Nitratireductor aquibiodomus gen. nov., sp. nov., a novel α-proteobacterium from the marine denitrification system of the Montreal Biodome (Canada)

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The Montreal Biodome operates a methanol-fed denitrification system that treats the water in its three million litre marine mesocosm. An unknown bacterium, named strain NL21^T, was isolated from this system on TSA and R2A agar. The organism is a Gram-negative, rod-shaped (1 × 3 μm) facultative aerobe. Optimal growth conditions on R2A agar are 30–35 °C, pH 7–7·5 and 1 % (w/w) NaCl. Phylogenetic analysis of the 16S rDNA sequence reveals that strain NL21^T forms a novel lineage in the family '*Phyllobacteriaceae*' within the α2 subgroup of the *Proteobacteria*. The closest related genera are *Aminobacter*, *Pseudaminobacter*, *Mesorhizobium* and *Defluvibacter*. Major cellular fatty acids are $C_{18:1}\omega7c$ (75 %), $C_{19:0}\omega8c$ cyclopropane (9·4 %) and $C_{18:0}$ (4·2 %). The DNA G+C content of strain NL21^T (57 mol%) differs from those of all other described members of the '*Phyllobacteriaceae*' (60–64 mol%). Strain NL21^T reduces nitrate to nitrite, but does not reduce nitrite to nitrogen gas. Only a few sugars and amino acids can serve as carbon sources. Strain NL21^T is able to grow without salt and tolerates up to 5 % NaCl. Phylogenetic analysis, as well as physiological and biochemical tests, showed that strain NL21^T was different from all other members of the '*Phyllobacteriaceae*' with validly published names. Strain NL21^T therefore represents a novel genus, for which the name *Nitratireductor aquibiodomus* gen. nov., sp. nov. is proposed, with the type strain NL21^T (=DSM 15645^T=ATCC BAA-762^T).

Nitrate is a pollutant that accumulates quickly in closed systems, such as marine aquariums. It is usually removed biologically in a denitrification system, where oxygen is replaced by nitrate as an electron acceptor in bacterial respiration. A large variety of bacterial species are able to reduce nitrate to nitrite or to molecular nitrogen [see Zumft (1997) for an exhaustive list]. Many of these species belong to the family '*Phyllobacteriaceae*' of the α-*Proteobacteria*, which comprises the genera *Phyllobacterium* (Knösel, 1984), *Aminobacter* (Urakami *et al.*, 1992), *Mesorhizobium* (Jarvis *et al.*, 1997), *Pseudaminobacter* (Kämpfer *et al.*, 1999), *Defluvibacter* (Fritsche *et al.*, 1999) and *Aquamicrobium* (Bambauer *et al.*, 1998). This family was originally described by Knösel (1984). It may also contain the uncharacterized strain B1G-2, which was isolated by Duncan *et al.* (2001), and the *Mesorhizobium* strain WG, which was isolated from a denitrification process by Costa *et al.* (2000).

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NL21^T is AF534573.

During the characterization of organisms that were isolated from the marine denitrification system of the Montreal Biodome (Labbé *et al.*, 2003), strain NL21^T was recovered on R2A and TSA agar. Sequence analysis of the 16S rRNA gene (rDNA) revealed that this bacterium was related to the family '*Phyllobacteriaceae*'. The goal of the present study was to further characterize strain NL21^T and to classify it within the family '*Phyllobacteriaceae*'.

Gram-staining was performed as described by Gerhardt *et al.* (1994). Cell morphology was observed under a Nikon light microscope at × 1000 with cells that were grown for 3 days at 35 °C. Physiological characteristics were investigated by using API systems: API 50CH strips inoculated with the medium described by Velázquez *et al.* (2001) were used for acid production and sugar assimilation, API 20NE strips were used for biochemical reactions (nitrate and nitrite reduction, urease and indole formation) and the assimilation of selected carbon sources and API ZYM strips were used to examine extracellular enzyme activity. Strips were incubated at 30 °C for 24 h (API ZYM) or 72 h (API 50CH and API 20NE). Strain NL21^T differs from most members of the family '*Phyllobacteriaceae*' by being positive

for citrate assimilation, negative for urease and positive for the presence of *N*-acetyl-β-glucosamidase (Table 1). Other differences in substrate assimilation between NL21^T and other representatives of the family '*Phyllobacteriaceae*' are shown in Table 1.

