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Aerobic Denitrifiers Isolated from Diverse Natural and Managed Ecosystems

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ABSTRACT

Twenty-eight bacterial strains were isolated from an ecosystem adapted to fluctuating oxic–anoxic conditions. This ecosystem comprised a mixture of different natural and wastewater treatment environments. Among the 28 strains isolated, 10 exhibited aerobic denitrifying activity, i.e., co-respiration of oxygen and nitrate and simultaneous production of nitrite by 4 of them and of nitrogen gas by the remaining 6. Comparisons between the 16S rDNA sequences of the 10 strains showed that 3 of them were identical to *M. aerodenitrificans,* whereas RAPD profiles showed that the 3 strains were identical to each other but that they were different from *M. aerodenitrificans.* This implies that alternating aerobic–anoxic conditions allowed the isolation of a new strain of this aerobic denitrifier. Moreover, other denitrifying bacteria belonging to the genera *Paracoccus, Thiobacillus, Enterobacter, Comamonas,* and *Sphingomonas* were isolated in this way. These data imply that a wide variety of bacteria are able to carry out this type of metabolism. *M. aerodenitrificans* was also detected in methanogenic, denitrifying, nitrifying, phosphate removal, and activated sludge ecosystems by two-step PCR amplification. After 4 months of acclimation to oxic–anoxic phases, the strain was also detected in a canal and in a pond. This suggests that there is no specific natural ecological niche for aerobic denitrifiers but, as soon as selective pressure such as alternating aeration conditions is applied, this metabolism is amplified.

Introduction

The biogeochemical cycling of nitrogen can be conveniently divided into different well-characterized biological reactions: nitrogen fixation, ammonium assimilation, ammonification, ammonium and nitrite oxidation (nitrification), dissimilatory reduction of nitrate to ammonium, and dissimilatory reduction of nitrate to nitrogen gas (denitrification). All of these processes are known to occur under well-defined conditions: for example, nitrification is performed by autotrophic aerobic microorganisms, whereas denitrification is carried out by heterotrophic bacteria under anoxic conditions. However, nitrogen levels either in the environment or in wastewater treatment processes can be affected by novel reactions which are unusual and have only recently been taken

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into consideration by the scientific community. For example, the newly discovered ANAMMOX process [27, 29] involves the biological conversion of ammonium to dinitrogen under anaerobic conditions in the presence of nitrite. In the same way, autotrophic nitrifiers are able to reduce nitrate or nitrite to nitric or nitrous oxide and nitrogen gas under oxygen-limited or anaerobic conditions [1, 3, 23, 24]. Finally, denitrification may proceed at substantial rates under aerobic conditions mediated by different genera of bacteria [5, 22, 25], suggesting that aerobic denitrification does occur and might have evolved several times. These different reactions may explain the over- or underestimations of the nitrogen concentrations that occur, for example, in wastewater treatment processes.

