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Functional Diversity in the Denitrifying Biofilm of the Methanol-Fed Marine Denitrification System at the Montreal Biodome

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Abstract Nitrate is a serious problem in closed-circuit public aquariums because its accumulation rapidly becomes toxic to many lifeforms. A moving bed biofilm denitrification reactor was installed at the Montreal Biodome to treat its 3,250-m^3 seawater system. Naturally occurring microorganisms from the seawater affluent colonized the reactor carriers to form a denitrifying biofilm. Here, we investigated the functional diversity of this biofilm by retrieving gene sequences related to *narG*, *napA*, *nirK*, *nirS*, *cnorB*, and *nosZ*. A total of 25 sequences related to these genes were retrieved from the biofilm. Among them, the corresponding *napA1*, *nirK1*, *cnorB9*, and *nosZ3* sequences were identical to the corresponding genes found in *Hyphomicrobium* sp. NL23 while the *narG1* and *narG2* sequences were identical to the two corresponding *narG* genes found in *Methylophaga* sp. JAM1. These two bacterial strains were previously isolated from the denitrifying biofilm. To assess the abundance of denitrifiers and nitrate respirers in the biofilm, the gene copy number of all the *narG*, *napA*, *nirS*, and *nirK* sequences found in biofilm was determined by quantitative PCR. *napA1*, *nirK1*, *narG1*, and *narG2*, which were all associated with either *Methylophaga* sp. JAM1 or *Hyphomicrobium* sp. NL23, were the most abundant genes. The other genes were 10 to 10,000 times less abundant. *nirK*, *cnorB*, and *nosZ* but not *napA* transcripts from *Hyphomicrobium* sp. NL23 were detected in the biofilm, and only the *narG1* transcripts from *Methylophaga* sp. JAM1 were detected in the biofilm. Among the 19 other genes, the transcripts of only two genes were detected in the biofilm. Our results show the predominance of *Methylophaga* sp. JAM1 and *Hyphomicrobium* sp. NL23 among the denitrifiers detected in the biofilm. The results suggest that *Hyphomicrobium* sp. NL23 could use the nitrite present in the biofilm generated by nitrate respirers such as *Methylophaga* sp. JAM1.

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Introduction

Nitrate is an anion that has proven to be toxic at high concentrations. In marine environments, the nitrate concentration should not exceed 20 mg NO_3^-–N/l to protect fish and invertebrates from its adverse effects [7]. In closed marine facilities such as public aquariums and aquaculture farms, the nitrate concentration often exceeds this limit [18, 36]. Maintaining a nitrate concentration below 20 mg NO_3^-–N/l currently involves frequent and expensive replacement of the seawater. Biological denitrification is a valuable alternative for removing nitrate from seawater [2, 18, 38, 44, 46]. Heterotrophic denitrification is a stepwise respiratory process in which microorganisms reduce nitrate (NO_3^-) or nitrite (NO_2^-) to nitric oxide (NO), nitrous oxide (N_2O), and dinitrogen (N_2) gases. The four reactions of the denitrification pathway are performed by nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase [51].

Few studies on the microbial diversity of denitrification systems treated wastewater highly charged in salt have been reported [9, 26, 48–50]. However, these studies were

mainly based on the analysis of the 16S ribosomal RNA (rRNA) gene. Heterotrophic denitrification is performed by a multitude of microorganisms belonging to diverse phylogenetic groups [42]. As a result, it is not ideal to use 16S rRNA gene sequences to identify denitrifying bacteria in the environment. During the last decade, detection of the functional genes encoding the different denitrification reductases, such as *narG*, *napA*, *nirK*, *nirS*, *cnorB, qnorB*, and *nosZ*, were shown to be a more effective way to study the diversity of denitrifying populations [4, 5, 14, 17, 20, 39].

In 1998, the Montreal Biodome established a methanol-fed denitrification reactor to control the concentration of nitrate in its 3-million-liter seawater system following an increase of the nitrate concentration to a critical level of 200 mg NO_3^-–N/l [36]. This completely mixed reactor contains a fluidized bed of plastic carriers that were colonized by naturally occurring microorganisms from the seawater affluent to form a denitrifying biofilm. In previous studies, we used culture-dependent and culture-independent methods to determine the composition of the microbial biota of this biofilm. Using the 16S rRNA gene library, the number of the most abundant bacterial species present in the biofilm was estimated to be between 15 and 20 [26], among which sequences related to *Hyphomicrobium* spp. and *Methylophaga* spp. were identified. Fluorescence *in situ* hybridization experiments confirmed that *Methylophaga* spp. and *Hyphomicrobium* spp. made up more than 50% of the biofilm microbiota [27]. Finally, the denitrifiers *Hyphomicrobium* sp. NL23 and the nitrate-reducing bacteria *Methylophaga* sp. JAM1 were isolated from the biofilm [1, 26].

