Desulfovibrio paquesii sp. nov., a hydrogenotrophic sulfate-reducing bacterium isolated from a synthesis-gas-fed bioreactor treating zinc- and sulfate-rich wastewater

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A hydrogenotrophic, sulfate-reducing bacterium, designated strain SB1^T, was isolated from sulfidogenic sludge of a full-scale synthesis-gas-fed bioreactor used to remediate wastewater from a zinc smelter. Strain SB1^T was found to be an abundant micro-organism in the sludge at the time of isolation. Hydrogen, formate, pyruvate, lactate, malate, fumarate, succinate, ethanol and glycerol served as electron donors for sulfate reduction. Organic substrates were incompletely oxidized to acetate. 16S rRNA gene sequence analysis showed that the closest recognized relative to strain SB1^T was *Desulfovibrio gigas* DSM 1382^T (97.5 % similarity). The G+C content of the genomic DNA of strain SB1^T was 62.2 mol%, comparable with that of *Desulfovibrio gigas* DSM 1382^T (60.2 mol%). However, the level of DNA–DNA relatedness between strain SB1^T and *Desulfovibrio gigas* DSM 1382^T was only 56.0 %, indicating that the two strains are not related at the species level. Strain SB1^T could also be differentiated from *Desulfovibrio gigas* based on phenotypic characteristics, such as major cellular fatty acid composition (anteiso-$C_{15:0}$, iso-$C_{14:0}$ and $C_{18:1}$ *cis* 9) and substrate utilization. Strain SB1^T is therefore considered to represent a novel species of the genus *Desulfovibrio*, for which the name *Desulfovibrio paquesii* sp. nov. is proposed. The type strain is SB1^T (=DSM 16681^T=JCM 14635^T).

Sulfate- and metal-rich wastewaters that are low in organic carbon are produced as a result of several industrial processes and pose a large environmental problem (Lens *et al.*, 1998; Johnson, 2000). Synthesis-gas-fed sulfate-reducing bioreactors have been shown to be suitable systems to remediate these types of wastewater (Boonstra *et al.*, 1999; van Houten *et al.*, 2006). In a previous study, the communities of two synthesis-gas-fed sulfate-reducing THIOPAQ bioreactors were found to be dominated by heterotrophic sulfate-reducing bacteria belonging to the genera *Desulfovibrio* and *Desulfomicrobium* (van Houten *et al.*, 2006). In order to perform growth kinetic and competition studies (van Houten, 2006), micro-organisms representing different trophic groups were isolated from these synthesis-gas-fed bioreactors.

A sulfate-reducing bacterium, designated strain SB1^T, was isolated from the sulfidogenic sludge of a full-scale THIOPAQ gas lift bioreactor, as described previously (van Houten *et al.*, 2006). This bioreactor, used for the remediation of wastewater from a zinc smelter (Budel-Dorplein, The Netherlands), was fed with hydrogen-rich synthesis gas and was operated at a temperature of 30–35 °C (van Houten *et al.*, 2006).

Sludge taken from the recycled material was crushed as described by Oude Elferink *et al.* (1995), serially diluted in defined liquid medium (Stams *et al.*, 1993) supplemented with sulfate (20 mM), acetate (4 mM) and an H_2/CO_2 gas-phase (ratio 4 : 1, pressure 1.7 kPa), and incubated at 30 °C. Strain SB1^T was obtained from the highest dilution showing growth (10^8), which contained micro-organisms

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Abbreviations: DGGE, denaturing gradient gel electrophoresis; rep-PCR, repetitive enterobacterial palindromic PCR.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strain SB1^T and *Desulfovibrio gigas* DSM 1382^T are AY726757 and DQ447183, respectively.

A rep-PCR pattern comparison of strain SB1^T and *Desulfovibrio* strains L3 and L7 is available as supplementary material with the online version of this paper.

with two distinct cell morphologies. This dilution was serially diluted in liquid medium and after incubation the highest dilution showing growth was serially diluted by using the agar roll-tube method (Hungate, 1969). A single well-separated colony was taken and serially diluted in liquid medium. The highest dilution showing growth was a pure culture, and this was designated strain SB1^T. The morphology of this micro-organism was the dominant one observed in the original highest dilution. Purity was confirmed by microscopic observation, from uniform colony formation in agar tubes and by testing for anaerobic contaminants on Wilkins–Chalgren anaerobe broth.

