from the 16S rRNA gene library and represented by IAFJAsip1, IAFJAsip3 and IAFJAsip4 is indicated. UB: uncultured bacterium.

represented more than 50% of the 16S rRNA gene libraries. Residual methanol was probably present in effluent of the deoxygenation system that favored the growth of *Methylophaga* spp. in the pipes. These results suggested that *Methylophaga* spp. could adapt to anoxic conditions. Unfortunately, the 16S rRNA gene sequences of this study were not available and no phylogenetic analysis could have been performed with our sequences.

This study is the first to reveal the capacity of a *Methylophaga* species to reduce nitrate into nitrite under denitrifying conditions. This was supported by growth assays, and the detection of two putative highly divergent *narG* gene sequences (*narG*1 and *narG*2) and their expression in strain JAM1 growing under denitrifying conditions. Some *Methylophaga* species have been previously reported to reduce nitrate into nitrite but only under aerobic conditions (Urakami and Komagata, 1987; Doronina *et al.*, 2003a). These studies, however, did not assay for the presence of the nitrate reductases *narG* or *napA*. No such genes were found in the only available *Methylophaga* genome sequence from *M. thiooxidans* DMS010 (GenBank accession number ABXT00000000), and there is no indication as to whether *M. thiooxidans* could reduce nitrate into nitrite (Schäfer, 2007). Finally, *narG* or *napA*, or any other denitrification genes were not detected in *M. alcalica* or *M. marina* with the primers used in our study.

The expression of *narG*1 and *narG*2 in strain JAM1 was also observed under aerobic conditions with or without nitrate addition, suggesting the constitutive expression of these genes. Aerobic nitrate respiration has been shown in a number of bacteria (Davies *et al.*, 1989; Robertson *et al.*, 1989).