Despite the abundance of *Methylophaga* spp. and *Hyphomicrobium* spp., we believed that other denitrifying bacteria and nitrate-reducing bacteria were present in the denitrifying biofilm and that these bacteria may play an important role in the denitrification process. In this paper, we further characterized the bacterial composition of the denitrifying biofilm by detecting functional genes encoding the different denitrification reductases and by determining whether they were expressed. Finally, the concentrations of the different *narG*, *napA*, *nirS*, and *nirK* sequences were determined by quantitative PCR (qPCR) to assess the level of denitrifiers and nitrate-reducing bacteria in the biofilm.

Materials and Methods

Denitrification System

The seawater system of the Montreal Biodome contains 3,250 m^3 of cold (10°C) artificial seawater (28–30‰). It comprises two life support systems and four pools, as described by Parent and Morin [36]. The denitrification system is operated in parallel to the main life support system. It consisted of a 1-m^3 fixed-bed deaeration tank, a 1-m^3 moving bed biofilm reactor (MBBR) for denitrification, and an overflow tank. The deaeration tank and the denitrification reactor were both methanol-fed. The denitrification reactor were filled with 0.3 m^3 of Bioflow® 9 mm carriers (1.020 density; Rauschert, Steinwiessen, Germany) for biofilm colonization. In the MBBR, carriers were continuously kept in motion by hydraulic flow, assuring that the carriers in the reactor were completely mixed. The water temperature in the reactor was 16–18°C, the pH was 7.8–7.9, and the salinity was 28–29‰. Twice a day, 370 mg of $FeSO_4{\cdot}7H_2O$, 130 mg of $MnSO_4{\cdot}4H_2O$, and 20 mg of $CuSO_4{\cdot}5H_2O$ were added to the system to enhance the denitrification rate [28]. At the sampling time, phosphate and nitrate concentrations in the seawater system averaged 18 PO_4–P mg/l (0.6 mM) and 50 NO_3–N mg/l (3.6 mM), respectively. The system's denitrification rates varied between 200 and 300 gday^{-1} NO_x–N. The dissolved oxygen concentration in the denitrification reactor was recorded below 0.5 mg/l during normal operation. All the carriers used in this study were taken at the same time and were frozen at −80°C without loss of activity [30].

DNA Extraction

DNA extraction from the biofilm was performed with three subsamples of the reactor using the biofilm carriers. For each subsample, the biofilm was scraped from five randomly selected carriers (to obtain a sufficient amount of DNA), pooled, and rinsed with TEN (50 mM Tris–HCl pH 8.0, 100 mM EDTA pH 8.0, and 150 mM NaCl). DNA was extracted by bead beating method, and the culture of *Hyphomicrobium* sp. NL23 and its DNA extraction were carried out as previously described [26].

Gene Libraries

Deduced amino acid sequences of *narG*, *napA*, *nirK*, and *cnorB* denitrification genes taken from type-strain bacteria were collected from a protein database [National Center for Biotechnology Information (NCBI) January 2006, http://www.ncbi.nlm.nih.gov/] and were aligned with ClustalW [45]. Highly degenerate primers were designed based on amino acid consensus sequences (Table 1) and were used for PCR amplifications to capture a high diversity of denitrification gene sequences. Optimization of PCR conditions (the annealing temperature, the concentration of primers, and the number of cycles) were performed with DNA extracted from *Pseudomonas aeruginosa* (kindly provided by Eric Déziel, INRS-Institut Armand-Frappier) for *narG*, *napA*, and *cnorB*, and *Hyphomicrobium* sp. NL23

Table 1 Degenerate oligonucleotides used as PCR primers

Name	Sequence (5′–3′)	Annealing temperature (°C)	Primer concentration (nM)	References
narG[a]				
narG-A241f	TTYTAYGAYTGGTAYGCNGA	46	500	[1]
narG-C241f	TTYTAYGAYTGGTAYTGYGA		500	
narG-631r	TTYTCYTGNCCNACRTARTG		1,000	
napA[a]				
napA-206f	GAYCCNAAYGCNMGNCAYTGYATGGC	51	1,000	[1]
napA-F457r	TGYTGRTTRAANCCCATNGTCCA		500	
napA-M457r	TGYTGRTTNATNCCCATNGTCCA		500	
nirS				
primer F1acd	TAYCACCCSGARCCGC	57	1,000	[20]
primer R4cd	CGTTGAACTTRCCGGTSGG			
nirK				
nirK-203f	TTYGTNTAYCAYTGYGCNCC	56	8,000	[1]
nirK-326r	TCNCCRTGNCCNCCDATNARRTGNGG			
cnorB[a]				
cnorB-V212f	TGGGTNGARGGNGTNTGGGA	51	500	[1]
cnorB-T212f	TGGGTNGARGGNACNTGGGA		500	
cnorB-355r	TANGCNCCRWARAANGC		1,000	
qnorB				
qnorB2F	GGNCAYCARGGNTAYGA	Touch down PCR	1,000	[5]
qnorB7R	GGNGGRTTDATCADGAANCC			
nosZ				
nos661f	CGGCTGGGGGCTGACCAA	56	400	[39]
nos1773r	ATRTCGATCARCTGBTCGTT			

[a] 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

for *nirK*. Further optimization was performed with the biofilm DNA. Previously published primers were used for the detection of *nirS*, *qnorB*, and *nosZ* (Table 1) [5, 20, 39].

