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# *Desulforhopalus vacuolatus* gen. nov., sp. nov., a new moderately psychrophilic sulfate-reducing bacterium with gas vacuoles isolated from a temperate estuary

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**Abstract** A new type of gas-vacuolated, sulfate-reducing bacterium was isolated at 10° C from reduced mud ($E_0 < 0$) obtained from a temperate estuary with thiosulfate and lactate as substrates. The strain was moderately psychrophilic with optimum growth at 18–19° C and a maximum growth temperature of 24° C. Propionate, lactate, and alcohols served as electron donors and carbon sources. The organism grew heterotrophically only with hydrogen as electron donor. Propionate and lactate were incompletely oxidized to acetate; traces of lactate were fermented to propionate, $CO_2$, and possibly acetate in the presence of sulfate. Pyruvate was utilized both with and without an electron acceptor present. The strain did not contain desulfoviridin. The G+C content was 48.4 mol%. The differences in the 16S rRNA sequence of the isolate compared with that of its closest phylogenetic neighbors, bacteria of the genus *Desulfobulbus*, support the assignment of the isolate to a new genus. The isolate is described as the type strain of the new species and genus, *Desulforhopalus vacuolatus*.

**Key words** Anaerobic sulfate reduction · Incomplete propionate oxidation · Marine environment · Low temperature · Psychrophilic bacteria · Growth yields · *Desulforhopalus vacuolatus*

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## Introduction

The most important substrates for the population of sulfate-reducing bacteria in marine environments are acetate and propionate, which provide about 50 and 10%, respectively, of the reducing equivalents for sulfate reduction (Sørensen et al. 1981; Parkes et al. 1989). Several genera are able to oxidize propionate completely to $CO_2$ (Widdel and Bak 1992), whereas only the few species of the genus *Desulfobulbus* are known to carry out an incomplete oxidation of propionate to acetate and $CO_2$ (Widdel and Pfennig 1982; Samain et al. 1984; Widdel and Bak 1992). Nevertheless, Parkes el al. (1993) have found that the incomplete oxidation of propionate by sulfate reducers dominates over the degradation of propionate in marine sediment slurries. Furthermore, Devereux and Mundfrom (1994) have found four new, *Desulfobulbus*-related 16s rRNA sequences after PCR amplification of nucleic acids extracted from marine sediment. Teske et al. (1996) have found two distantly *Desulfobulbus*-related strains by most-probable-number dilution from the water column of Mariager Fjord, Denmark. These results suggest high species diversity and ecological significance of *Desulfobulbus*-related, propionate-oxidizing sulfate reducers.

The *Desulfobulbus* species described so far are all mesophilic with a minimum growth temperature of 10° C or higher. Since the temperature in the marine environment generally is 5° C or lower (Herbert 1986), the incomplete degradation there must be carried out by heretofore unknown species or genera, or by new subspecies of known species. The present paper reports on the isolation and characterization of a new type of moderately psychrophilic, propionate-oxidizing, gas-vacuole-forming, sulfate-reducing bacterium that was isolated at 10° C from a temperate marine environment.

## Materials and methods

### Source of organism

Sediment samples from the shallow, but permanently water-covered coast of Kysing Fjord were collected at 1 m water depth. The temperature at the sediment surface at this locality varies between 0 and 20° C throughout the year (Jørgensen and Sørensen 1985). Enrichments were inoculated with sediment taken from the uppermost part of the black reduced zone.

### Media and conditions of cultivation

The sulfate-reducing bacteria were cultivated in a bicarbonate-buffered, sulfide-reduced mineral medium containing (g/l): NaCl, 15.0; $MgCl_2 \cdot 6H_2O$, 2.0; KCl, 0.5; $NH_4Cl$, 0.25; $KH_2PO_4$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.2; $NaHCO_3$, 1.75; $Na_2S \cdot 8H_2O$, 0.18; trace element solution SL 10a (Widdel and Bak 1992), 2 ml/l; vitamin solution (Widdel and Pfennig 1981), 2 ml/l. All chemicals were of analytical grade. The pH was adjusted to 7.2. The medium was prepared under an $O_2$-free $N_2$ atmosphere as described by Widdel and Bak (1992). Substrates (electron donors and acceptors) were added before inoculation from sterile neutralized stock solutions to give the desired concentrations. Prior to inoculation, all cultures received 5 µM sodium dithionite ($Na_2S_2O_4$) as an additional reducing agent. The sodium dithionite shortened the lag phase in batch cultures, but was not required for growth.

Pure cultures were obtained by repeated application of deep-agar dilutions as described by Widdel and Bak (1992). The method was modified to ensure that cells were kept cold whenever possible: a dilution series was made with enrichment culture in 10° C medium. Each of the dilutions (6 ml) was then dispersed in 3 ml 3% agar of approximately 45° C (resulting temperature of approximately 20–25° C) and quickly transferred to a 0° C cooling bath. The gas phase was then exchanged with oxygen-free $N_2/CO_2$. The agar tubes were incubated at 10° C. Isolated strains were checked for purity under the microscope and by growth tests in complex medium. Stock cultures were kept at 6° C in the dark and transferred to fresh medium at monthly intervals. The culture with the fastest growth at 10° C, strain ltk10, was chosen for further studies.

