***Denitrobacterium detoxificans* gen. nov., sp. nov., a ruminal bacterium that respire on nitrocompounds**

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A new group of anaerobic, Gram-positive, high G+C (56–60 mol%) bacteria was isolated from the bovine rumen. Of four strains characterized, all were non-motile and none produced spores. The isolates did not produce indole or H₂S and did not hydrolyse gelatin. Cells of each strain exhibited similar rod-shaped morphology (0.5–1.0 × 1.0–1.5 µm) although bulbous ends were sometimes present. None of the four strains were able to grow via oxidation of a variety of potentially fermentable substrates but rather obtained energy for growth via anaerobic respiration processes, oxidizing hydrogen, formate or lactate for reduction of various oxidized nitrogen compounds. Trimethylamine oxide and DMSO were also used as electron acceptor. All four strains shared greater than 99% 16S rRNA gene sequence identity. The closest match found between the 16S rRNA gene sequence of all four strains, NPOH1^T, NPOH2, NPOH3 and MAJ1, to sequences available in GenBank was that of *Coriobacterium glomerans* (86% sequence similarity), a phenotypically dissimilar anaerobe within the class *Actinobacteria*. To accommodate these bacteria the creation of a new genus and species, *Denitrobacterium detoxificans*, for placement within the family *Coriobacteriaceae* is proposed. The type strain, NPOH1^T (ATCC 700546^T), grew equally well over a narrow range of incubation temperatures tested (32–39 °C).

Keywords: anaerobic respiration, nitrocompound, *Denitrobacterium detoxificans*

INTRODUCTION

A bacterium capable of metabolizing the naturally occurring nitrotoxins 3-nitro-1-propanol and 3-nitro-1-propionate was recently isolated from a ruminal population enriched for enhanced rates of nitrotoxin metabolism (Anderson *et al.*, 1996). Ruminants gradually adapted to forages containing these toxins can acquire a tolerance resulting from enhanced ruminal detoxification and evidence suggests that bacteria like the one isolated by us may play a major role in this detoxification (Anderson *et al.*, 1998; Majak, 1992). Following characterization of the only available iso-

late, strain NPOH1^T, it became apparent that this anaerobe differed markedly from known bacteria (Anderson *et al.*, 1997). We now report results from phenotypic and genotypic characterizations of this and other recently isolated nitrotoxin-metabolizing bacteria. To accommodate these bacteria, which do not fit into any recognized genus, we propose a new taxon (*Denitrobacterium detoxificans* gen. nov., sp. nov.).

METHODS

Source of organisms. Strain NPOH1^T was isolated from a microbial population enriched via consecutive batch culture of rumen contents from a cow reared at the National Animal Disease Center (NADC) in Ames, IA, USA (Anderson *et al.*, 1996). Strain MAJ1 was isolated, following a similar enrichment, from rumen contents of a cow grazing on a milkvetch (*Astragalus miser* var. *serotinus*) range in British Columbia. The rumen fluid sample from Canada was provided by Walter Majak, Agriculture and Agri-Food

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The GenBank accession numbers for the 16S rDNA sequences of *Denitrobacterium detoxificans* strains NPOH1^T, NPOH2, NPOH3 and MAJ1 are U43492, AF079505, AF079506 and AF079507, respectively.

Canada, Kamloops, British Columbia, and had been preserved by freezing in 50% (v/v) glycerol during shipment and storage. Strains NPOH2 and NPOH3 were isolated from rumen contents originating from a cow at the NADC, although this animal was different from that which was the source of strain NPOH1^T. Both animals at the NADC received an alfalfa/corn (9:1) diet.

Strains NPOH2 and NPOH3 were isolated from a roll tube containing agar medium C (Anderson *et al.*, 1996) modified by adding a partially purified soy protein product (Amisoy; Quest International) at 0.08% (w/v) instead of the more complex component phytone that was usually used. This medium was supplemented with 9 mM 3-nitropropanol and the roll tube was inoculated with 2×10^{-4} ml non-enriched ruminal fluid. Colonies present after 14 and 22 d growth, respectively, were randomly picked and screened for nitrotoxin-reducing activity.

