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Comamonas nitrativorans sp. nov., a novel denitrifier isolated from a denitrifying reactor treating landfill leachate

Claudia Etchebehere,[1] María I. Errazquin,[1] Patrick Dabert,[2] René Moletta[2] and Lucía Muxí[1]

[1] Departamento de Microbiología, Facultad de Química y Facultad de Ciencias, Universidad de la República, Gral. Flores 2124, Montevideo, Uruguay

[2] Institute National de la Recherche Agronomique, Laboratoire de Biotechnologie de l'Environnement, Narbonne, France

Author for correspondence: Claudia Etchebehere. Tel: +598 2 9244209. Fax: +598 2 9241906. e-mail: cetchebe@bilbo.edu.uy

A group of Gram-negative denitrifying bacteria has been isolated from a denitrifying reactor treating landfill leachate. The new isolates produced both oxidase and catalase and showed growth on acetate, butyrate, n-caproate, i-butyrate, i-valerate, propionate, n-valerate, lactate, alanine, benzoate, phenylalanine and ethanol. No growth was observed on sugars. The bacteria could perform anoxic reduction of nitrate, nitrite and nitrous oxide to nitrogen, coupled to the oxidation of the same substrates as those used under aerobic conditions, except for aromatic compounds. They were very efficient denitrifiers, as estimated from the specific rate of N_2 gas production. All the strains showed the same 16S rDNA restriction profile and one of them, designated 23310^T, was selected for phylogenetic analysis. The organism clustered within the family *Comamonadaceae*, being related to *Comamonas terrigena* (95·8% sequence similarity). On the basis of the phylogenetic analysis, physiological characterization and the ability to efficiently reduce nitrate to N_2, it is proposed that the bacterium be assigned to a new species, *Comamonas nitrativorans*. The type strain is 23310^T (= DSM 13191^T = NCCB 100007^T = CCT 7062^T).

Keywords: *Comamonadaceae*, denitrification, specific denitrifying activity

INTRODUCTION

The need for the removal of inorganic nitrogen compounds from waste waters is being increasingly recognized. Ammonium removal can be accomplished by a combination of the biological processes of nitrification and denitrification, which convert it into gaseous products. Information on the microbiota present in denitrifying reactors is still scarce; a better understanding of the nature of nitrate-reducing organisms will contribute to an improvement in the operation and performance of these reactors.

Denitrifying bacteria belong to a biochemically and taxonomically diverse group of facultatively anaerobic bacteria, characterized by the ability to use nitrogen oxides (nitrate and nitrite) as electron acceptors, producing mainly N_2 as reduction product (Knowles, 1982). According to Mahne & Tiedje (1995) two criteria should be met to conclusively establish if an organism is a respiratory denitrifier, namely, that nitrogen recovery as N_2 plus N_2O from nitrate should exceed 80% and that the rate of N_2 gas production should exceed 10 μmol min^{-1} (g protein)$^{-1}$. Up to the last decade the most frequently isolated denitrifiers were classified as *Alcaligenes*, *Paracoccus*, *Pseudomonas* and *Rhodobacter* (Zumft, 1992); however, with the development of a classification based on 16S rDNA phylogenetic analysis, many denitrifying isolates have been shown to belong to new genera. Furthermore, organisms previously classified in the genus *Pseudomonas* were assigned to new genera (Anders *et al*., 1995).

During the characterization of the microflora of a denitrifying reactor treating landfill leachate we isolated a group of bacteria which persisted in the reactor and showed a high specific denitrifying activity. In this study the characterization of one of the strains is presented and, based on phenotypic and phylo-

Abbreviation: ARDRA, amplified 16S rDNA restriction analysis.

The EMBL accession number for the 16S rDNA sequence of strain 23310^T is AJ251577.

genetic properties, it is described as a novel species within the genus *Comamonas* for which we propose the name *Comamonas nitrativorans*.