### Determination of growth rates

Batch cultures of strain ltk10 were grown with 10 mM sulfate and 10 mM lactate at 10° C. The media were inoculated with bacteria collected in the late exponential growth phase. Growth was followed by measuring the optical density at 500 nm in a spectrophotometer. The growth rate, µ, of an exponentially growing culture was calculated from Eq. 1 by linear regression in a plot of ln(OD) as a function of time, *t*:

$$OD = OD_0 \exp(\mu t) \quad \text{(Eq. 1)}$$

where OD is the optical density at time *t* and $OD_0$ is the optical density at the start of the incubation. There were at least eight measurements for each regression.

### Stoichiometric measurements, cell material determination, and chemical determinations

The stoichiometry of substrate oxidation was determined in 0.5 l bottles sealed with rubber stoppers; the gas phase above the culture was kept very small. Growth was measured as OD throughout the experiment. The concentrations of sulfide, lactate, propionate, and acetate were measured at the start, twice in the exponential growth phase, and at the end of the experiments. Cell biomass was determined at the start and at the end of the experiment on an HCN analyzer (Carlo Erba Na 1500); cultures (10.0 ml) were filtered through GF/F filters (glass microfiber filters; Whatman), the filter was dried at 105° C for 24 h, and then the filter was analyzed for C and N content. The results were converted to biomass as described by Isaksen and Jørgensen (1996).

$H_2S$ was determined photometrically after the methylene blue method of Cline (1969). Lactate, propionate, and acetate were determined by HPLC (Sykam, Gilching, Germany) using an HPX-87H column at 60° C and UV detection. The eluent was 0.01 M $H_2SO_4$.

### Electron microscopy

Preparation of samples for electron microscopy was done as described by Paerl and Shimp (1973): 0.1–2.0 ml of an early exponential growth culture was filtered onto nucleopore filters (0.7 µm). The filters were fixed in 2% (by vol.) glutaraldehyde for 1 h at 4° C. After the fixation, synthetic sea water with decreasing salt concentration was passed through the filters (50%, 25%, and distilled water for 5 min each). The samples were then dehydrated with ethanol of increasing concentration (10, 25, 50, 75, and 100% for 10 min each). Samples were left in 75% ethanol for up to one week if critical-point drying could not be done shortly after the last step. Samples were filtered onto a polycarbonate filter (0.2 µm) and dried (critical-point drying). A sample on approximately 25 $mm^2$ was cut out and platinum-coated, and micrographs were taken with a scanning electron microscope (JEOL, J.S.M. 840).

### Isolation of nucleic acids and sequencing

Nucleic acids were isolated by lysozyme/proteinase K/SDS digest, followed by phenol extraction and ethanol precipitation as previously described (Sambrook et al. 1989). The 16S rRNA gene was amplified by PCR using primers GM3 (5′-AGAGTTTGATC-(A/C)TGGC-3′, corresponding to *Escherichia coli* positions 8–23) and GM4 (5′-TACCTTGTTACGACTT-3′, corresponding to *E. coli* positions 1492–1507). Each of the 30 PCR cycles started with 1 min denaturation at 95° C, followed by 2 min annealing at 40° C, and ending with 3 min elongation at 71° C. The reaction mixture (total volume: 50 µl) contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 200 µM of each deoxynucleotide (dGTP, dATP, dTTP, and dCTP), 0.5–1.0 units *Taq* DNA polymerase (SuperTaq; HT Biotechnology, Cambridge, UK), and 0.5 µM of each primer. The PCR products were analyzed by electrophoresis on 1% horizontal agarose gels in TAE buffer [20 mM Tris-acetate (pH 7.4), 10 mM acetate, 0.5 mM $Na_2EDTA$] using a defined 16S-PCR copy of 1.5 kb (*Desulfobulbus* sp.) as size marker. The PCR product was purified as follows: a small well was cut into the agarose gel in front of the selected PCR product; electrophoresis was continued until the PCR product migrated into the buffer-filled well, from where it was taken up with a pipette and transferred into an Eppendorf vial; the PCR product was precipitated for 1 h at –80° C with 0.1 vol. 5 M NaCl and 2.5 vol. ethanol and was pelleted by centrifugation; afterwards it was redissolved in 50 µl distilled water.

PCR products were sequenced directly: 10–100 ng purified PCR product was mixed with 2 µl sequencing buffer [200 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 250 mM NaCl], 1 µl 5% Nonidet P-40 (Sigma) aqueous solution, and 2 pmol primer, and was adjusted with distilled water to a final volume of 10 µl. The double-stranded PCR product was denatured by heating the 10 µl volume to 95° C for two 5-min periods, with a short centrifugation in between. The sequencing reaction was started by adding 1.0 µl 0.1 M dithiothreitol, 2.0 µl dNTP solution (200 nM dGTP, dATP, dTTP), 0.5 µl ($\alpha$-$^{33}$P)-dCTP; (10 µCi/µl; 3000 Ci/mmol), 2 µl 1 U Sequenase 2.0 (USB). After a short centrifugation to collect and mix the sample volume (15.5 µl), the sample was incubated for 5 min at 37° C. The sample volume was divided into four 3.5-µl portions and added to dideoxynucleotide termination solutions (2.5 µl, containing 80 µM each of dGTP, dATP, dTTP, dCTP, and 8 µM dideoxynucleotide). After 5 min incubation at 37° C, the reaction was stopped by adding 4 µl of a solution of 96% formamide and 20 mM EDTA.