Culture methods. Anaerobic methods (Bryant, 1972) were used for culture of the organisms and unless indicated otherwise, the bacteria were cultured at 39 °C. All strains were maintained by culture in medium B (Anderson *et al.*, 1996) supplemented with 16 mM formate and 9 mM 3-nitropropionate and these cultures were transferred at 3–4 d intervals. When the strains were inoculated into test media, cultures 24–48 h old were used as inocula. Tests for the ability of the strains to grow with potential hydrogen donors and with various electron acceptors were done using medium B; growth of cultures grown in 18 × 150 mm culture tubes was measured as optical density using a Spectronic 70 spectrophotometer (Bausch & Lomb) set at 600 nm. Substrates were added to the medium as small volumes (< 5%, v/v) of filter-sterilized (0.22 µm Millipore) concentrated stock solutions (420 mM) that were stored under N₂ gas. When potential donors other than H₂/CO₂ were tested, CO₂ was used as the gas phase. Tests for indole and H₂S production were performed using medium B supplemented with 9 mM nitropropanol, 5 mM formate, L-tryptophan (0.1%, w/v) or with ferrous sulfate and sodium thiosulfate (0.015%, w/v, each) as previously described (Anderson *et al.*, 1997). Medium B supplemented with 9 mM nitropropanol, 5 mM formate and 4% gelatin was used to test for gelatin hydrolysis (Anderson *et al.*, 1997). All culture tests were performed at least twice and mean results are reported.

Analytical methods. DMSO and dimethyl sulfide were analysed by GLC (model 5890; Hewlett Packard) using a flame-ionization detector set at 270 °C. Samples (3 µl) were injected at 120 °C onto a HP1 column (Hewlett Packard) containing cross-linked methyl silicone gum (30 mm × 0.53 mm × 3.0 mm) and eluted with N₂ at a flow rate of 6.5 ml min⁻¹. The oven temperature, initially set at 55 °C for 0.2 min, was programmed to increase 10 °C min⁻¹ to 140 °C, then 20 °C min⁻¹ to a final temperature of 180 °C. Total run time was 11.2 min. Total cellular fatty acid profiles were determined by GLC of methyl esters (Moore *et al.*, 1987) using the Microbial Identification System software package (MIDI).

DNA extraction and determination of mol% G + C. G + C compositions were determined spectrophotometrically during thermal denaturation (Johnson, 1981) using a Gilford Response II spectrophotometer (Ciba Corning Diagnostic) equipped with a thermal programmer. Reference melting temperature (*T_m*) was obtained using DNA from *Escherichia coli* HB101 and *Propionibacterium avidum* (ATCC 25577); DNA was extracted using the procedure of Marmur (1961). Two separate DNA extractions and thermal melt analyses

were performed for each strain and we report the mean from these two analyses, which differed from each other by no more than 2 mol%.

rRNA gene sequencing and phylogenetic analysis. Copies of the 16S rRNA gene of each strain were obtained by PCR amplification based on the procedure of Rainey *et al.* (1992); *E. coli* numbering (Brosius *et al.*, 1978) has been used to define primers. The forward primer 5'-GAGTTTGATC-(C/A)TGGCTCAG-3' and reverse primer 5'-GGTTACCTTGTTACGACTT-3', corresponding to positions 9–27 and 1510–1492, respectively, were used to amplify the gene of each strain. The amplified products were purified as described by Stanton *et al.* (1997) and sequenced at the Iowa State University Nucleic Acid Facility (Ames, IA) as described by Frothingham *et al.* (1994). For each strain, a partial sequence of the 16S rRNA gene product was obtained using in addition to the primers used for amplification, the forward primer 5'-ATTAGATACCCTGGTAG-3' and the reverse primer 5'-CCGTCGAATTCATTTGAGTTT-3', corresponding to positions 786–803 and 926–907, respectively. Two additional forward primers, 5'-CACATTGGGACTG-AGATAC-3' and 5'-ACCCGGTGGCCGAGAG-3', and a reverse primer, 5'-GCTCCCCACACCTAGT-3', were synthesized and used to determine remaining sequences of the 16S rRNA gene of each strain. These primers correspond to positions 311–329, 1015–1030 and 840–824, respectively. The total number of nucleotides sequenced was 1413, 1423, 1429 and 1426 for strains NPOH1^T, NPOH2, NPOH3 and MAJ1, respectively. The almost complete gene sequences of all four strains were compared to each other and to sequences in GenBank (Bilofsky & Burks, 1988) using the Basic Local Alignment Search Tool (Altschul *et al.*, 1990).