The fatty acid profile was traced with cells that were grown on TSA agar for 3 days at 35 °C. Analysis was carried out in accordance with the standard protocol of the Microbial Identification system (MIDI; Microbial ID). Major cellular fatty acids of strain NL21^T are $C_{18:1}\omega7c$ (75 %), $C_{19:0}\omega8c$ cyclo (9·4 %) and $C_{18:0}$ (4·2 %). This profile supports the affiliation of strain NL21^T to the '*Phyllobacteriaceae*', in which most species have $C_{18:1}\omega7c$ and $C_{19:0}\omega8c$ cyclo as major components. The fatty acid profile of strain NL21^T (Table 2) differs from those of all species of the genus *Mesorhizobium* (Tighe *et al.*, 2000) because of the absence of 11-methyl $C_{18:1}\omega7c$. The two genera also have different

Table 1. Physiological characteristics of the family '*Phyllobacteriaceae*'

Taxa: 1, strain NL21^T; 2, *Pseudaminobacter salicylatoxidans* DSM 6986^T; 3, *Aminobacter aminovorans* DSM 7048^T; 4, *Phyllobacterium myrsinacearum* LMG 2t2^T; 5, *Mesorhizobium tianshanense* DSM 11417^T; 6, *Defluvibacter lusatiensis* DSM 11099^T [data from Lechner *et al.* (1995) and Fritsche *et al.* (1999)]. All strains are positive for assimilation of D-glucose, D-arabinose, D-fructose, ribose, D-xylose and L-fucose and the presence of alkaline phosphatase, acid phosphatase, leucine arylamidase, cysteine arylamidase, trypsin, naphthol-AS-BI-phosphohydrolase and α-glucosidase. All strains are negative for indole formation, gelatinase, assimilation of caprate, phenylacetate, salicin, inulin, starch and erythritol and the presence of α-galactosidase, β-glucuronidase, α-mannosidase, α-fucosidase and lipase C14. +, Positive reaction; (+), weakly positive reaction; −, negative reaction; V, variable; ND, not determined.

Characteristic	1	2	3	4	5	6
Major fatty acids*	$C_{18:1}\omega7c$, $C_{19:0}\omega8c$ cyclo, $C_{18:0}$	$C_{18:1}\omega7c$, $C_{19:0}\omega8c$ cyclo, $C_{17:0}$	$C_{18:1}\omega7c$, $C_{19:0}\omega8c$ cyclo, $C_{16:0}$	$C_{18:1}\omega7c$, $C_{16:0}$, $C_{16:0}$ 3-OH	$C_{18:1}\omega7c$, $C_{19:0}\omega8c$ cyclo, $C_{16:0}$†	$C_{18:1}\omega7c$, $C_{19:0}\omega8c$ cyclo, $C_{16:0}$
Nitrate reduction	+	+	−	−	+	−
Nitrite reduction	−	−	−	−	+	−
Urease	−	+	+	+	+	−
Aesculin	−	−	−	(+)	+	−
Assimilation of:						
Adipate	(+)	(+)	(+)	−	−	−
Malate	(+)	(+)	−	+	+	v
Citrate	+	−	−	(+)	−	−
Sucrose	−	−	+	+	+	−
Gluconate	−	−	−	−	+	+
Adonitol	−	+	−	+	+	+
Trehalose	−	−	+	+	+	−
D-Xylose	−	+	+	−	v	v
Galactose	−	v	+	(+)	+	−
D-Turanose	−	−	+	+	−	−
Rhamnose	−	−	+	+	+	−
Mannitol	−	+	+	+	+	−
Maltose	−	−	+	+	+	−
Sorbitol	−	+	+	+	+	ND
Extracellular activity of:						
N-Acetyl-β-glucosamidase	+	−	−	−	+	ND
Esterase C4	+	−	+	+	+	ND
Esterase lipase C8	+	−	+	+	+	ND
β-Galactosidase	−	−	−	−	(+)	−
β-Glucosidase	−	−	−	−	+	ND
α-Chymotrypsin	−	−	−	+	−	ND
DNA G+C content (mol%)‡	57	63·9	62·5	60	61	61·4

*Data from Kämpfer *et al.* (1999) for *Pseudaminobacter salicylatoxidans* and *Aminobacter aminovorans*, from Mergaert *et al.* (2002) for *Phyllobacterium myrsinacearum* and from Tighe *et al.* (2000) for *Mesorhizobium tianshanense*.