### Phylogenetic tree inference

The 16S rRNA sequence of strain ltk10 was compared to those of the Ribosomal Database Project (RDP) database (Maidak et al. 1994) using the SIMILARITY_RANK tool of the RDP database to search for close evolutionary relatives of strain ltk10. Near-complete 16S rRNA sequences for the phylogenetic tree were obtained from the RDP database, including two *Desulfobulbus* sequences: *Desulfobulbus propionicus* strain Lindhorst 1pr3, a freshwater isolate, and *Desulfobulbus marinus* strain 3pr10, a marine strain (Widdel and Pfennig 1982; Widdel and Bak 1992). Sequences of *Desulforhopalus*- and *Desulfobulbus*-related molecular isolates were obtained from Genbank. Phylogenetic trees were inferred using the distance matrix programs DNADIST and FITCH as implemented in the PHYLIP program package version 3.5c by Felsenstein (1993). In the program DNADIST, a matrix of evolutionary distances was computed from the sequence alignment using the model of Jukes and Cantor (1969), which assumes independent change at all sites with equal probability. Phylogenetic trees were inferred from the distance matrix in FITCH, which uses the least-squares algorithm of Fitch and Margoliash (1967). The 16S rRNA sequence of *Desulforhopalus vacuolatus* is available from Genbank under accession number L42613.

### DNA base composition and characterization of pigments

G+C content determination was carried out at the DSM (Braunschweig, Germany). DNA of strain ltk10 was hydrolyzed with P1 nuclease, and the nucleotides were dephosphorylized with bovine alkaline phosphatase (Mesbah et al. 1989). The resulting deoxyribonucleosides were analyzed by HPLC (Tamaoka and Komagata 1984). For the detection of desulfoviridin, the fluorescence test of Postgate (1959) was applied.

## Results

### Enrichment and isolation

When culture media with lactate as sole electron donor and thiosulfate as sole electron acceptor were incubated with anoxic, reduced ($E_0 < 0$) mud from Kysing Fjord at 10° C, sulfide formation was detected within 1–2 weeks. The enrichment was dominated by gas-vacuolated, rod-shaped cells of constant width, but of variable length. Small, oval- to rod-shaped bacteria of the *Desulfobacter* type were also present. Strain ltk10 was isolated in pure culture through agar dilutions with lactate as electron donor and thiosulfate as electron acceptor. Only bacteria with morphology as strain ltk10 developed in the high dilutions of the agar shakes.

### Morphology and miscellaneous characteristics

The cells of strain ltk10 were rod-shaped, 3.0–5.0 μm long and 1.5–1.8 μm wide, nonmotile, and contained gas vacuoles (Fig. 1); they stained gram-negative. During exponential growth, cells were distributed evenly in the culture volume, whereas in the stationary growth phase, where long cells were observed, the cells formed a white to light-beige layer at the medium surface. Strain ltk10 did not have any flagella (Fig. 2). The DNA base ratio of strain ltk10 was 48.4 ± 0.3 mol% guanine+cytosine. No desulfoviridin was detected.

### Growth conditions and nutrition

Strain ltk10 grew fastest with a salinity of 2%, and it was able to grow in medium with a salinity of 0.5–5%; at 8% salinity, no growth was observed. At salinity lower than 1.5% and higher than 4%, however, the final optical density was less than half the optical density obtained with a salt concentration of 2–3%.

The optimal pH was at 6.8–7.2, and strain ltk10 was able to grow at pH from 5.7–8.0. At pH 8.2, no growth was observed. The final optical density of the culture was the same as at pH 6.1–7.8; below and above this value, the final optical density was approximately half the optimal value. At a pH from 5.3 to 5.7, the strain produced sulfide and grew slowly after 1 month of incubation.

Strain ltk10 grew at temperatures from 0 to 24° C with an optimum at 18–19° C. The doubling time with lactate and sulfate was 22.5 h at 19° C. Even though the strain did not grow at above 24° C, it was able to reduce sulfate at temperatures of up to 38° C. When the strain was exposed to these temperatures, the gas vacuoles disappeared.

With organic compounds other than lactate, growth and sulfide formation from sulfate were compared to blanks without added substrate. Optimal growth was obtained on lactate and propionate, but the strain was also able to grow on ethanol, propanol, and on hydrogen with 2 mM acetate as C source. Growth was also observed on pyruvate, but no sulfide was detected. No growth was observed on hydrogen without acetate (autotrophic), formate, acetate, butyrate, malate, fumarate, isopropanol, butanol, benzoate, 2,4,6-trimethoxybenzoate, glucose, or

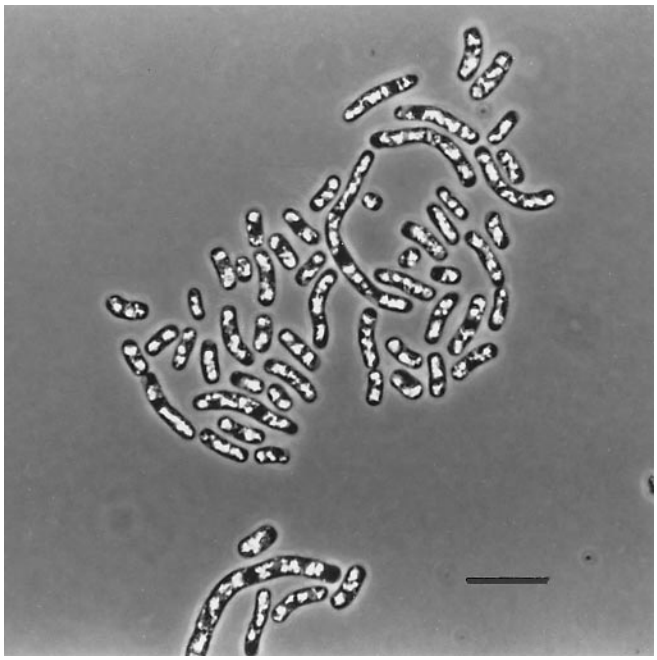

**Fig. 1** Phase contrast photomicrograph of the propionate-oxidizing sulphate reducer *Desulforhopalus vacuolatus* strain ltk10 with gas vacuoles. Note the longer cells typical for cells from the stationary phase (*Bar* 10 μm)