Nucleotide sequences were aligned using CLUSTAL W version 7 (Higgins *et al.*, 1992; Thompson *et al.*, 1994). The resulting alignment contained information on 1593 nucleotide positions for 24 micro-organisms. The DNADIST program from PHYLIP version 3.573 (Felsenstein, 1993) was used to generate a DNA distance matrix output using the nucleotide substitution model of Jukes & Cantor (1969). Trees were generated by the neighbour-joining method of Saitou & Nei (1987) using NEIGHBOR (PHYLIP 3.575). Bootstrap values, given as a percentage of 1000 replications, are indicated at the branching points.

RESULTS

Cells of all strains exhibited similar morphology and stained Gram-positive. Most cells were rod-shaped (0.5–1.0 × 1.0–1.5 µm) although cells with irregular ends were also observed. Motility was not observed in wet mount preparations. Growth of all four strains was supported when 3-nitropropanol, 3-nitropropionate, 2-nitropropanol, nitroethane, nitroethanol, 2-nitrobutane, 1-nitropropane, trimethylamine oxide or DMSO were supplied as electron acceptors (Table 1). The potential electron acceptors chlorate, fumarate, nitrite, perchlorate, sulfate and sulfite did not support growth of any of the strains. In agreement with an earlier report (Anderson *et al.*, 1997), growth of strain NPOH1^T was also supported by nitrate but none of the other three strains were able to grow with nitrate as a terminal electron acceptor (Table 1). In the case of strain NPOH1^T, nitrate was reduced to nitrite, which was then reduced to am-

Table 1. Maximum growth (OD₆₀₀) achieved by nitro-respiring bacteria during culture with various potential electron acceptors

Strains were cultured for at least 1 week in medium B supplemented with 16 mM formate, an H₂/CO₂ gas phase (1:1) and one of the following acceptors: 3-NPOH, 3-nitropropanol; 2-NPOH, 2-nitropropanol; 3-NPA, 3-nitropropionate; NETH, nitroethane; NEOH, nitroethanol; NO₃, nitrate; 2-NBUT, 2-nitrobutane; 1-NPRO, 1-nitropropane; DMSO, dimethyl sulfoxide; TMAQ, trimethylamine oxide. DMSO and trimethylamine oxide were supplied at 9 mM and all other acceptors were supplied at 5 mM.

Strain	None	3-NPOH	2-NPOH	3-NPA	NETH	NEOH*	NO ₃	2-NBUT*	1-NPRO*	DMSO	TMAQ
NPOH1 ^T	0.00	0.22	0.30	0.22	0.36	0.08	0.44	0.06	0.10	0.28	0.25
NPOH2	0.00	0.17	0.17	0.22	0.33	0.11	0.00	0.04	0.09	0.22	0.14
NPOH3	0.00	0.17	0.27	0.28	0.43	0.10	0.00	0.04	0.07	0.30	0.25
MAJ1	0.00	0.27	0.10	0.30	0.40	0.12	0.00	0.03	0.10	0.35	0.22

* The redox indicator (resazurin) within the medium turned pink subsequent to the cultures achieving their maximum optical density values thus indicating that a restrictively high E_h was encountered during reduction of the nitrocompounds (uninoculated tubes containing these nitrocompounds did not turn pink). Consequently, data pertaining to growth with these acceptors are interpreted qualitatively only.

monia, and DMSO was reduced to dimethyl sulfide. Aminopropanol and β -alanine, the respective products formed during reduction of nitropropanol and nitropropionate by mixed populations of ruminal microbes (Anderson *et al.*, 1993), were not detected in spent culture fluids of NPOH1^T.