Cells of strain SB1^T were vibrio- to spiral-shaped (1 × 5–8 μm), motile and appeared singly or in chains of up to eight cells. Strain SB1^T was desulfoviridin-positive and stained Gram-negative. Phase-contrast microscopy showed refractive structures, but spores were never observed. In solid medium the strain produced lens-shaped colonies.

For phylogenetic analysis, total DNA was extracted as described by Oude Elferink *et al.* (1997). 16S rRNA gene sequence analysis and denaturing gradient gel electrophoresis (DGGE) analysis were performed according to Roest *et al.* (2005). Two different 16S rRNA gene sequences were found for strain SB1^T, showing a single base pair difference at *Escherichia coli* position 1326. DGGE analysis with a primer pair amplifying the mismatch region also showed a profile with two dominant bands (Fig. 1), indicating that strain SB1^T has at least two different 16S rRNA operons within its genome. These two dominant bands were observed in the original sludge and also throughout the serial dilutions that were used to enrich strain SB1^T (Fig. 1).

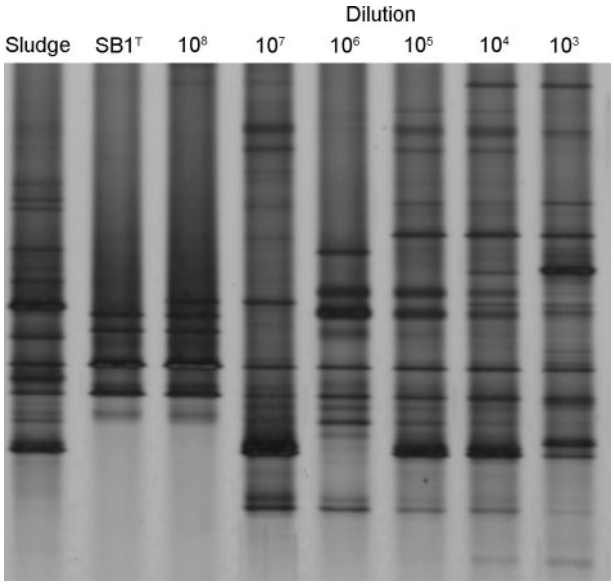

Fig. 1. 16S rRNA gene-based DGGE analysis of the bacterial diversity of the serial dilutions used to enrich strain SB1^T (*Desulfovibrio paquesii* sp. nov.).

This observation, together with the fact that strain SB1^T was obtained from a very high (10^8) dilution, indicates it was an abundant sulfate-reducing bacterium at the time of isolation.

16S rRNA gene sequence analysis by using the NCBI BLAST tool (McGinnis & Madden, 2004) indicated that strain SB1^T was most closely related to the sulfate-reducing strain L3 (GenBank accession number EF055876) isolated by Dar *et al.* (2007) (99 % sequence similarity). Interestingly, strain L3 was found to be a dominant sulfate reducer in an ethanol-fed sulfidogenic THIOPAQ bioreactor. Phylogenetic analysis confirmed strain L3 to be the closest relative of strain SB1^T (Fig. 2). No phenotypic differences were found between strains L3 and SB1^T based on either substrate utilization or morphological characteristics. A genomic comparison was made by using repetitive enterobacterial palindromic PCR (rep-PCR) as described by Dar *et al.* (2007); no differences were found in this comparison, indicating that strains L3 and SB1^T represent the same species. Results of this rep-PCR comparison are shown in Supplementary Fig. S1 in IJSEM Online.

Using the NCBI BLAST tool, the closest recognized relatives to SB1^T were found to be *Desulfovibrio indonesiensis* DSM 15121^T (89 % sequence similarity) and *Desulfovibrio giganteus* DSM 4370 (89 %). However, the 16S rRNA gene sequence of *Desulfovibrio gigas* DSM 1382^T (GenBank accession number M34400.1) contained 112 ambiguous nucleotides, which made an accurate phylogenetic comparison impossible with this potentially related organism. Therefore, the 16S rRNA gene of *Desulfovibrio gigas* DSM 1382^T was resequenced (DQ447183). This sequence was aligned with that of strain SB1^T by using the NCBI pairwise alignment BLAST tool, which revealed a similarity of 97.5 %. A 16S rRNA gene sequence similarity of 97 % is commonly considered as the upper limit for the definition of separate species (Stackebrandt & Goebel, 1994). Phylogenetic analysis confirmed *Desulfovibrio gigas* DSM 1382^T to be the closest recognized relative of strain SB1^T (Fig. 2). Analysis of the G+C content of the genomic DNA of SB1^T was performed at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany) according to recognized methods (Cashion *et al.*, 1977; Tamaoka & Komagata, 1984; Mesbah *et al.*, 1989); the DNA G+C content of strain SB1^T was 62.2 mol%. This is comparable with the value of 60.2 mol% found for *Desulfovibrio gigas* DSM 1382^T and falls within the 5 % range normally observed within the same species (Rosselló-Mora & Amann, 2001).

