***Comamonas denitrificans* sp. nov., an efficient denitrifying bacterium isolated from activated sludge**

Department of
Biotechnology, Royal
Institute of Technology,
KTH, S-100 44 Stockholm,
Sweden

Lena Gumaelius,† Gunnar Magnusson,† Bertil Pettersson
and Gunnel Dalhammar

Author for correspondence: Gunnel Dalhammar. Tel: +46 8 790 87 75. Fax: +46 8 790 93 06.
e-mail: Gunnel@biochem.kth.se

To find a biomarker for denitrification in activated sludge, five denitrifying strains isolated from three wastewater treatment plants were studied. These strains were selected from among 1500 isolates for their excellent denitrifying properties. They denitrify quickly and have no lag phase when switching from aerobic to anoxic conditions. All strains have the *cd₁*-type of nitrite reductase. The strains are Gram-negative rods and they all grow as filamentous chains when cultivated in liquid solution. The strains differ in colony morphology when grown on nutrient agar. Almost full-length 16S rDNA sequences were determined and phylogenetic analysis revealed that these strains are positioned among members of the genus *Comamonas* in the β -subclass of the *Proteobacteria*. Signature nucleotides and bootstrap percentages were also analysed to verify this position. Strains 110, 123^T, 2.99g, 5.38g and P17 were $\leq 96.7\%$ similar to known strains, but $\geq 99.7\%$ similar to each other, as judged from their 16S rDNA sequences, and grouped tightly together in the phylogenetic tree. Sequence motifs in the 16S rRNA gene were also found, suggesting the monophyletic origin of these strains. Nevertheless, some strains differed from the others, for example strain 110 branches early from the other strains and 5.38g is phenotypically more inert. Therefore, it is proposed that strains 110, 123^T, 2.99g and P17 are classified into a new species, *Comamonas denitrificans* sp. nov., while the taxonomic status of strain 5.38g will have to await the outcome of further studies. The type strain of *Comamonas denitrificans* is 123^T (ATCC 700936^T).

Keywords: *Comamonas denitrificans*, denitrification, 16S rRNA, phylogeny, activated sludge

INTRODUCTION

Denitrification is a major process in the global nitrogen cycle. During recent decades this process has been used as a method for biological nitrogen removal in wastewater treatment. This is to prevent eutrophication in the recipient [SNV (Svenska Naturvårdsverket, Swedish Agency for Environmental Protection), 1997]. The ability to denitrify is considered to be a common property in the bacterial flora of activated sludge,

though it is not yet clear what proportion of the bacterial flora possess this quality. It has been suggested that 10–70% of the cultivable heterotrophic flora establish denitrifying properties (Hallin *et al.*, 1996; Lemmer *et al.*, 1994; von Schmider & Ottow, 1986). In the literature, little effort has been put into the question of whether a large number of different species perform the bulk of the denitrification or if a low number of efficient denitrifiers are important for the overall process. One hypothesis is that a restricted number of bacterial species perform the bulk of the denitrification in wastewater treatment, using biological nitrogen removal (Magnusson *et al.*, 1998).

The overall aim of this study was to identify and characterize a relevant biomarker for denitrification in

†These authors contributed equally to this work.

The GenBank accession numbers for the 16S rRNA gene sequences of strains 110, 123^T, 2.99g, 5.38g and P17 are AF233876, AF233877, AF233878, AF233879 and AF233880, respectively.

Table 1. Physiological characters and phenotypic tests for denitrifying strains and *Comamonas terrigena*

All strains were sensitive to chloramphenicol (30 µg), erythromycin (15 µg) and streptomycin (30 µg) and showed a negative reaction for mannoic acid γ -lactone, L-arabinose, D-xylose, D-galactose, maltose, D-cellobiose, D-trehalose, palatinose, sucrose, D-lactose, melibiose, lactulose, β -gentiobiose, D-melezitose, L-raffinose, inosine, adonitol, *meso*-inositol, D-arabitol, glycerol, maltitol, D-sorbitol, dulcitol, L-sorbose, 2-deoxy-D-ribose, L-rhamnose, D-fucose, L-fucose, D-tagatose, D-amygdalin, arbutin, methyl- β -D-galactopyranoside, 5-keto-D-gluconate, D-gluconate, 6-O- α -D-galactopyranosyl-D-gluconic acid, D-galactonic acid γ -lactone, D-ribose, L-xylose, D-glucose, D-mannose, L-arabitol, *meso*-erythritol, D-mannitol, xylitol, D-fructose, 6-deoxy-D-galactose, 2'-deoxyinosine, inulin, methyl- α -D-mannopyranoside, methyl- α -D-xylopyranoside, methyl- α -D-galactopyranoside, starch, D-galacturonate, D-arabinose, D-turanose, D-glucuronolactone, glycogen, D-lyxose, *N*-acetyl-D-glucosamine, maltose, D-gluconate, caprate, adipate, maleate, phenylacetate, gelatin, *p*-nitrophenyl- β -D-galactopyranoside, L-tryptophan and arabic acid. S, Sensitive; R, resistant; +, significant reaction calculated as 3 standard deviations; (+), reaction calculated as 2–3 standard deviations.