In the presence of an appropriate electron acceptor, growth of all four strains was supported by hydrogen, formate or lactate with maximum OD₆₀₀ values ranging from 0.30 to 0.57. None of the four strains grew when these were replaced by any of the following: adonitol, citrate, glucose, glycerol, glycine, ethanol, fructose, malate, mannitol, methylamine, pyruvate, ribose, salicin or succinate (each at 20 mM). Within a narrow range of incubation temperatures tested (32, 37 and 39 °C), no difference was observed in the rate or extent of growth by strain NPOH1^T when cultured in our maintenance medium (data not shown). Nutritional factors supplied by supplementing phytone were important for all four strains and when phytone was omitted from medium B, growth was reduced by more than 45% during initial culture and by more than 80% during the next successive culture.

The fatty acid profiles from total cellular lipids of each of the nitro-respiring strains are presented in Table 2. Palmitic acid was the predominant fatty acid present in all four of the strains; however, considerable amounts of other straight- and branched-chain fatty acids, as well as aldehydes and dimethyl acetals, were present in all of the strains. No matches were found when these profiles were compared to profiles of other organisms contained in the MIDI Anaerobe Library or Aerobe Library databases.

The G+C content of strain NPOH1^T DNA was 60 mol% (Anderson *et al.*, 1996). Likewise, DNA from strains NPOH2, NPOH3 and MAJ1 also had high G+C contents, 58, 56 and 60 mol%, respectively. Analysis of the 16S rRNA gene sequence of each strain

revealed a close relationship (>99% sequence identity) between all four strains. The closest match (86% identity) found between the 16S rRNA gene sequence of strain NPOH1^T and sequences available in GenBank was that of *Coriobacterium glomerans*. The relatedness of strain NPOH1^T to *C. glomerans* and to some other organisms is depicted in Fig. 1.

DISCUSSION

We propose that based on their high mol% G+C content and their 16S rRNA gene sequence, the anaerobic Gram-positive bacteria described here be assigned to the class *Actinobacteria*, subclass *Coriobacteriidae*, order *Coriobacteriales*, family *Coriobacteriaceae* in accordance with the proposed classification scheme of Stackebrandt *et al.* (1997). While 16S rRNA sequence analysis indicated that strain NPOH1^T is most closely related to *C. glomerans*, their level of sequence similarity (Fig. 1) and their phenotypic dissimilarity suggest that these organisms are distantly related only. An anaerobe within the class *Actinobacteria*, *C. glomerans* is a fermentative bacterium (Hass & König, 1988; Rainey *et al.*, 1994) that does not metabolize 3-nitropropionate or 3-nitropropanol (data not shown). On the basis of their ability to metabolize various nitrocompounds and on other phenotypic characteristics, the bacteria we describe do not appear to fit into any currently recognized genus. We thus propose that these nitrocompound-respiring organisms be designated *Denitrobacterium detoxificans* gen. nov., sp. nov. Strain NPOH1^T, which has been characterized in this and in an earlier report (Anderson *et al.*, 1997), is proposed as the type strain of the species. Electron micrographs depicting cell morphology and cell wall structure of strain NPOH1^T have been published earlier (Anderson *et al.*, 1997).

Until the discovery of the organisms described in this report, the only ruminal bacteria known to obtain

Table 2. Total cellular fatty acid profiles

Bacteria were grown in BHI medium modified by inclusion of 9 mM 3-nitropropionate. Values outside parentheses are relative molar percentages with most abundant at 100 mol%; values within parentheses are percentages of total fatty acids. ND, Not detected.

