

# ***Denitrovibrio acetiphilus*, a novel genus and species of dissimilatory nitrate-reducing bacterium isolated from an oil reservoir model column**

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**A novel dissimilatory, nitrate-reducing bacterium, designated strain N2460<sup>T</sup>, was isolated from an oil reservoir model column. Strain N2460<sup>T</sup> is a mesophilic, obligately anaerobic, marine, Gram-negative bacterium. The cells are vibrio-shaped and motile by a bipolar flagellum. Strain N2460<sup>T</sup> reduces nitrate to ammonia in a mineral medium supplied by acetate. The presence of a 2-oxoglutarate dehydrogenase activity indicates that acetate is oxidized via the citric acid cycle. No growth is obtained on formate, higher fatty acids, malate, fumarate, benzoate, alcohols, sugar, yeast extract, crude oil, alkanes, proline, hydrogen, sulfur or thiosulfate with nitrate as electron acceptor. Oxygen, sulfate, thiosulfate and sulfur are not utilized as alternative electron acceptors. Strain N2460<sup>T</sup> grows fermentatively on fumarate, but not on pyruvate. The G+C content of the DNA is 42.6 mol%. 16S rRNA gene analysis shows that strain N2460<sup>T</sup> belongs to the *Bacteria* and that the closest relative is '*Geovibrio ferrireducens*' (sequence similarity 86.9%). On the basis of phylogenetic as well as phenotypic data, it is proposed that strain N2460<sup>T</sup> represents the type strain of a new genus and species, *Denitrovibrio acetiphilus* gen. nov., sp. nov.**

**Keywords:** *Denitrovibrio acetiphilus*, nitrate-reducing bacterium, oil reservoir model column

## **INTRODUCTION**

Reservoir souring, the production of H<sub>2</sub>S in oil wells by sulfate-reducing bacteria (SRB), is a detrimental process of great concern to the oil industry (Hamilton, 1985; Herbert, 1987; Beeder, 1996). Addition of nitrate to the injection water may reduce H<sub>2</sub>S production by stimulating a competing group of anaerobic bacteria, the nitrate-reducing bacteria (NRB) (Reinsel *et al.*, 1996). In order to investigate the potential of nitrate as an inhibitor of reservoir souring, an oil reservoir model column was constructed (S. Myhr, B.-L. P. Lillebø, E. Sunde, J. Beeder and T. Torsvik, unpublished results). The model column consisted of a sand-packed Perspex tube with crude oil as the sole carbon and energy source. The column was inoculated with a broad

spectrum of bacteria, including aerobic oil-degrading bacteria, NRB, SRB and methanogens. The system was flooded continuously with synthetic seawater medium. When stable H<sub>2</sub>S production was established, nitrate was added to the medium. During nitrate injection, several NRB were enriched and isolated from the column. In this paper, we describe one of these isolates, strain N2460<sup>T</sup>, which is an obligately anaerobic acetate specialist. It represents a new genus on the recently described phylogenetic line of '*Flexistipes sinusarabici*' (Fiala *et al.*, 1990), '*Geovibrio ferrireducens*' (Caccavo *et al.*, 1996) and *Deferribacter thermophilus* (Greene *et al.*, 1997).

## **METHODS**

**Source of organism.** Strain N2460<sup>T</sup> was isolated from an oil reservoir model column (S. Myhr, B.-L. P. Lillebø, E. Sunde, J. Beeder and T. Torsvik, unpublished results). The column consisted of a Perspex tube (9 × 192 cm) packed with fine-grained sand, saturated with crude oil and flooded with

**Abbreviations:** NRB, nitrate-reducing bacteria; SRB, sulfate-reducing bacteria

The GenBank accession number for the 16S rDNA sequence of strain N2460<sup>T</sup> is AF146526.

synthetic seawater (0.9 ml min<sup>-1</sup>). Excess oil was removed by flooding before the start of the experiment (residual oil, 0.65 l). The pore volume was 2.7 l.

The column was inoculated with enrichment cultures of aerobic oil-degrading bacteria, NRB, SRB and methanogens (enriched at 30 °C). Aerobic oil-degrading bacteria were enriched from two reverse-flooded water injectors (B02 and B26) at the Statfjord oilfield (North Sea) and a biological treatment plant for oil-polluted water (OMV oil refinery, Vienna, Austria). NRB were enriched from a nitrate bioreactor for removal of H<sub>2</sub>S from ballast water (Sture oil terminal, Norway). A mixed culture of SRB and methanogens was collected from two model columns constructed in a previous experiment designed to investigate microbial H<sub>2</sub>S production and methanogenesis at low sulfate concentrations (K. Drønen and T. Torsvik, unpublished). These columns were initially inoculated with the following bacteria: SRB and methanogens enriched from produced water (Statfjord oilfield, North Sea), ballast/process water from a biological treatment plant (Mongstad oil refinery, Norway) and a freshwater lake sediment (Tveitevatnet, Bergen, Norway); *Clostridium propionicum* DSM 1682<sup>T</sup>; *Desulfobacter vibrioformis* B54<sup>T</sup> (DSM 8776<sup>T</sup>); two SRB strains isolated from the injection water systems at the Gullfaks B and Statfjord B oil platforms (North Sea); *Methanosarcina barkeri* DSM 800<sup>T</sup>; and *Methanococcus vannielii* DSM 1224<sup>T</sup>.

