Desulfuromonas thiophila sp. nov., a New Obligately Sulfur-Reducing Bacterium from Anoxic Freshwater Sediment

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A mesophilic, acetate-oxidizing, sulfur-reducing bacterium, strain NZ27^T, was isolated from anoxic mud from a freshwater sulfur spring. The cells were ovoid, motile, and gram negative. In addition to acetate, the strain oxidized pyruvate, succinate, and fumarate. Sulfur flower could be replaced by polysulfide as an electron acceptor. Ferric nitrilotriacetic acid was reduced in the presence of pyruvate; however, this reduction did not sustain growth. These phenotypic characteristics suggested that strain NZ27^T is affiliated with the genus *Desulfuromonas*. A phylogenetic analysis based on the results of comparative 16S ribosomal DNA sequencing confirmed that strain NZ27^T belongs to the *Desulfuromonas* cluster in the recently proposed family “*Geobacteraceae*” in the delta subgroup of the *Proteobacteria*. In addition, the results of DNA-DNA hybridization studies confirmed that strain NZ27^T represents a novel species. *Desulfuromonas thiophila*, a name tentatively used in previous publications, is the name proposed for strain NZ27^T in this paper.

Bacteria that are able to grow by linking the oxidation of acetate to the reduction of elemental sulfur have been known since Pfennig and Biebl described the isolation of *Desulfuromonas acetoxidans* in 1976 (26). While a number of strains of the genus *Desulfuromonas* have been isolated and deposited in culture collections (33), *Desulfuromonas acetoxidans* (with type strain DSM 864) is the only species that has been validly published previously (26). Recently, two other species, the freshwater organism “*Desulfuromonas acetexigens*” (with type strain 2873 [= DSM 1397]) (10) and the marine organism “*Desulfuromonas palmitatis*” (with type strain SDBY1 [= ATCC 51701]) (4), have been characterized. Data obtained for a second freshwater strain (isolate NZ27^T), which was tentatively named *Desulfuromonas thiophila*, have been published in review articles (31, 33). However, a valid description of this strain has not been published previously.

In this paper we present a detailed characterization of *Desulfuromonas thiophila* NZ27^T, including a phenotypic description, the phylogenetic affiliation of this organism with known species of the recently described family “*Geobacteraceae*” (19), and the results of DNA-DNA hybridization studies performed with closely related species of the genus *Desulfuromonas*. Based on the genotypic and phenotypic differences between strain NZ27 and the other members of the genus *Desulfuromonas*, we propose that strain NZ27 is the type strain of a new species, *Desulfuromonas thiophila*.

MATERIALS AND METHODS

Source of the organism. Sulfur-reducing strain NZ27^T was isolated in 1979 by Bache and Pfennig (1) from anoxic mud from a freshwater sulfur spring at Ngawha Springs in Moerewa, New Zealand (North Island).

Media and cultivation conditions. The basal medium used for cultivation of freshwater strain NZ27^T contained (per liter) 0.5 g of NaCl, 0.2 g of MgCl₂ · 6H₂O, 0.1 g of CaCl₂ · 2H₂O, 0.1 g of NH₄Cl, 0.2 g of KH₂PO₄, and 0.25 g of KCl. After the medium was autoclaved and cooled under oxygen-free N₂, the following additions were made from sterile stock solutions: 30 ml of 1 M NaHCO₃ per liter, 3 ml of 0.5 M Na₂S per liter, 2 ml of trace element solution SL 10a (8) per liter, 1 ml of a vitamin B₁₂ solution per liter, 1 ml of a thiamine solution per liter, and 1 ml of a vitamin mixture per liter (32). After the NaHCO₃ was added, the medium was handled under a gas phase containing dinitrogen and CO₂ (90:10).

Finally, the medium was dispensed into 50-ml screw-cap bottles, leaving a small air bubble in each bottle.

Substrates were added to the basal medium from sterile stock solutions. Sterile suspensions of highly purified elemental sulfur (sulfur flower) and a polysulfide solution were prepared as described by Widdel and Pfennig (33). Elemental sulfur was aseptically added in pea size amounts to 50 ml of medium. Cultures were incubated in the dark at 28 or 30°C.