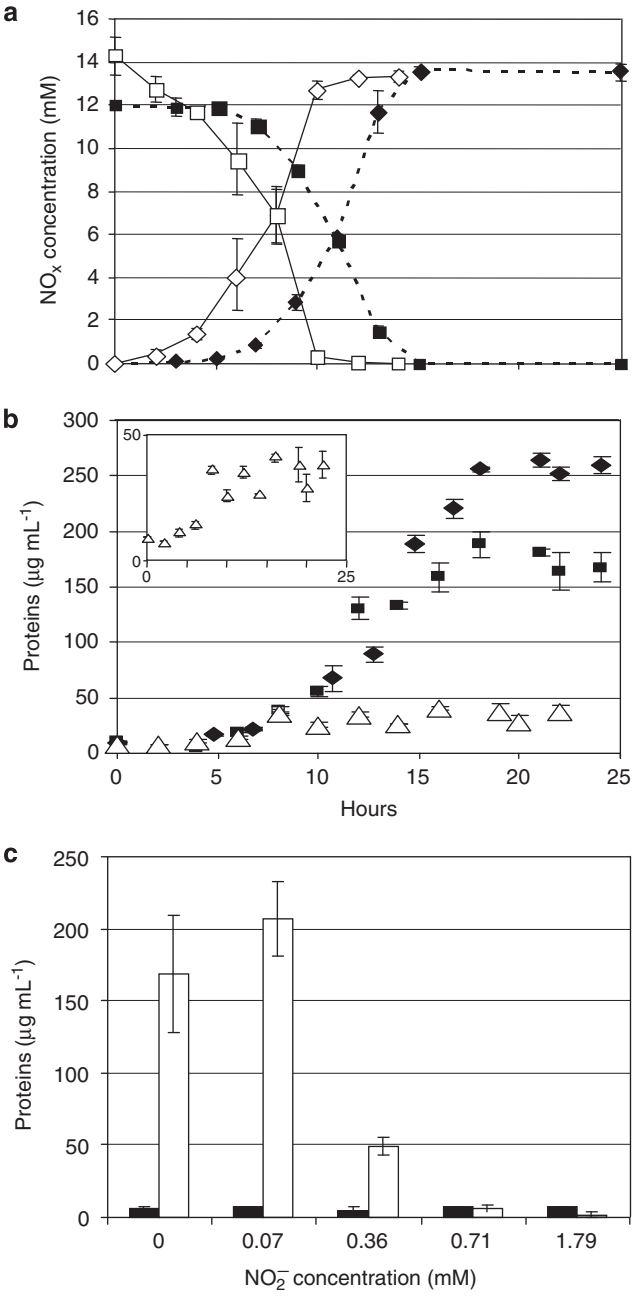

Figure 4 Growth of *Methylophaga* sp. strain JAM1 under aerobic and denitrifying conditions. (**a**) ASW medium supplemented with 200 mg NO_3-N/L was inoculated with strain JAM1 (10 µg protein per ml, final concentration). This was incubated at 30 °C for 3 days in the absence of oxygen (—) or in the presence of oxygen (- - -). At regular intervals, a 1-ml sample was taken and centrifuged. The supernatant was used for measuring the nitrate (square) and nitrite (diamond) concentrations by HPLC. (**b**) Strain JAM1 was cultured with methanol and nitrate in the absence of oxygen (triangle), with nitrate in the presence of oxygen (square) and with oxygen in the absence of nitrate (diamond). Growth was measured by protein concentration. The inserted graph is for better visualization of growth under denitrifying conditions. (**c**) Nitrite toxicity to *Methylophaga* sp. strain JAM1. Strain JAM1 was cultured aerobically in the absence of nitrate, but in presence of different concentrations of nitrite at the beginning of the culture. Growth was measured by protein concentration at time 0 (black bars) and after 24 h (white bars).

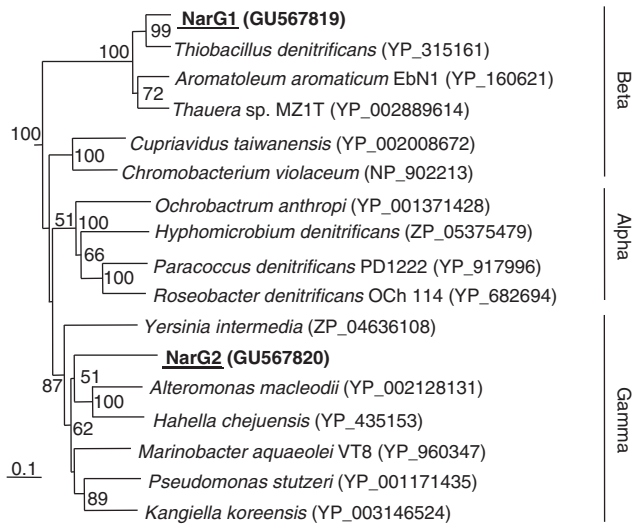

Figure 5 Phylogenetic tree of *narG* deduced amino-acid sequences of *Methylophaga* sp. strain JAM1. Unrooted phylogenetic trees showed the evolutionary relationship of NarG1 and NarG2, and representative NarG sequences affiliated with the Beta-, Alpha- and Gamma-Proteobacteria. Trees were inferred from a matrix of pairwise distance using aligned sequences containing 382 positions. The scale bar represents amino-acid substitutions per position. Bootstrap values above 50% are indicated at the branches. NarG sequences of *Bacillus licheniformis* (AAU40964) and *Mycobacterium tuberculosis* H37Rv (CAB09020) were used as outgroups. Outgroups were not included for clarity. GenBank accession numbers are indicated beside species name in parentheses.

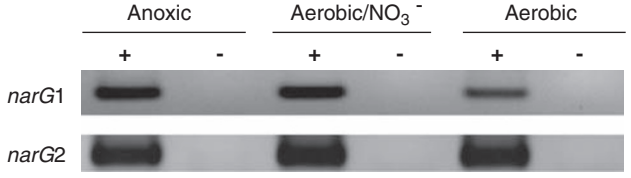

Figure 6 Expression of *narG*1 *and narG*2 in *Methylophaga* sp. strain JAM1. RT–PCR were performed on total RNA extracted from strain JAM1 cultured under anoxic (absence of oxygen and presence of nitrate) or aerobic (supplemented or not with nitrate) conditions. PCR products were run onto agarose gel electrophoresis +: RT–PCR with reverse transcriptase. −: RT–PCR without reverse transcriptase (negative control).

In *Thiosphaera pantotropha,* also known as *Paracoccus pantotrophus*, the simultaneous use of oxygen and nitrate increases the specific growth rate (Robertson and Kuenen, 1984; Kelly *et al.*, 2006). It has also been suggested that the presence of a constitutive enzyme allows for a better adaptation to oxygen concentration changes in the environment (Robertson and Kuenen, 1984). Recently, Gao *et al.* (2010) observed high rate of aerobic denitrification in marine coastal sediments, which may result from adaptation of denitrifying bacteria to tidally induced redox oscillations. Several lines of evidence have linked the aerobic nitrate-reducing activity to the periplasmic nitrate reductase (Nap) (Bell *et al.*, 1990; Carter *et al.*, 1995). The gene

encoding for NapA was not detected in strain JAM1 with our PCR primers. Another explanation for the nitrate reduction in the presence of oxygen is the formation of anoxic zone in the microbial clumps, allowing for nitrate reduction in the absence of oxygen inside the clumps.