The oil reservoir model column was first inoculated with enrichment cultures of oil-degrading bacteria to consume oxygen in the column. Subsequently, SRB and methanogens were added. After establishing an SRB biofilm and stable H<sub>2</sub>S production, the column was inoculated with NRB and 0.5 mM NaNO<sub>3</sub> was added to the synthetic seawater. The experiment was run at a low room temperature (17–20 °C).

**Enrichment and isolation.** NRB enrichments from the model column were initiated after 14 d with nitrate injection. NRB were enriched in an anoxic, reduced, marine mineral medium (NRB medium) containing the following components (l<sup>-1</sup>): 0.02 g Na<sub>2</sub>SO<sub>4</sub>, 1.0 g KH<sub>2</sub>PO<sub>4</sub>, 0.25 g NH<sub>4</sub>Cl, 20.0 g NaCl, 3.0 g MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.5 g KCl, 0.15 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.7 g NaNO<sub>3</sub>, 1 ml trace element solution SL10 (Widdel *et al.*, 1983) and 0.5 ml 0.2% resazurin. After autoclaving in a dispenser, the medium was cooled under nitrogen gas. Five ml vitamin solution (Pfennig, 1978), 30 ml 1 M NaHCO<sub>3</sub> and 4 ml 0.5 M Na<sub>2</sub>S were added aseptically and the pH was adjusted to 6.8–7.2 with HCl/Na<sub>2</sub>CO<sub>3</sub>. Finally, the medium was dispensed into nitrogen-flushed 50 ml serum bottles or 20 ml tubes and sealed by butyl rubber stoppers and aluminium crimp seals. Acetate (20 mM final concentration) was added from a sterile, anoxic stock solution prior to inoculation. A 2 ml inoculum of sand/water/oil (mixed from oxic and anoxic zones in the column) was added to 20 ml medium and incubated at 30 °C. A pure culture was obtained by the agar shake dilution method (Ljungdahl & Wiegand, 1986). The method involved a serial dilution of the enrichment culture in 45 °C medium containing 1.0% agarose (w/v) (washed five times with distilled water). The solidified tubes were incubated at 30 °C. Several colonies were picked and transferred to growth medium. All colonies examined consisted of similar vibrioid bacteria. To ensure that a pure culture was obtained, agar dilution was repeated twice for one of the colonies/cultures.

**Physiological studies.** Strain N2460<sup>T</sup> was tested for its ability

to use different electron donors and acceptors. An increase in cell number/OD<sub>600</sub> in three successive transfers (10% inoculum, v/v) was used as the criterion for growth. Electron donors were added to NRB medium from sterile, anoxic stock solutions, unless otherwise stated. The following final concentrations were applied: 20 mM acetate and lactate; 10 mM formate, propionate, butyrate, valerate, caproate, palmitate, malate and thiosulfate; 4 mM benzoate and fumarate; 0.1% methanol, ethanol, glucose, fructose, yeast extract, crude oil and 'alkane mixture' [equal amounts of heptane, octane, decane and dodecane; pristane (10%, v/v) added as internal standard]; 5 mM phenylacetate and heptanoate; 2.5 mM octanoate; 1.25 mM nonanoate and decanoate; 0.5 mM undecanoate; 1 mM proline. Hydrogen was added as an 80% H<sub>2</sub>/20% CO<sub>2</sub> headspace and elemental sulfur was added as a small amount of sterile powder. Different electron acceptors were added from sterile, anoxic stock solutions (except for sulfur) to medium with 20 mM acetate and no nitrate. The following final concentrations were used: 30 mM sulfate, 10 mM thiosulfate and ferric iron (ferric pyrophosphate) and 0.2 mM oxygen (air saturation). Sulfur was added as a small amount of sterile powder. Fermentative growth was tested in medium without nitrate, containing 10 mM pyruvate and 4 mM fumarate. Temperature, pH and salinity spectra for growth were determined in medium with 20 mM acetate and 8 mM nitrate. Except for the temperature experiment, all incubations were performed at 30 °C.

**Chemical measurements.** Nitrite was measured by a nitrite test tube (Hach). Ammonia was measured by the modified phenol/hypochlorite method (Solorzano, 1969).

