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Lithoautotrophic growth of sulfate-reducing bacteria, and description of *Desulfobacterium autotrophicum* gen. nov., sp. nov.

K. Brysch[1], C. Schneider[1], G. Fuchs[2], and F. Widdel[1,*]

[1] Fakultät für Biologie, Universität Konstanz, D-7750 Konstanz, Federal Republic of Germany
[2] Abteilung für Angewandte Mikrobiologie, Universität Ulm, Oberer Eselsberg, D-7900 Ulm, Federal Republic of Germany

Abstract. The capacity of mesophilic sulfate-reducing bacteria to grow lithoautotrophically with H_2, sulfate and CO_2 was investigated with enrichment cultures and isolated species. (a) Enrichments in liquid mineral media with H_2, sulfate and CO_2 consistently yielded mixed cultures of non-autotrophic, acetate-requiring *Desulfovibrio* species and autotrophic, acetate-producing *Acetobacterium* species (cell ratio approx. 20:1). (b) By direct dilution of mud samples in agar, various non-sporing sulfate reducers were isolated in pure cultures that did grow autotrophically. Two oval cell types (strains HRM2, HRM4) and one curved cell type (strain HRM6) from marine sediment were studied in detail. The strains grew in mineral medium supplemented only with vitamins (biotin, *p*-aminobenzoate, nicotinate). Carbon autotrophy was evident (i) from comparative growth experiments with non-autotrophic, acetate-requiring species, (ii) from high cell densities ruling out a cell synthesis from organic impurities in the mineral media, and (iii) by demonstrating that 96–99% of the cell carbon was derived from ^{14}C-labelled CO_2. Autotrophic growth occurred with a doubling time of 16–20 h at 24–28° C. Formate, fatty acids up to palmitate, ethanol, lactate, succinate, fumarate, malate and other organic acids were also used and completely oxidized. The three strains possessed cytochromes of the b- and c-type, but no desulfoviridin. Strain HRM2 is described as a new species of a new genus, *Desulfobacterium autotrophicum*. (c) The capacity for autotrophic growth was also tested with sulfate-reducing bacteria that originally had been isolated on organic substrates. The incompletely oxidizing, non-sporing types such as *Desulfovibrio* and *Desulfobulbus* species and *Desulfomonas pigra* were confirmed to be obligate heterotrophs that required acetate for growth with H_2 and sulfate. In contrast, several of the completely oxidizing sulfate reducers were facultative autotrophs, such as *Desulfosarcina variabilis, Desulfonema limicola, Desulfococcus niacini*, and the newly isolated *Desulfobacterium vacuolatum* and *Desulfobacter hydrogenophilus*. The only incompletely oxidizing sulfate reducer that could grow autotrophically was the sporing *Desulfotomaculum orientis*, which obtained 96% of its cell carbon from ^{14}C-labelled CO_2. *Desulfovibrio baarsii* and *Desulfococcus multivorans* may also be regarded as types of facultative autotrophs; they could not oxidize H_2, but grew on sulfate with formate as the only organic substrate.

Key words: Autotrophic growth – Sulfate-reducing bacteria – Carbon dioxide – Hydrogen – Formate – Homoacetogenic bacteria – *Desulfobacterium autotrophicum* – *Desulfovibrio*

During the first decades after the discovery of sulfate-reducing bacteria by Beijerinck (1895), microbiologists focused their interest on the dissimilatory capacities of these bacteria, viz. the reduction of sulfate to sulfide with an organic electron donor. When, later on, sulfate-reducing bacteria were shown to use in addition H_2 as an inorganic electron donor (Stephenson and Strickland 1931), also the assimilatory capacities became of interest. Carbon autotrophy in these bacteria was first supposed by Wright and Starkey (1945) when they obtained sulfate-reducing enrichment cultures with H_2, sulfate and CO_2. The first pure cultures reported to be autotrophic were four *Desulfovibrio* strains investigated by Butlin and Adams (1947). The enrichment and isolation for further autotrophic *Desulfovibrio* strains was reported by Sisler and ZoBell (1951). However, when Postgate (1960) and Mechalas and Rittenberg (1960) independently tested one of the *Desulfovibrio* strains (today *D. vulgaris*, strain Hildenborough) reported to be autotrophic (Butlin and Adams 1947), the earlier results could not be confirmed. With H_2 as electron donor, always less than 30% of the cell carbon was formed from labelled CO_2. Growth in mineral media was poor ($\leq$ 50 mg cell dry weight/l). For substantial yields, yeast extract had to be added as the major carbon source. Also, growth on propanol, butanol and isobutanol required an additional organic carbon source; these alcohols served merely as hydrogen donors because the formed corresponding fatty acids could not be metabolized by *Desulfovibrio*. The inability of *Desulfovibrio* strains to grow autotrophically was confirmed by Sorokin (1966a–c) and later by Badziong et al. (1978, 1979) and Brandis and Thauer (1981). These authors showed that acetate was required as a carbon source in addition to CO_2. Acetate was also required for growth on formate instead of H_2 as electron donor. Approximately two thirds of the cell carbon were formed from acetate and one third originated from CO_2, the molar assimilation ratio being 1:1. This ratio is explained by the reductive carboxylation of acetyl-CoA to pyruvate (pyruvate synthase reaction) as the key reaction of the anabolism. Furthermore, reactions of the Calvin cycle have been regarded as possible mechanisms that in *Desulfovibrio vulgaris* could allow CO_2 fixation to a certain

* *Present address and address for offprint requests:* Mikrobiologie, FB Biologie, Philipps Universität, Lahnberge, D-3550 Marburg, FRG

extent during assimilation of organic compounds as the major carbon sources (Alvarez and Barton 1977). In conclusion, the H_2-utilizing *Desulfovibrio* species have to be designated as lithoheterotrophs.

When new types of sulfate reducers differing from *Desulfovibrio* were isolated, a number of these turned out to grow in defined media with H_2 or formate, sulfate and CO_2 without an additional carbon source (Widdel 1980; Pfennig and Widdel 1981; Pfennig et al. 1981; Widdel et al. 1983; Imhoff-Stuckle and Pfennig 1983). *Desulfonema limicola, Desulfosarcina variabilis* and *Desulfococcus niacini* grew on H_2 or formate; *Desulfococcus multivorans* and *Desulfovibrio baarsii* lacked hydrogenase but grew on formate and sulfate in the absence of other organic compounds. Since the hydrogen-utilizing autotrophs grew very slowly or reached only low cell densities, the biochemistry of C_1-carbon assimilation was first investigated in *Desulfovibrio baarsii* using formate (Jansen et al. 1984, 1985). This sulfate reducer resembles morphologically "classical" non-autotrophic *Desulfovibrio* species, but differs from them completely by its degradative and assimilatory capacities. *D. baarsii* synthesized more than half of the cell carbon from CO_2, the rest was derived from formate. Enzyme activities and the incorporation of labelled C_1-compounds into amino acids furnished evidence for a pathway of acetyl-CoA synthesis from C_1-compounds similar to that in homoacetogenic bacteria or autotrophic methanogens (Fuchs 1986; Ljungdahl 1986; Wood et al. 1986). Thereafter, *Desulfotomaculum orientis*, a long known species cultivated so far routinely in complex media, was found to grow autotrophically with H_2, sulfate and CO_2 (Klemps et al. 1985). Without sulfate, *D. orientis* grew slowly by homoacetogenesis from methanol, methoxyl-groups or formate. Growth by acetate formation from H_2 and CO_2 in the absence of sulfate was very poor.