A total of 100 ng of DNA from each biofilm subsample or 25 ng of strain NL23 DNA was used as a template for the PCR detection of *narG*, *napA*, *nirS*, *nirK*, *cnorB*, *qnorB*, and *nosZ* using degenerate primers (Table 1). For *narG*, *napA*, *nirK*, and *cnorB*, PCR amplifications were performed in 50 µl reaction volume with 10 mM Tris–HCl pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl, 20 µg of bovine serum albumin (BSA), 200 µM dNTP, and 2.5 U of rTaq DNA polymerase (GE Healthcare, Buckinghamshire, UK). Amplifications were conducted as follows: 94°C for 5 min, annealing temperature for 5 min (Table 1), 30 cycles at 72°C for 1 min, 94°C for 1 min, annealing temperature for 1 min, and finally an extension period of 10 min at 72°C. Amplifications of *nirS*, *qnorB*, and *nosZ* were performed as described previously [5, 20, 39]. For each gene, the three PCR products from the biofilm subsamples were pooled. PCR products of the expected size were purified by agarose gel extraction with the QIAquick Gel Extraction Kit (Qiagen Inc., Mississauga, ON, Canada) before ligation.

PCR products were ligated in a T-vector (pGEM®-T Easy Vector System, Promega, Madison, WI, USA). DH5α™ Chemically Competent *Escherichia coli* (high efficiency 10^9 cfu/µg; Invitrogen Canada Inc., Burlington, ON, Canada) were transformed with DNA in accordance with manufacturer's instructions. Plasmids were isolated by the method described by Shergini et al. [41]. *In silico* digestions of published sequences were performed to determine which restriction enzymes were optimal to screen the clones using the online program RestrictionMapper (http://www.restrictionmapper.org/). Plasmids were screened for different inserts by restriction fragment length polymorphism (RFLP) using the restriction endonucleases AfaI for *narG*, *napA*, *nirK*, and *cnorB*, HhaI for *nirS*, and Sau3AI for *nosZ*. Inserts of representative clones based on restriction profiles were PCR amplified and sequenced in both directions using the primers SP6 and T7. For RFLP profiles represented by numerous clones, at least two clones were sequenced.

The deduced amino acid sequences of *narG*, *napA*, *nirS*, *nirK*, *cnorB*, and *nosZ* were compared against the protein databases with BLASTP (NCBI). The most related sequen-

ces of either type-strain organisms or uncultured organisms found in marine environments were selected. The deduced amino acid sequences were aligned with ClustalW [45]. The alignments were manually refined in BIOEDIT [19]. Phylogenic analysis was performed using PROTDIST (Jones–Taylor–Thornton distance method), FITCH (Fitch Margoliash method), and SEQBOOT/CONSENSE (bootstrap analysis with 1,000 replicates) programs in PHYLIP 3.65 software package [12].

Quantification of *narG*, *napA*, *nirS*, *nirK*, and *nosZ* in the Biofilm

Primers specific to the each of the *narG*, *napA*, *nirS*, and *nirK* sequences retrieved from the biofilm and to the *Hyphomicrobium* sp. NL23 *nosZ* sequence were designed for the qPCR assays (Table S1). Primer specificity was verified by PCR with plasmids carrying the respective gene sequences. No cross-reactivity was observed.

qPCR was performed with SYBR green in 20 µl reaction volume with 10 µl of PerfeCTa™ qPCR FastMix™ (Quanta BioSciences, Gaithersburg, MD, USA), 2 µl of DNA (biofilm or standard), and the specific primers (Table S1). The amplifications were performed at 95°C for 5 min, followed by 40 cycles at 95°C for 30 s, at the annealing temperature (Table S1) for 30 s, and at 72°C for 30 s. After PCR, specificity of the PCR products and the presence of primer dimers were verified by performing a melt curve by increasing the temperature from 65°C to 95°C by increments of 1°C per step with a pause of 5 s for each step. Specific amplifications with biofilm DNA were observed for each of the primers pairs, and no primer dimers were detected. Quantification was performed on each biofilm DNA subsample. Reactions were performed in a Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science, Sydney, Australia).

The PCR efficiency (E) for each primer pair was calculated from slope of their respective standard curve according to the following formula: $E = \left(10^{-1/\text{slope}}\right) - 1$. PCR-amplified fragments from plasmid DNA were used as standards. Plasmids carrying *narG*, *napA*, *nirS*, or *nirK* sequences retrieved from gene libraries or the *Hyphomicrobium* sp. NL23 *nosZ* sequence were PCR amplified with the SP6 and T7 primers as described above. PCR products were run on 1% agarose gel, and bands of expected size were extracted and purified with the QIAquick Gel Extraction Kit (Qiagen Inc.). DNA was quantified with Quant-iT™ PicoGreen® dsDNA Reagent and Kits (Invitrogen, Molecular Probe, Eugene, OR, USA) to determine the DNA copy number. Standard curves were obtained from a 10-fold dilution series of standard DNA (10^8–10^1 copies of the targeted gene).