Component*	Strain			
	NPOH1 ^T	NPOH2	NPOH3	MAJ1
C _{12:0} FAME	5 (0.9)	6 (1.0)	16 (3.6)	7 (1.3)
C _{13:0} iso-FAME	6 (1.2)	6 (1.0)	7 (1.5)	12 (2.3)
C _{13:0} anteiso-FAME	ND	ND	ND	ND
Unsep. ECL 12-925	16 (2.8)	15 (2.5)	17 (4.0)	18 (3.4)
Unsep. ECL 13-553	8 (1.5)	8 (1.4)	ND	7 (1.3)
C _{14:0} iso-FAME	21 (3.8)	18 (3.2)	16 (3.6)	29 (5.4)
C _{14:0} FAME	100 (17.7)	100 (17.4)	100 (23.0)	100 (18.7)
C _{13:0} iso-3OH-FAME	ND	ND	ND	4 (0.80)
C _{14:0} DMA	35 (6.1)	33 (5.7)	32 (7.3)	41 (7.7)
C _{15:0} iso-FAME	52 (9.2)	62 (10.4)	21 (4.9)	43 (8.1)
C _{15:0} anteiso-FAME	35 (6.2)	37 (6.4)	17 (3.8)	30 (5.7)
C _{16:0} aldehyde	22 (3.9)	21 (3.7)	17 (4.0)	14 (2.7)
C _{15:0} FAME	8 (1.4)	9 (1.5)	6 (1.5)	6 (1.1)
C _{15:0} iso-DMA	21 (3.8)	23 (3.9)	6 (1.5)	19 (3.6)
C _{15:0} anteiso-DMA	7 (1.3)	8 (1.3)	ND	6 (1.1)
Unsep. ECL 15-476	6 (1.0)	6 (1.1)	ND	6 (1.0)
C _{16:0} iso-FAME	9 (1.6)	8 (1.4)	ND	8 (1.4)
C _{16:1} <i>cis</i> -9-FAME	9 (1.5)	10 (1.8)	13 (3.0)	10 (1.9)
C _{16:0} FAME	56 (9.8)	54 (9.4)	42 (9.6)	40 (7.4)
UN, ECL 16-112	10 (1.9)	9 (1.6)	6 (1.4)	11 (2.0)
C _{16:1} <i>cis</i> -9-DMA	6 (1.0)	6 (1.0)	6 (1.4)	7 (1.3)
C _{16:0} DMA	88 (15.6)	88 (15.4)	54 (12.4)	62 (11.6)
C _{18:1} <i>cis</i> -9-FAME	8 (1.5)	9 (1.5)	13 (3.1)	9 (1.7)
C _{18:0} FAME	14 (2.5)	15 (2.7)	24 (5.5)	16 (3.0)
C _{18:1} <i>cis</i> -9-DMA	10 (1.8)	11 (1.9)	12 (2.8)	12 (2.3)
C _{18:0} DMA	13 (2.3)	15 (2.6)	16 (3.6)	17 (3.1)

* FAME, fatty acid methyl ester; DMA, dimethyl acetal; ECL, equivalent chain length; Unsep. ECL 12-925, C_{13:1} *cis*-12-FAME, C_{14:0} aldehyde and C_{11:1} 2OH-FAME unseparated; Unsep ECL 13-553, C_{15:0} aldehyde and unknown at ECL 13-570 unseparated; Unsep. ECL 15-476, C_{15:0} DMA and C_{14:0} 3OH-FAME unseparated; UN ECL 16-112, unidentified.

energy for growth strictly via anaerobic respiration processes were the methanogens, the sulfate-reducers and *Wolinella* species. The functional significance of the niche occupied by organisms such as strain NPOH1^T within the rumen is not completely understood but likely provides the host with a certain detoxification capability. The toxins 3-nitropropanol and 3-nitropropionate are constituents of many forages consumed by ruminants; notable among these are various *Astragalus* species (Majak & Pass, 1989). When absorbed into the bloodstream, 3-nitropropionate inhibits succinate dehydrogenase and while not toxic per se, 3-nitropropanol is converted to 3-nitropropionate by hepatic alcohol dehydrogenase (Majak & Pass, 1989). Rates of ruminal metabolism of both the nitrotoxins are enhanced when animals are fed sublethal amounts of milkvetch forage (*Astragalus miser* var. *serotinus*) or a non-toxic analogue (Majak, 1992; Majak & Pass, 1989). Thus a selection for, or

more appropriately, an *in vivo* enrichment of bacteria such as those we describe here may explain the host animal's acquisition of tolerance to the toxins.

Description of *Denitrobacterium* gen. nov.