The present study was undertaken to search for further new autotrophic species, to investigate the distribution of autotrophy among sulfate-reducing bacteria isolated on organic substrates, and to confirm autotrophic growth by labelling experiments. It is shown that this assimilatory capacity indeed occurs in various species that differ physiologically from the obligately heterotrophic *Desulfovibrio* species. The new strains comprehensively described in this paper have been presented briefly in recent studies on the menaquinones (Collins and Widdel 1986) and the terminal oxidation of acetyl-CoA (Schauder et al. 1986).

Materials and methods

Sources of organisms

Anaerobic marine or freshwater mud samples for enrichment and isolation of sulfate reducers were taken from Rio Marin (source of strains HRM2, HRM4, HRM6) and Rio di S. Giacomo, Venice (Italy), and from a waste water ditch near Konstanz.

Desulfovibrio africanus (strain Benghazi 1, NCIMB 8401), *Desulfovibrio salexigens* (strain British Guiana, NCIMB 8403) and *Desulfotomaculum orientis* (strain Singapore I, NCIMB 8382) were provided from the National Collection of Industrial and Marine Bacteria, Aberdeen, UK, by Prof. J. R. Postgate, University of Sussex. *Desulfovibrio desulfuricans* (strain Essex 6, DSM 642), *Desulfovibrio vulgaris* (strain Hildenborough, DSM 644), *Desulfovibrio gigas* (DSM 496), *Desulfovibrio* sp. (strain Norway 4, DSM 1741) and *Desulfomonas pigra* (DSM 749) were provided by Dr. H. Hippe, Deutsche Sammlung von Mikroorganismen, Göttingen, FRG. *Desulfobulbus propionicus* (strain 1pr3, DSM 2032), *Desulfobulbus* sp. (strain 2pr4, DSM 2033), *Desulfovibrio baarsii* (strain 2st14, DSM 2075), *Desulfococcus multivorans* (strain 1be1, DSM 2059), *Desulfococcus niacini* (strain NAV-1, DSM 2650) and *Desulfonema limicola* (strain 5ac10, DSM 2076) have been subcultured in the laboratory since their isolation (Widdel 1980; Pfennig and Widdel 1981; Pfennig et al. 1981; Imhoff-Stuckle and Pfennig 1983).

Desulfobacter hydrogenophilus (strain AcRS1, DSM 3380) was isolated with acetate from a heterogeneous sulfate-reducing enrichment on isovalerate as described elsewhere (Widdel 1987a). *Desulfobacterium vacuolatum* (strain IbRM, DSM 3385) was enriched and isolated with isobutyrate from marine mud from Venice (Widdel, unpublished work). Some characteristics of the latter species have already been described (Collins and Widdel 1986; for overview see Widdel 1987b).

Media and conditions of cultivation

All strains were grown under strictly anaerobic conditions in defined bicarbonate-containing, sulfide-reduced media (Widdel and Pfennig 1984) with an increased Na_2SO_4 concentration of 6 g/l. Glass vessels were prewashed with dilute HCl, stoppers were boiled in distilled water. In marine media used for new isolates and for *Desulfovibrio salexigens, Desulfococcus niacini, Desulfosarcina variabilis* and *Desulfonema limicola*, the amounts of NaCl and $MgCl_2 \cdot 6\,H_2O$ were increased to 20 and 3 g/l, respectively. In addition to the normal solution of trace elements, 1 ml from a solution of 6 mg $Na_2SeO_3 \cdot 5\,H_2O$, 8 mg $Na_2WO_4 \cdot 2\,H_2O$ and 0.5 g NaOH/l was added per liter of culture medium. For enrichment, isolation and routine growth of many strains, a mixture of seven vitamins (Widdel and Pfennig 1981) was added. The pure cultures of strains HRM2, HRM4 and HRM6 were supplemented only with biotin, *p*-aminobenzoic acid and nicotinic acid (10, 40 and 100 µg/l, respectively). For growth with H_2 as electron donor, stopper- or septum-sealed tubes or bottles were used with medium under a gas phase of 20% CO_2 in H_2 (liquid/gas ratio 2/3 or less), according to Bryant (1972) or Balch et al. (1979). For pH-tests, varying concentrations of CO_2 in H_2 were used. The vessels were either stationarily incubated and briefly shaken twice a day, or they were incubated on a slowly moving shaker (≤ 100 rpm). Usually, the culture vessels were flushed again once or twice during growth to provide new H_2 and CO_2 and to remove high, inhibitory H_2S concentrations. If indicated, organic substrates were added from separately sterilized 0.5 – 2 M stock solutions. Organic acids were neutralized with NaOH.

Isolation and maintenance of strains

Pure cultures were isolated via anaerobic agar (1%, wt/vol) dilution series under H_2 and CO_2 in tubes by modification of a method described elsewhere (Widdel and Pfennig 1984). Before use, the agar had been washed eight times in cold distilled water. Vitamins, but no other organic compounds were added. After dilution of the inoculum, tubes were kept nearly horizontal for 1 h at 20° C; the resulting large surface of solidified agar medium facilitated gas exchange with the

anaerobic atmosphere. Tubes were then incubated at 25–28°C with the seals downward so that accumulated water could be easily removed when the tubes were opened prior to picking of colonies. Strains isolated with H_2 were diluted in agar with organic electron donors such as malate. Picked colonies from these dilutions were grown again in mineral media with H_2 and CO_2.

Stock cultures of isolated strains were grown under H_2 and CO_2 and then stored under this gas phase at 4°C. Transfers were made every 4 weeks.

Chemical and other determinations

Dissolved H_2S was determined with an acidic $CuSO_4$ reagent as described by Cord-Ruwisch (1985). Volatile fatty acids were measured by gas chromatography (Widdel and Pfennig 1981) after acidification of the samples with 1 M formic acid (final concentration). To determine succinate in the culture medium after fermentative growth, *Propionigenium modestum* (Schink and Pfennig 1982) was added. The propionate formed by decarboxylation of succinate was measured gas-chromatographically. Benzoate was identified by its absorption spectrum (Imhoff-Stuckle and Pfennig 1983).

Tests for desulfoviridin were carried out according to Postgate (1959) and by recording of the absorption spectra of cell-free extracts against H_2O. Cytochrome analyses and recording of carbon monoxide difference spectra for detection of the sulfite reductase P582 were performed as described by Widdel and Pfennig (1981).