Aerobic denitrification has been well studied mainly through pure strains such as *Thiosphaera pantotropha* [26] and *Microvirgula aerodenitrificans* [19]. Aerobic denitrifying ability has been linked to the expression of a periplasmic nitrate reductase [2, 20] instead of the membrane-bound reductase usually expressed in anaerobic denitrifiers. Thus, the oxygen inhibitory effect on the nitrate–nitrite antiport system was prevented [11]. Oxygen was shown to regulate nitrate respiration by inhibiting enzyme synthesis: an FNR-like protein, required for anaerobic gene expression in *Escherichia coli,* is present in cells of *Paracoccus denitrificans, Pseudomonas aeruginosa,* and *Pseudomonas stutzeri* and was assumed to be activated after a sequence of events modifying the intracellular redox state [11, 15, 30]. The aerobic denitrifying enzymes of *T. pantotropha* [26] and *M. aerodenitrificans* [20] were shown to be expressed constitutively. Recent studies have focused on adaptation mechanisms of transcriptional activators (FnrP and NNR) in response to oxygen limitation or during anoxic–oxic transitions with common denitrifiers such as *P. denitrificans* [30] and *P. stutzeri* [10], and on characterizing electron transfer into the *cdl*-type nitrite reductase of *T. pantotropha* [14]. Although aerobic denitrification is now well known, many points remain unclear. How do the two systems (oxygen and nitrate respiration) work concomitantly (gene and enzyme regulation) as demonstrated through kinetic experiments [21]? What are the implications for wastewater treatment processes or soils? Is aerobic denitrification only observed with pure strains in laboratory experiments, or does this reaction occur naturally? Indeed, aerobic denitrifying activities were based on rates reported for *T. pantotropha* that have considerably decreased with time and have largely contributed to misconceptions about the phenomenon. With one exception [7], nothing is known about complex ecosystems. Furthermore, aerobic denitrification has always been linked to managed ecosystems. Indeed, bacteria isolated from natural soils and sediments, which reduce nitrate to nitrite aerobically [7], are thus not true aerobic denitrifiers. This study describes the isolation and partial characterization of denitrifying bacteria isolated from both natural ecosystems and wastewater treatment plants by applying alternating oxic–anoxic conditions in the presence of nitrate. Finally, the potential existence of aerobic denitrifying ecological niches is discussed based on studies on the origin of *M. aerodenitrificans.*

Materials and Methods

Sample Sites

Five samples were obtained from ecosystems where aeration conditions are assumed to be variable. Solid samples were collected from two contrasting soils (Narbonne, France): a mud–clayey soil (S1) collected after cereal cultivation, and a noncultivable clayey soil (S2). Triplicate soil samples were collected at 30 cm depth of each soil, mixed, and stored in sealed bottles at 4°C. An amount of 87 g of each soil was resuspended in 700 mL of nitrate broth Merck medium (see below) to obtain homogeneous soil suspension. Liquid samples were collected from the canal "la Robine" (R1), the pond "la Nautique" (N1), and the Mediterranean (M1) coast (Narbonne, France). Other samples were collected from different wastewater treatment processes dealing with vinasse (V1), yeast effluent (G1), pig manure (L1), and pharmaceutical effluent (T1). One additional sample came from a phosphate removal wastewater treatment plant in Roanne (France) and consisted of a mixture of aerobic, anoxic, and anaerobic sludge (P1). A further sample was collected from the aerobic basin of the municipal wastewater treatment plant of Narbonne, France (B1).

Enrichment and Isolation

One hundred mL of each of these 11 samples were used to inoculate 300 mL of nitrate broth Merck medium (1.5 g L^{-1} KNO_3, 8.6 g L^{-1} peptone, 6.4 g L^{-1} NaCl) to enrich denitrifying microflora. This rich medium was supplemented with carbon sources to prevent carbon source adaptation: 0.45 g L^{-1} sodium acetate, 0.25 g L^{-1} ethanol, and 0.35 g L^{-1} sodium propionate. The 500 mL sterilized bottles were simply sealed with a rubber stopper without bubbling with argon, implying the presence of air at the beginning of the culture. They were shaken in a rotary shaker (170 rpm) at 35°C.

After complete consumption of nitrate, 1 mL of R1, N1, B1, S1, and S2 culture was used to inoculate a 100 mL Penicillin flask filled with the growth medium described in [20] supplemented with 200 mg L^{-1} of $N\text{-}NO_3^-$ and 400 mg L^{-1} of C-carbon source (equal concentrations of acetate, propionate, and ethanol). The aeration conditions were the same as above. Nitrate consumption, pH increase and bubble formation indicated denitrification. Triplicate