Denitrobacterium (De.nit.ro.bac.te'ri.um. L. pref. *de* from; L. n. *nitro* nitrocompound; Gr. neut. dim. n. *bakterion* a small rod; M.L. neut. n. *Denitrobacterium* nitrocompound-reducing rod).

Cells are rod-shaped (0.5–1.0 × 1.0–1.5 µm); bulbous ends may be present. Gram-positive and non-motile. Spores are not formed. Strictly anaerobic chemo-organotroph; growth is dependent on the presence of a suitable electron acceptor and is proportional to the amount of acceptor reduced. The G+C content of DNA ranges from 56 to 60 mol% (thermal denaturation method). The genus comprises a single species, *Denitrobacterium detoxificans*.

Fig. 1. Phylogenetic relationship of *Denitrobacterium detoxificans* gen. nov., sp. nov. to selected bacteria based on analysis of almost complete 16S rRNA gene sequences. Bar, 10% difference in sequence homology. The GenBank accession numbers of the 16S rDNA sequences of *Denitrobacterium detoxificans* strains NPOH1^T (ATCC 700546^T), NPOH2 (ATCC 700547), NPOH3 (ATCC 700548) and MAJ1 (ATCC 700549) are U43492, AF079505, AF079506 and AF079507, respectively. The GenBank accession numbers of the reference micro-organisms used in the phylogenetic analysis are as follows: *Escherichia coli* (J01859); *Sphaerobacter thermophilus* (X53210); *Clostridium perfringens* (Y12669); *Lactobacillus acidophilus* (M58802); *Staphylococcus aureus* (D83357); *Bacillus subtilis* (X60646); *Rubrobacter xylanophilus* (X87135); *Atopobium parvulum* (X67150); *Atopobium rimae* (X67149); *Atopobium minutum* (X67148); *Eubacterium fossor* (L34620); *Coriobacterium glomerans* (X79048); *Frankia* sp. Cea1.3 (U72717); *Blastococcus aggregatus* (L40614); *Propionibacterium acidipropionici* (X53221); *Propionibacterium acnes* (M61903); *Propionibacterium freudenreichii* (X53217); *Mycobacterium smegmatis* (X52922); *Mycobacterium flavescens* (X52932); *Mycobacterium simiae* (X52931). Bootstrap values indicated at branching points are given as a percentage of 1000 replications.

Description of *Denitrobacterium detoxificans* gen. nov., sp. nov.

Denitrobacterium detoxificans (de.tox.i.f'i.cans. L. pref. de from; L. n. toxicum poison; L. neut. n. detoxificans poison-reducer).

Isolated from a population of ruminal microbes enriched for enhanced metabolism of 3-nitropropanol, the toxic aglycone of miserotoxin (3-nitro-1-propyl-β-D-glucopyranoside). Nitrate, 3-nitropropanol, 2-nitropropanol, 3-nitropropionate, nitroethanol, nitroethane, 1-nitropropane, 2-nitrobutane, DMSO and trimethylamine oxide may be used as electron acceptors. Hydrogen, formate and (DL)-lactate can be used as electron donor. Growth occurs in media containing clarified rumen fluid, peptone and a suitable electron acceptor. Indole and H₂S are not produced, gelatin is not hydrolysed. Little if any acid is produced during growth in medium with hydrogen or formate as electron donor. Acetate is the major product produced during growth on lactate; D-lactate is used more readily than L-lactate. The predominant cellular fatty acid of cells grown in modified brain heart infusion medium was palmitic acid, although an abundance of other straight- and branched-chain fatty acids, aldehydes and dimethyl acetals were present. A *c*-type cytochrome was found in the type strain, NPOH1^T, which was the only strain tested. Of four strains examined, all share close (>99%) 16S rRNA gene sequence similarity. All strains have been deposited in the American Type Culture Collection. The type strain, strain NPOH1^T (ATCC 700546^T), grew equally well at 32, 37 and 39 °C. Strain NPOH1^T differs from the others (strain NPOH2, ATCC 700547; strain NPOH3, ATCC 700548; and MAJ1, ATCC 700549) in that it has the ability to reduce nitrate.

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