The impact of the presence of co-extracted contaminants in biofilm DNA on qPCR efficiency was assessed. To avoid interference from the indigenous denitrification gene sequences, a DNA fragment not related to the denitrification genes sequences was spiked with biofilm DNA. qPCR assays were performed with a 10-fold dilution series of this DNA with 10 ng of biofilm DNA in each dilution. qPCR efficiencies ranged from 0.94 to 0.95 when 10 ng of biofilm DNA was present, with a linear range from 10^8 to 10^1 copies, and was 0.98 with no biofilm DNA added.

Reverse Transcriptase (RT)–PCR Assay

Hyphomicrobium sp. NL23 was cultured in *Hyphomicrobium* 337a medium [per liter—1.3 g of KH_2PO_4, 1.13 g of Na_2HPO_4, 0.50 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4{\cdot}7H_2O$, 3.09 mg of $CaCl_2{\cdot}2H_2O$, 2.0 mg of $FeSO_4{\cdot}7H_2O$, 1.0 mg of $Na_2MoO_4{\cdot}2H_2O$, and 0.88 mg of $MnSO_4{\cdot}4H_2O$] supplemented with $NaNO_3$ (400 mg of NO_3^-/l) and 0.2% methanol under denitrifying conditions for 64 h as previously described [1]. The cultures were then centrifuged at 5,000×g for 5 min and resuspended in TEN. One volume of acid phenol (pH 4.3) was added, and RNA was extracted as described below.

Biofilm was carefully scraped from carriers in an acid phenol (pH 4.3) bath. The phenol/biofilm mix was transferred to a screw-cap microcentrifuge tube containing 250 mg of autoclaved 0.1-mm glass beads (BioSpec Products, Inc., Bartlesville, OK, USA), and 1 vol of TEN was added. The biomass was disrupted with a Fast-Prep®-24 homogenizer (MP Biomedicals, Solon, OH, USA) twice for 20 s at 4.0 m/s. The lysate was centrifuged at 14,000×g for 15 min, and the supernatant was extracted three times with acid phenol/chloroform/isoamyl alcohol (25:24:1) and once with a chloroform/isoamyl alcohol (24:1). RNA was precipitated with 0.25 vol of 10 M ammonium acetate and 2 vol of 95% ethanol and then dissolved in diethyl pyrocarbonate-treated water. Total RNA (30 µg) was treated twice with 6 U of Turbo DNase (Ambion Inc., Austin, TX, USA) in 1× Turbo DNase buffer at 37°C for 3 h in a 300 µl volume. Turbo DNase was inactivated, after each treatment, by acid phenol/chloroform/isoamyl alcohol extraction (25:24:1) followed by a chloroform/isoamyl alcohol (24:1) extraction and RNA precipitation. The presence of DNA in the RNA samples was evaluated by performing RT–PCR without reverse transcriptase. No DNA contamination was observed.

Primers specific to the each of the *narG*, *napA*, *nirS*, *nirK*, *cnorB*, and *nosZ* sequences retrieved from the biofilm were designed for RT–PCR assays (Table S2). Primer specificity was verified by PCR with plasmids carrying the respective gene sequences. No cross-reactivity was ob-

served. All the primers showed PCR amplification of the expected size with biofilm DNA.

Reverse transcription (RT) of RNA (1 μg) was performed in a 50 μl reaction volume containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 200 μM dNTP, 200 U reverse transcriptase (SuperScript™ II RT, Invitrogen, Carlsbad, CA, USA), and 100 pmol of the reverse primer (Table S2). RT was performed at 65°C for 5 min and then at room temperature for 10 min, at 42°C for 60 min, and finally at 94°C for 5 min. Five microliters of the RT reaction was used for the PCR amplification. PCR was performed in 50 μl reaction volume as described before with the rTaq DNA polymerase. Primers are described in Table S2. The amplifications were performed at 94°C for 5 min, at the annealing temperature for 5 min (Table S2), followed by 30 cycles at 72°C for 45 s, at 94°C for 45 s, at the annealing temperature for 45 s, and finally at 72°C for an extension period of 10 min. RT–PCR products were verified by sequencing.

Results

Diversity of Denitrification Genes in the Denitrifying Biofilm

Degenerate primers targeting consensual sequences of *narG*, *napA*, *nirS*, *nirK*, *cnorB*, *qnorB*, and *nosZ* (Table 1) were used to PCR-amplify these sequences from the denitrifying biofilm and generate gene libraries. Although PCR products with the expected length were obtained for each gene, most of the PCR amplifications also contained nonspecific amplifications, which occurs often with the use of degenerate primers [4, 5, 20]. Cloning efficiency was variable from one gene to another, and a high proportion of clones with unspecific sequences was obtained for some genes (e.g., *nirS* and *cnorB*; Table S3). These problems could be related to the degenerate primers and unspecific amplifications. For instance, despite several cloning attempts, only 36 clones were obtained for *napA* or *nirK*, respectively. Among these clones, only one *napA*-related sequence (*napA1*, 14 clones; Table S3) and one *nirK*-related sequence (*nirK1*, 10 clones; Table S3) were obtained, which suggests very low diversity in the biofilm for these genes. Overall, 25 different sequences with more than 75% similarity to the different denitrification reductases were retrieved (Table S3). Seven different sequences related to *narG*, one related to *napA*, six related to *nirS*, one related to *nirK*, three related to *cnorB*, and seven related to *nosZ* were found (Fig. 1).

