ELSEVIER

FEMS Microbiology Ecology 35 (2001) 259–265

FEMS MICROBIOLOGY Ecology

www.fems-microbiology.org

Evaluation of the denitrifying microbiota of anoxic reactors

Claudia Etchebehere [a], Inés Errazquin [a], Elena Barrandeguy [a], Patrick Dabert [b], René Moletta [b], Lucía Muxí [a,*]

[a] *Cátedra de Microbiología, Facultad de Ciencas y Facultad de Química, General Flores 2124, Montevideo, Uruguay*
[b] *Institut National de la Recherche Agronomique, Laboratoire de Biotechnologie de l'Environnement (LBE), Narbonne, France*

Received 21 June 2000; received in revised form 17 January 2001; accepted 19 January 2001

Abstract

Removal of inorganic nitrogen compounds from wastewaters can be accomplished by a combination of the biological processes of nitrification and denitrification. The information on the microbiota present in denitrifying reactors is still scarce. In the present work the evaluation of the denitrifying microbiota of different reactor sludges was performed by specific activity measurements and MPN count of denitrifiers. We also present the isolation and physiological and phylogenetic characterisation of denitrifying bacteria from the anoxic reactor of a combined system treating landfill leachate. Specific denitrifying activity measurements were faster to perform and more reliable than MPN enumerations. 16S rDNA characterisation of the isolates showed that they belonged to the genera *Thauera*, *Acidovorax* and *Alcaligenes* and were closely related to microorganisms retrieved from ecosystems rich in recalcitrant compounds. Two of the isolates could grow on aromatic compounds as sole carbon source.

Keywords: Denitrifying potential; Denitrification; Landfill leachate; Nitrogen removal; 16S rDNA characterisation

1. Introduction

The anaerobic treatment of protein-rich wastes may efficiently reduce carbon contamination by conversion to methane and carbon dioxide. However, during the methanogenic process, the nitrogen originally present in proteins is turned into ammonium, which must then be removed in a post treatment stage. Among other environmental problems caused by inorganic N compounds, ammonia is toxic to fish and it increases the chemical oxygen demand (COD) of the receiving stream. A combination of the biological processes of nitrification (ammonia oxidation to nitrate or nitrite) and denitrification (nitrate reduction to N_2 or N_2O) is a convenient way to perform such post treatment. Although considerable effort has been made to improve designs for the efficient and economical removal of nitrate from wastewater by denitrification, the information on the microbiology of denitrifying reactors is still fragmentary. Denitrifiers belong to a biochemically and taxonomically diverse group of facultatively anaerobic bacteria, characterised by the ability to use nitrogen oxides (nitrate and nitrite) as electron acceptors, producing mainly N_2 as reduction product [1]. The population of such organisms in ecosystems may be evaluated by enumeration, in media with nitrate as an electron acceptor under anaerobic conditions, and by assessing activity. For the latter, several techniques have been developed: it has been estimated from N_2 flux measurements from aquatic samples [2] and by ^{15}N [3] and microsensor techniques [4,5] in sediments. Denitrifying activity has also been measured by the rate of nitrate depletion for anaerobic sludge [6,7]. In this method, however, the process of dissimilatory nitrate reduction to ammonium, that can occur under the same environmental conditions as denitrification, may lead to erroneous results, unless ammonia production is measured simultaneously. Finally, acetylene inhibition of the last step of denitrification, i.e. nitrous oxide reduction to dinitrogen, has been used to measure denitrification activity by pure cultures [8], activated sludge [9] and sediments [10–13].

In the last decade the denitrifying bacteria most frequently isolated from soil were classified as belonging to the genera *Alcaligenes*, and *Pseudomonas* [14,15]. With the incorporation of a classification based on the 16S rDNA phylogenetic analysis, organisms previously classified as

* Corresponding author. Tel.: +598 (2) 924 4209
Fax: +598 (2) 924 1906; E-mail: lmuxi@bilbo.edu.uy

0168-6496/01/$20.00
PII: S0168-6496(01)00098-8

Pseudomonas were assigned to new genera [16], and also new genera of denitrifiers were described [17,18]. 16S rDNA based phylogenetic analysis was also used to characterise the most efficient denitrifiers isolated from activated sludge, showing the predominance of members of the *Rubrivivax* subgroup in the β-purple subdivision, the *Rhodobacter* group in the α-purple subdivision and the *Pseudomonas* subgroup in the γ-purple subdivision [19].

In the present work we present results of the evaluation of the denitrifying microbiota of different reactor sludges by specific activity measurements, performed by the acetylene block technique, and by MPN count of denitrifiers. We also present the isolation and physiological and phylogenetic characterisation of denitrifying bacteria from the anoxic reactor of a combined system treating landfill leachate.