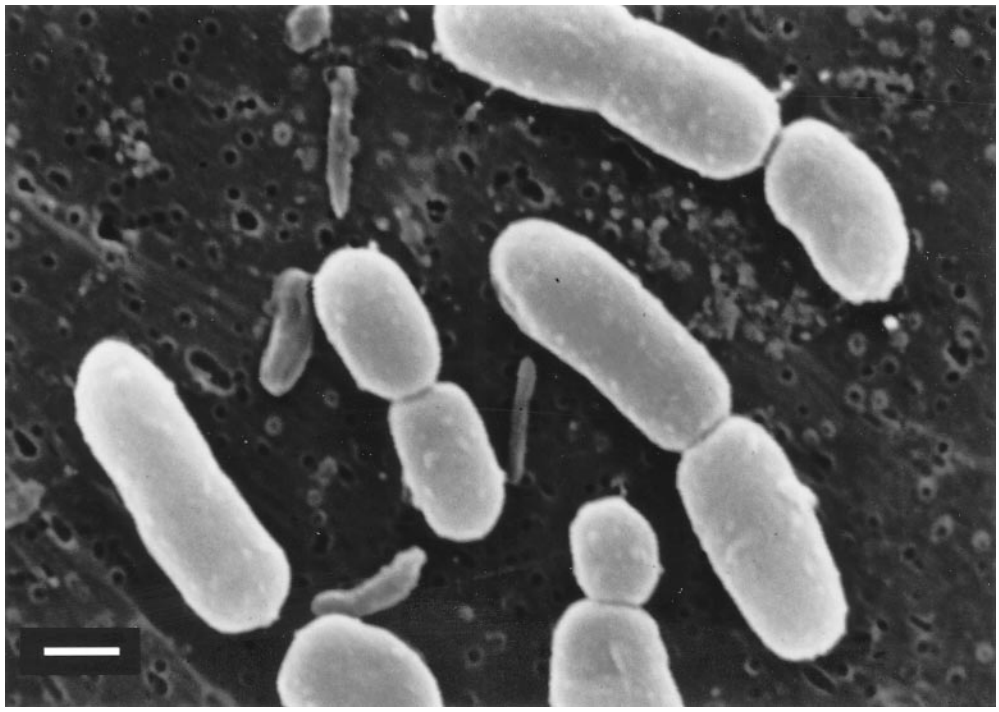

**Fig. 2** Scanning electron micrograph of *Desulforhopalus vacuolatus* (*Bar* 1 μm)

**Table 1** Stoichiometry of propionate conversion by *Desulforhopalus vacuolatus* strain ltk10 with sulphate as electron acceptor

| Propionate utilized (mmol/l) | Acetate excreted (mmol/l) | $H_2S$ formed (mmol/l) | Cell dry weight formed (mg/l) | Propionate consumed for cell material[a] (mmol/l) | Propionate oxidized by sulfate reduction (mmol/l) | Molar ratio of propionate oxidation $\frac{\text{Acetate}/H_2S}{\text{propionate}}$ | Growth yield (g dry weight per mol propionate utilized) |
|---|---|---|---|---|---|---|---|
| 11.8 | 11.3 | 8.5 | 45.6 | 0.5 | 11.3 | $\frac{1.00/0.75}{1}$ | 3.9 |

[a] Propionate consumed for cell material was calculated by the following equation: 17 $CH_3CH_2COO^-$ + 5 $HCO_3^-$ + 15 $H_2O \rightarrow 14(C_4H_7O_3)$ + 22 $OH^-$; thus, 0.0118 mmol propionate are required for 1.0 mg of cell dry weight

alanine. Growth by fermentation (no electron acceptor added) was observed on pyruvate and lactate. In the presence of propionate, growth was observed with sulfate, thiosulfate, and sulfite. Strain ltk10 did not grow with elemental sulfur, fumarate, or nitrate as electron acceptor. Nitrate was also tested in medium without sulfide, since sulfide can inhibit nitrate reduction (Dalsgaard and Bak 1994), but no growth was observed on this medium. Disproportionation on thiosulfate or on elemental sulfur in the presence of $Fe^{2+}$ was not carried out.

Tests for growth factor requirements revealed that of the nine vitamins originally added, the strain required only pyridoxamine, nicotinate, and 4-aminobenzoate. Stock cultures of the sulfate reducers were kept at 2–5° C and were transferred every month.