†Mean values from eight strains, including the type strain.

‡Data from Kämpfer *et al.* (1999) for *Pseudaminobacter salicylatoxidans*, from Urakami *et al.* (1992) for *Aminobacter aminovorans*, from Knösel (1984) for *Phyllobacterium myrsinacearum* and from Chen *et al.* (1995) for *Mesorhizobium tianshanense*.

hydroxy fatty acids. Compared to the genus *Pseudaminobacter*, strain NL21^T has a lower amount of $C_{17:0}$ and $C_{19:0}\omega8c$ cyclo and a higher amount of $C_{18:1}\omega7c$. Compared to the genus *Aminobacter*, strain NL21^T has a higher amount of $C_{18:1}\omega7c$, fewer unknown fatty acids and different hydroxylated fatty acids (Table 2). Compared

Table 2. Fatty acid profiles of bacteria in the family '*Phyllobacteriaceae*'

Taxa: 1, *Nitratireductor aquibiodomus* NL21^T; 2, *Pseudaminobacter salicylatoxidans* DSM 6986^T (data from Kämpfer *et al.*, 1999); 3, *Aminobacter aminovorans* DSM 7048^T (data from Kämpfer *et al.*, 1999); 4, *Phyllobacterium myrsinacearum* LMG 2t2^T (data from Mergaert *et al.*, 2002); 5, *Mesorhizobium tianshanense* (data from Tighe *et al.*, 2000); 6, *Defluvibacter lusatiensis* DSM 11099^T (data from Lechner *et al.*, 1995).

Fatty acid	1	2	3	4	5*	6
Saturated fatty acids:						
$C_{16:0}$	2·16	2·3	4·9	6	12·25	4·33
$C_{17:0}$	1·72	9·1	0·7		0·83	2·08
$C_{18:0}$	4·23	2·6	1·5	2·1	4·99	2·29
$C_{20:0}$				4·4		
Unsaturated fatty acids†:						
$C_{15:1}\omega8c$						
$C_{16:1}\omega7c$						1·22
$C_{17:1}\omega8c$	0·59	3·4	0·7		0·52	2·08
$C_{17:1}\omega6c$	0·54	1·2			0·11	1·01
$C_{18:1}\omega7c$‡	75·01			64·9		
$C_{18:1}\omega9c$	0·61					
$C_{19:1}\omega12t$		1·3				
$C_{20:1}\omega7c$	0·48					
$C_{20:1}\omega9t$		0·6	0·8			1·55
$C_{20:2}\omega6,9c$		0·6				
iso-$C_{15:0}$		1·2			0·16	
iso-$C_{17:0}$	1·93	1·5	1		3·34	
Hydroxy fatty acids:						
$C_{12:0}$ 3-OH			0·3		0·09	0·65
iso-$C_{13:0}$ 3-OH					0·27	
iso-$C_{15:0}$ 3-OH	1·86	0·5				
$C_{16:0}$ 3-OH				5·7		
$C_{18:0}$ 3-OH	0·52					
$C_{18:1}$ 2-OH				5·2		
Cyclopropane acids:						
$C_{17:0}$ cyclo			0·5		0·16	
$C_{19:0}\omega8c$ cyclo	9·35	25·4	13·7	4·8	12·37	3·92
Other:						
10-Methyl $C_{19:0}$					0·16	0·4
11-Methyl $C_{18:1}\omega8c$					9·99	
Summed feature 2§				4·7		
Summed feature 3	0·56	0·4	0·5	2·3	1·42	
Summed feature 5	0·42					
Summed feature 7		43·4	46·4		53·22	80·48
Unknown	1	6·7	26·4		0·11	

*Mean values from eight strains, including the type strain.