Enrichment and isolation. For enrichment cultures, the basal medium in 50-ml screw-cap bottles was supplemented with 5 mM acetate as an electron donor and carbon source and elemental sulfur as an electron acceptor. The bottles were inoculated with 2 ml of anoxic mud, incubated at 28°C in the dark, and thoroughly mixed each day. A slight turbidity and a marked increase in sulfide concentration were observed in consecutive passages, and these characteristics indicated that the enrichment cultures were positive. A pure culture was obtained by repeated use of deep agar dilution series (25). Agar deeps (1% agar) contained basal medium supplemented with elemental sulfur and 2 ml of polysulfide (from a 4 M stock solution) per liter, which resulted in preparations containing highly dispersed elemental sulfur and polysulfide. The solidified agar deeps developed an opaque lemon color. Appearance of pink to red colonies indicated that sulfur reducers of the *Desulfuromonas* type were present. In addition to microscopic examination, isolates were checked for culture purity in Difco AC medium.

Phenotypic characteristics. Gram staining of strain NZ27^T was performed by the method of Hucker, and flagellation was determined by the staining method of Leifson as described in the *Manual of Methods for General Bacteriology* (12). Motility was examined by phase-contrast microscopy.

Substrate tests. Potential electron donors and acceptors were tested in 20-ml screw-cap tubes, each of which contained a glass bead to disrupt clumps of elemental sulfur. Electron donors were tested with elemental sulfur as the electron acceptor. In the electron acceptor tests, pyruvate (5 mM) served as the electron donor. Unless otherwise indicated, the various electron acceptors were added at a concentration of 10 mM. Growth was monitored by measuring turbidity for 4 weeks.

Growth parameters. The pH tolerance, salt tolerance, and temperature tolerance of strain NZ27^T were determined by performing growth tests in which the changes in the optical density of the culture medium were monitored spectrophotometrically. Optimum growth was identified by comparing the optical densities of cultures after 3 days of incubation, and the growth range was determined after 28 days. In all of the tests, strain NZ27^T was grown in basal medium supplemented with elemental sulfur and pyruvate. We were careful not to resuspend the sulfur particles prior to the measurements.

To determine the pH range of strain NZ27^T, different amounts of sterile solutions of HCl (1 M) and Na₂CO₃ (1 M) were pipetted into inoculated 50-ml screw-cap bottles, and the contents were thoroughly mixed and transferred into 20-ml screw-cap test tubes. Finally, the pH of the liquid that remained in each 50-ml bottle was determined.

The salt tolerance of strain NZ27^T was determined by supplementing NaCl- and MgCl₂-free basal medium containing elemental sulfur, pyruvate, and inoculum (5% vol/vol) with different amounts of NaCl and MgCl₂ from sterile stock solutions so that the concentrations desired were obtained. The cultures used for salt and pH tolerance determinations were incubated at 30°C in the dark. The temperature range was determined by incubating inoculated test tubes in an

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insulated temperature gradient block. The lowest temperature tested was 0°C, and the highest temperature tested was 50°C.

Analytical procedures. Sulfide was quantified by the methylene blue method described by Cline (3). The presence of cytochromes was determined with whole cells harvested at the late exponential growth phase. Difference spectra were obtained with air-oxidized and dithionite-reduced cell suspensions. The guanine-plus-cytosine ratio was determined by the thermal denaturation method (23) and high-performance liquid chromatography (HPLC) separation followed by UV detection (29). Calibration and G+C content determinations by the latter method were performed as described by Mesbah et al. (24) at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany).