enrichments were set up under the same culture conditions to increase the population of denitrifiers. Concentrated cell suspensions of each of the five enriched cultures were mixed together and used to inoculate a 2-L Biolafitte reactor filled with 1.5 L of the growth medium described in [21] supplemented with 250 mg L^{-1} of $N\text{-}NO_3^-$ and 500 mg L^{-1} of C-carbon source (equal concentrations of acetate, propionate, and ethanol). The medium was stirred at a constant rate of 700 rpm. Temperature was regulated at 35°C. pH was measured using an Ingold pH electrode, connected to an Ingold transmitter 2300, and was maintained at 7 by addition of sterile hydrochloric acid (2 N) or sodium hydroxide (5 N) solution. A dilution rate of 0.033 h^{-1} was used throughout the study, corresponding to a hydraulic retention time of 30 h. Anoxic conditions were maintained for 1 month to achieve stable culture conditions. Then, the population was progressively adapted to aerobic denitrifying conditions by increasing the aerobic period time from 2 h per week to 1 week in 2. Nitrate was always added to the reactor. Aerobic and anoxic conditions were obtained by sparging the medium with, respectively, air and argon (DO concentrations of 7.5 and 0 mg L^{-1}). After 6 months of alternating phases, samples were taken at steady state during an aerobic and anoxic phase to evaluate the population and isolate strains. Enumeration and isolation were carried out on nutrient agar plates and nitrate broth Merck agar plates under aerobic conditions. The isolated strains were then tested for their ability to denitrify under aerobic batch culture conditions as described in [19]. The 2-L reactor was kept under alternating conditions and was used to evaluate the aerobic denitrifying activities of the consortium.

Similarly, after the first enriched culture on nitrate broth Merck medium, the 11 samples were individually adapted to aerobic/anoxic conditions. Cells were harvested by centrifugation, washed, and concentrated in 0.9% NaCl. They were then used to inoculate 1000-mL Erlenmeyer flasks filled with 400 mL of the growth medium described in [20] with 250 mg L^{-1} of $N\text{-}NO_3^-$ and 500 mg L^{-1} of C-carbon source (equal concentration of acetate, propionate, and ethanol). The cultures were kept under aerobic conditions during 1 week. Cells were again harvested, washed, concentrated, and used to inoculate a 500-mL Penicillin flask filled with the same medium, but bubbled with argon and sealed with rubber stoppers. Anoxic conditions were maintained during 1 week. This weekly oxic/anoxic transition was maintained during 6 months. Each enrichment step was carried out under sterilized conditions. Every 2 months, DNA extracts were taken in order to show the presence of *M. aerodenitrificans* by two-step PCR amplification.

Identification of Isolates (Phenotype and Genotype)

Oxidase activity was determined by monitoring the oxidation of tetramethyl-*p*-phenylenediamine on filter paper. Catalase activity was detected by bubble formation after addition of hydrogen peroxide 3% (v/v) to fresh colonies. Api 20 nE galleries (Biomerieux, SA, Lyon, France) were used to characterize the isolated strains. This system compares the carbon assimilation pattern (12 carbon sources) and 8 conventional tests on the unknown strain with a database containing essentially Gram negative, clinical non-Enterobacteria.

The 16S rRNA gene (rDNA) sequence of isolated strains was determined by sequencing PCR-amplified 16S rDNA. Genomic DNA extraction, PCR-mediated amplification of 16S rDNA, and purification of the PCR products were performed using previously described protocols [9]. The primers used for PCR amplification were W18 (5' GAGTTTGATCMTGGCTCAG 3' located at position 9 in *Escherichia coli*) and W2 (5' GNTACCTTGTTACGACTT 3' located at position 1509 in *E. coli*) [6]. Purified PCR products were sequenced using primer W7 (5' CTCGTTGCGGGACTTAAC 3' located at position 1072 in *E. coli*) and an ABI PRISM Ready Reaction dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequence reaction mixtures were electrophoresed with an Applied Biosystem model 373A DNA sequencer. The 16S rDNA sequences were compared with the Genbank database using Blast software. Sequence alignment and percentage divergence were obtained using CLUSTALW software [12]. RAPD profiles were obtained using standard protocol [28] with three sets of random primers.

