Pseudorhodoferax soli gen. nov., sp. nov. and *Pseudorhodoferax caeni* sp. nov., two members of the class *Betaproteobacteria* belonging to the family *Comamonadaceae*

Nadine Bruland,¹ Stephan Bathe,² Anne Willems³
and Alexander Steinbüchel¹

Correspondence

Alexander Steinbüchel
steinbu@uni-muenster.de

¹Institut für Molekulare Mikrobiologie und Biotechnologie, Westfälische Wilhelms-Universität Münster, Correnstrasse 3, 48149 Münster, Germany

²Institut für Ingenieurbiologie und Biotechnologie des Abwassers, Universität Karlsruhe, Am Fasanengarten, 76131 Karlsruhe, Germany

³Laboratory of Microbiology, Department of Biochemistry, Physiology and Microbiology, Universiteit Gent, K.L. Ledeganckstraat 35, 9000 Gent, Belgium

A novel betaproteobacterium, strain TBEA3^T, was isolated from soil using enrichment cultures with the organic thioether 3,3'-thiodipropionic acid as sole carbon and energy source. Analysis of the 16S rRNA gene sequence revealed 99.1 % sequence similarities to a poorly characterized member of the family *Comamonadaceae*, strain SB1^T, which had been previously isolated from activated sludge. Both strains showed highest gene sequence similarities (up to 96.9 %) to members of the genera *Rhodoferax* and *Curvibacter*. The DNA G + C contents of strains TBEA3^T and SB1^T were 69.1 and 70.1 mol%, respectively, and the DNA–DNA hybridization value between these two strains was 45.3 %. The predominant cellular fatty acids in both strains were C_{16:0}, C_{18:1ω7c} and summed feature 3 (C_{16:1ω7c} and/or C_{15:0} iso 2-OH). The major 3-hydroxy fatty acid was C_{10:0} 3-OH. Based on the genetic and chemotaxonomic data, strains TBEA3^T and SB1^T represent two novel species of a new genus within the family *Comamonadaceae*, for which the name *Pseudorhodoferax* gen. nov. is proposed. Strain TBEA3^T (=LMG 24555^T=DSM 21634^T) is assigned to *Pseudorhodoferax soli* sp. nov., as the type strain of the type species of the genus. Strain SB1^T (=LMG 24543^T=DSM 21598^T) is the type strain of *Pseudorhodoferax caeni* sp. nov.

The family *Comamonadaceae* belongs to the class *Betaproteobacteria* and contains numerous genera including *Variovorax*, *Rhodoferax*, *Curvibacter*, *Polaromonas* and *Acidovorax* (Willems & Gillis, 2005; Ding & Yokota, 2004). Recently, strain EMB320^T was proposed as belonging to a novel genus, *Caenimonas*, in this family (Ryu *et al.*, 2008). In this study, we describe the identification and characterization of two independently isolated strains, TBEA3^T and SB1^T. Strain SB1^T was isolated in a previous study from activated sludge after conjugal transfer of the aniline-

degradative plasmid pNB2 using enrichment cultures containing aniline, and was found to belong to the family *Comamonadaceae* (Bathe, 2004). Strain TBEA3^T was isolated in the present study from soil because it exhibited the ability to use the thioether 3,3'-thiodipropionic acid (TDP) as a sole source of carbon and energy. This organic sulfur compound (for details of the structure of the compound see Supplementary Fig. S1 in IJSEM Online) and its derivatives are used as antioxidants in the food and health sector as well as antioxidant stabilizers in various polymers (Weber *et al.*, 2006). Technical applications exploit the fact that polymer-bound TDP and its salts are found to be effective reductive quench reagents during the ozonolysis of olefins (Appel *et al.*, 1995). Furthermore, TDP is also being used as a precursor substrate for the bacterial synthesis of polythioesters (PTEs) containing 3-mercaptopropionic acid in *Ralstonia eutropha* (Lütke-Eversloh & Steinbüchel, 2004), now referred to as *Cupriavidus necator*. In contrast to 3-mercaptopropionic

Abbreviations: TDP, 3,3'-thiodipropionic acid; PTE, polythioester.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strain TBEA3^T and strain SB1^T are EU825700 and AJ606333, respectively.