Iron metabolism. Four 100-ml cultures of strain NZ27^T were grown with pyruvate as the electron donor and polysulfide as the electron acceptor. After 5 days, the cells were harvested by centrifugation at 5,000 × g and washed with anoxic bicarbonate buffer (2.5 g/liter; pH 7) prepared under N₂-CO₂ (80:20) as described previously (21). The cells were resuspended in bicarbonate buffer. The experiment was performed in duplicate in 15-ml serum vials containing 10 mM pyruvate and 10 mM ferric nitrilotriacetic acid [Fe(III)-NTA]. Fe(III)-NTA (pH 7) was prepared as described previously (27). The amount of HCl-extractable Fe(II) was determined by the ferrozine assay as described previously (20). Two controls were used; the first control was prepared like the test vials, but the cells were heat killed in an 80°C water bath for 5 min, and pyruvate was omitted from the second control. All of the vials were inoculated with 0.1 ml of the cell suspension and incubated at 30°C.

To test for growth, an inoculum was obtained by transferring strain NZ27^T from an active polysulfide-reducing culture into basal medium (10% inoculum) containing Fe(III)-NTA (10 mM) as the sole electron acceptor and pyruvate as the sole electron donor. When all of the Fe(III) was reduced (approximately 7 days), this culture was used to inoculate (10% inoculum) three 100-ml portions of basal medium containing 10 mM Fe(III)-NTA as the sole electron acceptor. Pyruvate (10 mM) was added to two of the vials but not to the third. All of the vials were incubated at 30°C. Growth was monitored by monitoring Fe(II) production and determining epifluorescent cell counts by acridine orange staining.

Phylogenetic analysis. DNA isolation, PCR-mediated amplification of the almost complete 16S rRNA gene, and a sequence analysis were performed as described by Liesack and Finster (18). Both strands of the 16S ribosomal DNA (rDNA) PCR product were completely sequenced by using a standard set of oligonucleotide primers (17). The 16S rDNA sequence of strain NZ27^T was manually aligned and compared with reference sequences of various members of the delta subgroup of the *Proteobacteria* obtained from the EMBL database (6) and the Ribosomal Database Project (22). The phylogenetic position of strain NZ27^T was deduced by comparing the 16S rDNA sequence of this organism with the 16S rDNA sequences of representative members of the recently proposed family "Geobacteraceae" (19). For tree construction we considered only those positions at which bases could be aligned unambiguously (1,224 nucleotide positions). Pairwise evolutionary distances were computed by using the correction of Jukes and Cantor (16) implemented in the DNADIST program of the PHYLIP (version 3.5) package (7). A tree on which the phylogenetic relationships were estimated was derived by using the FITCH program of the same package with a random-order input of sequences and the global rearrangement option. The accession numbers for the reference nucleotide sequences used to construct the phylogenetic tree are as follows: *Desulfuromonas acetoxidans*, M26634; "*Desulfuromonas acetexigens*," U23140; "*Desulfuromonas palmitatis*," U28172; *Desulfuromusa kysingii*, X79414; "*Geobacter chapeliei*," U41561; "*Geobacter hydrogenophilus*," U46860; *Geobacter metallireducens*, L07834; *Geobacter sulfurreducens*, U13928; *Pelobacter acetylenicus*, X70955; *Pelobacter acidigallii*, X77216; *Pelobacter carbinolicus*, X79419; *Pelobacter propionicus*, X70964; *Pelobacter venetianus*, U41562; and *Desulfomonile tiedjei*, M26635.

DNA-DNA hybridization. DNA-DNA hybridizations were carried out by workers at the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. Genomic DNAs were isolated by chromatography on hydroxyapatite by using the procedure of Cashion et al. (2). DNA-DNA hybridizations were performed by the spectroscopic method of De Ley et al. (5) with modifications described by Huss et al. (14) by using a spectrophotometer (Gilford System model 2600) equipped with a thermoprogrammer (Gilford model 2527-R) and a plotter. The DNA concentration was 50 µg/ml. The optimal temperature of renaturation (70°C) was calculated from the average G+C content of the samples by the formula of Gillis et al. (13). The DNA reassociation rate was calculated by performing a regression analysis between 10 and 30 min after start of the reaction by using the computer program TRANSFER.BAS (15). The homology values were calculated by using the formula of De Ley et al. (5). Each measurement was made three times.