2. Materials and methods

2.1. Samples

The following sources of sludge were tested for denitrifying potential:

Source 1: Sludge from the anoxic reactor (working volume 4.6 l) of a laboratory scale combination of three reactors – methanogenic, anoxic and aerobic – for carbon and nitrogen removal from the leachate of the sanitary landfill in the city of Montevideo. The reactor was fed with leachate and effluent from the methanogenic reactor and nitrate at a C/N ratio of 7 g COD (g $N\text{-}NO_3^-)^{-1}$ [20]. It was sampled at different moments: sample A was taken during full operation (nitrate removal 75%; carbon removal 35%). Sample B was taken after 1 month during which the reactor was not fed.

Source 2: Sludge from a methanogenic UASB reactor (3 m^3) treating malting wastewater at organic loads of 0.6–0.7 kg COD $(kg\ VSS)^{-1}\ (day)^{-1}$ (sample C) [21].

Source 3: Sludge from a laboratory scale anoxic reactor (working volume 4.6 l) inoculated with sludge from source 2 and fed with acetate and nitrate (C/N = 4 g COD (g $N\text{-}NO_3^-)^{-1}$). The reactor was sampled after 4 (sample D) and 5 months (sample E) of operation (nitrate removal 100%; carbon removal 85%).

2.2. Most probable number enumeration, isolation and characterisation of denitrifiers

Denitrifiers were enumerated by most probable number (MPN) in a basal medium (BC) supplemented with yeast extract (0.5 g l^{-1}), potassium acetate (1.84 g l^{-1}) and potassium nitrate (0.72 g l^{-1}) (BCY-acetate), as previously described [7]. Acetylene (purity: > 99.9%, AGA 10% v/v) was added to the headspace and tubes were considered positive for denitrification when accumulation of N_2O occurred in the headspace. Denitrifiers were isolated in tryptic soy agar (TSA, Difco) from the positive tubes of the highest dilutions of the MPN enumeration. The ability of pure cultures to denitrify was confirmed in BCY-acetate. A preliminary characterisation was performed by Gram stain, catalase and oxidase tests [22] and by commercial identification kits API 32GN and VITEK GN+ (bioMérieux).

Tests for growth of the isolates on aromatic compounds were performed in duplicate, aerobically in 10 ml of mineral BC medium supplemented with 2 mM of the respective substrate (phenol or benzoate) and anaerobically, under an N_2 atmosphere, in the same medium also supplemented with potassium nitrate (7 mM). Growth was measured spectrophotometrically (Genesys 5, Spectronic, Milton Roy) at 660 nm and the production of N_2O was determined in the anaerobic tubes.

The average individual cell weight of the isolates was calculated as the weight of dry cells (with the assumption that 1 g of dry cells corresponds to 0.5 g of proteins [23]) over the number of cells, in suspensions in anaerobic phosphate buffer (50 mM, pH 7.0). The number of cells was estimated by direct microscopical count and the protein concentration by the Lowry method [24].

2.3. Identification by 16S rDNA sequence analysis

Genomic DNA extraction and PCR mediated 16S rDNA amplification and purification were performed as described previously [25,26]. Bacterial universal primers used for PCR were: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) corresponding to positions 8–27 in forward *Escherichia coli* numbering and 1522R (5′-AAGGAGGTGATCCAGCCGCA-3′) corresponding to positions 1522–1542 in reverse *E. coli* numbering. Purified PCR products were sequenced (both strands) with the dye-terminator cycle-sequencing ready-reaction kit, with Amplitaq DNA polymerase FS (Perkin-Elmer) using internal primers. Sequence reaction mixtures were electrophoresed on the ABI model 373A sequencer stretch (Applied Biosystems, Perkin-Elmer). The 16S rDNA sequences were compared with all accessible sequences in databases using the BLAST server at NCBI (National Centre of Biotechnology Information).

The sequences were aligned with those belonging to representative organisms of the β-subclass of *Proteobacteria*. The alignments were performed and corrected by using Clustal W and Seq-pup software. An unrooted tree was constructed using DNADIST (Jukes and Cantor algorithm) and neighbour-joining programmes contained in the PHYLIP Phylogeny Inference Package, version 3.5 [27]. A bootstrap analysis of 100 replicates was also performed using programmes included in the same package.

Sequences were deposited at the EMBL Nucleotide Sequence Database with accession numbers as follows: strain O, AJ277704; strain 1917, AJ277705; strain N, AJ277706; strain 1916, AJ277707.