## Stoichiometry of propionate and lactate oxidation

When grown on propionate, strain ltk10 excreted acetate, which was not oxidized further. To estimate the amount of propionate incorporated into biomass, it was assumed that propionate was assimilated together with $CO_2$ as described in the equation in Table 1. The molar ratios of propionate dissimilated and of sulfide and acetate excreted (Table 1) are in good agreement with the following equation:

$$4\ CH_3CH_2COO^- + 3\ SO_4^{2-} \rightarrow 4\ CH_3COO^- + 4\ HCO_3^- + 3\ HS^- + 3HS^- + H^+ \quad \text{(Eq. 2)}$$
$$\Delta G^{\circ\prime} = -38.1\ \text{kJ/propionate oxidized}$$

The molar ratios of lactate oxidized and of sulfide and acetate excreted (Table 2) are in good agreement with the following equation:

$$2\ CH_3CHOHCOO^- + SO_4^{2-} \rightarrow 2\ CH_3COO^- + 2\ HCO_3^- + HS^- + H^+ \quad \text{(Eq. 3)}$$
$$\Delta G^{\circ\prime} = -80.3\ \text{kJ/lactate oxidized}$$

In the late exponential growth phase, strain ltk10 also excreted small amounts of propionate. Acetate and propionate are most likely produced by fermentation of lactate via a randomizing succinate pathway as described for *Desulfobulbus propionicus* (Stams et al. 1984). Two moles propionate, one mole acetate and $CO_2$ are formed from three moles lactate:

**Table 2** Stoichiometry of lactate conversion by *Desulforhopalus vacuolatus* strain ltk10 with sulfate as electron acceptor

| | Lactate utilized (mmol/l) | Acetate excreted (mmol/l) | Propionate excreted (mmol/l) | $H_2S$ formed (mmol/l) | Cell dry weight formed (mg/l) | Lactate consumed for cell material[a] (mmol/l) | Lactate oxidized by sulfate reduction (mmol/l) | Molar ratio of lactate oxidation $\frac{\text{Acetate}/H_2S}{\text{lactate}}$ | Growth yield (g dry weight per mol lactate utilized) |
|---|---|---|---|---|---|---|---|---|---|
| Measured reactants | 21.6 | 18.9 | 0.7 | 9.4 | 153.3 | 2.1 | | | |
| Fermentation | 1.1 | 0.4 | 0.7 | | | | | | |
| Respiration | 20.5 | 18.6 | | 9.4 | 153.3 | 2.1 | 18.4 | $\frac{1.01/0.51}{1}$ | 7.5 |

[a] Lactate consumed for cell material was calculated by the following equation: 17 $CH_3CHOHCOO^-$ + 8 $H_2O \rightarrow 12(C_4H_7O_3)$ + $HCO_3^-$ + 14 $OH^-$; thus, 0.0138 mmol lactate is required for 1.0 mg of cell dry weight