Chemicals. All of the chemicals were reagent grade and were obtained from commercial suppliers.

Nucleotide sequence accession number. The 16S rDNA sequence of strain NZ27^T has been deposited in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession no. Y11560.

FIG. 1. Phase-contrast photomicrograph of *Desulfuromonas thiophila* NZ27^T grown with pyruvate and polysulfide. Bar = 10 µm.

RESULTS

Enrichment and isolation. Enrichment cultures for sulfur-reducing bacteria were prepared with medium containing acetate (5 mM) and sulfur flower. Anoxic mud from Ngawha Sulfur Springs near Moerewa, New Zealand, was used as the inoculum. The incubation temperature was 28°C. Positive enrichment cultures were obtained after 2 weeks. These enrichment cultures were transferred every week. After four consecutive transfers, a subsample was used to inoculate deep agar dilution series. A few well-separated colonies were isolated from the third dilution series and transferred to liquid culture media supplemented with acetate (5 mM) and polysulfide. One of the cultures, strain NZ27^T, was maintained as a stock culture and was used for further studies.

Phenotypic characteristics. (i) Morphology. Cells of strain NZ27^T were ovoid rods that were 0.7 to 0.8 µm wide and 1.5 to 2 µm long (Fig. 1) and motile. Flagellar staining revealed the presence of one polar flagellum. The cells stained gram negative. In agar medium smooth pink to red colonies were formed.

(ii) Carbon and energy sources. Strain NZ27^T was able to grow with acetate, pyruvate, succinate, and fumarate in the presence of elemental sulfur. The following chemicals were tested but were not used by strain NZ27^T for growth when elemental sulfur was added as the electron acceptor (the concentration of the electron donor was 5 mM unless indicated otherwise): H₂, formate (10 mM), propionate, butyrate, caproate, oenanthal, palmitate (2 mM), myristate (2 mM), lactate, cyclohexanecarboxylate, citrate, malate, tartrate, malonate, glucose, fructose, mannitol, glycerol, glycolate, methanol, ethanol, *n*-propanol, butanol, benzoate (1 mM), betaine, alanine, aspartate, glutamate, Casamino Acids (0.1%), yeast extract (0.1%), and peptone (0.1%). A fermentative type of metabolism with fumarate, malate, or sugars was not observed. Elemental sulfur was the only electron acceptor with which sustained growth occurred. Fe(III)-NTA (10 mM) was reduced; however, this transformation was not coupled with growth (Fig. 2). Sulfate, sulfite, thiosulfate, nitrate, fumarate,

FIG. 2. Fe(II) concentrations (● and ○) and cell densities (▲ and △) as a function of time. Fe(III)-NTA was used as the electron acceptor, and pyruvate was used as the electron donor. Pyruvate-free preparations were used as controls.

malate, dimethyl sulfoxide, and trimethylamine-*N*-oxide dihydrate were not utilized as electron acceptors.

(iii) **Growth factor requirements and salt tolerance.** The complex vitamin solution which was originally used during enrichment and isolation of strain NZ27^T could be replaced by biotin. Biotin was essential for growth; however, the threshold concentration necessary for growth was not determined. Amino acids were not required.

Strain NZ27^T grew equally well in the presence of 0 to 0.3% NaCl and in the presence of 0.03 to 0.12% MgCl₂ · 6H₂O. Growth was inhibited by NaCl concentrations greater than 1.2% and by MgCl₂ · 6H₂O concentrations greater than 0.35%.

(iv) **pH and temperature range.** Strain NZ27^T grew at pH 6.5 to 8.2, and optimum growth occurred at pH 6.9 to 7.9. The minimum growth temperature was 10°C, and the maximum temperature was 39°C. After growth at 39°C, the cells were generally larger than cells grown at lower temperatures, and a large number of spheroplasts were observed after 1 week of incubation. After transfer to fresh medium and incubation at lower temperatures, cells having the original morphology developed. The optimum growth temperature was between 26 and 30°C. No growth occurred at temperatures above 40°C.

