

Pseudovibrio denitrificans gen. nov., sp. nov., a marine, facultatively anaerobic, fermentative bacterium capable of denitrification

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Two denitrifying strains of heterotrophic, facultatively anaerobic, marine bacteria, designated DN34^T and DN33, were isolated from sea-water samples collected in Nanwan Bay, Kenting National Park, Taiwan. They were Gram-negative. Cells in late exponential to early stationary phase of growth were predominantly straight or curved rods, but Y- or V-shaped forms were also observed. They were motile by means of one to several lateral or subpolar flagella. Both strains required NaCl for growth and exhibited optimal growth at about 30 °C, pH 8 and 3% NaCl. They were capable of anaerobic growth by carrying out denitrifying metabolism using nitrate, nitrite or nitrous oxide as terminal electron acceptors or, alternatively, by fermenting glucose, mannose, sucrose or trehalose as substrates. Anaerobic fermentative growth on glucose resulted in formation of various organic acids, including formate, lactate, acetate, pyruvate and fumarate. The major cellular fatty acids were 2-OH-14:0, 3-OH-14:0 and 16:0. DN34^T and DN33 had DNA G + C contents of 51.7 and 51.6 mol%, respectively. Physiological characterization, together with phylogenetic analysis based on 16S rRNA gene sequence analysis, revealed that the two denitrifying strains could be accommodated in a novel genus, for which the name *Pseudovibrio* gen. nov. is proposed. *Pseudovibrio denitrificans* sp. nov. is the type species, with DN34^T (= BCRC 17323^T = JCM 12308^T) as the type strain.

Denitrification is the dissimilatory reduction of nitrate (NO₃⁻) or nitrite (NO₂⁻) to the gaseous end product(s) nitrous oxide (N₂O) or dinitrogen gas (N₂). Many bacteria including autotrophs and heterotrophs perform this process to yield energy for living by oxidizing organic electron donors with NO₃⁻ or NO₂⁻ as the terminal electron acceptor. Denitrifying bacteria are usually facultative anaerobes, which normally gain energy by oxygen-dependent respiration under aerobic conditions and conduct denitrifying metabolism only when in an oxygen-depleted environment (Knowles, 1982). To our knowledge, *Azoarcus anaerobius* is the only strictly anaerobic, denitrifying bacterium unable to respire oxygen (Springer *et al.*, 1998).

Fermentation, like denitrification, is another type of metabolism that allows an organism to obtain energy for anaerobic growth. In fermentation processes organic compounds replace nitrogenous oxides as terminal electron

acceptors. Heterotrophic bacteria capable of fermentative metabolism are either facultative or strict anaerobes. The former may conduct aerobic respiration and fermentation simultaneously or they may shift their metabolism toward one or the other process, depending on the availability of oxygen. The latter, by contrast, tolerate no oxygen and are killed in air (MacFaddin, 1980). Documented heterotrophic denitrifiers that can ferment carbohydrates are weak fermenters (Tiedje, 1988). Several of these bacteria, such as some species of *Propionibacterium* and *Chromobacterium*, are not typical respiratory denitrifiers because they are only capable of NO₂⁻ detoxification by its reduction to N₂O (Grant & Payne, 1981; Tiedje, 1988; van Gent-Ruijters *et al.*, 1975). On the other hand, even the conventional denitrifying pseudomonads have been shown to survive in anaerobic and NO₃⁻-free environments by low-level fermentation (Jørgensen & Tiedje, 1993).

Heterotrophic, halophilic, facultatively anaerobic, fermentative, Gram-negative rods comprise a predominant bacterial group in many marine habitats (Simidu & Tsukamoto, 1985; Shieh *et al.*, 2000). They are currently placed in the genera *Vibrio* (Baumann *et al.*, 1984), *Photobacterium*

Published online ahead of print on 1 July 2004 as DOI 10.1099/ij.s.0.63107-0.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Pseudovibrio denitrificans* DN34^T is AY486423.