METHODS

Bacterial strains and culture conditions. The strains used in this study were isolated from sludge samples of the anoxic reactor 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 (Borzacconi, 1998). The denitrifying reactor was fed with leachate and KNO_3 [C/N = 4 g chemical oxygen demand (COD) (g $N/NO_3^-)^{-1}$] for the first year and with effluent from the methanogenic reactor and KNO_3 for the following 9 months. Samples were taken from the sludge and denitrifiers were enumerated by Most Probable Number (MPN) in a basal medium supplemented with potassium acetate (1·84 g l^{-1}) and potassium nitrate (0·72 g l^{-1}) (BCY acetate-nitrate) under an atmosphere of N_2, as previously described (Quevedo *et al*., 1996). Acetylene (10 %, v/v) was added to the headspace and tubes were considered positive for denitrification when accumulation of N_2O occurred in the headspace. The predominant denitrifiers were isolated from the positive tubes of the highest dilution of the MPN enumeration on Trypticase Soy Agar (TSA; Difco). The ability of pure cultures to denitrify was confirmed by N_2O accumulation when grown in BCY acetate-nitrate, under an atmosphere of N_2 with acetylene (10 %). The isolates with the ability to denitrify were stored in Trypticase Soy Broth (TSB; Difco) supplemented with glycerol (20 %, v/v) at −70 °C.

Amplified 16S rDNA restriction analysis (ARDRA). The bacterial isolates (18 strains) were differentiated by determining their ARDRA profiles. ARDRA was carried out as described by Fernandez *et al*. (1999) with primers specific for the 16S rRNA gene of the domain *Bacteria*, using *Hha*I and *Hae*III restriction enzymes (Amersham). Restriction patterns were normalized and compared by GelCompar software (version 4.1; Applied Maths). Pattern clustering was done by the UPGMA method applying the Dice coefficient. A maximum tolerance of 3·0 ± 0·5 % was used for band positions.

Phenotypic characterization. The strains were preliminarily characterized by Gram staining, an oxidation-fermentation test, presence of catalase and oxidase (Smibert & Krieg, 1994) and a VITEK GNI+ (bioMérieux) characterization kit. Cell morphology and motility were studied by phase-contrast microscopy (Axioplan; Zeiss). Tests for growth on different substrates were performed aerobically in tubes with 10 ml of a basal BCY medium (Quevedo *et al*., 1996) supplemented with 5 mM of the respective substrate and anaerobically under an N_2 atmosphere in the same medium also supplemented with potassium nitrate (5 mM). All growth tests were performed in duplicate.

The optimum temperature for growth was determined aerobically in duplicate in tubes with 10 ml TSB at different incubation temperatures (25, 30, 35, 40, 45 and 55 °C). The optimum pH was determined in TSB, previously adjusted to pH values ranging from 4 to 9, incubated at 35 °C. Growth was measured spectrophotometrically (Genesys 5; Spectronic) at 660 nm.

Electron microscopy. For ultrathin section microscopy, bacterial cultures were centrifuged at 3000 r.p.m. for 6 min at 4 °C, the supernatant was discarded and the pellet was fixed in 2·5 % (w/v) glutaraldehyde and 2 % (w/v) paraformaldehyde in 0·1 M potassium phosphate buffer (pH 7·4) for 2 h. Samples were post-fixed in 1·0 % (w/v) osmium tetroxide, dehydrated in an ascending gradient of ethanol (50, 70, 80, 90 and 95 %, v/v) and impregnated in propylene oxide. Finally, they were embedded in Poly/Bed 812 resin (Polysciences 18976-2590). Ultrathin sections were cut with an ultratome Super Nova (Reichter-Jung), stained with uranyl acetate and lead citrate, and examined under a JEM-1200 EX II (JEOL) transmission electron microscope. For negative staining the cell pellet was resuspended in PBS and mixed with an equal volume of 3 % (w/v) phosphotungstic acid (pH 6·5). A drop of this mixture was placed on a carbon/Formvar-coated 200 mesh copper grid. Grids were air-dried before examination in the transmission electron microscope.

Analyses of isoprenoid quinones. Quinones were characterized by HPLC using an EcoCart 125-3 (Lichrospher; RP-18, 5 μm) column and acetonitrile/2-propanol (65:35, v/v) as mobile phase at a flow rate of 0·5 ml min^{-1}. The column was kept at 40 °C. The ubiquinone was detected by a UV detector at 254 nm (Kroppenstedt, 1985). The analyses were performed at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany).