The *narG1* and *narG2* sequences were identical to the corresponding *narG* sequences found in *Methylophaga* sp. JAM1 (also named *narG1* and *narG2*; [1]). Deduced amino acid sequences of the five other *narG* sequences (Fig. 1a) were related to NarG sequences found in bacterial species affiliated with different sub-divisions of Proteobacteria. The *napA1* sequence was related to NapA sequences found in bacterial species affiliated with Alpha-Proteobacteria (Fig. 1b).

The deduced amino acid sequences of *nirS2* and *nirS34* were related to NirS sequences found in the Gamma-Proteobacterium *Kangiella koreensis* species. This bacterial species is a moderate halophilic bacterium known to perform denitrification [47]. NirS13 and NirS36 were related to NirS found in the denitrifying bacterium *Roseobacter denitrificans* and with NirS sequences retrieved from sediments in a mariculture environment (GenBank annotation). Finally, NirS5 and NirS32 were related to NirS sequences found in a marine environment (GenBank annotation). NirK1 was related to NirK sequences found in bacterial species affiliated with Alpha-Proteobacteria, including *Hyphomicrobium zavarinii*.

The three cNorB sequences identified in the biofilm were related to cNorB sequences found in bacterial species affiliated with Alpha-Proteobacteria. cNorB21 was nearly identical (99%) to the corresponding sequence in *Pseudovibrio* sp. JE062, a bacterium retrieved from a marine sponge [11]. No PCR amplification products were obtained from the biofilm DNA with primers used to amplify *qnorB*. NosZ3 was related to the *Hyphomicrobium denitrificans* NosZ. The six other sequences (NosZ1, NosZ5, NosZ8, NosZ21, NosZ25, and NosZ39) were related to the CLEM group of NosZ sequences retrieved from Pacific Ocean sediment (GenBank annotations and [40]).

As deduced amino acid sequences of *nirK1* and *nosZ3* were closely related to the corresponding sequences found in *Hyphomicrobium* species, we performed PCR assays with the degenerate primers on *Hyphomicrobium* sp. NL23. Sequences similar to *napA*, *nirK*, *cnorB*, and *nosZ* were retrieved. The *napA*, *cnorB*, and *nosZ* sequences were identical to the ones found in the biofilm (*napA1*, *cnorB9*, and *nosZ3*). There was only one nucleotide difference between *nirK* from strain NL23 and the *nirK1* retrieved from the biofilm. This difference could be an artifact introduced by PCR.

Quantification of the Nitrate- and Nitrite-Reductase Genes in the Biofilm

To assess the level of the denitrifying bacteria in the biofilm, the gene copy number of all the *narG*, *napA*, *nirS*, and *nirK* retrieved from the biofilm was determined by qPCR. We established that the *Methylophaga* sp. JAM1 *narG1* and *narG2* were dominant among all the *narG* genes

