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

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A novel betaproteobacterium, strain TBEA3T, was isolated from soil using enrichment cultures with the organic thioether 3,3-9-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 SB1T, 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 TBEA3T and SB1T 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 C16 : 0, C18 : 1v7c and summed feature 3 (C16 : 1v7c and/or C15 : 0iso 2-OH). The major 3-hydroxy fatty acid was C10 : 03-OH. Based on the genetic and chemotaxonomic data, strains TBEA3T and SB1T represent two novel species of a new genus within the family Comamonadaceae, for which the name Pseudorhodoferax gen. nov. is proposed. Strain TBEA3T (LMG 24555T = DSM 21634T) is assigned to *Pseudorhodoferax soli* sp. nov., as the type strain of the type species of the genus. Strain SB1T (LMG 24543T = DSM 21598T) 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 EMB320T 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, TBEA3T and SB1T. Strain SB1T 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 TBEA3T 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-mercaptocaprylic acid in Ralstonia eutropha (Lütke-Eversloh & Steinbüchel, 2004), now referred to as Cupriavidus necator. In contrast to 3-mercaptocaprylic acid, TDP served as a substrate for the synthesis of PTEs containing the thioether group.

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

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strain TBEA3T and strain SB1T 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-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 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 Pfu 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 Rhodoferax (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 (1 %) 20 μg biotin, 20 μg folic acid, 60 μg lipoic acid, 50 μg thiamine, 50 μg riboflavin, 50 μg nicotinic acid, 100 μg pyradoxal hydrochloride, 50 μg pantothenic acid, 50 μg pyridoxal 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 TBEA\textsuperscript{T} and SB1\textsuperscript{T} showed an oxidase-positive reaction with Bactident oxidase test strips (Merck) and a catalase-positive reaction after the addition of 3\% (v/v) H\textsubscript{2}O\textsubscript{2} 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 TBEA\textsuperscript{T} and SB1\textsuperscript{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 temperature range for growth was investigated on MSM agar plates containing 1\% (w/v) sodium gluconate at 4, 20, 30, 37, 40 and 70 \degreeCelsius. Best growth was observed for both strains between 20 and 30 \degreeCelsius, whereas no growth was observed at 4 \degreeCelsius or above 37 \degreeCelsius.

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 TBEA\textsuperscript{T} and SB1\textsuperscript{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 \textit{C. necator} strain H16 (Lütke-Eversloh & Steinbüchel, 2003), strains TBEA\textsuperscript{T} and SB1\textsuperscript{T} did not produce copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate.
Table 1. Morphological, nutritional and physiological characteristics of strains TBEA³T and SB¹T

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>TBEA³T</th>
<th>SB¹T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony morphology</td>
<td>White, circular</td>
<td>White–light yellow, circular</td>
</tr>
<tr>
<td>Utilization of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TDP</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>3-Sulfinopropionate</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Arabinose*</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Adipic acid*</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Citrate*</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>DNA G+C content (mol%)†</td>
<td>69.1</td>
<td>70.1</td>
</tr>
</tbody>
</table>

*Results obtained from API 20NE. †Data represent variations from four (TBEA³T) or six (SB¹T) 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 1 and/or C_{14:0} 3-OH. Summed feature 3 consisted of C_{16:1}ω7c and/or C_{15:0} iso 2-OH.

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, thiamine, 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.

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 TBEA³T and SB¹T in the family Comamonadaceae, where they showed similar sequence similarity levels with species of the genera Rhodofex (95.1–96.3%) and Curvibacter (95.5–96.5%). Both strains showed high amounts of fatty acids C_{16:0}ω7c and summed feature 3 (C_{16:1}ω7c and/or C_{15:0} iso 2-OH), a feature that has also been found for Rhodofex fermentans FR2³T (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 TBEA³T and SB¹T could be distinguished from the genera Rhodofex and Curvibacter by the occurrence of C_{10:0} 3-OH as the major 3-OH fatty acid, small amounts of C_{16:1}ω5c and the absence of C_{15:0}. Analysis of the DNA G+C contents revealed that strains TBEA³T and SB¹T had relatively high G+C values, 69.1 and 70.1 mol%, respectively, another notable difference from species of the genus Rhodofex, which showed lower DNA G+C contents: 60.1 mol% for R. fermentans FR2³T and 61.5 mol% for Rhodofex antarcticus ANT.BR³T (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 TBEA³T and SB¹T 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 TBEA³T and SB¹T 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<sup>T</sup> and SB1<sup>T</sup> and related genera

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell morphology</td>
<td>Rods</td>
<td>Rods</td>
<td>Curved rods</td>
<td>Curved rods</td>
</tr>
<tr>
<td>Colony morphology</td>
<td>White</td>
<td>White to light yellow</td>
<td>Peach–brown</td>
<td>Yellow–brown</td>
</tr>
<tr>
<td>Flagellation</td>
<td>Single, polar</td>
<td>Single, polar</td>
<td>Facultatively aerobic</td>
<td>Aerobic or microaerobic</td>
</tr>
<tr>
<td>Conditions for growth</td>
<td>Aerobic</td>
<td>Aerobic</td>
<td>Facultatively aerobic</td>
<td>Aerobic or microaerobic</td>
</tr>
<tr>
<td>Oxygen</td>
<td>Aerobic</td>
<td>Aerobic</td>
<td>Facultatively aerobic</td>
<td>Aerobic or microaerobic</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Enzyme activities</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Protease</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Assimilation of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arabinose</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Propionate</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>ND</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>69.1</td>
<td>70.1</td>
<td>60.1–61.5</td>
<td>62–66</td>
</tr>
</tbody>
</table>

### Description of *Pseudorhodoferax* gen. nov.

*Pseudorhodoferax* (Pseu.do.rho.do.fer.‘ax. Gr. adj. pseudes false; N.L. masc. n. Rhodoferax a bacterial genus name; N.L. masc. n. *Pseudorhodoferax* the false *Rhodoferax*).

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<sub>16:0</sub>, C<sub>18:1ω7c</sub> and summed feature 3 (C<sub>16:1ω7c</sub> and/or C<sub>15:0</sub> iso 2-OH). The major 3-hydroxy fatty acid is C<sub>10:0</sub> 3-OH. Poly(3-hydroxybutyrate) is accumulated as a storage compound.

The DNA G+C content is 69.1 mol%.

### Description of *Pseudorhodoferax* soli sp. nov.

*Pseudorhodoferax* 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 dehydrodase activities. Positive for the assimilation of D-mannitot, gluconate, malate, arabinose, adipic acid, acetate, D-fructose, taurine, propionate, succinate, 3-sulfonopropionate 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<sup>T</sup> (=LMG 24555<sup>T</sup>=DSM 21634<sup>T</sup>), 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 *Pseudorhodoferax* caeni sp. nov.

*Pseudorhodoferax* 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 dehydrodase activities. Positive for the assimilation of D-mannitot, 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-sulfonopropionate, 3-mercaptopropionic acid, mercaptosuccinic acid and thiolactic acid.

The type strain, SB1<sup>T</sup> (=LMG 24543<sup>T</sup>=DSM 21598<sup>T</sup>), was isolated from activated sludge from the municipal...
wastewater treatment plant at Grünbeck near Munich, Germany. The DNA G+C content of the type strain is 70.1 mol% (HPLC).

References


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