Denitrifying activity. 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 & Tiedje (1995). Briefly, the bacteria were grown in BCY acetate-nitrate medium under an N_2 atmosphere, anaerobically centrifuged (10000 r.p.m., 15 min) and resuspended in anaerobic potassium phosphate buffer (50 mM, pH 7·0). After determination of the protein concentration, the suspension was anaerobically dispensed under an N_2 atmosphere into 10 ml vials to give 0·7–1·5 mg protein. Then it was diluted to 4 ml with the same anaerobic potassium phosphate buffer supplemented with chloramphenicol (100 μg ml^{-1}). The vials were capped and acetylene (10 %) 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}. The specific denitrifying activity was also measured in TSB nitrate medium as described by Mahne & Tiedje (1995). All experiments were performed by duplicate and the standard deviation was always less than 5 %.

Aerobic denitrification. The ability to respire nitrogen oxides in the presence of oxygen was tested in batch cultures by measuring the reduction of nitrate under anoxic and aerobic conditions, according to the method described by Patureau *et al*. (1998). The strains were inoculated (1 %) into BCY acetate-nitrate and incubated with agitation (8000 r.p.m.) under anoxic and aerobic conditions at 35 °C. Nitrate concentrations were measured during growth.

Analytical procedures. Nitrate and nitrite were measured in the supernatant of centrifuged (10000 r.p.m.) samples by HPLC (Waters) using an IC-Pack Anion column (Waters) and UV detector (Shimadzu) at 210 nm. The solvent was potassium phosphate buffer (0·01 M, pH 6·8) at a flow rate of 1·2 ml min^{-1}. The column was kept at 40 °C. N_2O was measured by GC (Chrompack CP90001) with an electron

capture detector operating at 300 °C and with a Porapak Q 80100 (Chrompack) column. The carrier gas was N_2 and the oven temperature was 55 °C. The total N_2O content was calculated from the headspace concentration as described by Christensen & Tiedje (1988). Proteins were measured using the Lowry method with bovine serum albumin as standard (Daniels *et al*., 1994).

16S rDNA sequence analysis. The extraction of genomic DNA from strain 23310^T and the following PCR-mediated 16S rDNA amplification were performed as described by Sambrook *et al*. (1989) and Godon *et al*. (1997). Bacterial universal primers used for PCR were: forward 27F (5′-AGAGTTTGATCCTGGCTCAG-3′), corresponding to positions 8–27 (*Escherichia coli* numbering); and reverse 1522R (5′-AAGGAGGTGATCCAGCCGCA-3′), corresponding to positions 1522–1542. The PCR product was sequenced with the dye-terminator cycle-sequencing ready-reaction kit, with AmpliTaq DNA polymerase FS (Perkin-Elmer). Sequence reaction mixtures were electrophoresed on an Applied Biosystems/Perkin-Elmer model 373A sequencer. The 16S rDNA sequence was compared with all accessible sequences in databases using the BLAST server at NCBI (National Centre for Biotechnology Information, Bethesda, MD, USA). The sequence was aligned with sequences belonging to representative organisms of the *β*-subclass of the *Proteobacteria*. The alignments were performed and corrected by using CLUSTAL W and Seq-pup software. An unrooted tree was constructed using DNADIST (Jukes & Cantor algorithm) and neighbour-joining programs contained in the PHYLIP Phylogeny Inference Package, version 3.5 (Felsenstein, 1993). A bootstrap analysis of 100 replicates was also performed using programs included in the same package.

RESULTS

Screening of the isolates by ARDRA

The reactor was sampled six times during 1 year of operation; the 18 denitrifying strains isolated clustered in nine different ARDRA profiles. Strain K and strains 23310^T and 6 (isolated 8 and 9 months later than strain K, respectively) shared the same ARDRA profile (Fig. 1) and were selected for further characterization.