Supplementary figures showing the structural formula of TDP and 16S rRNA gene sequence phylogenies constructed with the maximum-likelihood and maximum-parsimony methods are available with the online version of this paper.

acid, the use of TDP has numerous advantages because the thioether has a low toxicity (LD_{50} oral for rats 3000 mg kg^{-1} ; Lehman *et al.*, 1951), it is odourless and inexpensive and is available on a large scale. To improve TDP-based biosynthesis of PTEs in bacteria, we investigated the hitherto-unknown microbial metabolism of TDP, starting with enrichment cultures containing this compound as the sole source of carbon and energy. As well as strain TBEA3^T, two strains of *Variovorax paradoxus* were also isolated and used for the elucidation of microbial TDP catabolism (Bruland *et al.*, 2009).

Strain TBEA3^T was isolated under mesophilic aerobic conditions from a soil sample. An enrichment culture was prepared by inoculating 50 ml mineral salts medium (MSM; Schlegel *et al.*, 1961) supplemented with 0.5 % (w/v) TDP as sole source of carbon and energy with a top-soil sample of approximately 2 g from an industrial area in Marschacht, Germany (53° 42' N 10° 38' E). The culture was incubated for 1 week at 30 °C on a rotary shaker. Afterwards, serial dilutions of the enrichment culture were spread onto MSM agar plates containing 0.5 % (w/v) TDP and incubated for 7 days at 30 °C. Colonies were repeatedly cultivated on identical agar plates until axenic cultures were obtained.

To analyse the utilization of TDP, strain TBEA3^T was cultivated for 72 h at 30 °C in MSM containing 1 % (w/v) TDP and samples of the cultivation broth were withdrawn every 24 h. Cells were disrupted for 10 min in a bead mill (type MM301; Retsch). Protein concentration was estimated by the method of Bradford (1976) using BSA as standard protein. TDP concentration was determined in the supernatant by HPLC using a LaChrom Elite HPLC (VWR-Hitachi International) consisting of a Metacarb 67H advanced C column (Varian; Bio-Rad Aminex equivalent) and a 22350 VWR-Hitachi column oven. The primary separation mechanism included ligand exchange, ion exclusion and adsorption. A VWR-Hitachi refractive index detector (type 2490) with an active flow cell-temperature control and automated reference flushing, eliminating temperature effects on the refractive index baseline, was used for detection. Aliquots of cell-free supernatant (20 µl) were injected and eluted with 0.0025 M sulfuric acid in double-distilled water at a flow rate of 0.8 ml min^{-1} . Online integration and analysis was performed with EZ Chrome Elite Software (VWR International). The results showed that strain TBEA3^T utilized $2.08 \text{ µM TDP h}^{-1}$ (µg protein^{-1}) under these incubation conditions.

To determine the sequence of the 16S rRNA gene, genomic DNA was extracted according to Marmur (1961). The gene sequence was amplified from the total genomic DNA by PCR using *Pfx* DNA polymerase (MBI Fermentas) and the oligonucleotide primers 27f and 1525r (Wübbeler *et al.*, 2006), which are complementary to conserved regions of the 16S rRNA gene of *Escherichia coli*. The 1502 bp PCR product was purified using the NucleoTrap kit (Machery

and Nagel) and sequenced as described in Wübbeler *et al.* (2006). The 16S rRNA gene sequences were analysed using the BLAST program (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov>) by running the nucleotide–nucleotide BLAST (BLASTN). The nucleotide sequences from strain TBEA3^T and related type strains belonging to the same phylogenetic group, as well as well-known representatives of the class *Betaproteobacteria*, were aligned using the default distance-matrix parameters of CLUSTAL_X version 1.83 (Thompson *et al.*, 1997). 16S rRNA gene sequences were retrieved from the EMBL database and from the Ribosomal Database Project (Maidak *et al.*, 1997). Phylogenetic trees were constructed using the neighbour-joining, maximum-parsimony and maximum-likelihood algorithms available in the PHYLIP software version 3.68 (Felsenstein, 2002). The resulting trees were displayed with TreeView (Page, 1996).