Genomic DNA–DNA hybridization was performed at the DSMZ according to De Ley *et al.* (1970) with the modifications suggested by Escara & Hutton (1980) and Huß *et al.* (1983). This revealed a level of DNA–DNA relatedness of 56.0 % between strain SB1^T and *Desulfovibrio gigas* DSM 1382^T, indicating that the two strains are not related at the species level (Wayne *et al.*, 1987). Differential morphological and physiological characteristics between

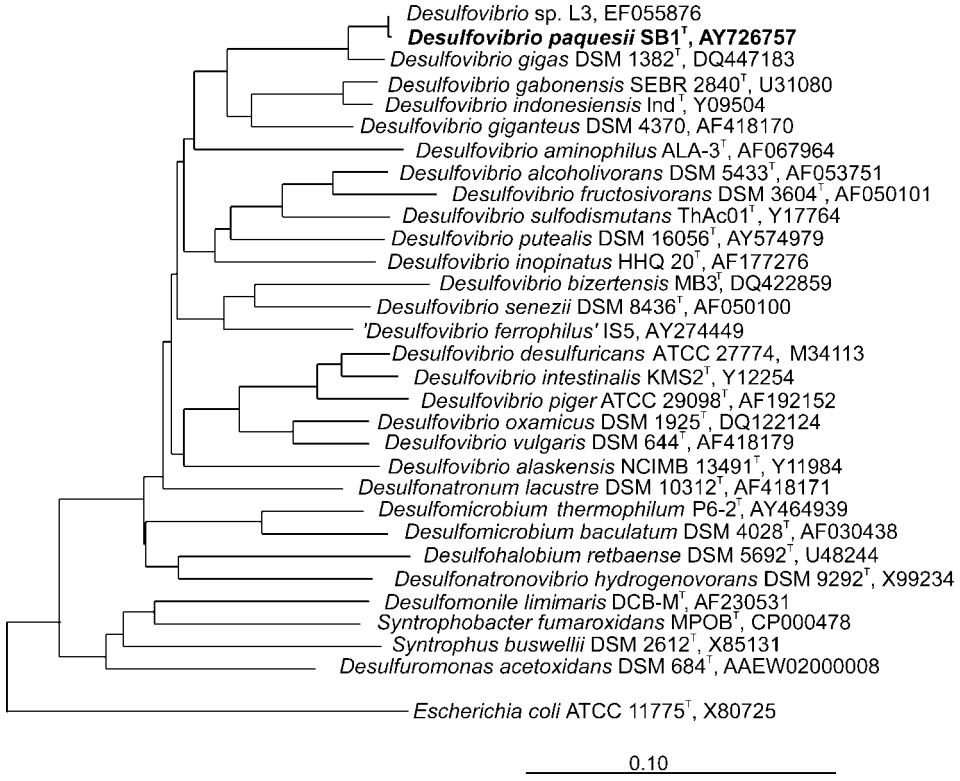

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences of selected *Deltaproteobacteria*. GenBank accession numbers of reference sequences are indicated. *E. coli* ATCC 11775^T was used as an outgroup. Phylogenetic analysis was based on release 93 of the SILVA database (Pruesse *et al.*, 2007). Sequences were aligned by using the ARB software package, with the FastAligner tool, and were checked manually according to secondary structures (Ludwig *et al.*, 2004). A phylogenetic tree was constructed with Felsenstein correction and a filter for *Deltaproteobacteria* in ARB, according to the neighbour-joining method (*E. coli* positions 80–1423). Bar, 10 % nucleotide sequence divergence.