**Morphology.** Cell morphology was examined by phase-contrast microscopy (Labolux K; Leitz) and transmission electron microscopy (TEM) (JEOL 100 CX 80 kV electron microscope). For TEM, cells were stained negatively with 1.5% (w/v) uranyl acetate in methanol on Formvar-coated grids. Gram-typing was performed by the KOH method described by Buck (1982).

**Enzyme assays.** Cells from 2 l of a culture of strain N2460<sup>T</sup> (grown to late exponential/early stationary phase) were harvested by centrifugation (8000 g for 20 min at 4 °C) and washed once in medium without substrate. The cell pellet (450 mg) was resuspended in 3 ml anoxic 0.1 M Tris/HCl buffer, pH 8.1. Cells were disrupted by sonication (2 × 30 s on ice) (Sonicel Disrupter; Du Pont Research). Cell debris and metal sulfides were removed by centrifugation (21 000 g for 20 min at 4 °C). The supernatant (cell extract) was transferred immediately to an anoxic serum bottle and flushed for 10 min with argon. The cell extract was stored on ice and assayed photometrically within an hour for activity of carbon-monoxide dehydrogenase and 2-oxoglutarate dehydrogenase, using the methods described by Beeder *et al.* (1994) and Zeikus *et al.* (1977). The assays were performed at room temperature. Proteins were measured according to Bradford (1976).

**Phospholipid fatty acids.** The composition of phospholipid fatty acids was analysed by the method described by Frostegård *et al.* (1993), modified by Odden (1998). The analysis was performed by M. Galteland and E. Odden (Norsk Hydro Research Centre, Porsgrunn, Norway).

**DNA base composition.** DNA was isolated by cell disruption in a French pressure cell and purification on hydroxyapatite according to the procedure of Cashion *et al.* (1977). The G+C content was determined by HPLC analysis as de-

scribed by Mesbah *et al.* (1989) using non-methylated lambda DNA as a standard. The analysis was performed by U. Mendrock (DSMZ).

**16S rDNA sequencing and phylogenetic analysis.** Extraction of genomic DNA, PCR-mediated amplification of the 16S rDNA and purification of the PCR products were carried out as described previously (Rainey *et al.*, 1996). Purified PCR products were sequenced using the ABI PRISM Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems) as directed in the manufacturer's protocol. Sequence reactions were electrophoresed using the Applied Biosystems 373A DNA sequencer. The resulting sequence data were put into the alignment editor ae2 (Maidak *et al.*, 1996), aligned manually and compared with representative 16S rRNA gene sequences of organisms belonging to the domain *Bacteria* (Maidak *et al.*, 1996). For comparison, 16S rRNA sequences were obtained from the EMBL database or RDP (Maidak *et al.*, 1996). The 16S rRNA gene similarity values were calculated by pairwise comparison of the sequences within the alignment. For construction of the phylogenetic dendrogram, operations of the PHYLIP package (Felsenstein, 1993) were used: (i) pairwise evolutionary distances were computed from percentage similarities by the correction of Jukes & Cantor (1969); and (ii) on the basis of the evolutionary distance values, a phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987). The root of the tree was determined by including the 16S rRNA gene sequence of *Aquifex pyrophilus* in the analysis. The 16S rDNA analysis was performed by C. Spröer (DSMZ).

**Polyclonal antibodies.** A polyclonal antiserum against strain N2460<sup>T</sup> was prepared by the method described by Christensen *et al.* (1992), with minor modifications (Beeder *et al.*, 1994). The titre and specificity of the serum were determined by the fluorescent antibody (FA)-DAPI double-staining technique (Hoff, 1988), performed on multi-well glass slides. A selection of other NRB and SRB isolates from the model column (N2461, N2660, S2550, S2450 and S2650) served as negative controls. Twenty µl samples of bacterial cultures (grown to late exponential phase) were applied to the slides and air-dried. Salt precipitates were carefully washed away with sterile-filtered distilled water. Fifty µl PBS-diluted antiserum was added to the wells. The slides were incubated in a moist chamber for 20 min to allow antibody binding. The antibody solution was then washed away with sterile-filtered distilled water and FITC-conjugated secondary antibodies (goat anti-rabbit IgG), diluted 1:40 in PBS, were added. The slides were incubated for 20 min in the moist chamber, protected from light. Finally, the conjugate solution was washed away and the bacteria were stained with DAPI (10 µg ml<sup>-1</sup> in sterile distilled water) for 2 min, washed and air-dried. The preparations were viewed with an epifluorescence microscope (Microphot; Nikon) with separate filters for FITC and DAPI signals. Only bacteria showing a clear halo with the FITC filter package and a blue signal with the DAPI filter package were regarded as positive.