2.4. Denitrification activity measurements

2.4.1. Sludge

The sludge sample was anaerobically transferred to 60 ml vials, under oxygen-free N_2, diluted to 10 ml, capped with butyl rubber stoppers and magnetically stirred. Acetylene (purity $>99.9\%$, AGA) was added to the headspace to reach a concentration of 10% (v/v). Substrates (acetate 40 μmol, or glucose 13 μmol) and KNO_3 (20 μmol) were added, from concentrated stock solutions, at time zero. The amount of sludge in the vial was adjusted to obtain the expected amount of N_2O in less than 60–80 min. All the experiments were performed in duplicate. The concentration of N_2O was periodically measured in samples of the headspace gas and the specific denitrification rate was calculated from the plot of produced N_2O versus time, and expressed as μmol N_2O ml^{-1} min^{-1}. The linear relationship between the rate of N_2O production and the amount of cells was verified by measuring the rate for different amounts of sludge and for a second feeding, after flushing the headspace of the vial with oxygen-free N_2. All the determinations were performed in duplicate.

Optimisation of the test conditions was performed on samples from the anoxic reactor of the leachate treating system, when it was in full operation.

2.4.2. Pure cultures

The specific denitrifying activity of the strains was measured by specific N_2O production rates by resting cells, using a modification of the method described by Mahne and Tiedje [8]. Briefly, the bacteria were grown in BCY-acetate–nitrate under an N_2 atmosphere, anaerobically centrifuged (10 000 rpm, 10 min), washed and re-suspended in anaerobic phosphate buffer (50 mM, pH 7.0). The suspension was anaerobically dispensed under an atmosphere of N_2, into 10 ml vials to give 0.7–1.5 mg protein and diluted to 4 ml with anaerobic phosphate buffer containing chloramphenicol (100 μg ml^{-1}). The vials were capped and acetylene (10% v/v) was added to the headspace. The assay mixture was magnetically stirred and, at time zero, nitrate (4 μmol) and potassium acetate (8 μmol) were added from an anaerobic stock solution. N_2O was measured at intervals in samples of the headspace, taken with a gas tight syringe (Hamilton). The specific denitrifying activity was calculated from the slope of the curve of N_2O production versus time, and expressed as μmol N_2O (g protein)$^{-1}$ min^{-1}. All the experiments were performed in duplicate.

2.5. Analytical procedures

Nitrate and nitrite were measured in the supernatant of centrifuged (10 000 rpm) samples by HPLC (Waters) using an IC-Pack Anion column (Waters) and a UV detector (Shimadzu) at 210 nm. The solvent was phosphate buffer (0.01 M, pH 6.8) at a flow of 1.2 ml min^{-1} at 40°C. N_2O was measured by GC (Chrompack CP90001) with an electron capture detector operating at 300°C, with a Porapak Q 80100 (Chrompack) column. The carrier gas was N_2 and the oven temperature was 55°C. Total N_2O content was calculated from the headspace concentration as described by Christensen and Tiedje [28].

Protein was measured using the method described by Lowry, with bovine serum albumin as standard [24].

3. Results and discussion

3.1. Optimisation of the specific denitrifying activity test conditions

Fig. 1 shows the curves corresponding to different samples of denitrifying sludges. In all cases, nitrous oxide accumulation was linear up to 60–80 min and N_2O recovery was complete. No nitrate was detected by HPLC in the sludge samples and neither nitrate nor nitrite was detected at the end of the experiments. The standard deviation of the values of the rate of nitrous oxide production, calculated from the slope of the curves, was always less than 10% for replicates (Table 1). The specific activities, calculated from the curves in Fig. 2, per ml in the assay mixture, showed no significant differences for the two amounts of sludge tested (0.089 μmol N_2O min^{-1} ml^{-1} and 0.080 μmol N_2O min^{-1} ml^{-1} for 2 and 4.5 ml of

Table 1
Specific denitrifying activity (SDA) and MPN count of sludges samples, comparison with SDA of the isolated denitrifying strains

	SDA[a] of the sludge (μmol N_2O ml^{-1} min^{-1})	Denitrifiers (MPN ml^{-1})	SDA[a] of the predominant denitrifier (μmol N_2O g biomass^{-1} min^{-1})	Weight of a cell (g)	Calculated SDA of the sludge (μmol N_2O ml^{-1} min^{-1})
A	0.086 ± 0.001	2.1×10^6	21.3 ± 0.5	2.0×10^{-13}	0.09×10^{-4}
B	0.033 ± 0.001	9.3×10^6	42.0 ± 0.8	1.8×10^{-13}	0.07×10^{-3}
C	0.022 ± 0.001	4.3×10^6	n.d.	n.d.	n.d.
D	0.096 ± 0.003	4.3×10^6	n.d.	n.d.	n.d.
E	0.132 ± 0.009	2.4×10^8	n.d.	n.d.	n.d.

Samples A and B, sludges from the anoxic reactor of the leachate treating system, sample C, sludge from a methanogenic reactor, samples D and E, sludges from the anoxic reactor fed with acetate.

N.d., not determined.

[a]Mean of two determinations ± standard deviation.