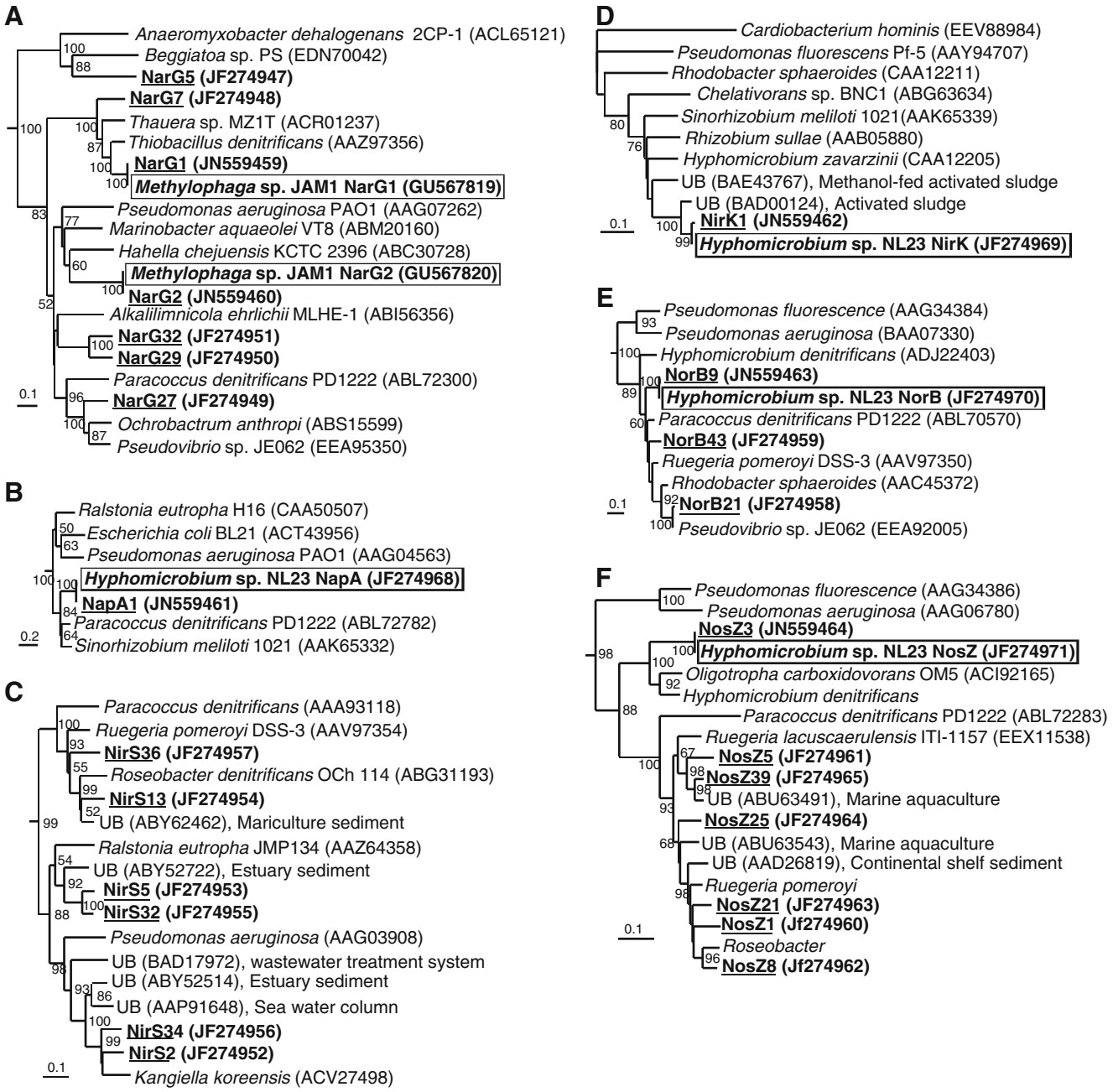

Figure 1 Phylogenetic analysis of the deduced reductase amino acid sequences retrieved from the denitrifying biofilm and the isolated strains. An unrooted phylogenetic tree demonstrated the evolutionary relationship of NarG (**a**), NapA (**b**), NirS (**c**), NirK (**d**), cNorB (**e**), and NosZ (**f**) sequences obtained in this study with representative reductase sequences affiliated with Proteobacteria. The *scale bar* represents amino acid substitutions per position. Bootstrap values (1,000 replicates) above 50% are indicated at the branches. GenBank accession numbers are indicated in *parentheses* beside the species name. Trees were inferred from a matrix of pairwise distances using aligned sequences containing 343, 189, 261, 109, 116, and 362 positions for NarG, NapA, NirS, NirK, cNorB, and NosZ, respectively. *Underlined* sequences are from the biofilm, and *boxed* sequences from the isolated strains. UB: uncultured bacteria

found in the biofilm, with 1.0×10^4 and 1.6×10^4 copies/ng biofilm DNA, respectively (Table 2). The other *narG* genes were approximately four to 500 times less abundant. The level of the *Hyphomicrobium* sp. NL23 *napA* was similarly abundant of *narG1* and *narG2*, with 4.5×10^4 copies/ng biofilm DNA. The abundance of the different *nirS* ranged from 8.9×10^0 to 4.4×10^2 copies/ng biofilm DNA, which were 100 to 10,000 less abundant than the *Hyphomicrobium* sp. NL23 *nirK*, with 8.4×10^4 copies/ng biofilm DNA. The *Hyphomicrobium* sp. NL23 *nosZ* was assayed to confirm the results obtained with *napA* and *nirK*, with 5.4×10^4 copies/ng biofilm DNA.

Table 2 Quantification of the denitrification gene sequences in the biofilm by qPCR

Gene	Gene copies[a]	SD[b]
Methylophaga sp. JAM1		
narG1	1.0×10^4	0.14×10^4
narG2	1.6×10^4	0.25×10^4
Hyphomicrobium sp. NL23		
napA	4.5×10^4	0.24×10^4
nirK	8.4×10^4	1.6×10^4
nosZ	5.4×10^4	0.65×10^4
Uncultured bacteria		
Nitrate reductase		
narG5	4.5×10^3	1.2×10^3
narG7	1.0×10^3	0.12×10^3
narG27	4.7×10^1	0.58×10^1
narG29	3.0×10^1	1.6×10^1
narG32	1.2×10^3	0.20×10^3
Nitrite reductase		
nirS2	4.4×10^2	0.85×10^2
nirS5	3.6×10^2	0.48×10^2
nirS13	1.6×10^2	0.21×10^2
nirS32	4.4×10^2	0.59×10^2
nirS34	3.6×10^2	1.0×10^2
nirS36	8.9×10^0	4.2×10^0