(Baumann & Baumann, 1984), *Listonella* (MacDonell & Colwell, 1985), *Salinivibrio* (Mellado *et al.*, 1996), *Moritella* (Urakawa *et al.*, 1998), *Enterovibrio* (Thompson *et al.*, 2002) and *Grimontia* (Thompson *et al.*, 2003). However, never have any recognized species of these genera been reported to be denitrifiers. A heterotrophic, halophilic, bacterium, designated strain DN34^T, was recovered previously by us from a sea-water sample collected in the shallow coastal region of Nanwan Bay, Kenting National Park, Taiwan. The isolate was shown to be capable of anaerobic growth by undertaking either denitrification or fermentation (Shieh & Liu, 1996). Evidence is presented here that this isolate and strain DN33, another denitrifying isolate also recovered from shallow sea-water in Nanwan Bay, may represent a novel species and deserve a novel genus rank. The name *Pseudovibrio denitrificans* gen. nov., sp. nov. is proposed for this novel species.

Bacterial cultures were maintained on Polypepton/yeast extract (PY) stabs and were cultivated in PY broth, Polypepton/yeast extract/nitrate (PYN) broth, Polypepton/yeast extract/glucose (PYG) broth or modifications thereof for growth and denitrification studies. PY broth contained the following constituents (per litre deionized water): 2 g Polypepton (Nihon Seiyaku, Japan), 0.5 g Bacto yeast extract (Difco), 30 g NaCl, 5 g MgCl₂·6H₂O, 0.005 g CaCl₂, 0.005 g Na₂MoO₄·7H₂O, 0.004 g CuCl₂·2H₂O and 0.006 g FeCl₃·6H₂O. The medium was adjusted to pH 8.0. Bacto agar (Difco) was added to this medium at 3 and 15 g l⁻¹ for the preparation of stab and plate media, respectively. PYN broth was prepared by adding Tris (Sigma-Aldrich) at 6 g l⁻¹ and KNO₃ at 2 g l⁻¹ (approximately 20 mM) to PY broth. PYG broth was prepared in two parts. The first part contained 2 g Polypepton, 0.5 g Bacto yeast extract, 30 g NaCl, 5 g MgCl₂·6H₂O, 0.005 g CaCl₂, 0.005 g Na₂MoO₄·7H₂O, 0.004 g CuCl₂·2H₂O, 0.006 g FeCl₃·6H₂O and 6 g Tris dissolved in 900 ml deionized water and adjusted to pH 8.0. The second part contained 5 g glucose dissolved in 100 ml deionized water. The two parts were autoclaved separately and mixed at room temperature. Polypepton/yeast extract/nitrate/glucose (PYNG) broth differed from PYG broth in containing KNO₃ (2 g l⁻¹) and bromothymol blue (0.03 g l⁻¹). Carbohydrate/mineral (CM) media were made up of two parts. Part I contained 0.54 g NH₄Cl, 30 g NaCl, 3 g MgCl₂·6H₂O, 2 g K₂SO₄, 0.2 g K₂HPO₄, 0.01 g CaCl₂, 0.006 g FeCl₃·6H₂O, 0.005 g Na₂MoO₄·7H₂O, 0.004 g CuCl₂·2H₂O and 6 g Tris dissolved in 900 ml water and adjusted to pH 8.0, whereas part II contained 5 g glucose or other test carbohydrates (D-arabinose, cellobiose, galactose, lactose, mannose, sucrose, trehalose, xylose, dulcitol, mannitol) dissolved in 100 ml deionized water. The two parts were autoclaved separately and mixed at room temperature.

Inoculum cultures of strains DN34^T and DN33 were routinely grown in PY broth at 30 °C under aerobic conditions. Late exponential to early stationary phase cultures (one loopful) were inoculated into tubes containing 5 ml

PYN broth or modifications thereof to determine the effects of pH, temperature and NaCl on aerobic growth. Anaerobic conditions for cultures grown in PY, PYN or PYG broth were established by flushing the culture tubes for 5 min with a high-purity (99.9%) argon gas, after sealing the tubes with rubber stoppers; each tube was shaken vigorously on a mixer during the flushing process. N₂O was injected at 102.4 µmol (2.5 ml at 1 atmosphere) into each of the tubes using a gas-tight syringe when necessary. Bacterial growth was monitored as the OD₆₀₀ using a Spectronic 20D⁺ spectrophotometer (Spectronic Instruments). All growth experiments were performed at 30 °C in the dark unless stated otherwise. All data presented here represent the means of three to five replicates.