Morphological characteristics

The three strains showed similar morphological characteristics. Strain 23310^T was a very motile, Gram-negative, slightly curved rod. Cells occurred singly or in pairs in young cultures. Colonies on TSA developed after a 24 h incubation period and were circular (1–2 mm diameter) and cream-coloured. Electron microscopy of thin sections of strain 23310^T revealed a typical Gram-negative cell wall structure with an undulating outer membrane. Cells presented flagella in bipolar tufts as shown by negative staining electron microscopy (Fig. 2).

Analyses of quinones

HPLC analysis of quinones from strain 23310^T revealed a unique peak corresponding to ubiquinone Q-8.

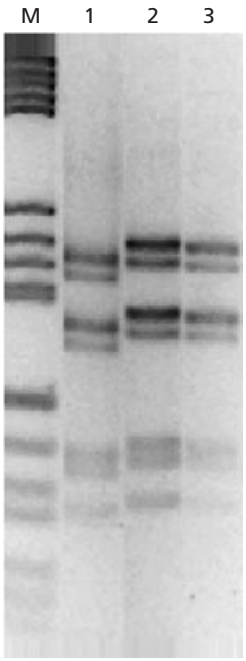

Fig. 1. ARDRA profile of strains K (lane 1) 6 (lane 2) and 23310^T (lane 3). Lane M, Smart Ladder DNA molecular mass marker (Eurogentec).

Physiological characterization

Strains 23310^T, 6 and K were heterotrophic microorganisms able to grow under aerobic and anoxic conditions. Under anoxic conditions nitrate, nitrite and N_2O were reduced to N_2; no N_2O was detected during growth in the absence of acetylene. They presented positive tests for oxidase and catalase, and were inactive in the oxidation fermentation test with glucose as substrate. The VITEK GNI+ test failed to identify the isolates, as the strains did not produce an adequate number of reactions on the GNI+ card to distinguish them from other glucose non-fermenting Gram-negative bacilli. As shown in Table 1, the three strains oxidized several organic acids and some amino acids, but no sugars. Under anaerobic conditions with nitrate as electron acceptor, growth was observed with the same substrates as the ones used under aerobic conditions, except for the aromatic compounds (benzoate, phenylalanine). The optimum temperature for growth of strain 23310^T was between 30 and 35 °C, but no growth was detected at 55 °C. Growth was observed between pH 5 and 9 with an optimum at pH 7.

Denitrifying ability

The three strains showed N_2O accumulation when grown in BCY supplemented with acetate and nitrate under an N_2 atmosphere in the presence of acetylene. To confirm the ability to perform respiratory denitrification, the rate of N_2O production by resting cells was evaluated in the presence of acetylene (Mahne &

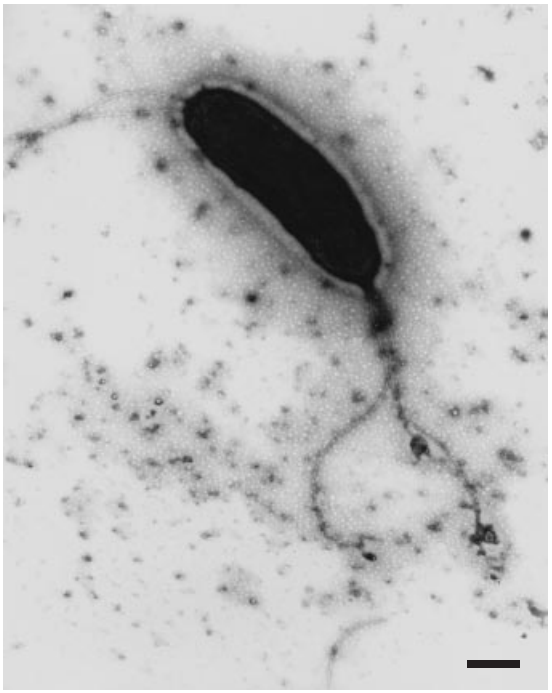

Fig. 2. Negative staining electron micrograph of strain 23310^T showing the presence of two tufts of bipolar flagella. Bar, 0·5 μm.