Character	110	123 ^T	5.38g	2.99g	P17	<i>Comamonas terrigena</i>
Nitrate reduced to N ₂	+	+	+	+	+	–
Pigment producer	–	–	–	–	+	–
Denitrification rate [mg nitrite (g bacteria) ⁻¹ min ⁻¹]	1.14 <i>s</i> = 0.15	0.95 <i>s</i> = 0.39	0.39 <i>s</i> = 0.17	0.54 <i>s</i> = 0.12	0.18 <i>s</i> = 0.065	– ND
Optimum pH	7.5*	ND	ND	ND	ND	ND
Optimum temperature	30 °C	30 °C	30 °C	30 °C	30 °C	ND
G + C (mol %)	60.4	60.8	60.3	60.5	60.5	65.2
Reaction to antimicrobial agents (amount per disk):						
Rifampicin (5 µg)	R	R	S	S	R	S
Sulfisoxazole (250 µg)	R	S	S	R	S	R
Tetracycline (30 µg)	S	S	S	S	S	R
Ampicillin (10 µg)	S	S	S	S	S	R
Penicillin G (10 µg)	R	S	S	S	S	S
Carbohydrate metabolism:						
Salicin	(+)	+	–	+	(+)	+
Citrate	+	+	(+)	+	–	+
Fumarate	+	+	+	+	+	+
L-Malinate	+	+	+	+	+	+
Malonate	+	+	(+)	+	(+)	+
Pyruvate	+	+	(+)	+	+	+
L-Tartarate	+	+	(+)	+	(+)	+
Urea	–	(+)	–	(+)	–	+
L-Ornithine	–	–	–	–	–	(+)
<i>m</i> -Hydroxybenzoate	+	+	–	(+)	–	+
<i>trans</i> -Aconitate	(+)	–	–	–	–	+
D-Glucuronate	+	+	–	+	(+)	+
Glycolate	+	+	–	+	+	+
D- β -Hydroxybutyrate	+	+	–	+	+	+
α -Ketovaleate	+	+	–	+	+	+
L-Lactate	+	+	–	+	+	+
Maleinate	(+)	–	–	–	–	+
D-Saccharate	+	(+)	–	+	(+)	+
Succinate	+	(+)	–	+	+	+
D-Tartarate	+	–	–	+	(+)	+
Gentisate	(+)	(+)	–	(+)	–	+
<i>p</i> -Coumarate	(+)	–	–	–	–	+
Hippurate	+	(+)	–	(+)	–	+
DL-2- γ -Aminobutyrate	+	(+)	–	+	–	+
L-Alanine	+	(+)	–	+	(+)	+
L-Arginine	+	(+)	–	(+)	(+)	+
L-Glutamate	+	+	–	+	+	+
L-Lysine	+	+	–	+	+	+
L-Serine	+	–	–	(+)	–	+
L-Histidine	–	–	–	–	–	(+)
Aesculin	(+)	–	–	(+)	–	+

* Data from Gumaelius *et al.* (1996).

activated sludge which could be used in biosensors analysing inhibition of denitrification (Gumaelius *et al.*, 1996) or denitrification status. About 1500 bacterial strains were isolated from five different wastewater treatment plants of which only efficient denitrifying strains were selected for partial 16S rDNA analysis. Five of these strains, 110, 5.38g, 123^T, P17

and 2.99g, possessed high sequence similarity. These strains included the most efficient denitrifiers of the selection and had no lag phase when switching from oxygen respiration to nitrite respiration. Considering this, these five strains were regarded as relevant potential biomarkers and for this purpose the five strains were further characterized.