**Construction of oligonucleotide probe.** Several potential probe sequences against the V3 region of the strain N2460<sup>T</sup> 16S rRNA gene were tested theoretically using the PROBE MATCH program (Ribosomal Database Project II; <http://www.cme.msu.edu/rdp>). For the probe N2460A (sequence: 5'-GAACCATTTCTTCCCTGCTG), no matches were found in the database. This probe was synthesized with a CY3 fluorochrome in the 5' end (Interactiva Biotechnology) and tested empirically in whole-cell hybridization on multi-well glass slides, by the method described by Amann (1995). According to the PROBE MATCH search results, all known bacterial sequences have at least three mismatches compared with the probe. One of the strains showing three mismatches, *Desulfonatronovibrio hydrogenovorans* (DSM 9292), served as a negative control together with the following NRB and SRB strains from the model column: N2660, S2251, S2250, S2450, S2550, S2551, S2650 and S2651.

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## RESULTS

### Enrichment and isolation

A rapidly moving vibrioid bacterium was observed in the enrichment bottle 2 d after inoculation. A dense culture was obtained after 5 d, consisting almost exclusively of the vibrio. Colonies that appeared in the agar tubes were off-white and diffuse. No gas production was detected. A strain designated N2460<sup>T</sup> was isolated in pure culture.

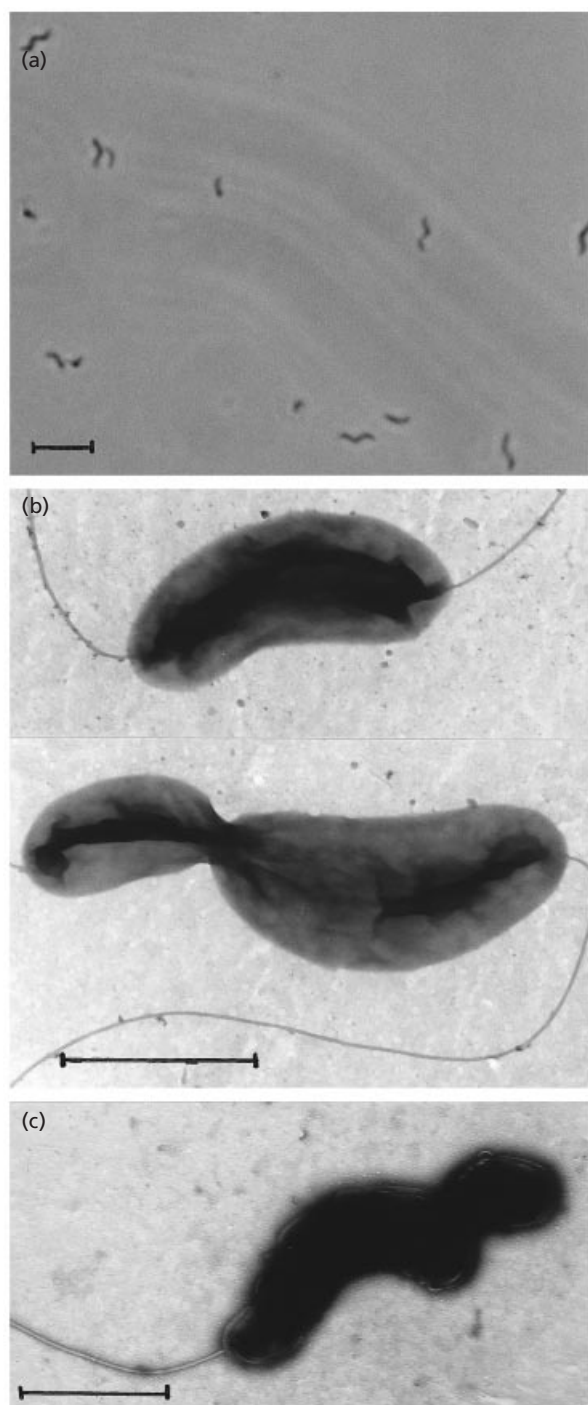
### Physiological studies

The isolated strain, N2460<sup>T</sup>, grew well on acetate with nitrate as electron acceptor, producing ammonia (nitrite was detected as an intermediate). The shortest doubling time at 35 °C was about 8 h. No growth was detected with the following electron donors: formate, lactate, propionate, butyrate, valerate, caproate, heptanoate, octanoate, nonanoate, decanoate, undecanoate, palmitate, malate, fumarate, benzoate, phenylacetate, methanol, ethanol, glucose, fructose, yeast extract, crude oil, alkanes (C<sub>7</sub>, C<sub>8</sub>, C<sub>10</sub> and C<sub>12</sub>), proline, hydrogen, sulfur and thiosulfate. Sulfate, thiosulfate, sulfur and oxygen were not utilized as alternative electron acceptors. Oxygen inhibited growth of strain N2460<sup>T</sup> and the strain was unable to grow in a non-reduced anoxic medium. This constituted a problem when testing ferric iron as electron acceptor: ferric pyrophosphate reacted chemically with sulfide in the medium, raising the redox potential and leading to inhibition of growth of the bacterium. It was therefore not possible to test iron reduction in a batch culture. Strain N2460<sup>T</sup> grew fermentatively on fumarate, while no growth was detected on pyruvate in medium without nitrate. Vitamins were required for growth.