The nucleotide sequence of the 16S rRNA gene of strain TBEA3^T showed high sequence similarities (98.2–99.8 %) to several uncultured bacteria. The closest similarity to a cultured bacterium was found for the poorly characterized *Comamonadaceae* isolate SB1^T (Bathe, 2004). Both strains showed highest sequence similarities to *Acidovorax caeni* R-24608^T (96.3 %), *Pseudacidovorax intermedius* CC-21^T (96.0 %) and members of the genera *Rhodofera* (95.4–96.6 %) and *Curvibacter* (96.6–96.9 %). The results of all three trees (Fig. 1, Supplementary Figs S2 and S3 in IJSEM Online) showed that strains TBEA3^T and SB1^T occupied a separate position within the family *Comamonadaceae*.

Strain SB1^T was not able to use TDP as sole source of carbon and energy and was therefore maintained on MSM agar plates containing 1 % (w/v) sodium gluconate. Long-term storage of the isolates was accomplished with glycerol (25 %, v/v) solution at –70 °C. Colonies of strain TBEA3^T were circular, convex and white. Colonies of strain SB1^T were circular and white to light yellow. Both strains grew on complex media within 1–2 days. To enhance growth on MSM, the medium was supplemented with yeast extract (0.1 % w/v) or with a vitamin solution containing (l^{-1}) 20 µg biotin, 20 µg folic acid, 60 µg lipoic acid, 50 µg thiamine, 50 µg riboflavin, 50 µg nicotinic acid, 100 µg pyridoxal hydrochloride, 50 µg pantothenic acid, 50 µg vitamin B₁₂ and 50 µg *p*-aminobenzoic acid (Mohn, 1995).

To analyse cell morphology using a light microscope, the cells were cultivated at 30 °C in liquid MSM containing 1 % (w/v) sodium gluconate and samples were withdrawn from different growth stages of the cultures. Flagella staining was done according to the method of Blenden & Goldberg (1965) and revealed single polar flagella. After cultivation under nitrogen-deficient conditions in MSM containing 1 % (w/v) sodium gluconate, the accumulation of polyhydroxyalkanoates was detected optically by microscopy and analysed by GC.

Gram staining was performed according to Gerhardt *et al.* (1994) and gave a negative reaction for both strains. Phenotypic characteristics were determined at 30 °C.

Strains TBEA3^T and SB1^T showed an oxidase-positive reaction with Bactident oxidase test strips (Merck) and a catalase-positive reaction after the addition of 3 % (v/v) H₂O₂ to freshly growing colonies. To determine the assimilation of some carbon sources and the presence of some important enzymes, the API 20NE and API 20E systems (bioMérieux) were used. The ability to utilize different carbon sources and growth at different temperatures were investigated in liquid MSM and on agar plates containing 0.2 % (w/v) of the respective carbon source, unless indicated otherwise in Table 1. Hugh–Leifson oxidative/fermentative tests were used to determine whether the strains were able to ferment D-fructose and D-glucose. No utilization of D-glucose by strains TBEA3^T and SB1^T could be observed, whereas D-fructose, succinate, gluconate, D-mannitol, malate, acetate and taurine were utilized as sole sources of carbon and energy under aerobic conditions.