The guanine plus cytosine content of the DNA was measured by thermal denaturation (Marmur and Doty 1962) with DNA from *Escherichia coli* K-12 as reference.

Growth of cells was followed in culture tubes by turbidity measurements at 578 nm in a Spectronic 70 photometer (Bausch and Lomb). Via calibration series, the values from the tubes were transformed to 1 cm cuvette values.

For determination of cell dry weight, cells were harvested by centrifugation and washed in 100 mM ammonium acetate buffer, pH 6.0–6.5, at 5°C. The recentrifuged cells were quantitatively transferred with distilled H_2O into light (2–4 g) glass beakers and dried at 80°C until the weight was constant.

Determination of $^{14}CO_2$ incorporation

To measure the incorporation of CO_2 into cell material, 0.2 ml of a filter-sterilized [^{14}C]-Na_2CO_3 solution (approx. 880 kBq) were injected into a 1250 ml septum bottle with 95 ml anaerobic medium under a gas phase of 20% CO_2 in H_2. Then, 5 ml fresh inoculum and 1 atm overpressure of the same gas mixture were added. The bottle was gently shaken and rotated to equilibrate the label with the gas phase. After 8 h of equilibration, approx. 50 ml gas phase were withdrawn and for determination of the specific radioactivity of CO_2 injected into a septum bottle with 10 ml 1 M carbonate-free NaOH solution as an absorbant. Growth started always after a lag phase and was negligible during the first 8 h when the label was equilibrated. The culture bottle was horizontally incubated for 4 days at 25°C in the dark and five times per day shaken for some minutes.

Before harvesting of the labelled culture by centrifugation, H_2S was removed with a stream of 20% CO_2 in N_2.

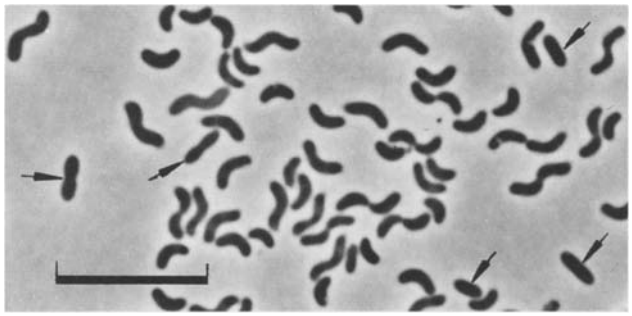

Fig. 1. Mixed culture of a heterotrophic *Desulfovibrio* sp. and *Acetobacterium* sp. (arrows) grown with H_2 and CO_2 in the presence of sulfate under autotrophic conditions. Phase contrast photomicrograph; bar, 10 μm

To obtain carbonate-free cell material, the pellet was resuspended and recentrifuged once in 2 ml 0.5 M $HClO_4$ and once in 2 ml distilled H_2O and then dried at 80°C. The ^{14}C-labelled cell material (around 8 mg dry mass) was subjected to wet combustion by the van Slyke and Folch method according to Simon and Floss (1967) and Watson and Williams (1970). The formed $^{14}CO_2$ was trapped in 10 ml carbonate-free NaOH solution. Controls were made with pure N_2.

For determination the specific radioactivity of $^{14}CO_2$ from the gas sample and from the combusted cells, 100 μl NaOH solution from each CO_2 trap were mixed in a scintillation vial with 0.2 ml H_2O and 10 ml Aqualuma. Determination of ^{14}C was by liquid scintillation counting. Fresh NaOH solution served as a control. The carbon content was determined gravimetrically after addition of 8 ml ^{14}C-containing NaOH solution to 16 ml of a reagent containing 1.5 g $BaCl_2 \cdot 2\,H_2O$, 2.7 g NH_4Cl and 1.2 ml ethanol per 100 ml H_2O (Simon and Floss 1967). The precipitated $BaCO_3$ was collected on a glass fiber filter (Schleicher & Schüll, No. 6), and washed with 10 ml H_2O and 4 ml acetone. The weight was determined after drying at 80°C. The procedure was also carried out with a fresh NaOH solution as a blank. The amounts of $BaCO_3$ obtained in the four experiments (Table 3) were between 32.3 and 60.5 mg; the blanks yielded 3.4 mg $BaCO_3$.

Results

Enrichments in liquid media under autotrophic conditions

Anaerobic enrichments in sulfate-containing mineral media were inoculated with 5% (vol/vol) ditch mud or marine mud and incubated at 25–28°C under an atmosphere of H_2 and CO_2 (80%/20%). An intense H_2S odor developed after 3–5 days. To establish autotrophic conditions, the original organic matter was diluted away in subcultures. The subcultures grew even within 2 days. The dominant cell types in these enrichments were vibrio-shaped motile bacteria. Furthermore, some rod-shaped, motile cells with round to pointed ends were observed. Agar dilution series under H_2 and CO_2 yielded two types of bacterial colonies. Most colonies were small (approx. 0.2 mm in diameter) and greyish to brownish with an uneven surface. From these, vibrio-shaped sulfate reducers, presumably *Desulfovibrio* species, were isolated that used H_2. However, they did not grow autotrophically but required acetate as a carbon source. The

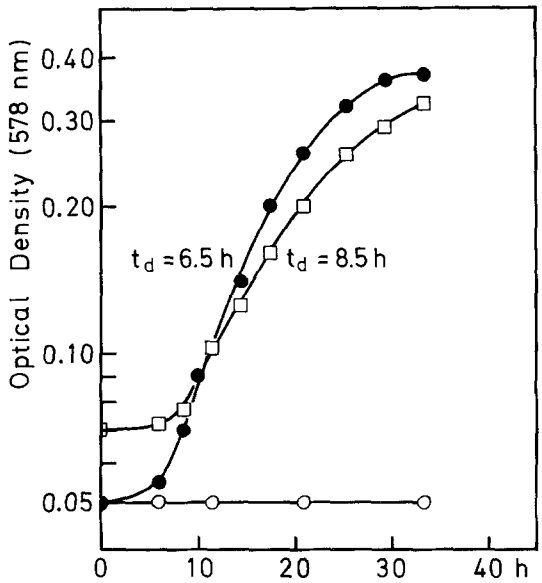

Fig. 2. Growth experiments with a heterotrophic *Desulfovibrio* sp. on H_2, CO_2 and sulfate (○) in pure culture under autotrophic conditions, (●) in pure culture in the presence of acetate (1.5 mM) and (□) in mixed culture with *Acetobacterium* under autotrophic conditions. The doubling time (t_d) is indicated

heterotrophic growth occurred with a doubling time of approx. 6.5 h (Fig. 2). In addition, somewhat larger whitish, smooth colonies were observed; these consisted of rod-shaped bacteria unable to reduce sulfate. Instead, they grew autotrophically by producing acetate from H_2 and CO_2. By their ability to grow well on trimethoxybenzoate or fructose, the rod-shaped bacteria were identified as members of the genus *Acetobacterium* (Bache and Pfennig 1981). These observations suggested that the growth of the sulfate-reducing enrichments under autotrophic conditions was due to a commensalism where *Desulfovibrio* was supplied with acetate synthesized by *Acetobacterium*. In fact, when the pure cultures of the sulfate reducer and the homoacetogen were recombined, a defined sulfide-producing mixed culture was obtained that like the enrichment grew under autotrophic conditions with a doubling time of 8.5 h (Fig. 2). The portion of *Acetobacterium* cells in the mixed culture was around 5% (Fig. 1).