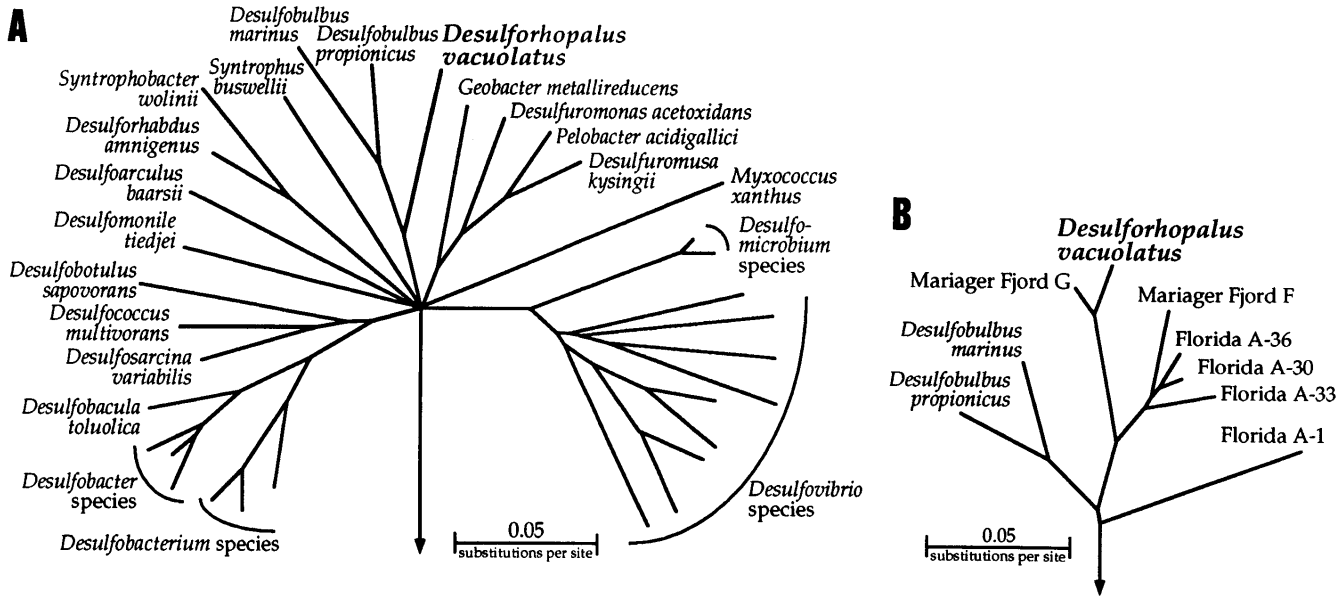


**Fig. 3 A, B** 16S rRNA distance trees of *Desulforhopalus vacuolatus*, related bacteria, and molecular isolates. *Bars* correspond to 0.05 substitutions per nucleotide position. Sequences used in preparing the figures were derived from Genbank or from the Ribosomal Database Project (RDP) on the anonymous ftp server at the University of Illinois, Urbana, Illinois, USA (Maidak et al. 1994). **A** Phylogenetic position of *Desulforhopalus vacuolatus* strain ltk10 in the delta subdivision of the Proteobacteria. The relative branching order of the *Desulfobulbus-Desulforhopalus* lineage, the *Desulfuromusa-Desulfuromonas* lineage, and of *Synthrophobacter*, *Desulfomonile*, *Desulfoarculus*, *Syntrophus*, and *Myxococcus* could not be determined unambiguously in the frame of this study. These lineages are, therefore, drawn converged to the basis of the delta subdivision, retaining their overall branch lengths. The tree is based on the 16S rRNA positions 24–1358 (*Escherichia coli* numbering) and is rooted with *Desulfotomaculum nigrificans* as outgroup. **B** *Desulforhopalus vacuolatus* strain ltk10, *Desulfobulbus propionicus*, *Desulfobulbus marinus*, and related molecular isolates A-1, A-30, A-33, and A-36 from Florida beach sediments (Devereux and Mundfrom 1994), and F and G from the water column of Mariager Fjord, Denmark (Teske et al. 1996). Genbank accession numbers: U08385 (A-1), U08387 (A-30), U08388 (A-33), U08391 (A-36), L40785 (F), L40786 (G). The tree is based on 16S rRNA nucleotide positions 384–896 (*E. coli* numbering) corresponding to the sequence length of the molecular isolates. For clarity, only the *Desulfobulbus-Desulforhopalus* lineage with related molecular isolates is shown, although the tree was inferred including all bacterial species of Fig. 3A as well

$$3\ CH_3CHOHCOO^- \rightarrow CH_3COO^- + 2\ CH_3CH_2COO^- + HCO_3^- + H^+ \quad \text{(Eq. 4)}$$
$$\Delta G^{\circ\prime} = -54.93\ \text{kJ/lactate fermented}$$

All the $\Delta G^{\circ\prime}$ values were calculated from data of Thauer et al. (1977)

Since the enzyme activities of the succinate pathway remain high even in the presence of sulfate (Stams et al. 1984), this fermentative pathway could still operate in the presence of sulfate, which is in agreement with the non-sulfate-reducing utilization of pyruvate by strain ltk10 in the presence of sulfate. It would be interesting to compare the contributions of sulfate-reducing and fermentative lactate utilization pathway under different sulfate concentrations.

The stoichiometric amounts of lactate used (and acetate excreted) to form 0.73 mmol/l of propionate are shown as „fermentation“, and the amounts are subtracted in the numbers presented in „respiration“, in Table 2. The stoichiometry is based on Eq. 4. To estimate the amount of lactate incorporated into biomass, it was assumed that lactate was assimilated as described in the equation in Table 2.

The growth yield of strain ltk10 was found to be 3.86 g dry weight/mol propionate oxidized at 15° C, similar to *D.*

*propionicus strain* 1pr3, whose growth yield on propionate oxidation was found to be 4.3–5.5 (Widdel and Pfennig 1982). Strain ltk10 had its highest growth yield at temperatures below 12° C (Isaksen and Jørgensen 1996). At 15° C, the growth yield was about 80% of the maximal yield. This may explain why the growth yield of strain ltk10 is lower than the yields of *D. propionicus*.

The growth yield of strain ltk10 on lactate was higher than on propionate, which is in accordance with the difference in free energy from the oxidation of lactate and propionate. *Desulfovibrio vulgaris* and *Desulfovibrio* (OttPd1) had a growth yield of 6.0–7.8 g cell weight/mol lactate dissimilated (Magee et al. 1978; Traore et al. 1981; Hansen 1994), which is comparable to the growth yield found for strain ltk10; whereas *Desulfovibrio gigas*, *Desulfovibrio desulfuricans*, *Desulfovibrio africanus*, and *Desulfovibrio* (DSM 3099) were found to have lower growth yields of 1.7–5.6 g dry weight/mol lactate dissimilated (Magee et al. 1978; Traore et al. 1982; Hansen 1994). The low values found for the last four species could be due to nonoptimal growth conditions.

### 16S rRNA sequence analysis

Strain ltk10 is a member of the delta subdivision of the Proteobacteria and forms a monophyletic group with *Desulfobulbus*; it is separated from other major groups of the delta-proteobacterial sulfate reducers, such as *Desulfovibrio*, the acetate oxidizers, *Desulfomonile*, *Desulfoarculus*, and other delta proteobacteria such as *Syntrophobacter*, *Desulfuromonas*, and *Pelobacter* (Fig. 3A). Strain ltk10 is separated from *Desulfobulbus* by a 16S rRNA distance of approximately 0.13 (Jukes-Cantor) and does not fall within the bifurcation of *D. propionicus* and *Desulfobulbus marinus*.

## Discussion

### Environment and temperature

Incompletely propionate-oxidizing sulfate reducers related to *Desulfobulbus* are capable of growth by sulfate reduction and by fermentation, and they catalyze the anaerobic turnover of propionate (and lactate) in marine environments (Parkes et al. 1993). Sulfate-reducing bacteria of the genus *Desulfobulbus* and strain ltk10 are widespread in marine sediments and in the marine water column; they were found as molecular isolates or by most-probable-number dilution series (Devereux and Mundfrom 1994; Teske et al. 1996). It seems surprising that, to our knowledge, only three *Desulfobulbus* species and one *Desulfobulbus propionicus*-related isolate have been described (Tasaki et al. 1990; Widdel and Bak 1992). The known *Desulfobulbus* species are not likely to be active at cool temperatures: *Desulfobulbus elongatus* did not grow below 20° C (Samain et al. 1984), and *D. propionicus* and *Desulfobulbus marinus* had a temperature minimum of 10° C (Widdel and Pfennig 1982). Such species can, therefore, not be responsible for the oxidation of propionate in marine ecosystems, since the temperature in most of the marine environment is below 5° C throughout the year (Herbert 1986) and the rest is cold in the winter period.