The polyester content of cells and its composition was determined upon methanolysis of 7–10 mg lyophilized cells in the presence of 15 % sulfuric acid by GC analysis of the resulting methyl esters as described elsewhere (Brandl *et al.*, 1988; Timm *et al.*, 1990). Cells of strains TBEA3^T and SBI^T were capable of synthesizing poly(3-hydroxybutyrate) as a storage compound when the cells were cultivated in liquid MSM under nitrogen-limited conditions and when 1 % (w/v) sodium gluconate was supplied as the sole source of carbon and energy. In contrast to *C. necator* strain H16 (Lütke-Eversloh & Steinbüchel, 2003), strains TBEA3^T and SBI^T did not produce copolymers of 3-hydroxybutyrate

Table 1. Morphological, nutritional and physiological characteristics of strains TBEA3^T and SB1^T

Both strains were positive for growth with D-mannitol, gluconate, malate, acetate, D-fructose, taurine and succinate. Both strains were negative for growth with D-glucose, maltose, D-mannose, inositol, sorbitol, rhamnose, sucrose, 3,3'-dithiodipropionic acid, L-cysteine, L-homocysteine, 3-mercaptopropionic acid, mercaptosuccinic acid and thiolactic acid. Both strains were negative for nitrate reduction, urease, protease, β -glucosidase, β -galactosidase, arginine dihydrolase, indole production and fermentative degradation of D-glucose and D-fructose. +, Positive, –, not detected.

Characteristic	TBEA3 ^T	SB1 ^T
Cell size (μ m)	0.8–0.9 $\times$ 1.4–2.5	0.9–1.0 $\times$ 1.5–3.3
Colony morphology	White, circular, convex	White–light yellow, circular
Utilization of:		
TDP	+	–
3-Sulfinopropionate	+	–
Arabinose*	+	–
Adipic acid*	+	–
Citrate*	–	+
Fatty acids (%)†		
C _{12:0}	3.4–6.3	3.3–5.4
C _{14:0}	0.8–1.4	1–1.7
C _{16:0}	21.2–27.4	20–25.3
C _{17:0}	–	0–0.3
C _{17:0} cyclo	4.9–15.8	1.2–8.7
C _{18:0}	0–0.6	0–0.3
C _{16:1} ω 5c	0–0.2	0–0.4
C _{18:1} ω 7c	6.7–10	11.3–16.4
C _{8:0} 3-OH	–	–
C _{10:0} 3-OH	3.5–8.6	3.2–6.6
C _{16:0} 3-OH	–	0–0.3
Summed feature 2‡	6.4–9.8	3.9–7.9
Summed feature 3‡	33.1–41.7	37.9–46.1
DNA G+C content (mol%)	69.1	70.1

*Results obtained from API 20NE.

†Data represent variations from four (TBEA3^T) or six (SB1^T) replicates.

‡Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 2 consisted of C_{12:0} aldehyde, C_{16:1} iso I and/or C_{14:0} 3-OH. Summed feature 3 consisted of C_{16:1} ω 7c and/or C_{15:0} iso 2-OH.

and 3-mercaptopropionate under the same conditions when TDP or 3-mercaptopropionate were provided as an additional carbon source.

For determination of the DNA G+C ratio, DNA was enzymically degraded into nucleosides as described by Mesbah *et al.* (1989). The nucleosides were then separated by using a Waters Breeze HPLC system and XBridge Shield RP18 column thermostabilized at 37 °C. The solvent was 0.02 M NH₄H₂PO₄ (pH 4.0) with 1.5 % acetonitrile. Non-

methyated λ phage (Sigma) and *E. coli* DNA were used as the calibration reference and control, respectively. The DNA G+C contents were 69.1 mol% for strain TBEA3^T and 70.1 mol% for strain SB1^T.

The separate phylogenetic position of strains TBEA3^T and SB1^T was confirmed by DNA–DNA hybridization. Experiments were performed at 42 °C with photobiotin-labelled probes in microplate wells as described previously (Goris *et al.*, 1998), using an HTS 7000 Bio Assay Reader (PE Applied Biosystems) for the fluorescent measurements. DNA–DNA hybridization between strains TBEA3^T and SB1^T yielded a relatedness value of 45.3 %.