Only one out of about 15 sulfate reducers isolated from the marine liquid enrichments, strain HRM1, was able to grow with H_2 and CO_2 without acetate. However, the autotrophic growth with a doubling time of 25 h yielded only low cell densities; growth was significantly stimulated by addition of acetate; in the presence of this, the doubling time was 13 h (Fig. 4A). Physiologically (Table 1) and morphologically (Fig. 3A), this "mini-autotroph" resembled common *Desulfovibrio* species. It used also lactate, but no fatty acids, and it contained desulfoviridin.

Direct isolation and characterization of new autotrophic sulfate reducers

Isolation of autotrophs. To circumvent that mixed *Desulfovibrio* and *Acetobacterium* cultures overgrew potential autotrophs from the mud, samples were directly diluted in anaerobic mineral agar under H_2 and CO_2 without pre-enrichment in liquid media. The agar had been carefully washed before use to keep development of heterotrophic bacteria as minute as possible. For control, agar dilution series were also incubated under N_2 and CO_2. After 8–15 days of incubation, various whitish, yellowish, brownish or greyish colonies with diameters of 0.2–2 mm were observed. As could be seen in the controls, several bacteria grew by utilization of agar or components therefrom. Only those types of colonies were picked that were not found in the dilutions under N_2 and CO_2. Such colonies of H_2-dependent bacteria often showed a brownish pigmentation, which is characteristic of many sulfate reducers. In the series from marine mud, these colonies were somewhat larger and more abundant than in corresponding dilutions from freshwater mud. Alternative agar dilutions were carried out with formate (15 mM) as the only electron donor. In these series, similar colonies grew as under H_2 and CO_2. Out of 86 colonies picked and transferred into liquid media, six isolates from the H_2/CO_2 tubes and eight isolates from the formate tubes continued to grow well and were microscopically homogeneous. These cultures all formed sulfide. Many isolates did not grow or grew very poorly in the liquid media, some colonies turned out to consist of more than one type of cells. Among the homogeneous isolates growing in liquid media, only one strain obtained with formate originated from freshwater mud, all other strains were of marine origin. The isolates were again purified by repeated application of the agar dilution method under the aforementioned conditions. All newly picked colonies again grew and formed sulfide under autotrophic conditions. The sulfate reducers isolated on formate grew also autotrophically with H_2 and CO_2. It was estimated from the number of isolated strains and the dilution steps of the first agar series that 1 ml marine mud contained at least 10^3-10^4 cells (or colony-forming units) of autotrophic sulfate reducers.

Purity control. Purity of the isolated strains and absence of homoacetogenic bacteria such as *Acetobacterium woodii* was verified by inoculating media containing yeast extract (1 g/l) and fructose (5 mM) or trimethoxybenzoate (3 mM) in the absence of sulfate (Bache and Pfennig 1981). No cell forms other than those inoculated were ever observed. Furthermore, three morphologically different isolates (strains HRM2, HRM4, and HRM6) were diluted in agar series under N_2 and CO_2 with sulfate and with malate as an organic substrate. Isolated colonies grew again under autotrophic conditions.

Morphological characteristics. The majority of the isolated autotrophs, namely 12 of the 14 strains, were oval, motile cells of 1.2–2.5 µm in length and about 1 µm in diameter, as represented by strain HRM2 (Fig. 3B, Table 1). One isolate, strain HRM4 (Fig. 3C, Table 1) had significantly larger, nonmotile cells, another isolate, strain HRM6 (Fig. 3D, Table 1) was a motile vibrio. Electron microscopy of negatively stained cells showed that all isolates, also the nonmotile strain HRM4, had single, polar flagella. Spores were never observed. When tested by Gram-staining or with dilute KOH (Gregersen 1978), the isolates behaved as Gram-negatives.

Growth conditions and nutrition. The effect of temperature and pH was investigated with nine well growing strains including HRM2, HRM4 and HRM6. The temperature optimum was throughout at 22–28°C which is somewhat

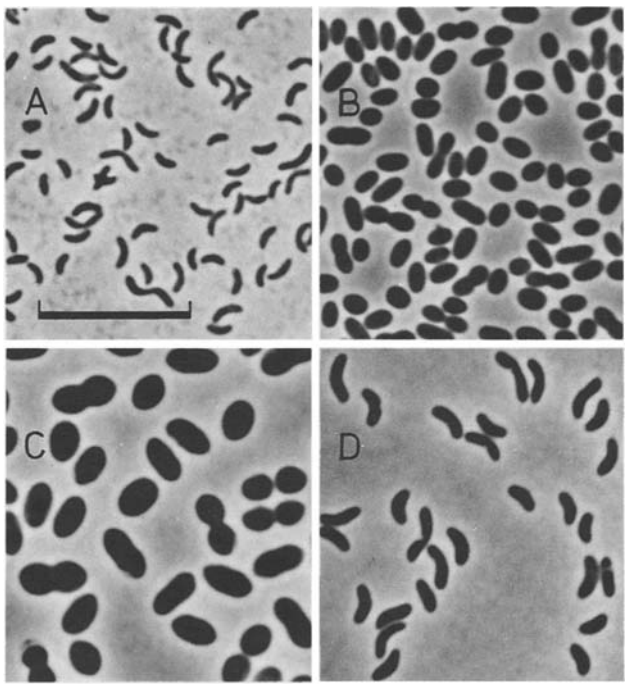

Fig. 3A–D. Newly isolated facultatively lithoautotrophic sulfate reducers grown with H_2, CO_2 and sulfate. Phase contrast photomicrographs; **A** Strain HRM1 (*Desulfovibrio* sp.); **B** strain HRM2 (*Desulfobacterium autotrophicum*); **C** strain HRM4 (*Desulfobacterium* sp.); **D** strain HRM6 (*Desulfobacterium* sp.); bar (for all photos), 10 μm

lower than that of many other mesophilic sulfate reducers (30–36°C; see e.g. Widdel and Pfennig 1984). Under autotrophic conditions, fastest growth occurred at pH 6.7–6.8. The CO_2 partial pressure at this pH and at the $NaHCO_3$ concentration (30 mmol/l) given in the medium was 0.25–0.3 atm (25–30 kPa). The consumption of sulfate caused an increase of the pH that was compensated to a certain extent by reflushing with H_2/CO_2 gas mixture.

The salt requirements were tested with three marine strains, HRM2, HRM4, and HRM6. When the original concentrations of NaCl (20 g/l) and $MgCl_2 \cdot 6\,H_2O$ (3 g/l) in the medium were decreased to 10 g and 1.5 g, respectively, growth was retarded, and no growth occurred in freshwater medium with 1 g and 0.5 g, respectively.