Strain N2460<sup>T</sup> grew at temperatures between 4 and 40 °C with an optimum at 35–37 °C. No growth was obtained at 45 °C. NaCl was not required for growth and strain N2460<sup>T</sup> grew well in medium containing up to 6% NaCl (w/v) (optimum at 2–4%). Growth occurred between pH 6.5 and 8.6. No growth was observed at pH 6.2 or 8.9.

### Morphology

As seen by phase-contrast microscopy, cells of strain N2460<sup>T</sup> were vibrio-shaped, measuring 0.5–0.7 × 1.7–2.0 µm, and showed a rapid corkscrew movement. The cells usually occurred singly or in pairs, but longer chains were sometimes observed. Pair of cells



**Fig. 1.** Morphology of strain N2460<sup>T</sup>. (a) Phase-contrast micrograph of cells. Bar, 5 μm. (b)–(c) Transmission electron micrographs of negatively stained cells. Bars, 1 μm.

resembled the silhouette of a flying gull (Fig. 1a). Electron microscopy (Fig. 1b, c) revealed bipolar flagellation for some cells (a single flagellum at each end) and showed that the cells grew by budding. No spores were observed. The KOH test showed that the cells were Gram-negative.

**Table 1.** Phospholipid fatty acids of strain N2460<sup>T</sup>

In the designations of the fatty acids, the first number represents the number of carbon atoms and the second number represents number of double bonds. The  $\omega$ -number states the position of double bonds (as the number of C-atoms from the carboxyl group); c, *cis*.

| Fatty acid*                            | Abundance (%) |
|----------------------------------------|---------------|
| C <sub>14:0</sub>                      | 4.7           |
| C <sub>15:0</sub>                      | 0.1           |
| C <sub>16:1<math>\omega</math>9</sub>  | 8.9           |
| C <sub>16:1<math>\omega</math>7c</sub> | 3.3           |
| C <sub>16:1<math>\omega</math>5</sub>  | 0.1           |
| C <sub>16:0</sub>                      | 24.4          |
| C <sub>17:1<math>\omega</math>8</sub>  | 0.1           |
| C <sub>17:0</sub>                      | 0.3           |
| C <sub>18:1<math>\omega</math>7</sub>  | 49.9          |
| C <sub>18:1</sub> *                    | 0.3           |
| C <sub>18:0</sub>                      | 3.4           |
| Unidentified (sum)                     | 4.5           |

\* Position of double bond is uncertain (probably  $\omega$ 5).

### Enzyme assays

The N2460<sup>T</sup> cell extract had a specific activity of 2-oxoglutarate dehydrogenase of 0.09  $\mu\text{mol min}^{-1} \text{mg protein}^{-1}$ . No activity of carbon-monoxide dehydrogenase was detected. A cell extract from an SRB strain designated S2550 (DSM 12861) (S. Myhr, B. E. Thorbjørnsen and T. Torsvik, unpublished) showed a high activity of carbon-monoxide dehydrogenase under the same conditions.

### Phospholipid fatty acids

Phospholipids of strain N2460<sup>T</sup> contained C<sub>16</sub> and C<sub>18</sub> fatty acids as the major components (Table 1).

### DNA base composition

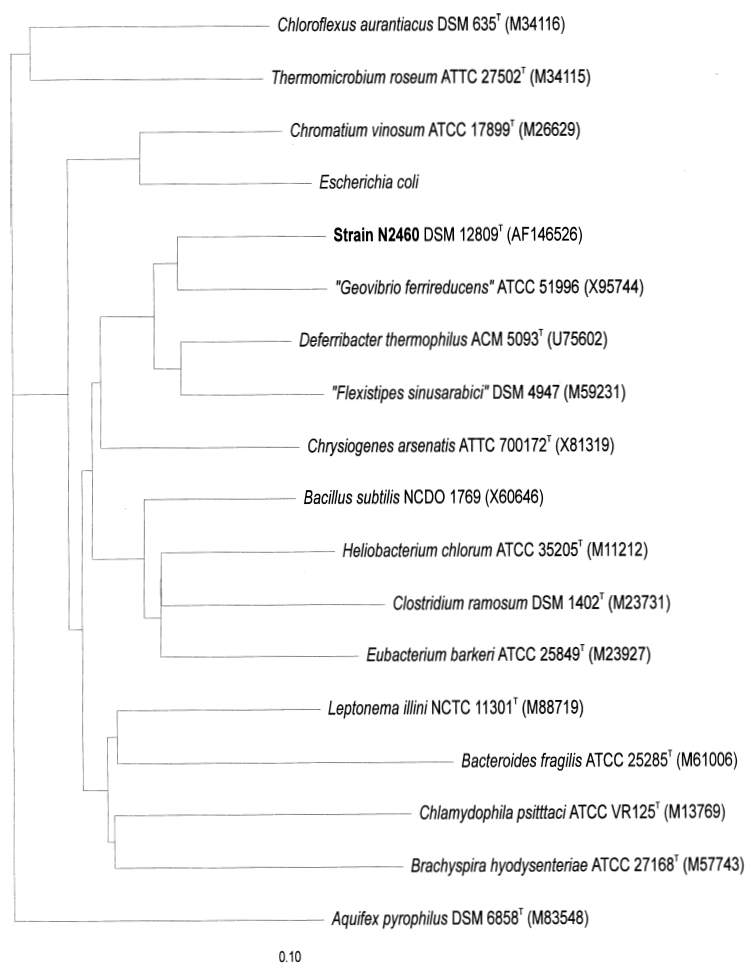
The G + C content of the DNA was  $42.6 \pm 0.4$  mol % (mean of three determinations).