(v) **Pigments.** The difference spectra of whole cells had maxima at 419, 523, and 553 nm. These absorption maxima are characteristic of type *c* cytochromes.

Genotypic characteristics. (i) **DNA-DNA hybridization.** DNA-DNA hybridization experiments were performed with strain NZ27^T, *Desulfuromonas acetoxidans* DSM 684^T, "*Desulfuromonas acetexigens*" DSM 1397, and "*Desulfuromonas palmitatis*" ATCC 51701^T. The levels of DNA-DNA hybridization with strain NZ27^T were between 22 and 31% (Table 1). These

values indicated that the strains tested belong to different species (30).

(ii) **DNA base composition.** The G+C content of the DNA of strain NZ27^T was 61 mol%, as determined by the thermal denaturation method. The same ratio was obtained by HPLC. The values previously reported for "*Desulfuromonas acetexigens*" (10) were also confirmed by the HPLC technique, while the value for *Desulfuromonas acetoxidans* was slightly different (Table 1).

Phylogenetic position. The comparative analysis of the almost complete 16S rDNA sequence confirmed that strain NZ27^T belongs to the *Desulfuromonas* cluster of the recently proposed family "*Geobacteraceae*" (19). The most closely related species was *Desulfuromonas acetoxidans* (level of similarity, 96.6%) (Fig. 3), as reported previously on the basis of the results of comparative 16S rRNA oligonucleotide cataloging studies (11). The levels of similarity between the 16S rDNA sequence of strain NZ27^T and the 16S rDNA sequences of "*Desulfuromonas acetexigens*" and "*Desulfuromonas palmitatis*" were 95.0 and 94.4%, respectively. The levels of similarity with *P. venetianus*, *P. carbinolicus*, and *P. acetylenicus* were 95.4, 94.8, and 94.4%, respectively, while the levels of similarity with members of the genus *Geobacter* were 93.1% or less. A bootstrap analysis based on 1,000 resamplings to test the statistical significance of the interior branches confirmed that the lineage formed by strain NZ27^T and *Desulfuromonas acetoxidans* was distinct, with a bootstrap value of 100 (expressed as a percentage of outcome). The general topology of the tree constructed in this study (Fig. 3) is almost identical to the topology of the tree published by Loneragan et al. (19); there are only slight differences in the interior branch points within the *Desulfuromonas* cluster.

DISCUSSION

In this paper we describe the isolation and identification of a mesophilic, obligately anaerobic, sulfur-reducing bacterium (strain NZ27^T) obtained from the anoxic sediment of a freshwater sulfur spring. The results of a previous study of the phylogenetic affiliation of this isolate with other sulfur-reducing bacteria indicated that strain NZ27^T is most closely related to *Desulfuromonas acetoxidans* and "*Desulfuromonas acetexigens*," with *S*_{AB} values of 0.64 and 0.59, respectively (11). The results of the comparative analysis of the almost complete 16S rDNA sequences confirmed the previous finding that strain NZ27^T and *Desulfuromonas acetoxidans* form a distinct line of descent. DNA-DNA hybridization studies of strain NZ27^T and the previously described species of the genus *Desulfuromonas*, including *Desulfuromonas acetoxidans*, gave reassociation values that were not greater than 31% (Table 1). These values were far below the DNA-DNA reassociation value of about 70% which is used to separate related organisms into different

TABLE 1. G+C contents of DNAs as determined by the thermal denaturation method and HPLC and DNA-DNA hybridization results

Strain	G+C content (mol%)		% Similarity to:		
	Thermal denaturation method	HPLC	<i>Desulfuromonas acetoxidans</i> DSM 684 ^T	" <i>Desulfuromonas acetexigens</i> " DSM 1397 ^T	" <i>Desulfuromonas palmitatis</i> " ATCC 51701 ^T
<i>Desulfuromonas acetoxidans</i> DSM 684 ^T	51 ± 1	53.6 ± 0.4			
" <i>Desulfuromonas acetexigens</i> " DSM 1397 ^T	62 ± 1	62.3 ± 0.4	20		
" <i>Desulfuromonas palmitatis</i> " ATCC 51701 ^T	ND	54.7 ± 0.2	22	37	
NZ27 ^T (= DSM 8987 ^T)	61 ± 1	61.6 ± 0.2	27	22	31

^a ND, not determined.