Fig. 1. A strain 110 bacterium picked from an overnight culture on nutrient agar. Flagella have been stained according to Gray (Gerhardt *et al.*, 1981). Bar, 1 μ m.

METHODS

Source of strains. The strains were all isolated from activated sludge in municipal wastewater treatment plants. Strains 110 and 123^T were isolated from Gustavsberg (Stockholm, Sweden), P17 from Písek (Slovakia), and 2.99g and 5.38g from Käppala treatment plant (Stockholm, Sweden). The wastewater treatment plants reported biological nitrogen removal. *Comamonas terrigena* (IAM 12052) was used as reference throughout the study.

Morphological analysis. Morphological features were studied using a phase-contrast microscope (Olympus BH-2). Photographs were made using an Olympus SC36 type 12 camera connected to the microscope. PJ800 or PJC1600 photographic films (Kodak) were used. Gram staining and flagella staining were performed using Gray's method. Staining of polysaccharides was done using Alcian blue and staining of inclusion bodies (polyhydroxyalkanoate; PHA) with Sudan black (Gerhardt *et al.*, 1981).

Physiological tests. Oxidase and catalase activities were analysed according to Gerhardt *et al.* (1981) and antibiotic sensitivity tests were performed using PDM Antibiotic Sensitivity Discs 2 (AB Biodisk) on nutrient agar plates (Difco). Saline tolerance was investigated using nutrient broth medium and nutrient agar (Difco) with the addition of NaCl.

Amplification of the nitrite reductase gene. For determination of nitrite reductase type a PCR-based method developed by Braker *et al.* (1998) was used. Primers used were either nirS1F and nirS6R for detection of the cd₁-type nitrite reductase or nirK1F and nirK5R for the Cu-type nitrite reductase. PCR reactions were run with 5 pmol of each primer. The program used was a three temperature cycling program that ran for 30 cycles starting with denaturation at 96 °C for 30 s followed by annealing at 41 °C for 40 s and extension at 72 °C for 40 s. Finally, a 10 min incubation was performed at 72 °C. *Pseudomonas stutzeri* (ATCC 14405) was used as positive control and *Alcaligenes faecalis* (ATCC 8750^T) was used as negative control.

Determination of the denitrification rate. The denitrification rate was measured as nitrite consumed over time. The method of Gumaelius *et al.* (1996) was used with some small modifications. Bacteria were added on a wet mass basis, 3.3 g bacteria per litre nutrient broth medium. Experiments

were performed in microtitre plates where each well contained 250 μ l bacterial suspension. Nitrite was added as NaNO₂ at a concentration of 50 mg nitrite per litre. The rate experiments were repeated 6–14 times for each species.

Biochemical characterization. Biochemical characteristics were analysed by using the API 20NE kit (BioMérieux) and the Phene Plate system (PhP; BioSys Inova). PhP is a miniaturized metabolic fingerprinting system designed to reveal bacterial strains at subspecies level.

Bacterial metabolism of various substrates was quantitatively measured via a pH indicator, thus creating a biochemical fingerprint (Möller *et al.*, 1993; Kühn *et al.*, 1991; Katouli *et al.*, 1997). The studied strains, 110, 123^T, 2.99g, 5.38g and P17, and *Comamonas terrigena* IAM 12052 were inoculated in duplicate into PhP microtitre plates, according to the manufacturer's instructions. Both PhP-48 and PhP-EX plates were used. Consequently, metabolic activity with 102 different carbon sources was investigated (see Table 1). Incubations were performed at 30 °C for 100 h and absorbance was measured with an Easy Reader EAR 340AT (SLT-Labinstruments). The spectrophotometer was engineered using the PhP system software, version 1.12 (BioSys Inova). Only statistically significant reactions, at the 99.7% level, were interpreted as positive. The significance level was calculated as 3 standard deviations from a mean value based on samples taken at time zero and reference samples. Results close to this level, between 2 and 3 standard deviations, were reported as (+).

Determination of the G+C content. Genomic DNA was prepared according to the procedure of Wilson (1987). The HPLC technique (Mesbah *et al.*, 1989) was used for the determination of the G+C content of strains 110, 123^T, 2.99g, 5.38g, P17 and the control strain, *Comamonas terrigena*.