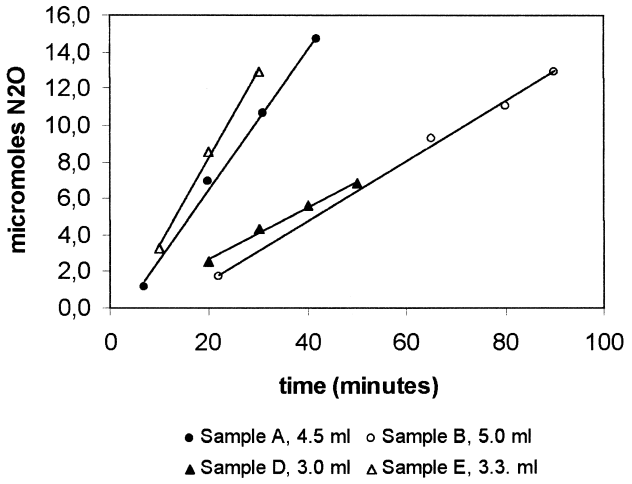

Fig. 1. N_2O production versus time for different sludge samples. A and B from the anoxic reactor of the leachate treating system. D and E from the anoxic reactor fed with acetate.

sludge sample, respectively), considering the standard deviation of 10% observed for replicates. This result shows that the amount of sludge was the limiting factor in the reaction, as required for a reliable estimation of the specific activity. Similar rates of N_2O production were obtained when testing C/N ratios of 5 and 9, whereas for the stoichiometric ratio, the rate was lower, and complete denitrification was not achieved (data not shown). These results are in agreement with reports on incomplete denitrification in reactors fed with low C/N ratios [30].

Glucose was a poor substrate for denitrification of the sludge under study, resulting in less than 10% (0.001 μmol N_2O min^{-1} ml^{-1}) of the specific activity measured with acetate. This suggests that glucose should not be used for testing potential denitrifying activity in reactors, as opposed to the usual nitrate reducing activity tests in soil, which are performed by addition of glucose and nitrate to the soil samples [31]. This result is in agreement with previous reports showing that acetate is a better carbon source than glucose to perform the enumeration of denitrifiers, in order to prevent competition for nitrate by ammonifying organisms [7]. Furthermore, the denitrifying cultures isolated from the denitrifying reactor were unable to utilise glucose as energy source, as indicated by the results of the characterisation kits.

3.2. Evaluation of denitrifying sludges by activity measurement and MPN enumeration

Table 1 shows the results for denitrifying activity and MPN enumeration of denitrifiers for the different sludge samples. Sample C (methanogenic reactor) showed denitrifying activity, as has been previously reported for other methanogenic ecosystems [7,30]. However, although the specific denitrifying activity was, as expected, significantly lower than that shown by all the other samples, the enumeration of denitrifiers showed no significant difference with the other samples, considering the range of the 95% confidence interval for the MPN method [29]. Similar results were obtained for samples from denitrifying reactors: sample A showed a higher SDA than sample B, as expected considering that the former corresponded to the reactor in full operation, whereas the latter was taken after 1 month during which the reactor was not fed. However, again the MPN enumerations did not reflect the actual reactor performance. The sludge from the methanogenic reactor (sample C) was used as inoculum for the denitrifying reactor from which samples D and E were taken. The results clearly show that, as expected, the SDA significantly increased during reactor start-up (samples D and E), however, again the MPN enumerations did not change accordingly. In order to compare the results of activity measurement and the activity of the isolates, the denitrifiers in samples A and B were enumerated, isolated from the positive tubes of the highest dilutions of the MPN enumeration (10^{-6}, 10^{-7}), and the specific denitrifying activity of the pure cultures was measured. The expected activity of the sludge was then calculated from the number of microorganisms (number of cells ml^{-1}), the activity of the predominant pure culture (μmol N_2O g $biomass^{-1}$ min^{-1}) and the estimated average cell weight of this culture (g $cell^{-1}$). As shown in Table 1, for both samples tested, the actual denitrifying activity of the sludge was about three to four orders of magnitude higher than the expected activity. This indicates that either (i) the enumeration method underestimated the population of denitrifiers in the sludge or (ii) the methodology failed in the isolation of a bacterium with a specific denitrifying activity much higher than that of the isolated strains. The denitrifying activity of the pure cultures was similar to the highest values reported by Mahne and Tiedje [8] for several denitrifying bacteria, therefore, it was concluded that the activity measurement was a more sensitive method to evaluate the performance of the denitrifying communities than MPN enumeration. The same conclusion has been reported for nitrifying biomass [32] and methanogenic sludges [33]. This may reflect the fact that the MPN meth-

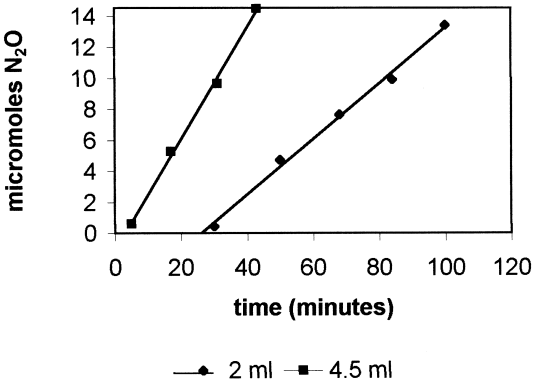

Fig. 2. Rate of N_2O production by anoxic sludge as a function of the amount of sludge in the assay.