The methods of Shieh *et al.* (2000), with some modifications, were used to test the phenotypic characteristics of strains DN34^T and DN33 and additional tests as cited below. Utilization of various carbohydrates as sole carbon and energy sources for growth was determined in CM media instead of the original use of GM-II medium and its modifications. H₂S production from thiosulphate was determined by inoculating a slant of triple-sugar iron agar (Smibert & Krieg, 1994) supplemented with NaCl (30 g l⁻¹). Positive reaction was indicated by a blackening of the butt, which was caused by the formation of black ferrous sulphide when ferrous ammonium sulphate reacted with H₂S. The possibility of simultaneous fermentation and denitrification was examined by inoculating one loopful of PY broth culture into a tube containing PYNG broth (5 ml) in which a Durham insert had been placed in an inverted position. The surface of the medium was overlaid with sterile liquid paraffin after inoculation. Occurrence of denitrification was indicated by production of gas(es) (N₂ or N₂O) accumulated in the Durham insert, and occurrence of glucose fermentation was indicated by a colour change of the culture from blue to yellow as a result of a decrease in pH. Antibiotic susceptibility tests were performed by disc diffusion methods as described by Shieh *et al.* (2003).

The DNA G+C content was determined according to the methods described by Shieh & Liu (1996).

Fatty acids of cells grown aerobically in PY broth at 30 °C were extracted, saponified and esterified following the Microbial Identification System (MIS) guidelines (MIDI, Inc.). GLC analysis of the fatty acid methyl esters was performed on a gas chromatograph (GC-14A; Shimadzu) equipped with a fused silica capillary column and a flame-ionization detector (Shieh & Jean, 1998).

Early stationary phase cultures grown anaerobically in PYG broth under argon at 30 °C were centrifuged to separate the cells. The supernatant, after filtering through Nucleopore membranes (pore size, 0.22 µm), was analysed for organic acids produced during anaerobic fermentative growth in the cultures. Analysis was carried out by HPLC with an Epic Polar 5 µm column (4.6 × 250 mm). Phosphoric acid solution (50 mM) was used as the eluent at a flow rate of

1 ml min⁻¹. The peaks were identified by retention time comparisons with authentic standards. Quantitative analysis of these peaks was performed with a chromatointegrator Hitachi D2500.

Amplification of the 16S rRNA gene sequence by PCR and purification of the PCR products were performed according to the methods of Hiraishi (1992). Sequencing reactions of the purified 16S rRNA gene samples were performed using an ABI PRISM Big Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems) and an ABI 3730 Genetic Analyzer (Applied Biosystems) following the manufacturer's instructions. The 16S rRNA gene sequences of strains DN34^T and DN33 were aligned using Genetics Computer Group (GCG) software provided by the National Institute of Health, Taiwan, and were compared with those of reference bacterial strains available in the GenBank database. Distance matrices for the aligned sequences were calculated with the PHYLIP program DNADIST (Felsenstein, 1989), using the Jukes & Cantor (1969) model. Thereafter, a phylogenetic dendrogram was reconstructed according to the estimated evolutionary distances, using the neighbour-joining method (Saitou & Nei, 1987).

The initial pH for PY and PYN broth media of 7.0 was found to be suitable for aerobic growth of strains DN34^T and DN33, but better growth was observed at pH 8.0. Growth was observed at pH 9.0 but not at pH 6.0. The strains grew aerobically in PY and PYN broth media over a temperature range of 20–35 °C, with optimal growth occurring at about 30 °C. Growth was not observed at 4 or 42 °C. Both strains showed similar results in additional growth studies; strain DN34^T was therefore selected as representative of the two strains when describing the following results. Strain DN34^T grew significantly in PYN broth under aerobic conditions with NaCl levels of 2–6% (about 0.34–1.03 M); growth occurred most rapidly at 3% (about 0.51 M). Growth was weak or negligible at 1 and 7% NaCl, and no growth was observed at 0 or 8% NaCl. Substitution of KCl (3–5%) for NaCl did not support growth, indicating that strain DN34^T required Na⁺ for growth and that the Na⁺ requirement was not for osmotic function.