Two-Step PCR Amplification

A two-step PCR amplification procedure with two nested pairs of primers specific for *M. aerodenitrificans* was applied in order to determine the origin of the aerobic strain. Specific primers were inferred from the 16S and 23S rDNA regions and 16S-23S rDNA intergenic spacer region (AF148122, AF148123) of *M. aerodenitrificans.* The first PCR was carried out with the primers W26 (5' ATGGAAGAGTGCCCGAAA 3') located at position 1011 in a specific region of the 16S rDNA of *M. aerodenitrificans* and W27 (5' ACTTACAGGTGTTTGCGAATAG 3') located at the 3' end of the 16S–23S spacer region. For the second PCR, W27 was combined with W38 (5' CATGGGAGTGGAATCCGC 3') located at position 1411 in the 16S rDNA region. PCR was performed in a final volume of 50 µL containing 1 to 2 µL of DNA, 5 µL of 25 mM $MgCl_2$, 5 µL of 10*PCR buffer [20 mM TRIS HCl, pH7.5 (25°C), 100 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 0.5% Tween 20 (V/V), 0.5% Nonidet P40 (V/V), 50% glycerol (V/V)], 5 µl of deoxynucleoside triphosphate mix (dGTP, dTTP, dATP, dCTP, each at 2.5 mM), 1.75 U of Expand high-fidelity polymerase, and 0.25 µg of each appropriate primer. For the second PCR, 1 µL of product from the first PCR was used as the template. The two-step PCR was performed as follows: denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min for 30 cycles and final extension at 72°C for 5 min. Five µL of the PCR products was analyzed on a 2% agarose gel by a standard protocol.

Results

Isolation and Characterization

The total numbers of culturable bacteria and culturable anoxic denitrifiers were determined for aerobic and anoxic samples from the mixed adapted ecosystem. Samples were taken at steady state during the weekly transition. The num-

ber of anoxic denitrifying bacteria (1.75×10^7 CFU mL^{-1}) remained the same, whatever the aeration conditions applied to the reactor. In contrast, the total viable population count varied from 9.33×10^8 to 3.8×10^8 CFU mL^{-1} for the oxic and anoxic periods, respectively. This implies that anoxic periods considerably reduced the total number of culturable bacteria by preventing the growth of obligate aerobes. In fact, denitrifying bacteria are facultative anaerobes that can use either oxygen or nitrate as electron acceptors, and consequently can be metabolically active under both conditions. In contrast, the growth of obligately aerobic bacteria is completely inhibited under anoxic conditions. Therefore, obligate aerobes and denitrifiers are counted during sampling in the aerobic reactor and spreading onto aerobic plates. The alternating phases applied to the continuous reactor will then enhance the selection of bacteria which are metabolically versatile, i.e., which are able to consume nitrate, or oxygen, or the two electron acceptors.

Further microbiological studies of the mixed system consisted of strain isolation on nitrate agar medium plates cultured under aerobic conditions. The presence of the two electron acceptors therefore maintained the selective pressure. A total of 28 strains were selected for further study on the basis of differences in colony morphology. They were characterized by Api 20 nE. This identification system revealed that 18 were able to reduce nitrate to nitrite or dinitrogen under anoxic conditions. The Api 20 nE characterization showed that 5 out of the 18 isolated strains exhibited a profile close to that found for *M. aerodenitrificans* [22]. The other profiles were totally unlike any profile in the database. The 18 anoxic denitrifiers were then studied for their aerobic-nitrate reduction ability under fully aerobic conditions by batch assays. Ten of the 18 isolated strains exhibited aerobic denitrifying activity with a nitrate reduction rate lower than that calculated for *M. aerodenitrificans* under the same culture conditions. Moreover, out of the 10 strains, 6 stoichiometrically reduced nitrate to nitrous oxide and dinitrogen gas (true aerobic denitrifiers) and four strains stoichiometrically reduced nitrate to nitrite under fully aerobic conditions (Table 1).

The partial 16S rDNA sequences of these 10 interesting strains revealed that 3 of them are identical to *M. aerodenitrificans* (0% divergence), 6 of them closely resemble known species (2 resemble *C. testosteroni*) and 1 (DS6) corresponds to a new species because of a high percentage divergence (Table 2). Nevertheless, RAPD profiles showed that the 3 strains identical to *M. aerodenitrificans* in terms of 16S rDNA are identical to each other but differ from *M. aerodenitrificans*. In fact, the Api profile of these 3 strains was also identical to that found for *M. aerodenitrificans*. The only difference in the carbon assimilation profile was that the 3 identical strains assimilate citrate, whereas *M. aerodenitrificans* does not.