Vitamin tests with strains HRM2, HRM4 and HRM6 revealed that from the initially added mixture of seven vitamins only biotin, *p*-aminobenzoate and nicotinate were required.

Growth on various electron donors was tested with the aforementioned selected nine strains and compared to inoculated blanks without organic substrates. All isolates grew well with H_2, formate, ethanol and higher alcohols, pyruvate, succinate, fumarate, malate and mostly also with lactate. The doubling times were between 12 and 25 h. Fastest growth was observed with pyruvate, lactate, fumarate or malate. The capacity for complete oxidation was obvious from sulfate reduction with acetate. This was observed with all except one of the isolates. However, growth on acetate was always poor and often only about 2 mM sulfide were produced during 3 weeks of incubation. By stoichiometric measurements with an alternative electron donor (see next paragraph), also the strain that did not oxidize acetate was shown to be capable of terminal oxidation. All strains used fatty acids up to palmitate (C_{16}) or sometimes stearate (C_{18}). Most strains oxidized propionate almost as slowly as acetate but grew readily with the higher fatty acids. Vice versa, the strain that did not use acetate and one poorly acetate-oxidizing strain grew well on propionate but very slowly on the higher fatty acids. Strain HRM4 (Table 1) used the cyclic fatty acid, cyclohexane-carboxylate, but no benzoate that was only degraded by a freshwater strain isolated on formate. The results obtained with strains HRM2, HRM4 and HRM6 are listed in Table 1.

The capacity for a terminal oxidation of organic electron donors was in addition confirmed by stoichiometric measurements with butyrate. An incomplete oxidation of 1 mol butyrate would lead to the formation of 2 mol acetate and only 0.5 mol H_2S. However, less than 1 mol acetate and more than 1.4 mol H_2S were formed per mol butyrate consumed. This was also observed with the one isolate that did not oxidize acetate. Previously reported measurements with strain HRM2 had shown a butyrate:acetate:H_2S ratio of 1:1:1.5, suggesting that one of the two acetate moieties derived from butyrate is easily oxidized to CO_2 (Schauder et al. 1986).

The ability to use electron acceptors other than sulfate was tested with strains HRM2, HRM4 and HRM6. Thiosulfate (5–10 mM) was used, too, but no sulfite (1.5–3 mM), sulfur or nitrate (5 mM). In the absence of an electron acceptor, these strains grew by fermentation of fumarate, malate or pyruvate. Strain HRM2 grew on pyruvate in the absence of sulfate nearly as fast as in the presence of the electron acceptor and fermented in addition lactate. The stoichiometry of L(−)-malate fermentation was tested with strain HRM4. The fermentation balance was best approximated by the equation

$$3\ \text{malate}^{2-} + 1.9\ H_2O \rightarrow 1.1\ \text{succinate}^{2-} + 0.3\ \text{propionate}^{-} + 2.05\ \text{acetate}^{-} + 2.6\ HCO_3^{-} + 1.15\ H^{+} \quad (1)$$

Chemical and biochemical characteristics. The guanine plus cytosine contents of the DNA of strains HRM2, HRM4 and HRM6 are listed in Table 1. In these strains, also pigments and menaquinones were examined. Each strain contained soluble and membrane-bound c-type cytochromes, and membrane-bound b-type cytochromes with acetone/HCl-extractable protoheme. Tests for the sulfite reductases, desulfoviridin and P582 in the three strains were negative. Also the other oval to rod-shaped autotrophic sulfate reducers isolated directly from mud lacked desulfoviridin. Strains HRM2, HRM4, and HRM6 contained menaquinone-7 (Collins and Widdel 1986).

Comparative growth experiments with various sulfate reducers under autotrophic and heterotrophic conditions

In addition to the newly isolated strains, several described sulfate reducers that had been isolated on organic substrates were included in the present study to examine and compare their carbon requirement during growth with H_2.

When grown under H_2 and CO_2, a strict requirement for acetate as an organic carbon source was observed with *Desulfovibrio desulfuricans*, *D. vulgaris*, *D. gigas*, *D. africanus*, *D. salexigens*, *Desulfovibrio* sp. strain Norway 4, *Desulfomonas pigra*, *Desulfobulbus propionicus*, and *Desulfobulbus* sp. strain 2pr4. These species are all incomplete oxidizers that degrade organic substrates to acetate as an end product.

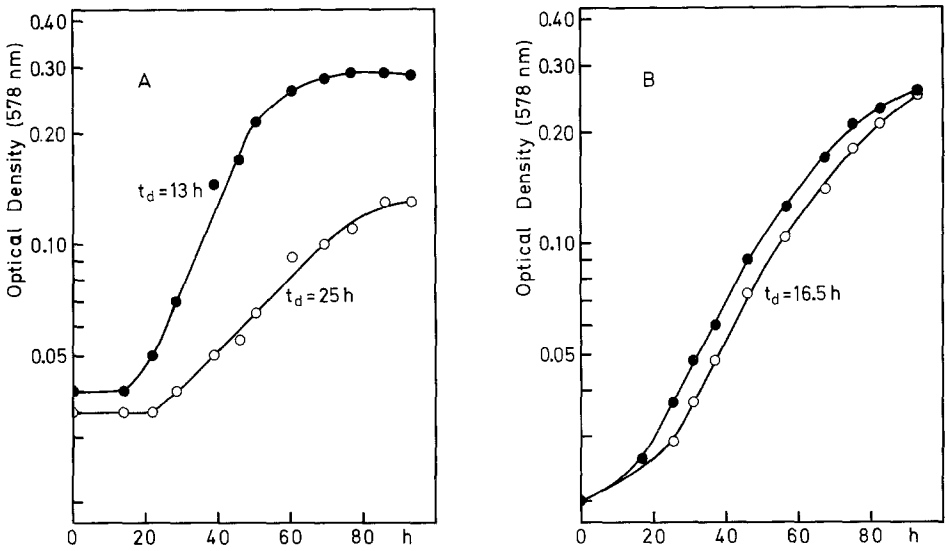

Fig. 4A, B
Growth of facultatively autotrophic sulfate reducers (○) under autotrophic conditions and (●) in the presence of 1.5 mM acetate. Formed H_2S was not removed. The doubling time (t_d) is indicated. **A** Strain HRM1 (*Desulfovibrio* sp.); **B** strain HRM2 (*Desulfobacterium autotrophicum*)

Table 1. Characteristics of newly isolated facultatively lithoautotrophic sulfate reducers