### Phylogenetic analysis

The 16S rRNA gene analysis showed that strain N2460<sup>T</sup> belongs to the *Bacteria*. The closest relatives are '*Geovibrio ferrireducens*', '*Deferribacter thermophilus*' and '*Flexistipes sinusarabici*' (Fig. 2), with respective gene similarity values of 86.9, 85.7 and 85.6%.

### Polyclonal antibodies

The antiserum generated against strain N2460<sup>T</sup> reacted strongly with its homologous strain (titre 100) in the FA-DAPI assay. The other control strains were negative.



**Fig. 2.** Phylogenetic dendrogram based on 16S rDNA data, showing the position of strain N2460<sup>T</sup> among the *Bacteria*. Bar, 10 nucleotide substitutions per 100 nucleotides.

### Oligonucleotide probe

The probe N2460A was specific for strain N2460<sup>T</sup>. The optimal conditions for whole-cell hybridization were 37 °C and 20 % formamide.

### DISCUSSION

The isolated strain, N2460<sup>T</sup>, is a strictly anaerobic acetate specialist. The presence of a 2-oxoglutarate dehydrogenase activity indicates that acetate is oxidized via the citric acid cycle. In metabolic aspects, strain N2460<sup>T</sup> is a nitrate-reducing analogue of the sulfate-reducing *Desulfobacter*. The *Desulfobacter* are, like strain N2460<sup>T</sup>, nutritionally specialized anaerobes with acetate as their characteristic substrate, though ethanol, lactate and hydrogen are used as electron donors by some species (Widdel & Pfennig, 1981; Widdel, 1988). *Desulfobacter* species grow relatively fast on acetate and oxidize the substrate via a modified citric acid cycle. This is in contrast to other acetate-oxidizing SRB, which use the acetyl CoA pathway (with carbon-monoxide dehydrogenase as a

key enzyme) and show much slower growth on the substrate (Widdel, 1988). The significantly shorter doubling time on acetate for strain N2460<sup>T</sup> (ca. 8 h) compared with *Desulfobacter* species (ca. 20 h) (Lien & Beeder, 1997) can be explained by the fact that nitrate is a much better electron acceptor than sulfate.

Most obligately anaerobic NRB perform dissimilatory nitrate reduction to ammonia (DNRA), rather than denitrification to N<sub>2</sub>/N<sub>2</sub>O (Tiedje, 1988; Zumft, 1992). The detection of ammonia as the end product from nitrate reduction in strain N2460<sup>T</sup> was therefore expected. The majority of DNRA bacteria gain energy only from the first reduction step (to nitrite). In these organisms, the further reduction of nitrite to ammonia probably serves as an electron sink for regeneration of oxidized co-enzymes or as a detoxification mechanism for nitrite (Tiedje, 1988). In some anaerobes, however, like *Desulfovibrio desulfuricans* (Steenkamp & Peck, 1981), *Desulfovibrio gigas* (Barton *et al.*, 1983), *Campylobacter sputorum* subsp. *bubulus* (de Vries *et al.*, 1980), *Wolinella succinogenes* (Bokranz *et al.*, 1983) and *Sulfurospirillum deleyianum* (Schumacher &