Table 1. Aerobic denitrifying ability of the 10 isolates compared to that of *M. aerodenitrificans*[a]

Isolated strains	Final denitrifying product	Nitrate reduction rate (mg $N\text{-}NO_3$ L^{-1} d^{-1})	Specific activity (g $N\text{-}NO_3$ (g protein)$^{-1}$ d^{-1})
M. aerodenitrificans	N_2	108	0.5
DS1	N_2	32.8	0.156
DB1	N_2	26.4	0.069
DB7	N_2, NO_2^-	32.8	n.d.
DB9	N_2	20.25	n.d.
DR7	N_2	18.8	0.06
DB2	N_2	18.3	0.043
DS6	NO_2^-	38.4	0.18
DR1	NO_2^-	12.9	0.074
DB6	NO_2^-	75.4	n.d.
DB13	NO_2^-	24.24	n.d.

[a] The nitrate reduction rate and specific activity were calculated during fully aerobic discontinuous culture. Partial aerobic batch cultures made it possible to determine the final denitrifying product through nitrogen balance calculations (for more details, see [19]). n.d.: not determined.

Moreover, many genera were represented: *Paracoccus, Thiobacillus,* and *Enterobacter,* which exhibit normal anoxic denitrification, and *Comamonas* and *Sphingomonas,* which are not classified as denitrifiers. These genera all belong to the *Proteobacteria* phylum, and within this phylum, they all fall into three of the five standard subclasses: α, β, γ.

Origin of M. aerodenitrificans

A two-step PCR amplification procedure was then used to determine from which ecosystem the *M. aerodenitrificans* came. The primer pairs W26–W27 and W38–W27 were tested for the specific detection of *M. aerodenitrificans* against different complex ecosystems such as methanogenic and nitrifying systems and against pure strains such as *C. testosteroni, Z. ramigera,* and *P. halodenitrificans.* The primer pairs exhibit an amplification profile characteristic of *M. aerodenitrificans,* with three fragments corresponding to different intergenic spacer regions depending on the presence of tRNA-Ile or tRNA-Ala (Fig. 1). The primer pairs W26–W27 and W38–W27 mainly amplify a fragment of 800 and 400 bp, respectively, which is consistent with the sizes pre-

Table 2. Phenotypic (Api 20nE results) and genotypic characterization of the 10 isolates[a]

Clones	Api 20nE results (discrimination criterion)	Closest microorganism (accession number)	% of divergence	Phylum
DS1	*C. testosteroni* (Cit+)	*M. aerodenitrificans* (U89333)	0	Proteo β
DB1	*C. testosteroni* (Cit+)	*M. aerodenitrificans* (U89333)	0	Proteo β
DB7	*C. testosteroni* (Cit+)	*M. aerodenitrificans* (U89333)	0	Proteo β
DB9	*C. testosteroni*	*C. testosteroni* (M11224)	2.6	Proteo β
DR7	*C. testosteroni*	*C. testosteroni* (M11224)	2.6	Proteo β
DB2	?	*Paracoccus* sp KS2 (V58016)	0	Proteo α
DS6	?	*Rhodospirillum salinarum* (D14432)	10.8	Proteo α
DR1	?	*Sphingomonas adhaesiva* (D13722)	2.4	Proteo α
DB6	?	*Thiobacillus* sp THI 051 (D32248)	3.2	Proteo β
DB13	?	*Enterobacter aerogenes* (AB004750)	0.2	Proteo γ

[a] Percentage divergence between the clones and the closest microorganisms given by CLUSTAL V software [12].