In vitro amplification of the 16S rRNA gene. The 16S rRNA genes were amplified directly from the bacterial genome in a semi-nested fashion (Pettersson, 1997). The first amplification of the 16S rRNA gene was performed with primers complementary to the regions close to the 5' and 3' termini of the gene. Freshly cultivated bacterial cells were added to the PCR mix. Amplification was performed using primers 605 and 621 (Pettersson, 1997). The PCR products were used as template in two subsequent and different reactions. The latter amplifications were performed using primer pairs 605/614B and 603/621B (Pettersson, 1997). This resulted in two different biotinylated PCR products, suitable for solid-phase DNA sequencing. The primer pair 605 and 614B generated a fragment with an approximate length of 900 bp and primers 603 and 621B gave a PCR product of about 1200 bp. These two products overlap by almost 600 nt. The PCRs were performed with 5 pmol of each primer for 30 cycles. The profile for each cycle was denaturation at 96 °C for 15 s, annealing at 65 °C for 30 s and extension at 72 °C for 60 s. Finally, a 10 min incubation was performed at 72 °C.

Automated solid-phase 16S rDNA sequencing. Automated solid-phase DNA sequencing with an Automated Laser Fluorescence System (Pharmacia Biotech) was performed on each strand after separation with streptavidin-coated super paramagnetic beads (Dynabeads M-280 Streptavidin; Dynal). The sequences were determined in both directions by analysis of both the immobilized and eluted strands (Hultman *et al.*, 1991). Sequencing primers used were USP, 622F, 596F, 585F, 538F and 597F (Pettersson, 1997).

Fig. 2. Growth of strain 123^T in nutrient broth under aerobic conditions. Samples were taken at 1 (a), 2 (b) or 4 h (c) after inoculation. Bar, 5 µm.

Phylogenetic analysis. The sequences generated in this work were all searched against GenBank using the advanced gapped BLAST option (Altschul *et al.*, 1997) at the NCBI home page (<http://www.ncbi.edu>). Selected sequences were retrieved and aligned manually to a set of operational taxonomic units (OTUs), which were downloaded from the Ribosomal Database Project (Maidak *et al.*, 1999; Ribosomal Database Project RDP-II, 1999). The alignment was performed using the Genetic Data Environment (GDE) (Smith, 1992). A secondary structure of the 16S rRNA molecule of *Escherichia coli* was used to facilitate identification of stems and loops in the alignment procedure. Phylogenetic trees were inferred using programs contained in the phylogenetic inference package, PHYLIP version 3.573, (Felsenstein, 1993). Evolutionary distance trees were constructed from distances corrected by the two-parameter model of Kimura (1980) with a transition/transversion ratio set to 2.0 using the neighbour-joining algorithm (Saitou & Nei, 1987). Statistical tests were performed using the bootstrap option (Felsenstein, 1985). The construction of the maximum-likelihood tree was performed with the DNAML program using the F84 model of molecular evolution with a transition/transversion ratio of 2.0 and applying global rearrangement for finding the optimal tree.

Nucleotide accession numbers. The GenBank accession numbers for the 16S rRNA sequences of the reference strains used for comparison in this study are as follows: *Acidovorax avenae* subsp. *avenae* ATCC 19307^T, AB021421; *Acidovorax konjaci* ATCC 33996^T, AF078760; *Acidovorax* sp. strain IMI 357678, AF078763; *Alcaligenes faecalis* ATCC 8750^T, M22508; *Aquaspirillum metamorphum* ATCC 15280^T, AF078757; *Aquaspirillum psychrophilum* ATCC 33335^T,

AF078755; *Bordetella bronchiseptica* S-1, X57026; *Brachymonas denitrificans* JCM 9216^T, D14320; *Comamonas* sp. strain 12022, AF078773; *Comamonas terrigena* ATCC 8461^T, AF078772; *Comamonas terrigena* IAM 12052, AB021418; *Comamonas testosteroni* NCTC 10698^T, M112-24; *Delftia acidovorans* ATCC 15668^T, AF078774; *Hydrogenophaga taeniospiralis* ATCC 49743^T, AF078768; *Hydrogenophaga palleronii* ATCC 17724^T, AF078769; *Ideonella dechloratans* CCUG 30898^T, X72724; *Lautropia mirabilis* NCTC 12852^T, X73223; *Leptothrix discophora* ATCC 51168, L33974; *Polaromonas vacuolata* ATCC 51984^T, U14585; *Rhodoferrax fermentans* JCM 7818^T, D16211; *Roseateles depolymerans* DSM 11813^T, AB003623; *Rubrivivax gelatinosus* ATCC 17011^T, D16213; *Sphaerotilus natans* strain 565, Z18534; *Sutterella wadsworthensis* ATCC 51579^T, L37786; *Variovorax paradoxus* MBIC 3839, AB008000; *Xylophilus ampelinus* ATCC 33914^T, AF078758; isolate rA3, AB021355; isolate rA6, AB021357.