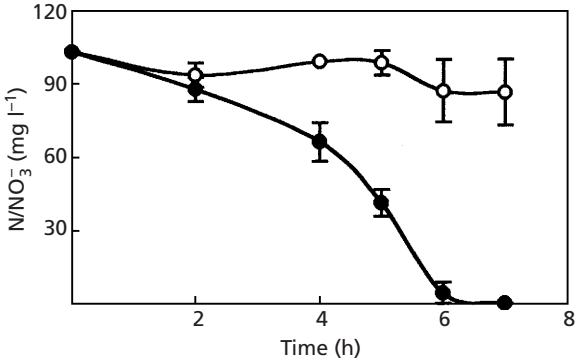

Fig. 3. Nitrate reduction in batch cultures with strain 23310^T in BCY acetate-nitrate medium. ●, Anoxic conditions; ○, aerobic conditions. Data are means ± SD of a duplicate assay.

Tiedje, 1995). The three strains showed no significant differences in the specific rate of N_2O production with acetate as electron donor [98 ± 3 μmol N_2O (g protein)$^{-1}$ min^{-1}] with almost complete nitrogen recuperation as N_2O. The denitrification rate in TSB for strain 23310^T was 183 ± 8 μmol N_2O (g protein)$^{-1}$ min^{-1}.

Aerobic denitrification

The ability to co-respire oxygen and nitrate was tested for strain 23310^T. Fig. 3 shows that there was no reduction of nitrate under aerobic conditions, whereas total nitrate depletion occurred under anoxic conditions in 6 h.

Table 1. General and nutritional characteristics of strains 23310^T, 6 and K and comparison with members of the genus *Comamonas*

All strains were positive for propionate, lactate but negative for glucose, arabinose, fructose, galactose, xylose, mannitol, malonate, tartrate and *p*-aminobenzoate. W, Weak; NR, not reported.

Character	Strains 23310^T, 6 & K	*C. terrigena**	*C. testosteroni*†
Nitrate to N_2	+	−	−
Growth on:			
Acetate	+	+	±
Gluconate	−	+	+
Butyrate	+	+	NR
Caproate	+	−	NR
i-Butyrate	+	+	NR
i-Valerate	+	+	NR
n-Valerate	+	+	NR
Maleate	W	W	NR
Pyruvate	−	+	NR
Alanine	+	+	−
Citrate	−	−	+
Benzoate	+	−	+
L-Phenylalanine	+	−	−
Ethanol	+	NR	−

* Data from De Vos *et al.* (1985).

† Data from Tamaoka *et al.* (1987) and Patureau *et al.* (1998).