dicted from the sequence data of 16S–23S spacer regions. The detection threshold was determined by mixing nitrifying DNA with a dilution of *M. aerodenitrificans* DNA. A single PCR allowed the amplification of 2×10^3 equivalent cells, whereas the two-step PCR allowed the detection of 20 equivalent cells (data not shown). The two-step PCR amplification revealed that *M. aerodenitrificans* was present in laboratory wastewater treatment processes such as a methanogenic reactor (V1) and a methanogenic/denitrifying process (L1). *M. aerodenitrificans* was also detected in a phosphate removal sludge process (P1) and an activated sludge plant (B1), which were not studied in our laboratory. However, *M. aerodenitrificans* was not detected in the natural ecosystems tested such as soils, ponds, canal, and sea. However, after 4 months of weekly oxic/anoxic transition, *M. aerodenitrificans* was detected in the canal and pond ecosystems as well as in the four other managed ecosystems.

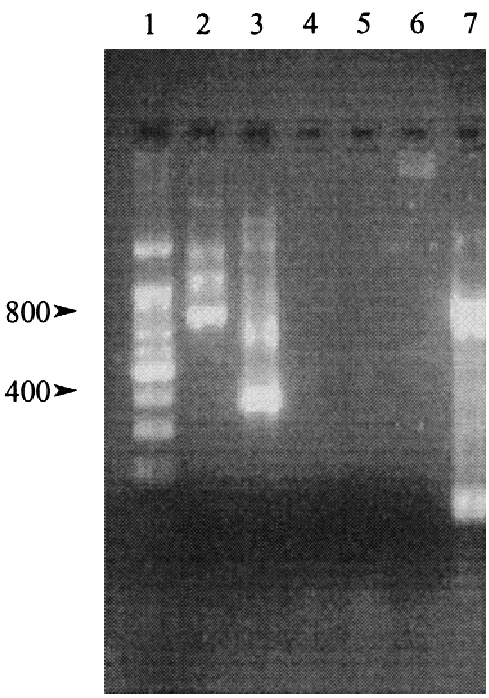

Fig. 1. Polyacrylamide gel electrophoresis of singe and double PCR products amplified from DNA extracts from *M. aerodenitrificans* and anaerobic digester. Lane 1 contains 100 bp DNA ladder (Promega). Lanes 2, 4, 6: W26–W27 PCR product from DNA extracts of *M. aerodenitrificans* (lane 2) and anaerobic digester (lane 6), and from no DNA (lane 4). Lanes 3, 5, 7: (W26–W27) × (W27–W38) PCR product from DNA extracts of *M. aerodenitrificans* (lane 3) and anaerobic digester (lane 7), and from no DNA (lane 5). Arrowheads indicate the positions of the 400- and 800-bp DNA products.

Discussion

High Diversity and High Frequency for Aerobic Denitrifiers

It can be concluded from this study that "Aerobic denitrification is the rule rather than the exception," as Lloyd et al. [16] postulated. Indeed, it is clearly possible to isolate many aerobic denitrifiers from various environments such as canals, ponds, soils, or activated sludges. These isolates belong to different genera (*Paracoccus, Thiobacillus, Enterobacter, Comamonas, Sphingomonas*) of the α, β, γ groups of *Proteobacteria.* Lukow and Diekmann [18] isolated a strain (TL1) from the nitrification step of a leachate-treatment plant which is able to simultaneously reduce oxygen and nitrate and which belongs to the β group of *Proteobacteria* and is possibly related to *Zoogloea* strains. *T. pantotropha* belongs to the α group of *Proteobacteria* [17]. Carter et al.[7] isolated strains belonging to *Pseudomonas, Moraxella,* and *Aeromonas* genera capable of aerobic nitrate respiration from soils and sediments. Moreover, the common anoxic denitrifiers also belong to different genera and are mainly

found in the *Proteobacteria* group. This implies that aerobic denitrification is not a function linked to a particular group or to specific strains, as in the case for nitrification, but it may be a metabolic pathway that has evolved parallel to anoxic denitrification in strains that have been exposed to alternating aerobic/anoxic phases. Another surprising result was that a new strain of *M. aerodenitrificans* was isolated three times from the consortium. This suggests that *Microvirgula* is present in one or all of the ecosystems constituting the consortium (see discussion below).