†For unsaturated fatty acids, the position of the double bond is located by counting from the methyl (ω) end of the carbon chain. Isomers (*cis* and *trans*) are indicated by the suffixes *c* and *t*, respectively.

‡This unsaturated fatty acid is also included in summed feature 7.

§Summed feature 2 comprises any combination of $C_{12:0}$ aldehyde, $C_{14:0}$ 3-OH and/or iso-$C_{16:1}$. Summed feature 3 comprises any combination of iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$. Summed feature 5 comprises any combination of $C_{18:0}$ ANTE and/or $C_{18:2}\omega6,9c$. Summed feature 7 comprises any combination of $C_{18:1}\omega7c$, $C_{18:1}\omega9t$ and/or $C_{18:1}\omega12t$.

to the genus *Phyllobacterium*, strain NL21^T has 17-carbon fatty acids ($C_{17:0}$, $C_{17:1}$, iso-$C_{17:0}$ and $C_{17:0}$ cyclo) and different hydroxy fatty acids. Finally, it differs from the genus *Defluvibacter* in that it lacks the fatty acids $C_{16:1}\omega7c$, $C_{20:1}\omega7c$, $C_{12:0}$ 3-OH and 10-methyl $C_{19:0}$. Additional differences in fatty acid profiles are shown in Table 2.

DNA extraction was performed by dispersing colonies of strain NL21^T in 250 µl TEN buffer (50 mM Tris/HCl, pH 8·0; 10 mM EDTA, pH 8·0; and 150 mM NaCl) and then mixing them with 250 mg sterilized 0·4–0·5 mm glass beads (B. Braun Melsungen). The DNA pellet was prepared as described by Labbé *et al.* (2003) and was then washed with 70 % ethanol and resuspended in 50 µl water.

The DNA G+C content of strain NL21^T was determined as described by Mesbah *et al.* (1989) by using two 15 cm C18 columns. λ-Phage DNA was used for calibration, whilst salmon sperm served as a control. The DNA G+C content of strain NL21^T was lower than those of other characterized members of the family '*Phyllobacteriaceae*' (57 vs 60–64 mol%). The 16S rDNA sequence of NL21^T was determined as reported previously (Labbé *et al.*, 2003) (GenBank accession no. AF534573); both DNA strands of the resulting PCR product were sequenced.

Sequences were analysed by using BioEdit software, version 5.0.9.1 (http://www.mbio.ncsu.edu/bioedit/page2.html). Comparisons of bacterial sequences in gene databases were done with the BLASTN program (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/). The CLUSTALW 1.4 program (included in the BioEdit software package) was used to align sequences. Phylogenetic analyses were carried out with software applications from the PHYLIP package, version 3.5 (http://evolution.genetics.washington.edu/phylip.html). Bootstrap values (1000 replicates) were derived by using the SEQBOOT program. The DNADIST program was used to generate a distance matrix for each bootstrap replicate. Each number in the matrix represented the distance between a pair of sequences. The FITCH program was used to calculated a tree according to the Fitch–Margoliash algorithm. Lastly, the CONSENSE program was used to derive consensus trees. Strain designations and GenBank accession numbers for 16S rDNA sequences of reference strains are given in Fig. 1.

Representative members of the family '*Phyllobacteriaceae*' showed 16S rDNA sequence similarity values of approximately 95 % with strain NL21^T. The closest species with validly published names to strain NL21^T were *Mesorhizobium tianshanense* and *Mesorhizobium chacoense* (95·1 %). However, phylogenetic analysis showed that strain NL21^T is not related closely to any genus within the family '*Phyllobacteriaceae*' (Fig. 1). Parsimony and neighbour-joining analyses were also done with PHYLIP software (DNAPARS and NJ). These two methods produced phylogenetic trees that were similar to the consensus tree. Sequence similarity analysis also revealed that strain NL21^T shows 99·8 % similarity (only three different nucleotides) to strain TUT1018 (GenBank accession no. AB098586), which was isolated from a biowaste sequence-batch composting system (Hiraishi *et al.*, 2003).