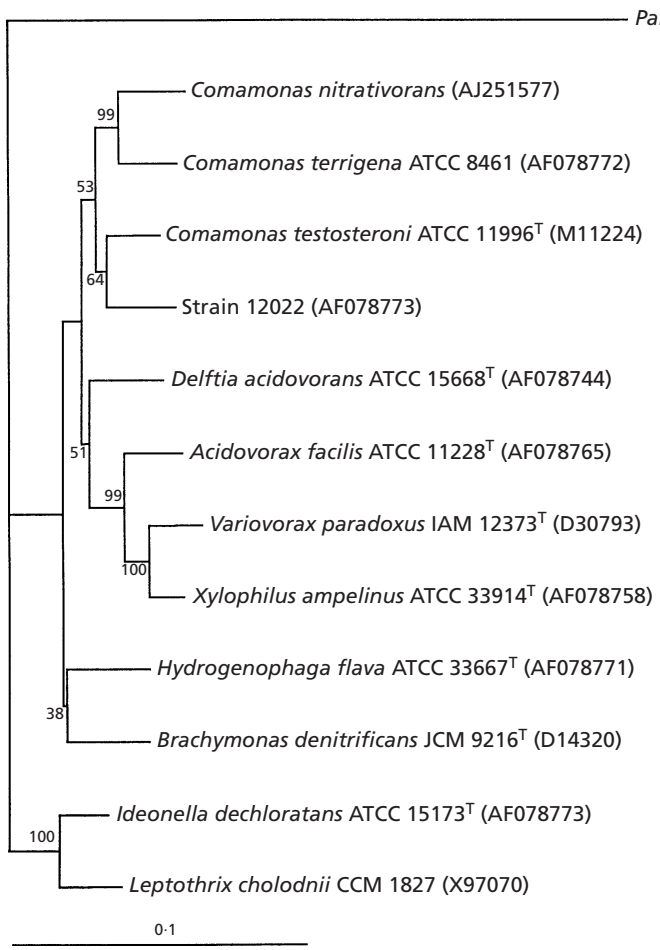

Fig. 4. Phylogenetic dendrogram derived from 16S rDNA sequence analysis, showing the position of strain 23310^T within the genus *Comamonas* in the family *Comamonadaceae*. Numbers at branches represent bootstrap values (percentages; 100 replicates). The sequence from *Paracoccus versutus* was used as outgroup. The bar corresponds to an evolutionary distance of 0·1.

Phylogenetic analysis

A total of 1498 nt of the 16S rDNA of strain 23310^T was sequenced. Comparison with rDNA sequences available in databases revealed that strain 23310^T was related to sequences belonging to the genus *Comamonas* in the β-subclass of the *Proteobacteria*. An evolutionary distance phylogenetic dendrogram (Jukes & Cantor) was constructed with the neighbour-joining method using 1405 unambiguously aligned positions. 16S rDNA similarity values between the sequence of strain 23310^T and the sequences included in the analysis were 95·8 % for *Comamonas terrigena*, ATCC 8451^T, 94·5 % for *Comamonas testosteroni* ATCC 11996^T and 94·4 % for strain 12022. According to the phylogenetic analysis, strain 23310^T and *C. terrigena* are in the same branch supported by a high bootstrap value of 99 % (Fig. 4).

DISCUSSION

Strains 23310^T, K and 6 were isolated at different times during the operation of the denitrifying reactor. They shared the same ARDRA profile (Fig. 1) and the same substrate range and showed similar specific denitrifying rates. These results suggest that the three strains may be different isolates belonging to the same species, which persisted in the reactor, and probably play an important role in this denitrifying ecosystem. However, further studies are needed to demonstrate if the three strains are a single species. Commercial identification kits failed to identify these environmental strains, probably due to their physiological incapacity to grow on sugars.

The 16S rDNA phylogenetic analysis clearly showed the position of strain 23310^T, within the family *Comamonadaceae*, in the genus *Comamonas* (Fig. 4). Recently, a report about the phylogenetic relationships among members of the *Comamonadaceae* showed that *C. testosteroni* and *C. terrigena* form a deeply branched cluster and that *Comamonas acidovorans* (now *Delftia acidovorans*) occurs on a very deep branch and cannot be considered a member of the genus *Comamonas* (Wen *et al.*, 1999). According to this, the genus *Comamonas* is represented by only two species, *C. terrigena* and *C. testosteroni* and by the as yet unidentified strain *Comamonas* sp. strain 12022 (Koivula & Hantula, 1997). A third species, *Comamonas denitrificans*, has now been described by Gumaelius *et al.* (2001). The similarity values between the sequence of strain 23310^T and the sequences belonging to *C. terrigena* and *C. testosteroni* demonstrate that it represents a new species in this genus.

Several physiological differences between strain 23310^T and the other representatives of the genus *Comamonas* confirm this result. Strain 23310^T showed the ability to denitrify in BCY acetate-nitrate, accumulating N_2O during growth with acetate and nitrate in the presence of acetylene, an inhibitor of the last step of denitrification. This trait was confirmed by the high specific rate of N_2O production by resting cells, compared to the data reported by Mahne & Tiedje (1995), and by the wide range of substrates utilized under denitrifying conditions. Neither *C. terrigena* nor *C. testosteroni* show this capacity (Wen *et al.*, 1999). Currently, the only denitrifying micro-organisms described within the family *Comamonadaceae* are *Brachymonas denitrificans* (Hiraishi *et al.*, 1995), phylogenetically distant from strain 23310^T (Fig. 4), and *C. denitrificans* (Gumaelius *et al.*, 2001).