The deduced amino-acid sequence of *narG*1 showed a high identity percentage (88%) with the corresponding part of NarG of *T. denitrificans*. This species belongs to the Beta subdivision of Proteobacteria, which suggests that strain JAM1 acquired *narG* by horizontal gene transfer. NarG2 clustered with the NarG sequences of the Gamma-Proteobacteria, suggesting that this gene is indigenous to the genus *Methylophaga*, or was acquired from another Gamma-Proteobacterium. Multiple copies of *narG,* with some of which clustered distantly from each other, have been observed in *Escherichia coli, Burkholderia pseudomallei* and *Ralstonia pickettii* 12J (Philippot, 2002; Palmer *et al*., 2009). Palmer *et al*. (2009) suggesting that two *narG* genes would originate from different lineage if their sequences were $<67\%$ similar in their nucleic acid sequence. As *narG*1 and *narG*2 are 61% similar in their nucleic acid sequences, it reinforces the hypothesis of horizontal gene transfer of *narG*1 to strain JAM1.

A substantial number of bacterial species, many in marine environments (Zobell, 1946), are able to reduce nitrate into nitrite. Nitrite can be converted into N_2 by denitrification and by anaerobic ammonium oxidation (anammox), or into ammonium by means of dissimilatory nitrate reduction to ammonium. Low nitrite concentration initially added to the medium affected the growth of strain JAM1. However, nitrite accumulation during the SIP cultures was not observed, which suggests that denitrifying microorganisms in the biofilm used nitrite produced by the strain JAM1 and thus prevented its toxic effect.

The 16S rRNA gene library from the ^{13}C-DNA showed the affiliation of one other 16S rRNA gene sequence (IAFJAsip4) with the genus *Methylophaga*. In the 16S rRNA gene library derived from the biofilm, Labbé *et al*. (2003) reported one other sequence, besides IAFDn39, affiliated with the genus *Methylophaga* (IAFDn2). These results suggest that several *Methylophaga* species lived in the biofilm. On the basis of their low abundance in the two screened 16S rRNA gene libraries, we hypothesize that these *Methylophaga* species were not capable of using nitrate for their growth as observed with strain JAM7. Residual dissolved oxygen (0.5 p.p.m.) was present in the denitrification unit of the Montreal Biodome, which may have initially sustained the growth of non-nitrate-reducing bacteria such as JAM7 (Labbé *et al*., 2007). Cross-feeding could explain their presence in the SIP assay where absence of residual oxygen occurred.

Neufeld *et al*. (2007) performed SIP experiments with ^{13}C-methanol to examine the diversity of methylotrophic bacteria in coastal seawater. Besides finding 16S rRNA gene sequences affiliated with the genus *Methylophaga*, a distinct clade closely related to *Methylophaga* spp. was found. The authors suggested that these sequences could represent a methanol-specific clade of marine methylotrophs. Phylogenetic analyses showed that IAFJAsip3 and IAFDn7 clustered with this clade (Figure 3). This suggests that the denitrifying biofilm could be the reservoir of uncharacterized marine methylotroph bacteria.

A number of studies have highlighted the implication of *Methylophaga* species in the biogeochemical cycling of methanol in marine environments using independent-cultured techniques (Neufeld *et al*., 2007, 2008a, b). Regarding the presence of *Methylophaga* spp. in such environments and the demonstration of the anaerobic nitrate-reducing activity of *Methylophaga* sp. JAM1, *Methylophaga* spp. could contribute, by the formation of nitrite, to the nitrogen dissimilation in the oxygen-limited zone of the ocean. For instance, they could provide the nitrite needed by the anammox bacteria (Strous *et al*., 1999; Lam *et al*., 2009) or be reduced into N_2 by denitrifying bacteria.

Approximately 10% of the 16S rRNA gene library of the ^{13}C fractions was related to species found in marine environments. With the exception for the clone IAFJAsip25 affiliated with *Sedimenticola selenatireducens,* which is known to reduce nitrate and nitrite under denitrifying conditions (Narasingarao and Haggblom, 2006), none of clones were affiliated with bacteria known to denitrify or to reduce nitrate into nitrite. However, one sequence was related to *Bacteriovorax* sp., which suggests that bacteria devourers were present in the biofilm that probably fed on the denitrifying methylotrophic bacteria. *Hyphomicrobium* spp. was shown to represent 8% of the bacterial population in the biofilm as determined by FISH (Labbé *et al*., 2007). The absence of detected isotope incorporation into the DNA of *Hyphomicrobium* spp. during the SIP experiments could be connected with their slow growth rate (division time of about 10 h) (Matzen and Hirsch, 1982).

This study presents the first direct evidence of the adaptation of a *Methylophaga* strain in a limited oxygen environment. The fitness of *Methylophaga* spp. for marine environments could have allowed efficient colonization on the fluidized supports by strain JAM1 in the reactor. Furthermore, the abundance of methanol in the denitrification system and the presence of denitrifying bacteria such as *Hyphomicrobium* spp. that can consume the toxic nitrite could have allowed the development of a high proportion of this *Methylophaga* strain in the biofilm. In return, it could have provided subproducts of their metabolism to the denitrifying bacteria.

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