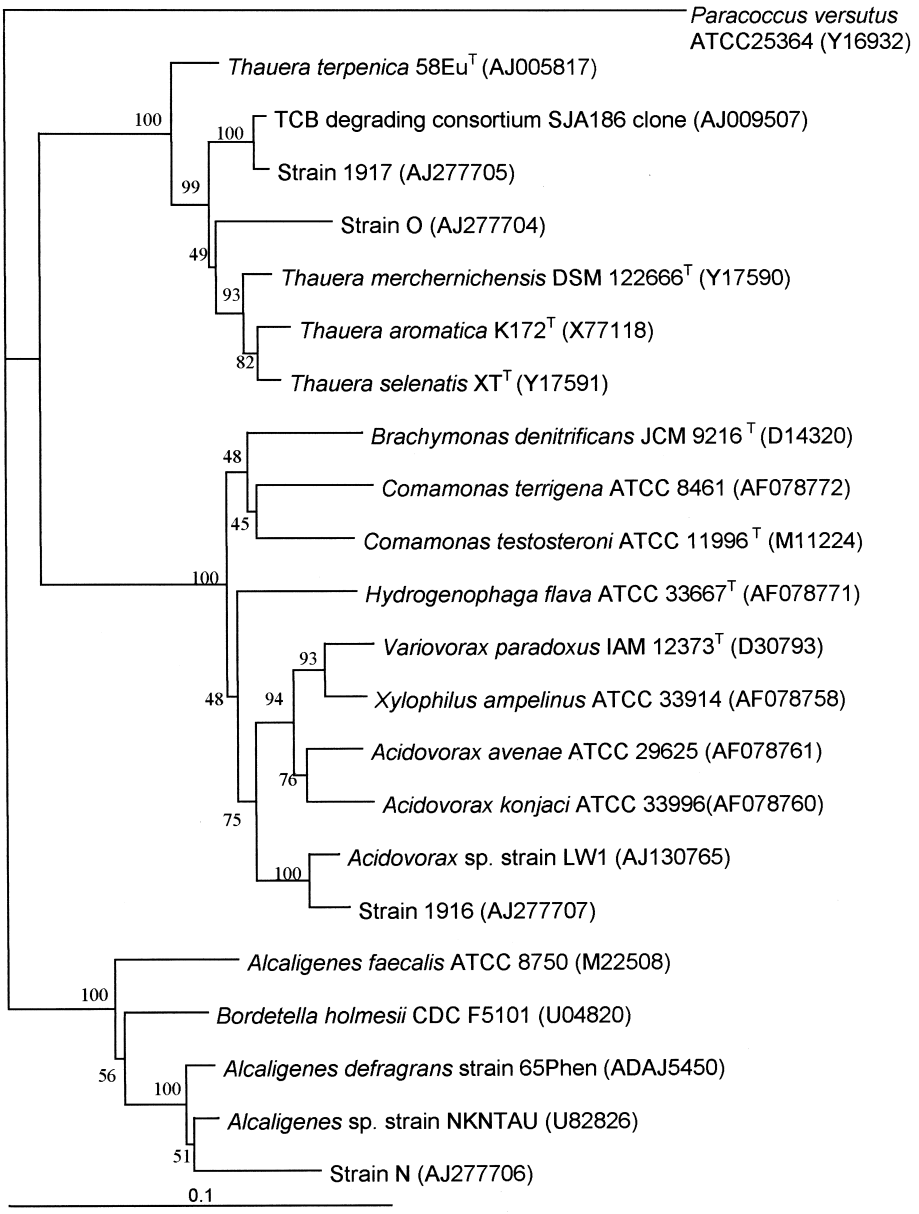

Fig. 3. Phylogenetic dendrogram derived from 16S rDNA sequence analysis showing the positions of strains O, 1917, 1916, N. The numbers next to the nodes represent bootstrap values of 100 replicates. Sequence from *Paracoccus versutus* was used as outgroup. The scale bar represents 10 nucleotide substitutions per 100 nucleotides.

od is only a rough estimate of the concentration of cells, as indicated by the broad range of the 95% confidence interval (i.e. lower limit: 35, upper limit: 470 for MPN = 210/100 ml [29]). Another explanation may be the existence of metabolically active cells that are non-culturable under the conditions of the enumeration.