**Table 2.** Comparison of habitats and some characteristics of strain N2460<sup>T</sup> and the most-closely related bacteria

| Characteristic               | Strain N2460 <sup>T</sup>  | ' <i>Geovibrio ferrireducens</i> '<br>ATCC 51996   | ' <i>Flexistipes sinusarabici</i> '<br>DSM 4947 | <i>Deferribacter thermophilus</i><br>ACM 5093 <sup>T</sup> |
|------------------------------|----------------------------|----------------------------------------------------|-------------------------------------------------|------------------------------------------------------------|
| Habitat                      | Oil reservoir model column | Surface sediment of hydrocarbon-contaminated ditch | Atlantis II Deep brines of the Red Sea          | Produced water from a North Sea oil reservoir              |
| Gram reaction                | Negative                   | Negative                                           | Negative                                        | Negative                                                   |
| Morphology                   | Vibrio                     | Vibrio                                             | Straight to bent rods                           | Straight to bent rods                                      |
| Metabolism                   | Respiratory/fermentative   | Respiratory                                        | Fermentative                                    | Respiratory                                                |
| Optimum temperature (°C)     | 35–37                      | 35                                                 | 45–50                                           | 60                                                         |
| Salinity range (% NaCl, w/v) | 0–6                        | 0–2                                                | 3–10                                            | 0–5                                                        |
| DNA G + C content (mol %)    | 42.6                       | 42.8                                               | 38.6                                            | 34                                                         |
| Electron acceptors:          |                            |                                                    |                                                 |                                                            |
| NO <sub>3</sub> <sup>-</sup> | +                          | –                                                  | NT                                              | +                                                          |
| Fe(III)                      | NT                         | +                                                  | NT                                              | +                                                          |
| Mn(IV)                       | NT                         | –                                                  | NT                                              | +                                                          |
| Sulfur                       | –                          | +                                                  | (+)                                             | –                                                          |
| O <sub>2</sub> tolerance     | –                          | –                                                  | –                                               | (+)*                                                       |
| Electron donors:             |                            |                                                    |                                                 |                                                            |
| Acetate                      | +                          | +                                                  | (+)                                             | +                                                          |
| Lactate                      | –                          | +                                                  | –                                               | +                                                          |
| Propionate                   | –                          | +                                                  | NT                                              | NT                                                         |
| Fumarate                     | –                          | +                                                  | NT                                              | –                                                          |
| Proline                      | –                          | +                                                  | NT                                              | NT                                                         |
| Yeast extract                | –                          | +                                                  | +                                               | +                                                          |
| H <sub>2</sub>               | –                          | +                                                  | –                                               | +                                                          |

\* Grows in presence of 3% O<sub>2</sub>, provided that Fe(III), Mn(IV) or nitrate is added as electron acceptor.

NT, Not tested.

Kroneck, 1992; Schumacher *et al.*, 1992), the entire reduction process is coupled to electron transport phosphorylation. This may also be the case in strain N2460<sup>T</sup>. Since acetate is a non-fermentable substrate, it is unlikely that the nitrite–ammonia reduction step serves solely as an electron sink.

The 16S rDNA analysis showed that strain N2460<sup>T</sup> belongs to the '*Flexistipes*' group of the *Bacteria*. This is a recently discovered cluster of bacteria, consisting until now of only three genera and species: '*Flexistipes sinusarabici*' (Fiala *et al.*, 1990), '*Geovibrio ferrireducens*' (Caccavo *et al.*, 1996) and *Deferribacter thermophilus* (Greene *et al.*, 1997). The fatty acid composition has only been investigated for '*Flexistipes sinusarabici*' and strain N2460<sup>T</sup>. In both strains, C<sub>16</sub> and C<sub>18</sub> fatty acids are major components of the polar lipids. In strain N2460<sup>T</sup>, however, the C<sub>18</sub> fatty acids are predominantly of the unsaturated form (one double bond), while the saturated form is dominant in '*Flexistipes sinusarabici*'. '*Flexistipes sinusarabici*' also differs from strain N2460<sup>T</sup> in having *iso*-branched C<sub>14</sub>, *anteiso*-branched C<sub>15</sub> and C<sub>17</sub> fatty acids as important components. It should be noted, though, that all polar lipids were analysed for '*Flexistipes sinusarabici*', while only the phospholipids (normally a major component

of the polar lipids) were analysed for strain N2460<sup>T</sup>. Table 2 compares some characteristics of strain N2460<sup>T</sup> and the three established species in the '*Flexistipes*' cluster, as well as their habitats. It is evident that only two characteristics are shared by all four strains: they are obligate anaerobes and Gram-negative. Strain N2460<sup>T</sup> and the closest relative (according to the 16S rDNA data), '*Geovibrio ferrireducens*', are the only mesophiles. The two strains have nearly identical G + C values and both are vibrios. Strain N2460<sup>T</sup> differs, however, in showing bipolar flagellation and a budding form of growth. Major differences in salinity range and spectra of electron donors and acceptors utilized also clearly distinguish the two strains. '*Geovibrio ferrireducens*' is an iron-reducing, freshwater bacterium and exhibits a much broader substrate spectrum than strain N2460<sup>T</sup>. *Deferribacter thermophilus* reduces nitrate but differs from strain N2460<sup>T</sup> in most of the other characteristics listed, except for salinity range. '*Flexistipes sinusarabici*' is a fermentative, halophilic and slightly thermophilic bacterium that shares few phenotypic traits with strain N2460<sup>T</sup>.