Strain DN34^T grew significantly in PYN and PYG broth media under anaerobic conditions (maximal OD₆₀₀ > 0.35), whereas anaerobic growth in PY broth was weak (maximal OD₆₀₀ 0.10–0.12) unless N₂O was present in the culture systems (Fig. 1). This indicated an insufficiency of NO₃⁻ and fermentable substrates for the strain in PY broth cultures. The PYN broth cultures showed remarkable decreases in the OD₆₀₀ within 24 h after entering the stationary phase of growth, indicating rapid cell lysis in these cultures. Our previous report (Shieh & Liu, 1996) has revealed that strain DN34^T possessed all reductases necessary to reduce NO₃⁻ to N₂, and that the N₂O produced via reduction of NO₃⁻ was not further reduced to N₂ when C₂H₂ was injected into the culture systems. Growth in PYN broth under argon and in PY broth under argon in the presence of N₂O (Fig. 1) confirmed that strain DN34^T was

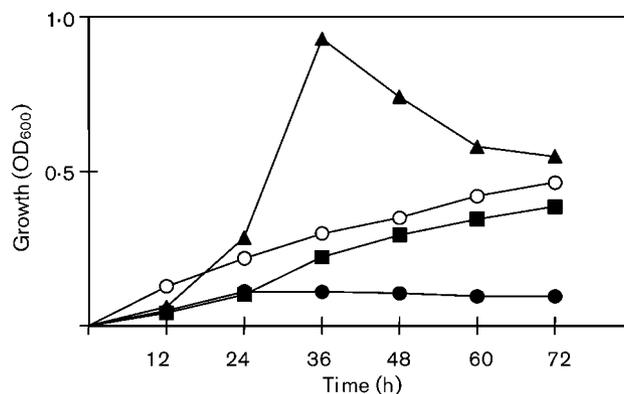


Fig. 1. OD₆₀₀ during anaerobic growth of strain DN34^T in PY (●, ○), PYN (▲) and PYG (■) broth media under argon in the presence (○) or absence (●, ▲, ■) of N₂O (102.4 μmol per tube).

capable of anaerobic growth by carrying out denitrifying metabolism with NO₃⁻ or N₂O as the terminal electron acceptor. Glucose-dependent anaerobic growth (anaerobic growth in PYG broth) was accompanied by a remarkable decrease in medium pH from 8.0 to 5.8 within 72 h (data not shown), regardless of the large buffer content (about 50 mM Tris) of the medium. It was evident that strain DN34^T achieved anaerobic growth in PYG broth by fermenting glucose, with considerable production of organic acids. In addition to glucose, the strain also achieved anaerobic growth by fermenting mannose, sucrose or trehalose as substrates. However, no other test substrate (D-arabinose, cellobiose, dulcitol, glycerol, lactose, inositol, mannitol, sorbitol and xylose) supported anaerobic fermentative growth (data not shown).

Strains DN34^T and DN33 were revealed to be mixed acid fermenters. Organic acids produced from anaerobic fermentative growth in PYG broth included formate (39.1–45.5 mol%), lactate (26.9–38.2 mol%), acetate (20.9–24.4 mol%), pyruvate (1.7–2.9 mol%) and fumarate (0.16–0.22 mol%). Both strains contained 2-OH-14:0 (60.4–60.5 mol%), 3-OH-14:0 (13.0–14.8 mol%) and 16:0 (9.4–13.3 mol%) as the major fatty acids. Other cellular fatty acids present at levels greater than 1 mol% included 17:0 (1.8–1.9 mol%), 3-OH-12:0 (1.0–1.3 mol%), 18:0 (1.4–3.2 mol%), 18:2ω9,12 (2.0–5.4 mol%) and 20:0 (2.7–2.9 mol%).

Strains DN34^T and DN33 were mesophilic, halophilic, Gram-negative bacteria. The cells grown in PY and PYN broth media were predominantly straight or curved rods, but V- and Y-shaped forms were also observed. They were motile by means of one to several lateral or subpolar flagella (for electron micrographs see Shieh & Liu, 1996). Colonies produced on PY plate medium were circular, translucent and non-luminescent. Oxidase and catalase tests were both

positive. Both strains were resistant to the vibriostatic agent O/129 at 10 and 150 µg per disc. Both denitrification and glucose fermentation were detected in PYNG broth cultures grown for 24–36 h under oxygen-deficient conditions. The results suggested that the strains were able to conduct denitrification and fermentation simultaneously in an environment depleted of oxygen, but in the presence of both NO_3^- and a fermentable carbohydrate(s). Additional phenotypic characterization data are given below in the species description.

Strains DN34^T and DN33 had DNA G + C contents of 51.7 and 51.6 mol%, respectively.