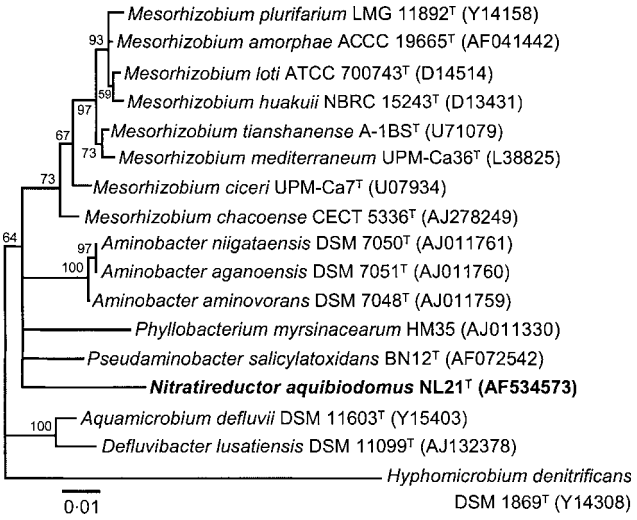

Fig. 1. Phylogenetic analysis of 16S rDNA sequences. Evolutionary distances among 16S rDNA of strain NL21^T and all species of the family '*Phyllobacteriaceae*' with validly published names are illustrated (*Hyphomicrobium denitrificans* was used as the outgroup species). The tree was inferred from a matrix of pairwise distances by using 1400 nt (DNADIST). The FITCH program was used to derive the best phylogenetic tree for each replicate. Lastly, the CONSENSE program was used to derive the consensus tree. Numbers indicate percentages of 1000 bootstrap resamplings; only values >50 % are shown. Bar, 0·01 nucleotide substitution per site.

Description of *Nitratireductor* gen. nov.

Nitratireductor (Ni.tra.ti.re.duc′tor. N.L. n. *nitras* nitrate; L. v. *reducere* to bring back, reduce; N.L. masc. n. *Nitratireductor* nitrate-reducing bacterium).

Gram-negative rods, 1 µm in diameter and 2–3 µm in length. Multiplies by budding. Cells are pleomorphic in rapid growth, motile and oxidase- and catalase-positive. Colonies on R2A agar are white, 2–3 mm in diameter, smooth, circular and convex. Optimum growth conditions are 30–35 °C and pH 7–7·5. No growth occurs at pHs lower than 7. Major fatty acids are $C_{18:1}\omega7c$ (75 %), $C_{19:0}\omega8c$ cyclo (9·4 %) and $C_{18:0}$ (4·2 %). DNA G+C content is 57 mol%. Physiological characteristics are shown in Table 1. Phylogenetically, the genus is a member of the α-subclass of the *Proteobacteria*. The type species is *Nitratireductor aquibiodomus*.

Description of *Nitratireductor aquibiodomus* sp. nov.

Nitratireductor aquibiodomus (a.qui.bi.o.do′mus. L. fem. n. *aqua* water; N.L. fem. n. *biodomus* Biodome; N. L. gen. n. *aquibiodomus* of the water of the Montreal Biodome).

Description is the same as that given for the genus. Cells can reduce nitrate to nitrite, but not nitrite to nitrogen gas. NaCl is not required for growth, but 1 % NaCl stimulates growth. Strain NL21^T grows at 0–50 g NaCl l^{-1}. Physiological characteristics are shown in Table 1.

The type strain is NL21^T (=DSM 15645^T=ATCC BAA-762^T). Isolated from the marine denitrification system of the Montreal Biodome, Canada.

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References

Bambauer, A., Rainey, F. A., Stackebrandt, E. & Winter, J. (1998). Characterization of *Aquamicrobium defluvii* gen. nov. sp. nov., a thiophene-2-carboxylate-metabolizing bacterium from activated sludge. *Arch Microbiol* **169**, 293–302.

Chen, W., Wang, E., Wang, S., Li, Y., Chen, X. & Li, Y. (1995). Characteristics of *Rhizobium tianshanense* sp. nov., a moderately and slowly growing root nodule bacterium isolated from an arid saline environment in Xinjiang, People's Republic of China. *Int J Syst Bacteriol* **45**, 153–159.

Costa, C., Dijkema, C., Friedrich, M., García-Encina, P., Fernández-Polanco, F. & Stams, A. J. (2000). Denitrification with methane as electron donor in oxygen-limited bioreactors. *Appl Microbiol Biotechnol* **53**, 754–762.