strain SB1^T and *Desulfovibrio gigas* DSM 1382^T are given in Table 1; other characteristics are given in the species description below. Strain SB1^T showed differences with regard to the utilization of malate, glycerol and pyruvate. Strain SB1^T also differed from *Desulfovibrio gigas* DSM 1382^T with respect to major cellular fatty acids. Cellular fatty acid composition analysis was performed at the DSMZ according to Kaksonen *et al.* (2006). Based on the

Table 1. Comparison of the morphological and physiological characteristics of strain SB1^T (*Desulfovibrio paquesii* sp. nov.) and *Desulfovibrio gigas* DSM 1382^T

Data for *Desulfovibrio gigas* DSM 1382^T are from Edlund *et al.* (1985), Esnault *et al.* (1988), Kremer *et al.* (1989) and Dar *et al.* (2007). Tests for utilization of carbon and energy sources were performed in duplicate by using defined medium (Stams *et al.*, 1993) at 37 °C. +, Positive; −, negative. The combination of substrate conversion, product formation and an increase in turbidity was considered to indicate positive growth.

Characteristic	Strain SB1^T	*D. gigas* DSM 1382^T
Cell size (μm)	1 × 5–8	0.8–1 × 6–11
Major cellular fatty acids	anteiso-$C_{15:0}$, iso-$C_{14:0}$, $C_{18:1}$ *cis* 9	iso-$C_{15:0}$, $C_{16:0}$, iso-$C_{17:0}$
Electron donors used for sulfate reduction		
Malate (20 mM)	+	−
Glycerol (20 mM)	+	−
Fermentative growth with:		
Malate (20 mM)	−	+
Pyruvate (20 mM)	+	−

genotypic and phenotypic data presented, strain SB1^T is considered to represent a novel species of the genus *Desulfovibrio*, for which the name *Desulfovibrio paquesii* sp. nov. is proposed.

Description of *Desulfovibrio paquesii* sp. nov.

Desulfovibrio paquesii (pa.que′si.i. N.L. gen. n. *paquesii* of Paques, named after Jos Paques, a Dutch biotechnologist and entrepreneur, in recognition of his contribution to the application of the biological sulfur cycle in anaerobic wastewater treatment).

Cells are Gram-negative, non-spore-forming, motile, vibrio- to spiral-shaped (1×5–8 μm), and occur singly or in chains of up to eight cells. Grows between pH 6.5 and 8.5 and at 10–45 °C. Strictly anaerobic; reduces sulfate, sulfite and thiosulfate, producing sulfide. Organic substrates are incompletely oxidized to acetate. Utilizes hydrogen, formate, pyruvate, fumarate, lactate, succinate, malate, ethanol and glycerol as electron donors for sulfate reduction. Acetate, propionate, butyrate, methanol, cysteine and choline do not serve as electron donors. Malate and choline are not used fermentatively. Nitrate is not used as an electron acceptor. Positive for desulfoviridin-type sulfite reductase. Major cellular fatty acids are anteiso-$C_{15:0}$ (38.5 %), iso-$C_{14:0}$ (18.5 %) and $C_{18:1}$ *cis* 9 (11.3 %). The DNA G+C content of the type strain is 62.2 mol%.

The type strain, SB1^T (=DSM 16681^T=JCM 14635^T), was isolated from a sulfidogenic synthesis-gas-fed bioreactor used for remediation of wastewater from a zinc smelter (Budel-Dorplein, The Netherlands).

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References

Boonstra, J., van Lier, J., Janssen, G., Dijkman, H. & Buisman, C. J. N. (1999). Biological treatment of acid mine drainage. In *Biohydrometallurgy and Environment Toward the 21st Century. Process Metallurgy 9B*, pp. 559–567. Edited by R. Amils & A. Ballister. Amsterdam: Elsevier.

Cashion, P., Holder-Franklin, M. A., McCully, J. & Franklin, M. (1977). A rapid method for the base ratio determination of bacterial DNA. *Anal Biochem* **81**, 461–466.

Dar, S. A., Stams, A. J. M., Kuenen, J. G. & Muyzer, G. (2007). Co-existence of physiologically similar sulfate-reducing bacteria in a full-scale sulfidogenic bioreactor fed with a single organic electron donor. *Appl Microbiol Biotechnol* **75**, 1463–1472.

De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.