Strains DN34^T and DN33 might have been identified as members of the family *Vibrionaceae* given that they were mesophilic, halophilic, facultatively anaerobic, motile, Gram-negative rods, and were capable of both respiratory and fermentative metabolism (Baumann & Schubert, 1984). However, several characteristics contradict this common rule. Both strains were distinct from all species of the *Vibrionaceae* in their formation of irregular rods, including V- and Y-shaped cells, and in their denitrification ability (Baumann & Schubert, 1984). They were also distinguished from most halophilic species of the *Vibrionaceae* by their resistance to the vibriostatic agent O/129 (Baumann *et al.*, 1984; Holt *et al.*, 1994; Shieh *et al.*, 2000). Moreover, both strains had DNA G + C contents greater than all known halophilic species included in the *Vibrionaceae* (51.6–51.7 versus 38–51 mol%).

Almost complete 16S rRNA gene sequences of strains DN34^T and DN33 (92.9–93.0%; estimated by comparison with the *Escherichia coli* sequence J01859) were determined. They were aligned and compared with all bacterial sequences available in the GenBank database. The results of our phylogenetic analysis are shown in Fig. 2. The sequences of the two strains were identical except for one nucleotide difference (99.9% similarity). The two strains formed a cluster at sequence similarity levels of 98.3–99.9% with the α -proteobacteria MBIC3368 (J. Hamada, unpublished), NW001 (Webster & Hill, 2001) and SB89 (Hentschel *et al.*, 2001). MBIC3368, NW001 and SB89 (redesignated MBIC3368 in the GenBank database) were all isolated from marine sponges and none has been previously phenotypically characterized. MBIC3368 was an actual isolate whereas the other two were clones rather than isolated strains. Strains DN34^T and DN33 did not share more than 95% 16S rRNA gene sequence similarity with any known bacterial species, including those belonging to the family *Vibrionaceae*. Their closest species were *Stappia aggregata* (Uchino *et al.*, 1998), *Roseibium hamelinense* (Suzuki *et al.*, 2000) and *Stappia stellulata* (Uchino *et al.*, 1998). The sequence similarities between the present two denitrifying strains and *Stappia aggregata*, *Roseibium hamelinense* and *Stappia stellulata* were 94.5, 93.6 and 91.8%, respectively. No other known species shared more than 90% sequence similarity with the two strains. Strains DN34^T and DN33 are distinguished from *Roseibium* (Suzuki *et al.*, 2000) in that

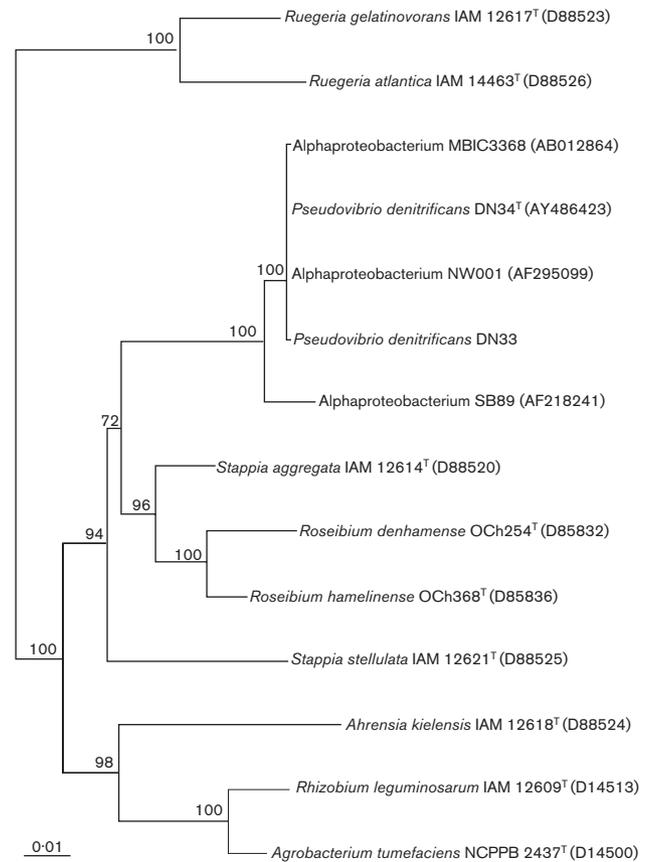


Fig. 2. Unrooted phylogenetic tree derived from neighbour-joining analysis of the 16S rRNA gene sequences of strain DN34^T and other members of the α -Proteobacteria. Numbers above nodes represent bootstrap confidence values obtained with 100 resamplings; values below 80 are not shown. Bar, 1% estimated sequence divergence.