Ten percent of the isolates were "true aerobic denitrifiers," i.e., able to reduce nitrate to nitrous oxide and dinitrogen gas under fully aerobic conditions at significant rates. By extrapolating the 10% isolation rate to the total viable population of the consortium, culturable bacteria capable of aerobic denitrification may represent in fact a significant fraction of the total culturable population (9.3 to 3.8×10^7 per mL of suspension). Thus, the aerobic denitrifying population is approximately two- to five-fold more abundant than the anoxic denitrifying population. In the same way, 10^4 to 10^6 aerobic nitrate-respiring bacteria were counted per gram of soil or sediment. This represents 10% of the total culturable population and in the case of fresh sediment was 40-fold higher than culturable anoxic denitrifiers [7]. It has therefore been clearly demonstrated that aerobic denitrifiers or aerobic nitrate-respiring bacteria may be isolated at a high frequency from various ecosystems as soon as a selective criterion such as anoxic/oxic transition is applied. Indeed, (1) exerting selective enrichment pressure, i.e., alternating oxic/anoxic phases, combined with anaerobic [8] or aerobic/nitrate isolation (this study), or (2) exerting no selective culture conditions pressure combined with selective enrichment isolation, i.e., oxic growth on butyrate plus nitrite accumulation [7], led to the isolation of various genera of bacteria. The population cultured exclusively under anoxic conditions would not allow the isolation of strains such as *Comamonas* or *Sphingomonas* because they are not recognized as being denitrifiers. On the other hand, the population cultured exclusively under aerobic conditions in the presence of nitrate would have been sufficient to isolate aerobic denitrifiers. However, as oxygen remains the preferred electron acceptor, in the long run the isolated strains would have lost their ability to co-utilize the two electron acceptors. For these reasons, transition between oxic and anoxic culture conditions was proposed as the best selective criterion for isolating aerobic denitrifiers.

In addition to the isolation of true aerobic denitrifiers, four different strains were able to strictly reduce nitrate to nitrite under fully aerobic conditions (accumulation of nitrite in the medium). Production of nitrite could be due to the assimilatory reduction of nitrate. However, this pathway is assumed to be inhibited by the presence of ammonium, and usually the assimilatory reduction of nitrate and that of nitrite are simultaneous reactions such that nitrite does not accumulate. The dissimilatory pathway usually occurs under reducing conditions, which did not correspond to our experiments. Therefore, nitrate reduction to nitrite may only be due to a respiratory process. These results are consistent with those found by Carter et al. [7], who isolated strains using two criteria: butyrate utilization and nitrite accumulation under oxic conditions. This made it possible to identify denitrifiers with a periplasmic nitrate reductase at a high frequency with a significant rate of nitrate reduction to nitrite under aerobic conditions. In the same way, Bonin et al. [5] showed that the nitrite reductase of *Pseudomonas nautica* was more oxygen-sensitive than the nitrate reductase. Further physiological investigations on the enzymes of these four strains may help us to provide answers to some of these questions. Nevertheless, according to the results of the aerobic denitrifying activities of the consortium, nitrite never accumulated. In fact, in our consortium, co-metabolism prevents the accumulation of nitrite because, for example, *M. aerodenitrificans* is able to aerobically denitrify with nitrite as an electron acceptor [20]. Strain 1 isolated by Frette et al. [8] was characterized by a more effective nitrite reduction than nitrate reduction under aerobic conditions. These different results show the high flexibility of aerobic denitrifying metabolism and the great potential for adapting to each condition. This underlines again the importance of taking aerobic denitrification in environments into consideration because it could contribute to the atypical accumulation of nitrite or nitrous oxide if the reaction is incomplete. This is all the more true since the nitrate reduction rates calculated for the consortium were comparable to those found in the literature for standard denitrifying processes [unpublished data].

Ecological Niches for Aerobic Denitrification?