Duncan, K. E., Sublette, K. L., Rider, P. A., Stepp, A., Beitle, R. R., Conner, J. A. & Kolhatkar, R. (2001). Analysis of a microbial community oxidizing inorganic sulfide and mercaptans. *Biotechnol Prog* **17**, 768–774.

Fritsche, K., Auling, G., Andreesen, J. R. & Lechner, U. (1999). *Defluvibacter lusatiae* gen. nov., sp. nov., a new chlorophenol-degrading member of the alpha-2 subgroup of proteobacteria. *Syst Appl Microbiol* **22**, 197–204.

Gerhardt, P., Murray, R. G. E., Wood, W. A. & Krieg, N. R. (1994). *Methods for General and Molecular Bacteriology*. Washington, DC: American Society for Microbiology.

Hiraishi, A., Narihiro, T. & Yamanaka, Y. (2003). Microbial community dynamics during start-up operation of flowerpot-using fed-batch reactors for composting of household biowaste. *Environ Microbiol* **5**, 765–776.

Jarvis, B. D. W., Van Berkum, P., Chen, W. X., Nour, S. M., Fernandez, M. P., Cleyet-Marel, J. C. & Gillis, M. (1997). Transfer of *Rhizobium loti*, *Rhizobium huakuii*, *Rhizobium ciceri*, *Rhizobium mediterraneum*, and *Rhizobium tianshanense* to *Mesorhizobium* gen. nov. *Int J Syst Bacteriol* **47**, 895–898.

Kämpfer, P., Müller, C., Mau, M., Neef, A., Auling, G., Busse, H.-J., Osborn, A. M. & Stolz, A. (1999). Description of *Pseudaminobacter* gen. nov. with two new species, *Pseudaminobacter salicylatoxidans* sp. nov. and *Pseudaminobacter defluvii* sp. nov. *Int J Syst Bacteriol* **49**, 887–897.

Knösel, D. H. (1984). Genus IV. *Phyllobacterium* (ex Knösel 1962) nom. rev. (*Phyllobacterium* Knösel 1962, 96). In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 254–256. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

Labbé, N., Juteau, P., Parent, S. & Villemur, R. (2003). Bacterial diversity in a marine methanol-fed denitrification reactor at the Montreal Biodome, Canada. *Microb Ecol* **46**, 12–21.

Lechner, U., Baumbach, R., Becker, D., Kitunen, V., Auling, G. & Salkinoja-Salonen, M. (1995). Degradation of 4-chloro-2-methylphenol by an activated sludge isolate and its taxonomic description. *Biodegradation* **6**, 83–92.

Mergaert, J., Cnockaert, M. C. & Swings, J. (2002). *Phyllobacterium myrsinacearum* (subjective synonym *Phyllobacterium rubiacearum*) emend. *Int J Syst Evol Microbiol* **52**, 1821–1823.

Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.

Tighe, S. W., de Lajudie, P., Dipietro, K., Lindstrom, K., Nick, G. & Jarvis, B. D. (2000). Analysis of cellular fatty acids and phenotypic relationships of *Agrobacterium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* species using the Sherlock Microbial Identification System. *Int J Syst Evol Microbiol* **50**, 787–801.

Urakami, T., Araki, H., Oyanagi, H. Suzuki K. & Komagata, K. (1992). Transfer of *Pseudomonas aminovorans* (den Dooren de Jong 1926) to *Aminobacter* gen. nov. as *Aminobacter aminovorans* comb. nov. and description of *Aminobacter aganoensis* sp. nov. and *Aminobacter niigataensis* sp. nov. *Int J Syst Bacteriol* **42**, 84–92.

Velázquez, E., Igual, J. M., Willems, A. & 9 other authors (2001). *Mesorhizobium chacoense* sp. nov., a novel species that nodulates *Prosopis alba* in the Chaco Arido region (Argentina). *Int J Syst Evol Microbiol* **51**, 1011–1021.

Zumft, W. G. (1997). Cell biology and molecular basis of denitrification. *Microbiol Mol Biol Rev* **61**, 533–616.