The fatty acid analysis was carried out with a loopful of well-grown cells from cultures incubated for 24 h on tryptic soy agar. Fatty acid methyl esters were prepared, separated and identified using the Microbial Identification System (Microbial ID) as described (Vandamme *et al.*, 1992). The results are shown in Table 1.

The phylogenetic analysis of 16S rRNA gene sequences placed strains TBEA3^T and SB1^T in the family *Comamonadaceae*, where they showed similar sequence similarity levels with species of the genera *Rhodoferax* (95.1–96.3 %) and *Curvibacter* (95.5–96.5 %). Both strains showed high amounts of fatty acids C_{16:0}, C_{18:1} ω 7c and summed feature 3 (C_{16:1} ω 7c and/or C_{15:0} iso 2-OH), a feature that has also been found for *Rhodoferax fermentans* FR2^T (Hiraishi *et al.*, 1991; Hiraishi & Imhoff, 2005) and members of the genus *Curvibacter* (Ding & Yokota, 2004; <http://www.ccug.se>), but notable differences were revealed in the fatty acid profiles of members of these genera. Strains TBEA3^T and SB1^T could be distinguished from the genera *Rhodoferax* and *Curvibacter* by the occurrence of C_{10:0} 3-OH as the major 3-OH fatty acid, small amounts of C_{16:1} ω 5c and the absence of C_{15:0}. Analysis of the DNA G+C contents revealed that strains TBEA3^T and SB1^T had relatively high G+C values, 69.1 and 70.1 mol%, respectively, another notable difference from species of the genus *Rhodoferax*, which showed lower DNA G+C contents: 60.1 mol% for *R. fermentans* FR2^T and 61.5 mol% for *Rhodoferax antarcticus* ANT.BR^T (Madigan *et al.*, 2000). The reported G+C contents of members of the genus *Curvibacter* range from 63 to 66 mol% (Ding & Yokota, 2004).

Because of the genotypic and phenotypic differences with other described species of the family *Comamonadaceae* (Table 2), it is proposed that strains TBEA3^T and SB1^T represent a novel genus, for which the name *Pseudorhodoferax* gen. nov. is proposed. Sequence similarity of the 16S rRNA gene between the two strains was 99.1 % but DNA–DNA hybridization showed a relatedness value of 45.3 %. The strains could also be distinguished from each other by the characteristics given in Table 1. It is therefore proposed that strains TBEA3^T and SB1^T each represent a novel species, for which the names *Pseudorhodoferax soli* sp. nov. and *Pseudorhodoferax caeni* sp. nov. are proposed, respectively.

Table 2. Differential phenotypic and genotypic characteristics of strains TBEA3^T and SB1^T and related genera

Taxa: 1, TBEA3^T; 2, SB1^T; 3, *Rhodoferrax* (data from Hiraishi *et al.*, 1991; Hiraishi & Imhoff, 2005; Madigan *et al.*, 2000; Finneran *et al.*, 2003; Ryu *et al.*, 2008); 4, *Curvibacter* (Ding & Yokota, 2004; Pot & Gillis, 2005; Ryu *et al.*, 2008). All taxa are mesophilic as regards temperature for growth, all require growth factor (as supplied in the vitamin solution), all are oxidase-positive and all are able to assimilate gluconate. +, Positive; –, negative; ND, not detected/not reported.