All pairs of specific primers showed a linear response of the Ct versus the copy numbers of added standard DNA (R^2 >0.99) and exhibited PCR efficiency ranging between 0.89 and 1.06

[a] Average quantification of the three biofilm DNA subsamples. Unit: gene copies per nanogram of biofilm DNA

[b] Standard deviation

Transcription of Denitrification Genes in the Bacterial Strains and in the Denitrifying Biofilm

The four denitrification genes carried by *Hyphomicrobium* sp. NL23 were transcribed in pure cultures (Table 3). However, in the biofilm, *napA* transcripts were not detected, but the other three were detected. The two *Methylophaga* sp. JAM1 *narG* were transcribed in pure cultures [1], but only *narG1* transcripts were detected in the biofilm (Table 3). Among the 19 denitrification-related gene sequences associated with the uncultured bacteria in the biofilm, only the *narG7* and *cnorB21* transcripts were detected in the biofilm (Table 3).

Discussion

Twenty-five putative denitrification gene sequences originating from the methanol-fed denitrifying biofilm were retrieved. This number of gene sequences may seem relatively low for an environmental microbiota, but it is probable that other denitrifying genes were present but not detected. At the time that the degenerate PCR primers were designed, the sequences available in the gene databases were not necessarily representative of all respective denitrifying genes, such as those found in Archaea. In addition, the degeneration of the primers could have affected the sensitivity of detecting such genes. On the other hand, the low number of denitrification gene sequences could reflect the level of diversity of the biofilm, which was estimated to have between 15 and 20 bacterial species. The biofilm has developed under controlled conditions (affluent from a closed-circuit aquarium made with artificial seawater, 18°C, methanol as carbon source, and denitrifying conditions), which may have limited the number of denitrifying bacterial species that could have been established. All these gene sequences are more closely related with corresponding genes found in bacteria affiliated with Proteobacteria. Moreover, most, if not all, of these sequences were related to corresponding reductases retrieved from marine environments.

Among the 25 gene sequences, six are identical to the corresponding genes in *Hyphomicrobium* sp. NL23 and *Methylophaga* sp. JAM1. Our study shows that *Hyphomicrobium* sp. NL23 does possess a complete set of denitrifying genes (*napA*, *nirK*, *cnorB*, and *nosZ*). Very few studies have reported a complete survey of denitrifying

Table 3 Detection of denitrification gene transcripts in the bacterial strains and in the biofilm by RT–PCR

	Hyphomicrobium sp. NL23		*Methylophaga* sp. JAM1		Uncultured[b]
	Pure culture	Biofilm	Pure culture[a]	Biofilm	Biofilm
narG	NA	NA	*narG1*	*narG1*	*narG7*
			narG2	–	
napA	+	−	NA	NA	NA
nirK	+	+	NA	NA	NA
nirS	NA	NA	NA	NA	None
cnorB	+	+	NA	NA	*cnorB21*
nosZ	+	+	NA	NA	None

NA not applicable

[a] From Auclair et al. [1]

[b] Only the *narG*- and *cnorB*-related sequences with positive RT–PCR signals were entered in this column. No RT–PCR signal was obtained with the others

genes in *Hyphomicrobium* spp. Kloos et al. [25] and Fesefeldt et al. [13] reported the presence of *narG*, *nirK*, and *nosZ*, but not that of *nirS*, in several *Hyphomicrobium* species and strains. However, *narG* was not detected in several of these strains, and, as *napA* had not been discovered at the time these studies were conducted, it is possible that such strains contained *napA*. Recently, two complete genomes from *H. denitrificans* and *Hyphomicrobium* sp. MC1 were available in gene databases. Gene sequences related to the nitrate reductase/molybdopterin oxidoreductase *narG* and *nasA* (involved in nitrate assimilation) are present in each genome, but not *napA*. Therefore, our study shows for the first time that *napA* is associated with a *Hyphomicrobium* strain.

Seven sequences related to *narG* were retrieved from the biofilm. Some of the *narG* could come from the same microorganisms since multiple copies of distantly related *narG* have been reported for some bacterial species [35]. Indeed, two *narG* sequences retrieved from the biofilm are identical to the two *narG* found in *Methylophaga* sp. JAM1. Furthermore, some evidence supports the coexistence of both *narG* and *napA* in the same microorganism [37]. DNA from *Hyphomicrobium* sp. NL23, which contains the only *napA* found in the biofilm, did not amplify *narG* sequences with the *narG* primers used in this study.

Sequences related to nitrite reductases, key enzymes of the denitrification pathway, were detected in the biofilm DNA, of which six sequences were related to *nirS* and one to *nirK*. By compiling data of diversity studies in which genes encoding the nitrite reductases were used as functional markers of denitrification, Jones and Hallin [23] found that most *nirK* sequences were derived from soil but that most *nirS* sequences were prominently derived from marine and estuarine environments. Moreover, Hallin et al. [21] observed that the addition of methanol to a pilot-scale plant treating wastewater resulted in an increase of the *nirS*-type denitrifiers diversity. Yoshie et al. [50] observed that the diversity of *nirS* was higher than the diversity of *nirK* in two acetate-fed anaerobic reactors used to remove nitrate from metallurgic saline wastewater. Our results are consistent with these observations, as more *nirS* than *nirK* was found in the marine methanol-fed denitrifying biofilm. *nirS13* and *nirS36* were related to *nirS* belonging to the *Roseobacter* clade, which is prominent in coastal waters and in surface waters of the open ocean [32] and was associated with an industrial saline wastewater denitrification system [9]. With our degenerate primers, only the *Hyphomicrobium* sp. NL23 *nirK* was found in the biofilm. *Hyphomicrobium* spp. has been associated with marine methanol-fed denitrification systems [16, 33, 43].