The different bacteria were isolated from the mixed consortium. It would therefore be interesting to know which ecosystems contribute most to increasing the aerobic denitrifying population and thus to answer the question of whether there is a specific ecological niche for aerobic denitrification. Based on the literature results, it is a difficult question to answer. *T. pantotropha* [26] was isolated from a denitrifying/

sulfate treatment plant, *M. aerodenitrificans* [19] from an upflow anoxic–aerobic filter, strain 1 [8] from an alternating activated sludge plant, and strain TL1 [18] from the oxic tank of a nitrification plant. Carter et al. [7] isolated 29 strains from 3 cultivated soils and a freshwater sediment which are completely different. By using two-step PCR amplification, the strain *M. aerodenitrificans* was found in methanogenic and nitrifying/denitrifying ecosystems. To find denitrifiers in a methanogenic microflora may be surprising. However, it is consistent with results obtained by Jorgensen et al. [13], who reported a high frequency of denitrifiers and isolated species of *Pseudomonas* and *Alcaligenes* in anaerobic, nitrate-free habitats. The presence of *M. aerodenitrificans* in these two laboratory systems may be explained by cross-contamination between them. However, the presence of *M. aerodenitrificans* was also observed in a phosphate removal wastewater treatment plant in Roanne, France, and in the aerobic basin of the wastewater treatment plant in Narbonne, France. This confirms the fact that *M. aerodenitrificans* is not linked to our laboratory. It is well known that in phosphate removal ecosystems, diverse microbiota are present: phosphate-removing bacteria, nitrifiers, fermentative bacteria, denitrifiers, and, in these systems, activated sludge are subjected to cyclical anaerobic and aerobic conditions [4]. It is therefore not surprising to find *M. aerodenitrificans* in this alternating system. For the other natural ecosystems, the results are quite different. In fact, although the threshold of the two-step PCR amplification is very low, the presence of *M. aerodenitrificans* in completely natural ecosystems was not detectable. However, after 4 months of acclimation, natural ecosystems such as the canal and the pond were found to contain *M. aerodenitrificans.* Soils that would be suitable ecological niches for aerobic denitrifiers gave negative results at the start as well as at the end of the experiment. It would be interesting to test other types of soils where the aeration conditions are much more favorable than in a clay soil. For example, Carter et al. [7] isolated many nitrate-respiring bacteria from a humo-ferric podzol, a brown earth, and a gley.

These observations imply that *M. aerodenitrificans* is not restricted to a precise natural ecological niche. However, as soon as alternating oxic–anoxic phases are applied, the ability to simultaneously reduce nitrate and oxygen is amplified, and this selective pressure is sufficient to reisolate the type strain *M. aerodenitrificans* and other strains. Because most conventional wastewater treatment removal systems are based on either recycling biomass between aerobic and anoxic zones or applying alternating aerobic and anoxic conditions, it is obvious that microorganisms with this flexible nitrate-oxygen metabolism will be found in these environments. As a consequence, it is clear now that this atypical metabolism has to be taken into account when calculating nitrogen balances.

To conclude, it appears that wherever there is anoxic denitrification or the potential presence of anoxic denitrifying microflora, and wherever alternating aeration conditions are applied or occur naturally, aerobic denitrification may exist. The existence of a constitutive enzyme system (demonstrated for *T. pantotropha* and *M. aerodenitrificans*) offers a physiological advantage when subjected to fluctuating aerating conditions. Indeed, the permanent expression of denitrifying enzymes of aerobic strains prevents the reinduction of the enzymes of common denitrifiers after an oxic period, implying rapid adaptation and response to aeration changes. Moreover, another physiological explanation for aerobic denitrification is the ability to dispose of excess reducing equivalents, i.e., an increasing carbon load implies an increasing aerobic denitrifying rate of the consortium. By combining these two ideas, aerobic denitrification may be found in environments where fluctuating oxygen concentrations and/or reduced carbon are available. This is often the case in wastewater treatment plants, implying that aerobic denitrification has to be taken into consideration when calculating nitrogen balances.

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