Characteristic	1	2	3	4
Cell morphology	Rods	Rods	Curved rods	Curved rods
Colony morphology	White	White to light yellow	Peach–brown	Yellow–brown
Flagellation	Single, polar	Single, polar	Polar	Polar or none
Conditions for growth				
Oxygen	Aerobic	Aerobic	Facultatively aerobic	Aerobic or microaerobic
Fermentative degradation of	–	–	+	–
D-glucose or D-fructose				
Nitrate reduction	–	–	+	–
Enzyme activities				
Protease	–	–	+	–
β-Galactosidase	–	–	+	+
Assimilation of:				
Arabinose	+	–	+	–
D-Fructose	+	+	+	ND
D-Glucose	–	–	+	–
D-Mannitol	+	+	+	–
Propionate	+	+	–	ND
DNA G + C content (mol%)	69.1	70.1	60.1–61.5	62–66

Description of *Pseudorhodoferrax* gen. nov.

Pseudorhodoferrax (Pseu.do.rho.do.fer'ax. Gr. adj. *pseudes* false; N.L. masc. n. *Rhodoferrax* a bacterial genus name; N.L. masc. n. *Pseudorhodoferrax* the false *Rhodoferrax*).

Cells are Gram-negative-staining, short rods, motile by a single polar flagellum. They are aerobic and oxidase- and catalase-positive. Optimal growth occurs at 30 °C and growth is supported by the addition of yeast extract. No fermentative growth is observed with D-glucose or D-fructose. The major fatty acids are C_{16:0}, C_{18:1ω7c} and summed feature 3 (C_{16:1ω7c} and/or C_{15:0} iso 2-OH). The major 3-hydroxy fatty acid is C_{10:0} 3-OH. Poly(3-hydroxybutyrate) is accumulated as a storage compound. The DNA G + C content is 69–70 mol%. The type species is *Pseudorhodoferrax soli*.

Description of *Pseudorhodoferrax soli* sp. nov.

Pseudorhodoferrax soli (so'li. L. gen. n. *soli* of soil, the source of the type strain).

Colonies are circular, convex and white. Cells are motile rods, approximately 0.8–0.9 µm in width and 1.4–2.5 µm in length. Negative for nitrate reduction, indole production and urease, protease, β-glucosidase, β-galactosidase and arginine dehydrolase activities. Positive for the assimilation of D-mannitol, gluconate, malate, arabinose, adipic acid, acetate, D-fructose, taurine, propionate, succinate, 3-sulfinothiopropionate and 3,3'-thiodipropionic acid. Negative

for growth with D-glucose, maltose, D-mannose, inositol, sorbitol, rhamnose, sucrose, citric acid, 3,3'-dithiodipropionic acid, L-cysteine, L-homocysteine, 3-mercaptopropionic acid, mercaptosuccinic acid and thiolactic acid.

The type strain, TBEA3^T (=LMG 24555^T=DSM 21634^T), was isolated from a soil sample collected in Marschacht near Hamburg, Germany. The DNA G + C content of the type strain is 69.1 mol%.

Description of *Pseudorhodoferrax caeni* sp. nov.

Pseudorhodoferrax caeni (cae'ni. L. gen. n. *caeni* of sludge, the source of the type strain).

Colonies are circular and white to light yellow. Cells are motile rods, approximately 0.9–1.0 µm in width and 1.5–3.3 µm in length. Negative result in tests for nitrate reduction, indole production and urease, protease, β-glucosidase, β-galactosidase and arginine dehydrolase activities. Positive for the assimilation of D-mannitol, gluconate, malate, citrate, acetate, fructose, taurine, propionate and succinate. Negative for growth with D-glucose, maltose, D-mannose, inositol, sorbitol, rhamnose, sucrose, arabinose, adipic acid, 3,3'-thiodipropionic acid, 3,3'-dithiodipropionic acid, L-cysteine, L-homocysteine, 3-sulfinothiopropionate, 3-mercaptopropionic acid, mercaptosuccinic acid and thiolactic acid.

The type strain, SB1^T (=LMG 24543^T=DSM 21598^T), was isolated from activated sludge from the municipal

wastewater treatment plant at Grüneck near Munich, Germany. The DNA G+C content of the type strain is 70.1 mol% (HPLC).

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