Most quantification studies performed to date have used universal primers to quantify the entire denitrifying population and then compare the sampling sites [6, 22, 24, 31, 34]. Here, we performed a quantification of specific genes in an attempt to establish the contribution of each gene to the overall functional population of the biofilm. Assays have targeted *narG* and *napA*, which can represent either nitrate-reducing or denitrifying bacteria, *nirS* and *nirK*, which were used as markers of denitrifying populations. The most abundant genes in the biofilm were associated with the genes found in *Methylophaga* sp. JAM1 (*narG1* and *narG2*) and in *Hyphomicrobium* sp. NL23 (*napA*, *nirK*, and *nosZ*). The other genes from the uncultured bacteria were 10 to 10,000 less abundant than both strains. These results confirmed our previous studies [1, 26, 27] that *Methylophaga* spp. and *Hyphomicrobium* spp. are predominant in the biofilm. The low abundance of the other genes suggests minor involvement of those in the denitrification process in the biofilm at the time of the sampling.

napA, *nirK*, *cnorB*, and *nosZ* of *Hyphomicrobium* sp. NL23 were transcribed in pure cultures under denitrifying conditions, which suggests its capacity to reduce nitrate into N_2. However, only the *Hyphomicrobium* sp. NL23 *nirK*, *cnorB*, and *nosZ* transcripts were detected in the biofilm. Contrary to pure cultures, the *napA* transcripts were not observed in the biofilm, suggesting that strain NL23 could use the nitrite produced by the nitrate reducers present in the biofilm to support its metabolism.

Methylophaga sp. JAM1 has been shown to reduce nitrate into nitrite in denitrifying conditions, and this activity was related to the presence of both *narG* genes [1]. We previously showed that the two genes were transcribed in pure cultures regardless of the incubation conditions (denitrifying or aerobic conditions), suggesting a constitutive expression of these genes [1]. Furthermore, the *narG1* gene product is 88% identical to NarG of the obligate chemolithoautotrophic, sulfur-compound-oxidizing Beta-Proteobacterium *Thiobacillus denitrificans*, one of the rare bacteria species known to couple denitrification and sulfur-compound oxidation. It has been proposed that *Methylophaga* sp. JAM1 acquired *narG1* by horizontal gene transfer [1]. The "recent" acquisition of *narG1* could have allowed *Methylophaga* sp. JAM1 to adapt to the Biodome denitrification system. Curiously, only *narG1* transcripts were detected in the denitrifying biofilm, suggesting that it is differently regulated from *narG2*. Similarly, *Escherichia coli* has two homologous membrane-bound nitrate reductases, NRA and NRZ, which are encoded by *narGHJI* and *narZYWV*, respectively [3]. Both enzymes are expressed at basal levels, with higher expression observed at stationary growth phase, but they are regulated differently. NRA expression is stimulated by anaerobiosis and nitrate under the control of oxygen-responsive regulator FNR and the nitrate/nitrite-responsive regulator NarXL, respectively. NRZ expression is rather dependent of the stationary phase regulatory factor RpoS

and is not influenced by anaerobiosis and nitrate [8]. RpoS is a stress response regulator associated with the stationary phase and is responsible for the expression of numerous genes conferring more resistance to bacteria under environmental stress conditions [29].

Among the 19 putative denitrification genes associated with the uncultured bacteria, only the *narG7* and *cnorB21* transcripts were detected by RT–PCR. The 17 other genes were not transcribed, or the levels of their transcripts were below the detection limit. RT–PCR primers used for these genes were able to detect the corresponding sequences in the DNA biofilm, which thus excludes a problem related to the primers' sensitivity.

Conclusions

Our results confirmed that *Methylophaga* sp. JAM1 and *Hyphomicrobium* sp. NL23 are predominant in the biofilm, with strain JAM1 expressing *narG1* and strain NL23 expressing *nirK*, *cnorB*, and *nosZ*. Bacteria only able to reduce nitrate to nitrite are widespread in many environments and are abundant in wastewater treatment plants [10, 15]. The low level of *napA* transcripts of *Hyphomicrobium* sp. NL23 may suggest that this strain directly used the nitrite present in the biofilm, generated by nitrate respirers such as *Methylophaga* sp. JAM1. Other sequences related to denitrification genes from uncultured bacteria were found in the biofilm. Although present in low amounts, bacteria carrying these genes could play an important role in the denitrification process of the biosystem, thus allowing the biofilm to adapt quickly upon changing conditions.

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