Considering the facts that two of the closest relatives of strain N2460<sup>T</sup> are iron reducers and that several other

NRB also perform iron reduction (Sørensen, 1982; Jones *et al.*, 1983, 1984; Lovley & Phillips, 1988), we wanted to test strain N2460<sup>T</sup> for this trait. As mentioned, however, ferric pyrophosphate reacted chemically with sulfide in the medium, raising the redox potential and causing growth inhibition of the bacterium. The chemical reaction between ferric iron and sulfide, leading to sulfur and FeS, has been described previously (Widdel, 1988). In spite of this chemical reaction, it should be possible for sulfide and ferric iron to co-exist in a natural gradient/biofilm when complex microbial populations are involved. Ferrous iron can be reoxidized to the ferric state by both aerobic (Wood, 1988; Emerson & Revsbeck, 1994a, b) and anaerobic (Straub *et al.*, 1996; Hafenbradl *et al.*, 1996) bacteria and hydrogen sulfide can be supplied continuously from SRB in the layer beneath. Accordingly, our results do not exclude the existence of a niche for iron reduction by strain N2460<sup>T</sup> in the environment.

The oil reservoir model column was initially inoculated with enrichment cultures from several different sources. NRB were selectively enriched only from the nitrate bioreactor, which is an analogue system to the model column, containing oil, sulfide, seawater and externally added nitrate. It therefore seemed likely that the bioreactor was the source of strain N2460<sup>T</sup>. Yet, efforts to isolate the strain from the bioreactor 3 years after the first enrichment failed (unpublished data). This may have been due to operating problems and reinoculation of the reactor during this period. It is, however, also possible that strain N2460<sup>T</sup> originated from one of the other inocula, as acetate is a common fermentation product in almost any anoxic environment (Gottschalk, 1986). The most likely inocula would be the marine/brackish ones that were used for anaerobic enrichments, produced water (sampled from mesophilic parts of the pipelines) and ballast/process water from the Mongstad biological treatment plant. The polyclonal antibodies and oligonucleotide probe that we developed can be used for screening of environmental samples for strain N2460<sup>T</sup>.

Based on the 16S rDNA analysis as the main criterion, together with the phenotypic differences between strain N2460<sup>T</sup> and '*Geovibrio ferrireducens*' (as well as the other relatives), we suggest that strain N2460<sup>T</sup> represents the type strain of a new genus and species, *Denitrovibrio acetiphilus*.

#### Description of *Denitrovibrio* gen. nov.

*Denitrovibrio* (De.ni.tro.vi'bri.o. L. pref. *de* from; M.L. n. *nitrum* nitrogen; L. v. *vibrare* vibrate; M.L. n. *vibrio* that which vibrates; *Denitrovibrio* a vibrio that reduces nitrate).

Cells are Gram-negative and vibrio-shaped, occurring singly, in pairs or in chains. No spores are formed. Strictly anaerobic, heterotrophic growth with nitrate as electron acceptor. Phylogenetically, the genus

belongs to the *Bacteria*, clustering together with the genera '*Flexistipes*', '*Geovibrio*' and *Deferribacter*.

#### Description of *Denitrovibrio acetiphilus* sp. nov.

*Denitrovibrio acetiphilus* (a.ce.ti'phi.lus. L. n. *acetum* vinegar; M.L. n. *acidum aceticum* acetic acid; Gr. v. *philein* to like, love; M.L. adj. *acetiphilus* loving or requiring acetate).

Cells are 0.5–0.7 × 1.7–2.0 µm and motile by bipolar flagella. Growth occurs between 4 and 40 °C (optimum at 35–37 °C), with NaCl concentrations ranging from 0 to 6% (optimum at 2–4%) and at pH 6.5–8.6. Acetate is the only electron donor utilized with nitrate as electron acceptor. Nitrate is reduced to ammonia, with nitrite as an intermediate. Sulfate, thiosulfate and sulfur are not used as alternative electron acceptors. Fumarate can be fermented, while pyruvate is not utilized. Vitamins are required. Phospholipids contain C<sub>16:0</sub>, C<sub>16:1ω9</sub> and C<sub>18:1ω7</sub> fatty acids as the principal compounds. The DNA G + C content is 42.6 mol %.

The type strain, *Denitrovibrio acetiphilus* N2460<sup>T</sup>, was isolated from an H<sub>2</sub>S-producing oil reservoir model column flooded by nitrate-enriched synthetic seawater. Strain N2460<sup>T</sup> has been deposited at the DSMZ (DSM 12809<sup>T</sup>).

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