Strain ltk10, isolated from Kysing Fjord, a shallow but permanently water-covered estuary, is able to grow at temperatures as low as 0° C. It has a lower temperature optimum than the incompletely propionate-oxidizing species described so far; furthermore it shows short-term tolerance of elevated temperatures. The seasonal temperature at this locality varies between 0 and 20° C, which is within the temperature range for growth of strain ltk10. Furthermore, strain ltk10 is able to survive at temperatures above 25° C for some hours and can thereby survive a warm, sunny summer day on which the water and sediment in the shallow estuary can be heated up further.

Bacteria from many different genera form gas vacuoles (Walsby 1994). The function for planktonic phototrophic bacteria is probably to maintain the buoyancy of cells and thereby ensure that they are placed optimally in the water column where light and nutrients are available. It is remarkable, however, that some strictly anaerobic bacteria possess gas vacuoles (e.g., see Zhilina and Zavarzin 1987). Gas vacuoles on endospores can have a spreading function. The vacuoles can increase the possibility for transport out in the water phase since spores are not harmed by oxygen. The function of gas vacuoles in vegetative cells is, however, less obvious. The gas vacuoles of strain ltk10 apparently did not have any effect on the buoyancy of the cells, since the cells were evenly distributed in the medium bottle in the exponential growth phase. At the stationary phase, however, the cells formed clumps at the surface, which could result from the formation of longer cells.

Strikingly, vacuolated types are the most abundant sulfate-reducing bacteria in the surface layer (0–2 cm) of Kattegat sediment (Jørgensen and Bak 1991). Vacuolated sulfate reducers have been isolated either from the highest dilutions of most-probable-number (MPN) counts, with acetate or with hydrogen as electron donors, or from psychrophilic enrichments when low temperature inhibited the growth of mesophilic competitors. Cell numbers are highest in the oxic surface layer (0–1 cm) and in the suboxic chemocline layer (1–2 cm), reaching $2 \times 10^6$ cells/ml. The low in situ temperatures (5.5° C) in the sediment and, possibly, also the prevalence of oxic and suboxic conditions could select for vacuolated types of sulfate-reducing bacteria. Since *Desulforhopalus vacuolatus* was isolated from a similar marine environment, the shallow Kattegat tributary Kysing Fjord, it is a likely member of the psychrophilic vacuolated sulfate reducers that dominate sulfate-reducing bacterial populations of Kattegat sediment surfaces.

Interestingly, high numbers of bacteria with gas vacuoles have also been found in sea ice and in the water column of Antarctica (Staley et al. 1989). A possible function of gas vacuoles in these cold environments could be to increase the cell surface without increasing the actual

volume of the cytoplasm. This would increase transport of solutes over the cell membrane, which could be an adaptation to low enzymic activity at low temperature. Strain ltk10 was isolated from a temperate estuary, and the temperatures in these environments are below 10–15° C for most of the year. The ability to form gas vacuoles could also be an adaptation to low temperatures in this environment.

## Phylogeny and taxonomy

Strain ltk10 is a member of the delta subdivision of the Proteobacteria and is affiliated, although not closely, to the genus *Desulfobulbus*, represented by *D. propionicus* and *D. marinus* (Fig. 3A). Strain ltk10 is separated from both *Desulfobulbus* species by 16S rRNA Jukes-Cantor distance of approximately 0.13, which is higher than the 16S rRNA distances between *D. propionicus* and *D. marinus* (approximately 0.085). Within the sulfate-reducing bacteria of the delta subdivision, 16S rRNA sequence distance of approx. 0.10–0.15 has been discussed as the upper limit for genus range (Devereux et al. 1990). The genetic distance of strain ltk10 to *Desulfobulbus* is reflected not only by 16S rRNA sequences, but also by the G+C content of the genome: the DNA base ratio of strain ltk10, 48.4 ± 0.3 mol% G+C, is lower than the base ratio of *D. propionicus* (60 mol% G+C) and of *Desulfobulbus elongatus* (59 mol% G+C) (Samain et al. 1984; Widdel and Bak 1992).

The 16S rRNA molecular isolates A-30, A-33, and A-36, obtained from sandy marine sediment in Florida (Devereux and Mundfrom 1994), form a monophyletic group together with strain ltk10 and separate from *D. propionicus* and *D. marinus*. A 16S rRNA sequence G, obtained from dilution cultures of the water column of Mariager Fjord, Denmark (Teske et al. 1996), is closely affiliated to strain ltk10. A second sequence (F) from the same source clusters with the Florida sediment molecular isolates (Fig. 3B). These findings indicate abundant, undescribed bacteria related to strain ltk10 and *Desulfobulbus* species in nature. The molecular isolates from Florida and from Mariager Fjord and strain ltk10 do not share the 16S rRNA sequence motif CAGAGGGGAAAGUGGAAUUC (*Escherichia coli* numbering 660–679), the target sequence of a *Desulfobulbus*-directed 16S rRNA hybridization probe (Devereux et al. 1992). This probe has been designed on the basis of the previously published 16S rRNA sequence of *D. propionicus* and *D. marinus*. A more complete collection of bacteria related to strain ltk10 and