Characteristics	Strain HRM1 (*Desulfovibrio* sp.)	Strain HRM2 (*Desulfobacterium autotrophicum*)	Strain HRM4 (*Desulfobacterium* sp.)	Strain HRM6 (*Desulfobacterium* sp.)
Cell shape	curved	oval	oval	curved
Width × length (μm)	0.7–1.2 × 1.5–2.5	1–1.5 × 1.5–2.5	1.5–2 × 2.5–3.5	1 × 2–3
Flagella; motility	single polar; motile	single polar; motile	single polar; nonmotile	single polar; motile
Temperature optimum (°C)	28–34	25–28	25–28	25–28
pH Optimum (on H_2/CO_2)	approx. 7.0	6.7	6.8	6.7
mol% G + C of DNA	n.t.	47.6	45.3	47.6
Compounds tested as electron donors[a]				
H_2 (autotrophically)	+	++	++	++
Formate (10)	+	++	++	++
Acetate (10)	–	+	+	+
Propionate (10)	–	+	+	+
Fatty acids C_4–C_{16} (≥ 0.5)	–	++	++	++
Stearate (1)	–	–	–	+
Isobutyrate (5)	–	++	++	++
2-Methylbutyrate (5)	n.t.	+	–	+
3-Methylbutyrate (5)	n.t.	–	+	–
3-Phenylpropionate (3)	n.t.	–	++[b]	–
Cyclohexane-carboxylate (2)	n.t.	–	++	–
Ethanol (10)	+	++	++	++
Propanol (5)	–	++	++	++
Butanol (5)	–	++	++	++
Lactate (10)	++	++	++	–
Pyruvate (10)	++	++	++	++
Succinate (5)	–	++	++	++
Fumarate (5)	–	++	++	++
Malate (10)	–	++	++	++
Glutamate (5)	–	+	++	–

[a] Added concentrations of organic compounds (in parentheses) are given in mM. Symbols: ++, doubling time 15–21 h, growth density > 0.2 (OD_{578}, 1 cm); +, doubling time > 21 h, growth density mostly < 0.2; –, no growth; n.t., not tested. Compounds tested but not utilized: Methanol (5), fructose (5), glucose (5), benzoate (3)
[b] Phenylpropionate was oxidized to benzoate as an end product

With inocula of 5% (vol/vol) into mineral media, already the second passage after growth on the organic substrate (propionate for *Desulfobulbus* spp., otherwise lactate) failed to grow: If acetate (≥ 1 mM) was supplemented, good growth occurred with a doubling time of 5 h (*Desulfovibrio vulgaris*) to 14 h. These results are consistent with earlier reports on some of the species (Sorokin 1966a, b, c; Brandis and Thauer 1981; Widdel and Pfennig 1982) and also gave proof of the purity of the mineral media used for the autotrophy experiments.

Table 2. Comparison of growth characteristics of sulfate-reducing bacteria able to use CO_2 or formate as sole carbon source. The first four species were isolated during the present study

Species, strain	Substrate (+ sulfate) for original isolation	Oxidation of organic substrates	Growth[a] without acetate on		Growth on $H_2 + CO_2$ or formate + 1 mM acetate	By-products (mM) synthesized from $H_2 + CO_2$ or formate[b]	
			$H_2 + CO_2$	formate		Acetate	Propionate
Desulfovibrio sp., HRM1	$H_2 + CO_2$	incomplete	++[c]	+	stimulated[c]	n.t.	n.t.
Desulfobacterium autotrophicum, HRM2	$H_2 + CO_2$	complete	++[c]	++	unchanged[c]	0.4	0
Desulfobacterium sp., HRM4	$H_2 + CO_2$	complete	++	++	unchanged	0.4[d]	0
Desulfobacterium sp., HRM6	$H_2 + CO_2$	complete	++	++	unchanged	0.8	0.2
Desulfobacterium vacuolatum	isobutyrate	complete	++	++	unchanged	n.t.	n.t.
Desulfococcus niacini	nicotinate	complete	+	+	unchanged	n.t.	n.t.
Desulfonema limicola	Acetate + fatty acids	complete	+	+	stimulated	0.2	< 0.1
Desulfosarcina variabilis	benzoate	complete	+	+	stimulated	0.5[e]	0.4[f]
Desulfotomaculum orientis	lactate	incomplete	++	++	unchanged	≤ 1.0[g]	0
Desulfobacter hydrogenophilus	acetate	complete	++	−	unchanged	0	0
Desulfovibrio baarsii	stearate	complete	−	++	unchanged	3.2	< 0.1
Desulfococcus multivorans	benzoate	complete	−	+	stimulated	2.6[e]	0.2

[a] Symbols: ++, fair to good growth, doubling time ≤ 30 h; +, slow growth, doubling time > 30 h; −, not used
[b] Unless otherwise indicated, the values were measured after 2 weeks of incubation in batch cultures with limiting sulfate concentration (2 mM) in the absence of other substrates
[c] See Fig. 4
[d] In sulfate-limited chemostat, 4 mM acetate
[e] After 8 weeks, 3.4 mM acetate
[f] After 8 weeks, 0.8 mM propionate
[g] From Klemps et al. (1985); in sulfate-limited chemostat, ≤ 14.5 mM acetate (Cypionka and Pfennig 1986)

Species that in addition to the new isolates grew with H_2 and CO_2 or formate in the absence of other carbon sources are listed in Table 2. Cultivation in mineral media was possible for an indefinite number of passages after transfer from the original media containing organic substrates.

The autotrophic H_2-using sulfate reducers except *Desulfobacter hydrogenophilus* were all able to use formate; two formate-utilizing species, *Desulfovibrio baarsii* and *Desulfococcus multivorans* could not grow with H_2 because they lacked hydrogenase (Schauder et al. 1986).

Autotrophic growth was drastically slowed down and ceased after some days when 5–10 mM H_2S were formed, even if H_2 and CO_2 were still present or newly injected. However, growth started again when H_2S was removed by anaerobic flushing with H_2 and CO_2. Autotrophically growing species were more sensitive to H_2S than heterotrophically growing ones.

In the autotrophic batch cultures that were reflushed once per day with H_2 and CO_2, the highest cell density and fastest growth (13–24 h doubling time) was obtained with strains HRM2, HRM4, HRM6, *Desulfobacterium vacuolatum*, *Desulfotomaculum orientis* (Klemps et al. 1985) and *Desulfobacter hydrogenophilus*. Addition of acetate did not stimulate these species (e.g. Fig. 4B). The growth rate and final cell density of some other species was increased by small amounts of acetate (Table 2).

Strains HRM2 and HRM6 were also grown in a 500 ml fermenter culture that was permanently sparged with H_2 and CO_2 (50 ml/min; gentle magnetic stirring with 120 rpm). The CO_2 content was controlled to keep the pH at 6.9. Under these conditions, the concentration of the otherwise inhibitory H_2S was kept at approx. 0.5 mM. The cell densities of strains HRM2 and HRM6 reached an OD (578 nm, 1 cm) of 1.2 and 1.0, respectively, corresponding to approx. 265 and 225 mg cell dry weight per liter. The gassing and stirring caused lysis of a part of the cells at high densities. The obtained high yields cannot be explained by a cell synthesis from organic impurities in the mineral media and furnish evidence for the assimilation of CO_2 as the carbon source. In the gassed culture, the doubling time of strain HRM6 at 25° C was 15 h.