Recently, it has been shown that some organisms can reduce oxygen and nitrate simultaneously up to oxygen-saturated conditions (Robertson *et al.*, 1988, 1989, 1995; Patureau *et al.*, 1998). However, no co-respiration of nitrate and oxygen was detected for strain 23310^T. This trait has been shown for *Paracoccus pantotropha* (Robertson & Kuenen, 1984; Rainey *et al.*, 1999), *Microvirgula aerodenitrificans* (Patureau *et al.*, 1998) and the recently characterized *Thauera mechernichensis* (Scholten *et al.*, 1999) which were isolated from denitrifying environments exposed to oxygen. Strain 23310^T was isolated from a reactor maintained under anoxic conditions for a long time; probably the property of aerobic denitrification was not selected in this reactor.

Strain 23310^T, like *C. terrigena* and *C. testosteroni*, presented only one type of quinone, ubiquinone Q-8, as opposed to *Brachymonas denitrificans* which presented both ubiquinone Q-8 and rhodoquinone RQ-8 (Hiraishi *et al.*, 1995).

Strain 23310^T shared the same polar flagella morphology, optimum temperature and pH with *C. terrigena* (De Vos *et al.*, 1985), but several differences were observed in the substrate range (Table 1). Differences in substrate utilization between strain 23310^T and members of the genus *Comamonas* were found for gluconate, pyruvate, alanine, citrate, benzoate, phenylalanine and ethanol. Several substrates were also utilized, coupled to nitrate reduction under denitrifying conditions, by strains 23310^T, K and 6. This result confirms the ability of these strains to perform respiratory denitrification with different substrates. According to this we can hypothesize that these strains were selected in the reactor because of the ability to couple oxidation of substrates present in the leachate with nitrate reduction, thus playing an important role in the elimination of nitrate and organic compounds.

According to the physiological and phylogenetic characteristics of strain 23310^T, we propose the creation of a new species within the genus *Comamonas* for which the name *Comamonas nitrativorans* is proposed.

Strain 23310^T was deposited at the DSMZ as strain DSM 13191^T, at the NCCB (Netherlands Culture Collection of Bacteria) as NCCB 100007^T and at the CCT (Coleçao de Culturas Tropical, Brazil) as CCT 7062^T. The 16S rDNA sequence was deposited at EMBL (AJ251577). During the course of this work, the sequences of strain 110 (AF233876) and the closely related sequences of strains P17 (AF233880), 5.38g (AF233879), 2.99g (AF233878) and 123^T (AF233877) were also deposited at EMBL (see Gumaelius *et al.*, 2001). As these sequences showed high similarity values to the sequence of strain 23310^T (97% in a BLAST search), further phenotypic characterization is needed to determine their relatedness to 23310^T, 6 and K. Moreover, DNA–DNA hybridization or another genetic techniques, such as repetitive extragenic palindromic (REP)-PCR, randomly amplified polymorphic DNA (RAPD) or ARDRA, of the 16S rDNA, ITS and part of the 23S rDNA are needed to determine if all the strains belong to the same species.

Description of *Comamonas nitrativorans* sp. nov.

Comamonas nitrativorans (ni.tra.ti.vo′rans. N.L. *nitras* nitrate; L. adj. part. *vorans* devouring, digesting; N.L. adj *nitrativorans* nitrate-consuming).

Gram-negative, curved rod-shaped cells, occurring singly or in pairs. Very motile, with two tufts of polar flagella. Oxidase-positive, catalase-positive. Colonies in TSA medium (24 h) are cream-coloured, circular, 1–2 mm diameter. Aerobic and chemo-organotrophic metabolism, can grow on acetate, butyrate, n-caproate, i-butyrate, i-valerate, propionate, n-valerate, lactate, alanine, benzoate, phenylalanine and ethanol. No growth is observed on sugars. Anoxic reduction of nitrate, nitrite and nitrous oxide to nitrogen. Rate of nitrogen gas production is 95 μmol N_2O (g protein)$^{-1}$ min^{-1} on acetate. Optimum pH and temperature are 7 and 30 °C, respectively. Phylogenetically, closely related to *C. terrigena* within the family *Comamonadaceae* in the *β*-subclass of the *Proteobacteria*. Isolated from a denitrifying reactor from a landfill leachate treatment system in Montevideo, Uruguay. Type strain is 23310^T (= DSM 13191^T = NCCB 100007^T = CCT 7062^T).

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