Edlund, A., Nichols, P. D., Roffey, R. & White, D. C. (1985). Extractable and lipopolysaccharide fatty acid and hydroxy acid profiles from *Desulfovibrio* species. *J Lipid Res* **26**, 982–988.

Escara, J. F. & Hutton, J. R. (1980). Thermal stability and renaturation of DNA in dimethyl sulfoxide solutions: acceleration of the renaturation rate. *Biopolymers* **19**, 1315–1327.

Esnault, G., Caumette, P. & Garcia, J.-L. (1988). Characterization of *Desulfovibrio giganteus* sp. nov., a sulfate reducing bacterium isolated from a brackish coastal lagoon. *Syst Appl Microbiol* **10**, 147–151.

Hungate, R. E. (1969). A roll tube method for cultivation of strict anaerobes. *Methods Microbiol* **3B**, 117–132.

Huß, V. A. R., Festl, H. & Schleifer, K. H. (1983). Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* **4**, 184–192.

Johnson, D. B. (2000). Biological removal of sulfurous compounds from inorganic wastewaters. In *Environmental Technologies*, pp. 175–206. Edited by P. Lens & L. Hulshoff Pol. London: International Association on Water Quality.

Kaksonen, A. H., Spring, S., Schumann, P., Kroppenstedt, R. M. & Puhakka, J. A. (2006). *Desulfotomaculum thermosubterraneum* sp. nov., a thermophilic sulfate-reducer isolated from an underground mine located in a geothermally active area. *Int J Syst Evol Microbiol* **56**, 2603–2608.

Kremer, D. R., Nienhuis-Kuiper, H. E., Timmer, C. J. & Hansen, T. A. (1989). Catabolism of malate and related dicarboxylic acids in various *Desulfovibrio* strains and the involvement of an oxygen-labile NADPH dehydrogenase. *Arch Microbiol* **151**, 34–39.

Lens, P. N. L., Visser, A., Janssen, A. J. H., Hulshoff Pol, L. & Lettinga, G. (1998). Biotechnological treatment of sulfate-rich wastewaters. *Crit Rev Environ Sci Technol* **28**, 41–88.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S. & other authors (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32**, 1363–1371.

McGinnis, S. & Madden, T. L. (2004). BLAST: at the core of a powerful and diverse set of sequence analysis tools. *Nucleic Acids Res* **32**, W20–W25.

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

Oude Elferink, S. J. W. H., Maas, R. N., Harmsen, H. J. M. & Stams, A. J. M. (1995). *Desulforhabdus amnigenus* gen. nov. sp. nov. a sulfate reducer isolated from anaerobic granular sludge. *Arch Microbiol* **164**, 119–124.

Oude Elferink, S. J. W. H., Rinia, H. A., Bruins, M. E., de Vos, W. M. & Stams, A. J. M. (1997). Detection and quantification of *Desulforhabdus amnigenus* in anaerobic granular sludge by dot blot hybridization and PCR amplification. *J Appl Microbiol* **83**, 102–110.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W., Peplies, J. & Glockner, F. O. (2007). SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* **35**, 7188–7196.

Roest, K., Heilig, H. G. H. J., Smidt, H., de Vos, W. M., Stams, A. J. M. & Akkermans, A. D. L. (2005). Community analysis of a full-scale anaerobic bioreactor treating paper mill wastewater. *Syst Appl Microbiol* **28**, 175–185.

Rosselló-Mora, R. & Amann, R. (2001). The species concept for prokaryotes. *FEMS Microbiol Rev* **25**, 39–67.

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

Stams, A. J. M., Van Dijk, J. B., Dijkema, C. & Plugge, C. M. (1993). Growth of syntrophic propionate-oxidizing bacteria with fumarate in the absence of methanogenic bacteria. *Appl Environ Microbiol* **59**, 1114–1119.

Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.

van Houten, B. H. G. W. (2006). *Microbial aspects of synthesis gas fed bioreactors treating sulfate and metal rich wastewaters*. PhD thesis, Wageningen University.

van Houten, B. H. G. W., Roest, K., Tzeneva, V. A., Dijkman, H., Smidt, H. & Stams, A. J. M. (2006). Occurrence of methanogenesis during start-up of a full-scale synthesis gas-fed reactor treating sulfate and metal-rich wastewater. *Water Res* **40**, 553–560.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.