FIG. 3. Phylogenetic tree constructed for strain NZ27^T and 13 reference organisms belonging to the family "Geobacteraceae" of the delta subgroup of the *Proteobacteria*. The 16S rDNA sequence of *Desulfomonile tiedjei* was used as an outgroup sequence. Scale bar = 2% difference in nucleotide sequences as determined by measuring the horizontal branch lengths connecting two species.

species (30). Thus, these results suggest that strain NZ27^T is a new species of the genus *Desulfuromonas*. The relationship between the overall similarity of the 16S rDNA sequences and the DNA-DNA reassociation values obtained for strain NZ27^T and the other members of the genus *Desulfuromonas* confirmed the general finding that strains with levels of 16S rDNA similarity below the threshold value of 97% never exhibit DNA-DNA reassociation values of 70% or more (28).

Strain NZ27^T and all previously described members of the genus *Desulfuromonas* have the ability to oxidize acetate with concomitant reduction of elemental sulfur to hydrogen sulfide (33). This characteristic has also been found in members of the recently described genera *Desulfurella* and *Desulfuromusa* (1a, 9, 18). In contrast to all of the other members of the family "Geobacteraceae" tested for the ability to grow by reducing iron (19), strain NZ27^T was not able to grow by reducing this element, but used only elemental sulfur as an electron acceptor. However, it is able to reduce Fe(III)-NTA.

Like type strain *Desulfuromonas acetoxidans* DSMZ 684 cells, cells of strain NZ27^T are ovoid to rod shaped and motile and form smooth pink to red colonies in agar medium. They also require biotin for growth. Like the other members of the *Desulfuromonas* cluster, strain NZ27^T contains type *c* cytochromes.

Based on the results of the polyphasic approach described above, we propose the new species *Desulfuromonas thiophila* for strain NZ27^T.

Description of *Desulfuromonas thiophila* sp. nov. *Desulfuromonas thiophila* (thi.o.phi'la. Gr. n. thios, sulfur; Gr. adj. philos, loving; M.L. fem. adj. thiophila sulfur loving). Cells are ovoid, 0.7 to 0.8 µm wide by 1.5 to 2.0 µm long. Multiplication is by binary fission. Cells are motile by means of polar flagella. Obligately anaerobic. Chemotrophic growth occurs in mineral medium supplemented with 0.01 to 0.03% Na₂S · 9H₂O as a reductant. Growth is inhibited by NaCl concentrations greater than 1.2%. Biotin is required as a growth factor. Acetate, pyruvate, succinate, and fumarate are used as electron donors and carbon sources in the presence of 30 mM hydrogen carbonate. Elemental sulfur is as the only electron acceptor utilized for growth. Fe(III)-NTA is reduced with pyruvate; this reduction does not sustain growth. The pH range is 6.5 to 8.2; the optimum pH is 6.9 to 7.9. The optimum growth temperature is 26 to 30°C.

Accumulations of cells in colonies or pellets are pink to reddish; under reducing conditions cell suspensions exhibit absorption spectra characteristic of type *c* cytochromes (absorption maxima at 419, 523, and 553 nm).

The G+C content of the genomic DNA of strain NZ27^T is 61.0 ± 1 mol% as determined by the thermal denaturation method and 61.6 ± 0.2 mol% as determined by HPLC.

Phylogenetically, strain NZ27^T belongs to the *Desulfuromonas* cluster of the recently proposed family "Geobacteraceae" in the delta subgroup of the *Proteobacteria* and is most closely related to *Desulfuromonas acetoxidans*.

The habitat is anoxic mud of a freshwater sulfur spring.

The type strain was isolated from anoxic mud from a freshwater sulfur spring at Ngawha Springs near Moerewa, New Zealand.

The type strain is strain NZ27, which has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, as strain DSMZ 8987.

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