3.3. Identification of the bacterial isolates

Five strains were isolated from the most diluted tubes of the MPN technique in samples A (strains O, N and Q, from dilution 10^{-6}) and B (strains 1916 and 1917, from dilution 10^{-7}). All the bacterial isolates were Gram-negative, and gave positive results in oxidase and catalase tests. The conventional identification performed by API 32GN or VITEK GN+ (bioMérieux) identification kits presented low acceptable profiles because there were not enough positive results to have a good identification, probably due to the asaccharolytic behaviour of these strains. Difficulties for the identification of denitrifying strains isolated from wastewater treatment systems using phenotypic based kits were also found by Merzouki et al. [34] and Scholten et al. [35].

The 16S rDNA of four of the strains was sequenced (ca. 1100 bp) and compared with sequences of representatives of the domain *Bacteria* in databases (NCBI, Blast search). An evolutionary distance phylogenetic dendrogram (Jukes and Cantor) was constructed with the neighbour-joining

method using 1001 unambiguously aligned positions. The four sequences clustered in different branches within the β-subclass of *Proteobacteria* (Fig. 3) and presented high phylogenetic distance similarity values (>95%) with sequences available in databases.

Sequences from strains O and 1917 clustered within the genus *Thauera* in the β-subclass of *Proteobacteria* (Fig. 3). The sequence of strain O was closely related to that of *Thauera aromatica* strain K172^T (95.4%). The sequence of strain 1917 presented very high similarity (99.3%) with a sequence belonging to a not yet cultured microorganism (SJA-186 clone), retrieved from an anaerobic trichlorobenzene transforming reactor [36]. Most of the species described in the genus *Thauera* have been reported to degrade aromatic compounds coupled to the reduction of nitrate [37].

Strain N was positioned within the genus *Alcaligenes*, closely related (96.1%) to *Alcaligenes* sp. strain NKNTAU [38], a respiratory denitrifier with the ability to perform 2-aminoethanosulfonate (taurine) oxidation. The same branch includes the 16S rDNA from *Alcaligenes defragrans* strain 65Phen, isolated on alkenoic monoterpenes and nitrate [39].

The sequence of strain 1916 (from sludge sample B) showed a high similarity level (98.16%) with *Acidovorax* sp. strain LW1 [40] within the family *Comamonadaceae* in the β-*Proteobacteria*. This strain was isolated from a highly contaminated river in Germany and has the ability to use 1-chloro-4-nitrobenzene as sole source of carbon, nitrogen and energy.

From these results, it can be inferred that the organisms isolated from the anoxic system under study, are related to microorganisms selected in ecosystems rich in nitrate and aromatic compounds. In order to detect this property in the isolated denitrifiers, a test for growth on phenol and benzoate as sole carbon source was performed under denitrifying and aerobic conditions. Strain O showed the ability to grow on benzoate under denitrifying conditions and aerobically, as indicated by an increase in optical density at 660 nm (0.332 ± 0.007 for aerobic conditions, 0.150 ± 0.003 for denitrification; no growth was detected in control tubes with no added substrate). Strain Q could grow on both substrates aerobically (optical density at 660 nm was 0.120 ± 0.019 for benzoate, and 0.115 ± 0.005 for phenol). A variety of bacteria have been reported to catalyse aromatic compound degradation coupled to denitrification, suggesting a role for denitrification in the anaerobic mineralisation of such compounds [41]. These findings suggest that the denitrifiers in the anoxic reactor under study not only contribute to inorganic N elimination, but may also remove aromatic compounds which have been reported as common contaminants in landfill leachate [42]. More physiological studies on the isolated denitrifiers are necessary to postulate a role of these strains in the denitrifying ecosystem.

Although MPN count is a common method to evaluate the proportion of one physiological group of microorganisms in a particular ecosystem, the results of the present work show that activity measurements gave a more realistic view of the denitrifying populations under study, probably due to the bias in the culture based methods. More studies applying molecular techniques are necessary to detect the presence of microorganisms that are non-culturable under the conditions of the enumeration, which may contribute to the denitrifying activity.

Acknowledgements

This investigation was financially supported by International Foundation for Science (IFS), by PEDECIBA-Química (a fundation for the development of basic science in Uruguay) and by CONICYT (Consejo Nacional de Investigación Científica) Fondo Clemente Estable.