they are not strictly aerobic, bacteriochlorophyll-containing bacteria and that they do not have DNA G + C contents in the range 57.6–63.4 mol%. They are also differentiated from *Stappia* (Uchino *et al.*, 1998), because *Stappia* includes only strict aerobes with G + C contents of about 59 mol%. 16S rRNA gene sequence analysis, together with comparisons of G + C contents and phenotypic characteristics, strongly support the creation of a novel genus outside the family *Vibrionaceae*. *Pseudovibrio* gen. nov. is proposed to accommodate the two denitrifying strains. *Pseudovibrio denitrificans* sp. nov. is the type species of this genus, with DN34^T as the type strain.

Pseudovibrio denitrificans is considered to be of marine origin, because it is unable to grow in the absence of NaCl and requires a NaCl level similar to that of natural sea water for optimal growth. It provides evidence that heterotrophic, halophilic, facultatively anaerobic, fermentative bacteria include denitrifying species. The abundance and distribution of *Pseudovibrio denitrificans* and other halophilic,

facultatively anaerobic, fermentative denitrifiers and their importance in the marine denitrification process remain to be further investigated.

Description of *Pseudovibrio* gen. nov.

Pseudovibrio (Pseu.do.vib'ri.o. Gr. adj. *pseudes* false; N.L. n. *vibrio* a name of a bacterial genus; N.L. masc. n. *Pseudovibrio* false *Vibrio*).

Members are heterotrophic, Gram-negative bacteria belonging to the α -Proteobacteria. Cells in exponential to early stationary phase of growth are predominately straight or curved rods, but Y- and V-shaped forms also occur. They are motile by means of one to several lateral or subpolar flagella. Oxidase and catalase tests are both positive. Facultatively anaerobic, growing aerobically by carrying out oxygen respiration and growing anaerobically by undertaking denitrification and/or fermentation. Mesophilic, growing at 20–35 °C but not at 4 or 45 °C. Halophilic, growing in 2–6 % NaCl; no growth in the absence of NaCl. The type species is *Pseudovibrio denitrificans*.

Description of *Pseudovibrio denitrificans* sp. nov.

Pseudovibrio denitrificans (de.ni.tri'fi.cans. N.L. v. *denitrifico* to denitrify; N.L. part. adj. *denitrificans* denitrifying).

Description is as for the genus with the following additional characteristics. Irregular rod-shaped cells, approximately 2.0–4.5 μm long by 0.6–0.8 μm wide. Colonies produced on plate media are circular, translucent and non-luminescent, with an entire margin. Swarming does not occur. Capable of complete denitrification, i.e. capable of reducing NO_3^- to N_2 via NO_2^- and N_2O . Able to ferment glucose, mannose, sucrose and trehalose with production of acid but no gas. Unable to ferment D-arabinose, cellobiose, dulcitol, glycerol, inositol, lactose, mannitol, sorbitol or xylose. Optimal growth occurs at about 30 °C, pH 8 and 3 % NaCl. Unable to grow at 42 °C or at 8–10 % NaCl. Major cellular fatty acids are 2-OH-14:0, 3-OH-14:0 and 16:0. Resistant to the vibriostatic agent O/129 at 10 and 150 μg . DNase, gelatinase and lipase tests are positive, but agarase, amylase, arginine dihydrolase and lysine and ornithine decarboxylase tests are negative. H_2S is not produced from thiosulphate. Galactose, glucose, mannose, sucrose and trehalose can be utilized as sole carbon and energy sources, but cellobiose, lactose, xylose, dulcitol and mannitol can not. Susceptible to ampicillin (10 μg), carbenicillin (100 μg), cephalothin (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), neomycin (30 μg), novobiocin (30 μg) and vancomycin (30 μg); intermediate susceptibility to nalidixic acid (30 μg) and tetracycline (30 μg); resistant to clindamycin (2 μg), colistin (10 μg), gentamicin (10 μg), lincomycin (2 μg), penicillin G (10 U), polymyxin B (300 U) and streptomycin (10 μg). Strain DN34^T has a DNA G + C content of 51.7 mol%.

The type strain is DN34^T (= BCRC 17323^T = JCM 12308^T).

Acknowledgements

We thank Dr J.-S. Chen, School of Medicine, China Medical University, for advice and critical reading of the manuscript. This work was supported by grant NSC92-3114-B-002-014 from the National Science Council.

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