RESULTS AND DISCUSSION

Morphology and growth rate

All strains appeared as Gram-negative rods when grown on nutrient agar plates. Several flagella were present attached at the polar ends (Fig. 1) and all strains were motile. These features are in good agreement with an earlier morphological description of the *Comamonas* genus (Wen *et al.*, 1999). A thick layer of polysaccharide is produced around the cell chains. Inclusion bodies are also produced in liquid culture.

Table 2. Differentiation of the three *Comamonas* species

+, Present in all strains; (+), present in 90–99 % of the strains; D, present in 11–89 % of the strains; (–), absent in 90–99 % of the strains; –, absent in all strains; S, sensitive; R, resistant.

Characteristic	<i>Comamonas denitrificans</i> *	<i>Comamonas testosteroni</i> †	<i>Comamonas terrigena</i> †
Production of inclusion bodies containing PHA	+	ND	ND
Growth on:			
Glycerol	–	D	(–)
5-Keto-D-gluconate	–	(–)	–
Citrate	+	+	(–)
Pyruvate	+	D	D
<i>m</i> -Hydroxybenzoate	D	+	D
Aconitate	D	D	D
Glycolate	+	+	(–)
L-Alanine	+	D	D
L-Arginine	+	–	(–)
L-Serine	D	(–)	–
L-Histidine	–	+	(–)
D-Gluconate	–	+	D
Caprate	–	D	D
Sensitivity to penicillin	S	R	D
Nitrate reduction	+	D	(+)
Nitrite reduction	+	–	(–)
G + C content (mol %)	60.3–60.8	62.5–64.5	59.7–66.7

* As determined in this study for the strains 110 (ATCC 700937) 123^T (ATCC 700936^T), 2.99g (ATCC 700940) and P17 (ATCC 700939).

† Data from Willems *et al.* (1991).

The results obtained after staining with Sudan black suggested the content to be PHA. This sort of inclusion body has been reported for *Comamonas acidovorans* (Sudesh *et al.*, 1998), later renamed as *Delftia acidovorans* (Wen *et al.*, 1999). When cultivated aerobically in liquid nutrient broth medium, long chains of filamentous clusters were formed for all strains (Fig. 2).

When grown on nutrient agar, all strains formed yellow-white colonies, although strain P17 produced a pigment that made the agar brownish and bacterium 5.38g formed dryer colonies that adhered more to the agar than the other strains.

Physiological analysis

The strains were oxidase and catalase-positive. Bacterial growth was detected at 20, 30 and 37 °C but not at 4 °C. Growth was also detected on nutrient agar with 2 % NaCl. All strains survived in 5 % NaCl, but not in 9 % NaCl. Eight different antimicrobial agents were tested (Table 1). Strain 110 was resistant to three of the eight agents, whereas the others were resistant to only one or none. Three out of five strains showed no sensitivity towards rifampicin.

Characteristics of denitrification

Nitrite reductase is the first enzyme in the denitrification process, reducing nitrite to NO. Two types of nitrite reductase have been found so far. One containing a copper centre (NirK) and one containing haems c and d₁ (NirS). It is not yet established how these enzymes are distributed among taxonomic families; however, they are both regarded to be widespread (Zumft, 1997). The two types seems to be mutually exclusive in a given species, but both types have been found at the genus level (Coyne *et al.*, 1989). Among the denitrifiers studied all five strains contained the cd₁-type of nitrite reductase, even though the strains were isolated at different locations.

Denitrification rates are reported in Table 1. Strains 110 and 123^T showed higher denitrification rates than the others.