A phenomenon obviously associated with the capacity for autotrophic growth was the *de novo* synthesis of acetate as a by-product from H_2 and CO_2 or from formate; this

Table 3. Incorporation of ^{14}C-labelled CO_2 during growth of autotrophic sulfate reducers with H_2 and sulfate as energy source. Each culture was grown in 100 ml mineral medium under a gas phase of 1150 ml H_2/CO_2 mixture (80/20, vol/vol). All data have been corrected by the values from control assays that were prepared by using pure N_2 instead of labelled CO_2 (see Text). Also, the amount of nonlabelled cell carbon added with the inoculum (5%, vol/vol) has been subtracted

Organism	Specific radioactivity (cpm/μmol)		Part (%) of cell carbon derived from CO_2	
	Of CO_2 in the culture vessel	Of formed cell carbon[a]	No isotope fractionation	Isotope fractionation of 4.6% assumed
Strain HRM2	3880	3547	91.4	95.6
Strain HRM4	4016	3813	94.9	99.3
Strain HRM6	3813	3499	91.8	96.0
Desulfotomaculum orientis	4055	3716	91.6	95.8

[a] After chemical reoxidation to CO_2

was observed especially in sulfate-limited cultures (Table 2). Sometimes, also small amounts of propionate were found. In sterile mineral media incubated in the same way, neither acetate nor propionate were detectable; this showed that these acids did not originate from other sources such as the stoppers. The synthesis of acetate as a by-product was first observed with *Desulfovibrio baarsii* (Jansen et al. 1984) and thereafter with *Desulfotomaculum orientis* (Klemps et al. 1985).

Cell carbon derived from CO_2

The capacity for autotrophic growth was finally substantiated by growing the newly isolated strains HRM2, HRM4 and HRM6 and *Desulfotomaculum orientis* in the presence of ^{14}C-labelled CO_2. The sulfate reducers were grown for approx. four generations with ^{14}C-labelled CO_2 as sole carbon source. The cells were combusted and the specific radioactivity of the CO_2 derived from cell carbon and from the head space of the culture vessels were determined. The results are obvious from Table 3. Without considering an isotope fractionation, the calculated part of cell carbon derived from CO_2 would be 91.4–94.9%. In *Methanobacterium thermoautotrophicum* grown with $^{12}CO_2$ and $^{13}CO_2$, a fractionation of 2.3% in favour of the lighter isotope was measured (Fuchs et al. 1979). Hence, a fractionation between $^{12}CO_2$ and $^{14}CO_2$ of 4.6% appears realistic for the sulfate reducers studied here. With this fractionation, the part of cell carbon originating from CO_2 would be 95.6–99.3%.

Discussion

Physiology of autotrophic sulfate reducers

The present investigation shows that diverse physiological and morphological types of facultative lithoautotrophs occur among the sulfate-reducing bacteria. For enrichment of these species from mud samples, liquid media turned out to be useless since they selected for mixed populations of lithoheterotrophic *Desulfovibrio* species and acetate-producing, non-sulfate-reducing homoacetogens. This selection is explained in terms of growth rates. In batch cultures, all autotrophic sulfate reducers studied grew relatively slowly, the fastest growth rate being $\leq 0.053\ h^{-1}$ ($t_d \geq 13$ h; Klemps et al. 1985). Under the same conditions in the presence of acetate, the heterotrophic *Desulfovibrio* species studied here grew faster; *D. vulgaris*, strain Hildenborough, reached a growth rate of $0.14\ h^{-1}$ ($t_d = 5$ h). Also the mixed cultures of heterotrophic *Desulfovibrio* species and *Acetobacterium* grew faster than any of the autotrophic sulfate reducers (Figs. 2, 4). In continuously gassed fermenter cultures at low H_2S concentration, growth of sulfate reducers may be favoured. However, even the well growing autotrophic *Desulfotomaculum orientis* has a lower growth rate ($0.09\ h^{-1}$ or $t_d = 7.7$ h; Cypionka and Pfennig 1986) in such a fermenter than e.g. the heterotrophic *Desulfovibrio vulgaris*, strain Marburg ($0.15\ h^{-1}$ or $t_d = 4.6$ h; Badziong and Thauer 1978).

The low *Acetobacterium/Desulfovibrio* cell ratio in the cocultures (Fig. 1) grown under autotrophic conditions is explained by two factors. Firstly, the product from the catabolism of *Acetobacterium*, acetate, is the substrate for the anabolism of *Desulfovibrio*. If one assumes that half of the cell material (as dry mass) of *Desulfovibrio* is carbon and that two thirds of this are derived from acetate, the assimilation of 1 mmol acetate would yield 72 mg cell dry mass. The production of 1 mmol acetate by *Acetobacterium* allows the synthesis of only 2.7–4.2 mg cell dry mass (Tschech and Pfennig 1984). Secondly, the growth rate of the mixed culture in mineral medium is limited by the acetate production and growth rate of *Acetobacterium*. This is obvious from the growth experiments shown in Fig. 2. The heterotrophic *Desulfovibrio* grew somewhat faster in batch culture with acetate than with *Acetobacterium* as a producer of acetate. Hence, *Acetobacterium* can never overgrow *Desulfovibrio*.

With the exception of *Desulfotomaculum orientis* and the poorly growing autotrophic *Desulfovibrio* sp. strain HRM1, the investigated autotrophic sulfate reducers differed by their nutrition significantly from the "classical", heterotrophic *Desulfovibrio* species. In contrast to the latter, the autotrophs utilized a variety of fatty acids and were capable of a terminal oxidation of organic compounds to CO_2.

Biochemical studies on the assimilation of CO_2 and other C_1-compounds and on the oxidation of organic substrates by the facultatively autotrophic sulfate reducers have been carried out so far with *Desulfobacter hydrogenophilus* (Schauder et al. 1987) and *Desulfovibrio baarsii* (Jansen et al. 1984, 1985). There is a striking similarity between the pathway of acetyl-CoA oxidation on the one hand and the pathway of CO_2 fixation on the other hand. *Desulfobacter hydrogenophilus* possesses all enzymes of a complete citric acid cycle that during growth on acetate is used for acetyl-CoA oxidation, as it also occurs in the hydrogenase-negative, obligately acetate-oxidizing *Desulfobacter postgatei* (Brandis-Heep et al. 1983; Gebhardt et al. 1983). This pathway includes some interesting modifications in comparison to the cycle in other bacteria and higher organisms. During lithoautotrophic growth of *Desulfobacter hydrogenophilus*, the cycle appears to operate in a reductive direction to assimilate CO_2. Completely oxidizing sulfate reducers of other genera, probably the majority, lack a complete citric acid cycle. There is evidence that the terminal

oxidation in these types occurs via the acetyl-CoA pathway (Schauder et al. 1986); the key reaction is the direct cleavage of acetyl-CoA into a methyl group and bound carbon monoxide that are separately oxidized to CO_2. It has been shown with *Desulfovibrio baarsii* that the acetyl-CoA pathway operates in a reductive direction for carbon assimilation during growth on formate as sole electron donor and carbon source (Jansen et al. 1984, 1985). Probably, also all other facultatively autotrophic sulfate reducers except *Desulfobacter hydrogenophilus* use the acetyl-CoA pathway for CO_2 fixation during autotrophic growth (Schauder and Fuchs, unpublished work), similar to homoacetogens and autotrophic methanogens (Fuchs 1986; Ljungdahl 1986; Wood et al. 1986). In conclusion sulfate reducers seem to use central metabolic pathways in both directions, depending on the growth conditions. It is not understood why the well growing autotrophic *Desulfotomaculum orientis* that probably uses the acetyl-CoA pathway for CO_2 fixation cannot perform a terminal oxidation of organic substrates.