**Table 3** Characteristics of *Desulforhopalus vacuolatus* strain ltk10 and three *Desulfobulbus* species (*ND* not determined)

| Characteristics | *Desulforhopalus vacuolatus* Strain ltk10 | *Desulfobulbus propionicus* Strain 1pr3[1] | *Desulfobulbus marinus* Strain 3pr10[1] | *Desulfobulbus elongatus*[2] |
|---|---|---|---|---|
| Width × length (μm) | 1.5–1.8 × 3.0–5.0 | 1.0–1.3 × 1.8–2.0 | 1.0–1.3 × 1.8–2.0 | 0.6–0.7 × 1.5–2.5 |
| Flagellation | No flagellum, nonmotile | No flagellum, nonmotile | Single polar, motile | Single polar, motile |
| Vacuoles | Yes | No | No | No |
| Gram stain | Negative | Negative | Negative | Negative |
| Growth factor requirement | 4-Aminobenzoic acid, pyridoxamine, nicotinate | 4-Aminobenzoic acid | 4-Aminobenzoic acid | 4-Aminobenzoic acid |
| Temperature range of growth (°C) | 0–24 | 10–43 | 15–36 | 20–40 |
| Temperature optimum (°C) | 18–19 | 39 | 29 | 35 |
| pH range of growth | 5.7–8.0 | 6.0–8.6 | 6.6–8.1 | 6.0–7.8 |
| pH optimum | 6.8–7.2 | 7.1–7.5 | 7.4 | 7.0 |
| Salt requirement | NaCl: 5 g/l | None | NaCl: 15 g/l | ND |
| Compounds used as electron donors and carbon sources: | | | | |
| $H_2$ + $CO_2$ + acetate | + | + | + | + |
| Formate + acetate | – | – | + | – |
| Acetate | – | – | – | – |
| Propionate | + | + | + | + |
| Butyrate | – | (+) | – | – |
| Ethanol | + | + | + | + |
| Propanol | + | + | + | + |
| Pyruvate | + | + | + | + |
| Lactate | + | + | + | + |
| Compounds used as electron acceptors | Sulfate, thiosulfate, sulfite | Sulfate, thiosulfate, sulfite, nitrate | ND | Sulfate, thiosulfate, sulfite |
| Sulfur disproportionation | No | Yes | ND | ND |
| G+C mol % | 48.4 | 59.9 | ND | 59.0 |

[1] Data from Widdel and Pfennig (1982)
[2] Data from Samain et al. (1984)

*Desulfobulbus* species and 16S rRNA sequences will be required to provide a broader basis for probe design.

Strain ltk10 shares some traits with the genus *Desulfobulbus*: strain ltk10 is a gram-negative, non-spore-forming, sulfate-reducing bacterium with respiratory/fermentative metabolism; it oxidizes propionate and other fermentation products incompletely to acetate and $CO_2$. In contrast to *Desulfobulbus*, strain ltk10 is moderately psychrophilic and is able to grow at 0° C, but not above 24° C. Morphologically, it differs by having large cells with gas vesicles and by forming long cells in stationary growth phase. Furthermore, strain ltk10 requires NaCl and two more vitamins than *Desulfobulbus propionicus*, the type strain for *Desulfobulbus*.

Considering these genomic and physiological differences (Table 3), it appears justified to establish a new genus for the propionate-oxidizing, moderately psychrophilic, gas-vacuolated sulfate reducers, with strain ltk10 as the type strain. Based on the morphology, we propose the name *Desulforhopalus vacuolatus* for the new genus and strain.

Description of *Desulforhopalus* gen. nov.

De.sul.fo.rhó.pa.lus. L. pref. *de* from; L. n. *sulfo* sulfur; L. n. *rhopalus* cudgel; M.L. masc. n. *Desulforhopalus* cudgel-formed sulfate reducer.

Oval-shaped, variable length, vacuolated, single or in chains. Nonmotile with no flagella. Gram-negative.

Anaerobic chemo-organotroph; metabolism respiratory and fermentative. Sulfate and other sulfur compounds serve as electron acceptors and are reduced to sulfide. Propionate and other fermentation products are used as electron donors and carbon sources. Acetate is formed as end product of incomplete oxidation.

The G+C content of the type species is 48.4 ± 0.3 mol%.

The type species is *Desulforhopalus vacuolatus*.

Description of *Desulforhopalus vacuolatus* sp.nov.

va.cu.o.la′tus.L. vacuolated due to the morphology of the cells.

Rod-shaped with gas vacuoles, 3.0–5.0 by 1.5–1.8 μm with rounded ends. In stationary growth phase, longer cells formed. Nonmotile. No spore formation.

Propionate, lactate, and ethanol are used as electron donors and carbon sources. Acetate is formed as end product of incomplete oxidation. Chemolithotrophic growth with hydrogen as electron donor and acetate as carbon source. Lactate and pyruvate are fermented. Sulfate, sulfite, and thiosulfate serve as electron acceptor and are reduced to $H_2S$; elemental sulfur, nitrate fumarate, and oxygen are not utilized. Growth requires mineral medium with sulfide as reductant and not less than approximately 5 g NaCl/l and 1 g $MgCl_2 \cdot 6H_2O$/l. Pyridoxamine, nicotinate, and 4-aminobenzoate are required as growth factors. Selective enrichment with thiosulfate and lactate or propionate at higher NaCl and $MgCl_2$ concentration and at temperature below 10° C.

pH Range: 5.7–8.0, optimum at 6.8–7.2. Temperature range: 0–24° C, optimum at 18–19° C.

Desulfoviridin absent.

The G+C content of the DNA of the type strain is 48.4 ± 0.3 mol% (as determined by HPLC).

The type strain is DSM 9700 (= ltk10), deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

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