Biochemical characterization

The strains studied were tested for a total of 102 carbon sources in the API 20 NE kit and in the PhP system. Results are presented in Table 1. Only 29 of the compounds gave a positive reaction for any of the strains tested. Out of these 29, 5 were exclusively positive for *Comamonas terrigena*. This bacterium was

Fig. 3. A phylogenetic tree derived by neighbour-joining (Saitou & Nei, 1987) showing the position of strains 110, 123^T, 2.99g, 5.38g and P17 within the *Comamonas* species group. A member of the β -subclass of the *Proteobacteria*, *Alcaligenes faecalis* ATCC 8750^T, served as outgroup. Statistical support for the topology was obtained from bootstrap analysis performed by 500 resamplings of the data set and by using maximum-likelihood (indicated by percentage values and ML, respectively, at the nodes regarded as relevant for this study). The *Comamonas* species group is indicated by a vertical bar. The scale bar denotes the number of substitutions per nucleotide position.

shown to be the most metabolically active of the strains. Strains 110, 2.99g and 123^T showed similar patterns with 15–22 carbon sources. Strain P17 showed significant activity with only 10 carbon sources, while strain 5.38g showed significant metabolic activity with only two tested carbon sources.

Determination of G + C content

The strains tested were shown to be very similar in their G + C content, between 60.3 and 60.8 mol%, compared to *Comamonas terrigena* with a value of 65.2 mol%. The values of the individual strains, a mean of three independent analyses of the same DNA sample, are presented in Table 1. These similarities further strengthen the idea that the strains belong to the same species. A compilation of physiological features for the three species of the genus *Comamonas* is shown in Table 2. Results not evaluated in this study have been collected from Willems *et al.*, (1991).

Phylogenetic analysis

Nucleotide sequences of the 16S rRNA gene were determined for strains 110, 123^T, 2.99g, 5.38g and P17. The obtained primary structures were 1443, 1442, 1446, 1444 and 1441 bp in length, respectively. Unambiguous sequences were obtained by applying the direct solid-phase 16S rDNA sequencing technique using both strands (Hultman *et al.*, 1991). The sequences differed slightly from each other with 16S

rDNA sequence similarity values ranging between 99.7 and 99.9% (not shown), with strain 110 being the most divergent. The resulting sequences were checked for their uniqueness against deposited data in GenBank and the 100 best hits, including those for non-cultivated and non-taxonomically characterized organisms, were retrieved and merged into the alignment.

Different data sets were generated, correcting for gaps and ambiguously aligned positions; sets without any position removed were used to establish evolutionary relationships. Moreover, changes in the number of OTUs and outgroups were determined. A representative tree inferred by neighbour-joining is presented in Fig. 3 and its overall topology resembles that published previously by Wen *et al.* (1999). *Alcaligenes faecalis* served as outgroup. The tree was inferred from a data set only consisting of nucleotide positions having a conserved composition in more than 50% of the used sequences. Statistical testing of the stability of the nodes was performed by bootstrap analysis by re-sampling the data set 500 times. Also, the maximum-likelihood method resulted in a topology essentially congruent with that produced by neighbour-joining (Fig. 3). The obtained bootstrap value of 57% showed that the phylogenetic grouping of *Comamonas* species shown in Fig. 3 has relatively low statistical support. Despite the relatively low bootstrap value for the *Comamonas* species group, a maximum-likelihood tree supported this phylogenetic entity (not shown). Shifts of the internal branching order within some of the clades could be observed, but this was not further

studied in this work. Strains 110, 123^T, 2.99g, 5.38g and P17 grouped tightly together and with species of the genus *Comamonas*. The *Comamonas* species group has been indicated in Fig. 3. Strain 110 formed an early branch of the subclade containing denitrifying strains in all trees (Fig. 3). Interestingly, the closest neighbour was found to be a non-characterized isolate from the aeration tank of an activated sludge process in Japan (Watanabe *et al.*, 1999), sharing 16S rDNA sequence similarity of 96.6–96.7% (not shown). Furthermore, the five strains in this study were shown to be 94.5–95.6% (not shown) similar to other members of the *Comamonas* species group, thus falling below the upper and general limit of 97% used as a cut-off value for species delineation (Stackebrandt & Goebel, 1994).

As noted above, the *Comamonas* species group was supported by only moderate bootstrap values. However, by removing *Brachymonas denitrificans* from the data set, the tree in Fig. 3 gained a marked increase in the node stability of the *Comamonas* branch, raising the bootstrap support value from 57% to 68% (not shown). This reflects that the 16S rDNA sequences of the members of the family *Comamonadaceae* have a somewhat low resolving power. Nevertheless, we believe that strains 110, 123^T, 2.99g, 5.38g and P17 have been placed in the *Comamonas* species group with a high cladistic confidence. Besides sharing an ancestral branch in all computed trees, the members of this group showed a unique base pair composition, G·C_(603·635), in the resulting 16S rRNA molecule (*Escherichia coli* numbering; Brosius *et al.*, 1978). This feature is generally not seen among taxa of the β - and γ -*Proteobacteria*, thus being idiosyncratic for the *Comamonas* species group.