The ability of autotrophic sulfate reducers to synthesize organic compounds from CO_2 or formate was also evident from the excretion of acetate; this occurred especially with limiting sulfate concentration and an excess of H_2 and CO_2 or formate. Growth due to acetate formation in the absence of sulfate has been observed only with *Desulfotomaculum orientis* (Klemps et al. 1985). However, growth was very poor so that *D. orientis* would not be able to compete in the absence of sulfate with *Acetobacterium* or other homoacetogens. The formation by *Desulfotomaculum orientis* of 1.3 mol acetate during fermentation of 1 mol lactate indicated a *de novo* synthesis of acetate from the excess of reducing equivalents and CO_2 (Klemps et al. 1985). Also the balance of malate fermentation measured in the present study with strain HRM4 [Eq. (1)] furnishes evidence for CO_2 reduction to acetate to a certain extent during the sulfate free growth. A fermentation of 3 mol malate (or fumarate) via a pathway including only the reduction of fumarate and the oxidation of malate and of the intermediary pyruvate would yield 2 mol succinate (or propionate by decarboxylation) and 1 mol acetate.

The physiological and ecological significance of the capacity of sulfate-reducing bacteria to grow autotrophically is barely understood. The estimated 10^3-10^4 colony-forming units of facultatively autotrophic sulfate reducers per milliliter showed that they were relatively abundant in the marine organic-rich sediments. Cell counts of the common non-autotrophic *Desulfovibrio* species range at 10^3-10^5 colony-forming units per milliliter of marine sediment (see e.g. Jørgensen 1978; Laanbroek and Pfennig 1981). It has been calculated that the counting techniques for *Desulfovibrio* may underestimate their real cell numbers in marine sediment by orders of magnitudes (Jørgensen 1978). The underestimation may result e.g. from the natural growth in cell aggregates (microcolonies) and attachment to particles. Hence, not only the actual cell number of *Desulfovibrio* species but also of autotrophic sulfate reducers such as strain HRM2 may be significantly higher than estimated. In view of the nutritional versatility of the facultatively autotrophic sulfate reducers it appears likely that they do not only use H_2 but also fatty acids and other organic compounds that usually occur in anaerobic sediments. Under such conditions there seems to be no obvious benefit from the capacity for autotrophic growth. One may, therefore, argue that autotrophy of the sulfate reducers is a secondary effect of the coincidence of hydrogenase and enzymes that primarily function in the oxidation of acetyl-CoA. However, this argument does not hold in case of *Desulfotomaculum orientis*. Hence, the acetyl-CoA pathway in sulfate reducers may also be regarded as a mechanism preserved from purely fermentative ancestors where it served for an energetically favourable disposal of reducing equivalents. Indeed, a striking relationship, that may be interpreted from the viewpoint of phylogeny, can be seen especially between *Desulfotomaculum orientis* and *Acetobacterium* or homoacetogenic *Clostridium* species. They not only are able to grow autotrophically, but also utilize methanol or methoxylated aromatic compounds. Moreover, *Desulfotomaculum* species are the only sulfate reducers that, like the homoacetogens, belong to the division of the Gram-positive eubacteria (Fowler et al. 1986).

Taxonomic status of the newly isolated autotrophs

Most of the newly isolated autotrophic sulfate reducers were nutritionally and morphologically very similar. A well growing representative is strain HRM2. A clear affiliation of this type to one of the existing genera established on the basis of nutritional, morphological and chemical characteristics is not possible. Strain HRM2 differs significantly from the incompletely oxidizing, heterotrophic *Desulfovibrio* and *Desulfobulbus* species. The formerly isolated *Desulfovibrio baarsii* that grows autotrophically on formate (Pfennig and Widdel 1981; Jansen et al. 1984) still has an exceptional position in the genus and may be considered for a future reclassification. As already indicated and discussed elsewhere (Schauder et al. 1986; Bak and Widdel 1986; Widdel 1987b), the new genus *Desulfobacterium* is proposed for various completely oxidizing sulfate reducers with oval to rod-shaped cells that use a number of fatty acids and may grow autotrophically. Strain HRM6 resembles *Desulfovibrio* species only morphologically. However, nutritionally (Table 1) and biochemically (Schauder et al. 1986) strain HRM6 is very similar to strains HRM2 and HRM4. All three isolates may be regarded as species of the genus *Desulfobacterium* which, therefore, also includes certain curved cell types. Strain HRM2 represents the most common cell type isolated in this study. Furthermore, by being more lysis-resistant than strains HRM4 and HRM6, strain HRM2 is better suited for studies in the laboratory. Therefore, the latter strain is proposed as type strain and type species, *Desulfobacterium autotrophicum*. The genus description has been given already with the description of the recently isolated *D. indolicum* (Bak and Widdel 1986).

Desulfobacterium autotrophicum

Au.to.tro'phi.cum. Gr. pronoun *autos* by itself. Gr.n. *trophe* nurishment. *Desulfobacterium autotrophicum* a rod-shaped sulfate reducer that nourishes itself from inorganic compounds.

Oval, usually motile cells, 1–1.5 μm in diameter and 1.5–2.5 μm in length, with single polar flagellum. Good growth with a doubling time of 15–25 h occurs in the presence of sulfate on H_2 and CO_2, formate, butyrate and higher fatty acids up to palmitate, ethanol and higher alcohols, lactate, pyruvate, succinate, fumarate and malate. Acetate and propionate are slowly utilized. Lactate, pyruvate, fumarate and malate can be fermented in the absence of

sulfate. Cell synthesis is possible with CO_2 as the only source of carbon.

Biotin, *p*-aminobenzoate and nicotinate, and 20 g NaCl and 3 g $MgCl_2 \cdot 6\,H_2O$ per liter are required for growth. The temperature optimum is at 25–28° C. At higher temperature, cells tend to lyse. The pH optimum for autotrophic growth is around 6.7.

Cells contain b- and c-type cytochromes, menaquinone-7 and high activity of carbon monoxide dehydrogenase.

The G + C content of the DNA is 47.6 mol%.

The type strain has been isolated from a marine sediment. Similar types with somewhat slower growth may occur in anaerobic freshwater habitats.

Type strain: HRM2, DSM number 3382, deposited in the Deutsche Sammlung von Mikroorganismen, Göttingen, FRG.

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