References

[1] Knowles, R. (1982) Denitrification. Microbiol. Rev. 46, 43–70.
[2] Nowicki, B.L. (1994) The effect of temperature, oxygen, salinity, and nutrient enrichment on estuarine denitrification rates measured with a modified nitrogen gas flux technique. Estuar. Coast. Shelf Sci. 35, 137–156.
[3] Koike, I. and Hattori, A. (1978) Simultaneous determinations of nitrification and nitrate reduction in coastal sediments by a 15-N dilution technique. Appl. Environ. Microbiol. 35, 853–857.
[4] Binnerup, S.J., Jensen, K., Revsbech, N.P., Jensen, M.H. and Sfrensen, J. (1992) Denitrification, dissimilatory reduction of nitrate to ammonium and nitrification in a bioturbated estuarine sediment as measured with ^{15}N and microsensor techniques. Appl. Environ. Microbiol. 58, 303–313.
[5] Jensen, K., Revsbech, N.P. and Nielsen, L.P. (1993) Microscale distribution of nitrification activity in sediment determined with a shielded microsensor for nitrate. Appl. Environ. Microbiol. 59, 3287–3296.
[6] Akunna, J.C., Bizeau, C. and Moletta, R. (1994) Nitrate reduction by anaerobic sludge using glucose at various nitrate concentrations: ammonification, denitrification and methanogenic activities. Environ. Technol. 15, 41–49.
[7] Quevedo, M., Guynot, E. and Muxí, L. (1996) Denitrifying potential of methanogenic sludge. Biotechnol. Lett. 18, 1363–1368.
[8] Mahne, I. and Tiedje, J.M. (1995) Criteria and methodology for identifying respiratory denitrifiers. Appl. Environ. Microbiol. 61, 1110–1115.
[9] Hallin, S. and Pell, M. (1994) Acetylene inhibition for measuring denitrification rates in activated sludge. Water Sci. Technol. 30, 161–167.
[10] Sörensen, J. (1978) Denitrification rates in a marine sediment as measured by the acetylene inhibition technique. Appl. Environ. Microbiol. 36, 139–143.
[11] Bonin, P. and Raymond, N. (1990) Effects of oxygen on denitrification in marine sediments. Hydrobiologia 207, 115–122.
[12] Kaspar, H.F. (1982) Denitrification in marine sediments. Measurement of capacity and estimate of in situ rates. Appl. Environ. Microbiol. 43, 522–527.
[13] Jorgensen, K. and Tiedje, J.M. (1993) Survival of denitrifiers in nitrate-free anaerobic environments. Appl. Environ. Microbiol. 59, 3297–3305.