Nucleotide motifs of the 16S rRNA gene were also found for strains 110, 123^T, 2.99g, 5.38g and P17. Totalling four, the positions of these attributes could be regarded as two adjacently positioned canonical base pairs in the matured 16S rRNA molecule. The composition of the actual pairs are U·A_(953·1228) and C·G_(954·1226) (position 1227 is a non-paired unilaterally bulged residue). These nucleotide compositions are commonly found in the domain *Bacteria*, but not in the family *Comamonadaceae* and have not been found in the domain *Archaea*. The pair U·A_(953·1228) was shared with *Streptosporangium* and relatives, class *Mollicutes* (except for the hominis and the spiroplasma phylogenetic groups), *Aeromicrobium erythreum* subgroup (three species) and *Brachybacterium faecium* subgroup (four species). Only species belonging to the genus *Rubrobacter* (two species) showed identical composition in both these pairs. As well as the species of these phylogenetic entities, exceptions were only found in a few other 16S rDNA sequences. Consequently, it is likely that the denitrifying strains 110, 123^T, 2.99g, 5.38g and P17 are detected to the exclusion of most other prokaryotes by the construction of probes harbouring the sequence motifs 5'-GATG-ATGTTCTTTAATTC-3' and/or 5'-GGCTAGAAA-CGTC-3'.

Taxonomic considerations

As judged from the morphology, physiology, phenotypic tests and genetic analysis based 16S rDNA and the G+C content, strains 110, 5.38g, 123^T, P17 and 2.99g can be regarded as constituting a somewhat heterogenic subclade within the *Comamonas* species group. The most divergent strains were 110 and 5.38g. Strain 110 formed an early branch of the actual subclade in all derived trees and it also showed a slightly altered physiological response to the eight antimicrobial agents used for testing. Strain 5.38g differed from the others by forming dryer colonies and showed a marked difference in biochemical features as judged by phenotypic testing. Despite being closely related with regard to 16S rRNA gene similarity (Fig. 3) we stress that the taxonomy of strain 5.38g should await further genetic data such as DNA–DNA re-association, PFGE, amplified fragment length polymorphism (AFLP) and enterobacterial repetitive intergenic consensus (ERIC)-, BoxA subunit (BOX)-, repetitive extragenic palindromic (REP)- and/or randomly amplified polymorphic DNA (RAPD)-PCR. Preferably, these studies should be performed including more isolates of this group to discern the range of heterogeneity among the members of these denitrifying strains. Therefore, we propose that strains 110, 123^T, P17 and 2.99g should be classified in a new species for which we propose the name *Comamonas denitrificans* sp. nov. The type strain of *Comamonas denitrificans* is 123^T (= ATCC 700936^T).

Description of *Comamonas denitrificans* sp. nov.

Comamonas denitrificans (de.ni.tri'fi.cans. L. prep. de away from; L. n. *nitrum* soda; N.L. n. *nitrum* nitrate; M.L. v. *denitrifico* to denitrify; N.L. part. adj. *denitrificans* denitrifying).

Straight or slightly curved rods, 2–6 μ m long and 1–2 μ m wide. When grown on nutrient agar plates appear as single cells or as filaments, motile by means of polar flagella, form yellow-white colonies and strain P17 produces a brownish pigment. Gram-negative, oxidase- and catalase-positive. Grow at 20, 30 and 37 °C but not at 4 °C. Reduce nitrate to nitrogen gas (the only species in the genus *Comamonas* to do so) and contain cd₁-type nitrite reductase. All strains utilize fumarate, L-malonate, pyruvate, glycolate, D- β -hydroxybutyrate, α -ketovalerate, L-lactate, L-glutamate and L-lysine. Grow in 2% saline solution and survive in 5% NaCl, but not in 9% NaCl. Three of the four strains are rifampicin-tolerant. Found in activated sludge with biological nitrogen removal properties. The DNA G+C content varies between 60.4 and 60.8 mol %. Type strain is 123^T (= ATCC 700936^T).

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