[14] Tiedje, J.M. (1988) Ecology of denitrification and dissimilative nitrate reduction to ammonium. In: Biology of Anaerobic Microorganisms (Zehnder, A.J.B., Ed.), pp. 179–243. Wiley, New York.
[15] Zumft, W.G. (1992) The denitrifying prokaryotes. In: The Prokaryotes, 2nd Edn. (Balows, A., Träper, H.G., Dworkin, M., Harder, W. and Shleifer, K.-H., Eds.), pp. 554–582. Springer, New York.
[16] Anders, H.J., Kaetzke, A., Kämpfer, P., Ludwig, W. and Fuchs, G. (1995) Taxonomic position of aromatic-degrading denitrifying pseudomonad strains K 172 and KB 740 and their description as new members of the genera *Thauera*, as *Thauera aromatica* sp. nov., and *Azoarcus*, as *Azoarcus evansii* sp. nov., respectively, members of the beta subclass of the *Proteobacteria*. Int. J. Syst. Bacteriol. 45, 327–333.
[17] Wagner, M., Nielsen, P.H., Schmid, M., Purkhold, U., Daims, H., Juretschko, S., Lee, N. and Scleifer, K.-H. (1998) New insights in the structure and function of microbial populations active in biological nitrogen removal plants: *Nitrosococcus mobilis*, *Nitrospira*-like bacteria and *Azoarcus* sp. as dominant populations. In: European Conference on New Advances in Biological Nitrogen and Phosphorus Removal for Municipal or Industrial Wastewaters, pp. 27–30. Narbonne, France.
[18] Song, B., Lily, Y.Y. and Palleroni, J. (1998) Identification of denitrifier strain T1 as *Thauera aromatica* and proposal for emendation of the genus *Thauera* definition. Int. J. Syst. Bacteriol. 48, 889–894.
[19] Magnusson, G., Edin, H. and Dhalhammar, G. (1998) Characterization of efficient denitrifying bacteria strains isolated from activated sludge by 16S-rDNA analysis. Water Sci. Technol. 38, 63–68.
[20] Borzacconi, L. (1998) Sistema de tratamiento de lixiviado de relleno sanitario. PhD Thesis. Facultad de Ingeniería, Universidad de la República.
[21] Martinez, J., Lopez, I., Giani, L. and Borzacconi, L. (2000) Blanket development in a malting wastewater anaerobic treatment. In: Anais da VI Oficina e Seminario Latino-Americano de Digestao Anaerobia, pp. 83–89. Recife, Brasil.
[22] Simbert, R.M. and Krieg, N.R. (1994) Phenotypic characterization. In: Methods for General and Molecular Bacteriology (Gerhardt, P., Murray, R.G.E., Wood, W.A. and Krieg, N.R., Eds.), pp. 514–554. American Society for Microbiology, Washington, DC.
[23] Macy, J.M., Schröder, I., Thauer, R.K. and Kröger, A. (1986) Growth of *Wolinella succinogenes* on H_2S plus fumarate and on formate plus sulfur as energy sources. Arch. Microbiol. 144, 147–150.
[24] Daniels, L., Hanson, R.S. and Phillips, J.A. (1994) Chemical analysis. In: Methods for General and Molecular Bacteriology (Gerhardt, P., Murray, R.G.E., Wood, W.A. and Krieg, N.R., Eds.), pp. 514–554. American Society for Microbiology, Washington, DC.
[25] Sambrook, J., Fritsh, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[26] Godon, J.J., Zumstein, E., Dabert, P., Habouzit, F. and Moletta, R. (1997) Molecular microbial diversity of an anaerobic digestor as determined by small-subunit rDNA sequence analysis. Appl. Environ. Microbiol. 63, 2802–2813.
[27] Felsfelstein, J. (1993) Phylip (Phylogeny Inference Package) version 3.5. Distributed by the author. Department of Genetics, University of Washington, Seattle, WA.
[28] Christensen, S. and Tiedje, J.M. (1988) Sub-parts-per-billion nitrate method: Use of an N_2O-producing denitrifier to convert NO_3^- or $^{15}NO_3^-$ to N_2O. Appl. Environ. Microbiol. 54, 1409–1413.
[29] APHA (1985) Standard Methods for the Examination of Water and Wastewater, 16th Edn. American Public Health Association, Washington, DC.
[30] Akunna, J.C., Bizeau, C. and Moletta, R. (1992) Denitrification in anaerobic digesters: possibilities and influence of wastewater COD/N-NO_x ratio. Environ. Technol. 13, 825–836.
[31] Murray, R., Feig, Y. and Tiedje, J.M. (1995) Spatial heterogeneity in the distribution of denitrifying bacteria associated with denitrification activity zones. Appl. Environ. Microbiol. 61, 2791–2793.
[32] Belser, L.W. and Mays, E.L. (1982) Use of nitrifier activity measurements to estimate the efficiency of viable nitrifier counts in soils and sediments. Appl. Environ. Microbiol. 43, 945–948.
[33] Dolfing, J. and Bloemen, W. (1985) Activity measurements as a tool to characterise the microbial composition of methanogenic environments. J. Microbiol. Methods 4, 1–12.
[34] Merzouki, M., Delgenès, J.P., Bernet, N., Moletta, R. and Benlemlih, M. (1999) Polyphosphate-accumulating and denitrifying bacteria isolated from anaerobic-anoxic and anaerobic-aerobic sequencing batch reactors. Curr. Microbiol. 38, 9–17.
[35] Scholten, E., Lukow, T., Auling, G., Kroppenstedt, R.M., Rainey, F.A. and Diekman, H. (1999) *Thauera mechernichensis* sp. nov., an aerobic denitrifier from a leachate treatment plant. Int. J. Syst. Bacteriol. 49, 1045–1051.
[36] Wintzingerode, F., Selent, B., Hegemann, W. and Göbel, U. (1999) Phylogenetic analysis of an anaerobic, trichlorobenzene-transforming microbial consortium. Appl. Environ. Microbiol. 65, 283–286.
[37] Song, B., Palleroni, J.N. and Häggblom, M.M. (2000) Description of strain 3CB-1, a genomovar of *Thauera aromatica*, capable of degrading 3-chlorobenzoate coupled to nitrate reduction. Int. J. Syst. Evol. Microbiol. 50, 551–558.
[38] Denger, K., Laue, H. and Cook, A.M. (1997) Anaerobic taurine oxidation: a novel reaction by a nitrate-reducing *Alcaligenes* sp. Microbiology 143, 1919–1924.
[39] Foss, S., Heyen, U. and Harder, J. (1998) *Alcaligenes defragrans* sp. nov., a description of four strains isolated on alkenoic monoterpenes ((+)-menthene, alpha-pinene, 2-carene, and alpha-phellandrene) and nitrate. Syst. Appl. Microbiol. 21, 237–244.
[40] Katsivela, E., Wray, V., Pieper, D.H. and Wittich, R.M. (1999) Initial reactions in the biodegradation of 1-chloro-4-nitrobenzene by a newly isolated bacterium, strain LW1. Appl. Environ. Microbiol. 65, 1405–1412.
[41] Zumft, W.G. (1997) Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. 61, 533–616.
[42] Wang, Y.S. and Barlaz, M.A. (1998) Anaerobic biodegradability of alkylbenzenes and phenol by landfill derived microorganisms. FEMS Microbiol. Ecol. 25, 405–408.