Properties of *Desulfovibrio carbinolicus* sp. nov. and Other Sulfate-Reducing Bacteria Isolated from an Anaerobic-Purification Plant

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Several sulfate-reducing microorganisms were isolated from an anaerobic-purification plant. Four strains were classified as *Desulfovibrio desulfuricans*, *Desulfovibrio sapovorans*, *Desulfobulbus propionicus*, and *Desulfovibrio* sp. The *D. sapovorans* strain contained poly-β-hydroxybutyrate granules and seemed to form extracellular vesicles. A fifth isolate, *Desulfovibrio* sp. strain EDK82, was a gram-negative, non-spore-forming, nonmotile, curved organism. It was able to oxidize several substrates, including methanol. Sulfate, sulfite, thiosulfate, and sulfur were utilized as electron acceptors. Pyruvate, fumarate, malate, and glycerol could be fermented. Because strain EDK82 could not be ascribed to any of the existing species, a new species, *Desulfovibrio carbinolicus*, is proposed. The doubling times of the isolates were determined on several substrates. Molecular hydrogen, lactate, propionate, and ethanol yielded the shortest doubling times (3.0 to 6.3 h). Due to the presence of support material in an anaerobic filter system, these species were able to convert sulfate to sulfide very effectively at a hydraulic retention time as short as 0.5 h.

Anaerobic treatment of wastewater rich in organic, sulfurous compounds, inorganic sulfurous compounds, or both usually results in the formation of considerable quantities of hydrogen sulfide, along with a whole range of other reduced sulfur-containing (organic) substances (4, 7, 10, 14, 21, 32, 33). In methanogenic digesters low sulfide concentrations (up to approx. 1 mM) have been shown to stimulate the anaerobic mineralization. This is quite understandable because its presence ensures strictly anaerobic conditions, several methanogens require sulfide for growth (2, 24, 36), and toxic metal ions are precipitated (15, 19, 23). However, high sulfide concentrations have been shown to reduce the rate of methanogenesis (13, 14, 24, 30) and to cause unacceptable contamination of the biogas with H₂S (13, 14). Moreover, the sulfide present in the effluent of a methanogenic digester results in an enhanced oxygen demand of the effluent, thus causing additional pollution of recipient waters. Therefore, in methanogenic digesters the sulfide concentration ($H_2S + HS^- + S^{2-}$) should be kept well below 1 mM.

Wastewater of the potato starch-producing factory of Avebe (De Krim, The Netherlands) is very rich in readily degradable organic matter (27, 28). After an initial deproteination treatment, 7 to 8 g of organic carbon per liter remains in the effluent, together with up to approximately 4 mM sulfate. The aim of the additional steps in the purification process is to bring down the chemical oxygen demand of the wastewater to an acceptable level and to remove all the sulfate. Following passage through a sedimentation pond, further fermentation takes place in an anaerobic upflow reactor with very little sludge retention. Finally, methane is produced in an upflow anaerobic slude blanket reactor. The hydraulic retention times in the different stages are approximately 3.25, 9.5, and 20 h (27). To remove the sulfide that is produced in the first upflow reactor, a sulfide stripper operates between this reactor and the methane reactor. In this manner the occurrence of appreciable quantities of

sulfide in the methane reactor and in the biogas can be prevented, provided that all sulfate is converted into sulfide before the sulfide stripper. In the first reactor the wastewater contains several compounds that are suitable as electron donors for the bacterial reduction of sulfate, such as ethanol, acetate, propionate, and butyrate (27, 46; F. Widdel, Ph.D. thesis, University of Göttingen, Göttingen, Federal Republic of Germany, 1980). Yet, at the hydraulic retention times reported above, only a partial conversion of sulfate takes place in the first reactor (27). In this study we attempted to answer the question of which factors are responsible for the incomplete removal of sulfate. To this end we investigate which species represent the dominating sulfate-reducing bacteria (SRB) in the purification plant and which compounds can be used by them as electron donors. This resulted in the isolation of several SRB and the description of a new, methanol-oxidizing, sulfate-reducing species.

MATERIALS AND METHODS

Media and cultivation. A basal bicarbonate-buffered (pH 7.0) freshwater medium with vitamins and trace elements was used as described previously (26). Acetate was supplied only as indicated in the text. The amounts of yeast extract and Casamino Acids (Difco Laboratories, Detroit, Mich.) were both reduced from 0.025 to 0.010 g/liter. These two components were added as chelating agent and additional carbon source, respectively, although all strains grew well without these additions. Resazurin was omitted from the medium used for the growth of cells for the determination of desulfoviridin and cytochromes. Substrates were added from sterile 0.5 to 1.0 M stock solutions. With the exception of some thermolabile substrates that were filter sterilized (pore size, 0.2 μm), all substrates were heat sterilized. Thermostable substrates with low solubilities were autoclaved together with the salts. Crystalline elemental sulfur was converted into a colloidal, amorphous suspension by the method of Brierley, as described by Madigan (M. Madigan, Ph.D. thesis, University of Wisconsin, Madison, 1974), prior to its addition to the basal medium. Just before the media were inoculated, a strong reductant, dithionite,

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was added to a final concentration of approximately 0.1 mM (44).

All experiments, including those with the anaerobic filter system, were conducted at 35°C. Substrate tests and enrichments in batch cultures were done in completely filled serum bottles, with the exception of those for hydrogen utilization, which were performed in 600-ml bottles with 200 ml of medium and a gas phase consisting of 80% H_2 and 20% CO_2. The pH range for growth was estimated in media in which the pH was adjusted by variation of the KH_2PO_4-Na_2HPO_4 or $NaHCO_3$-Na_2CO_3 concentration in such a way that the total phosphate or inorganic carbon concentration amounted to approximately 50 mM at any given pH.

To determine the bacterial doubling times in batch cultures, serum bottles (volumes, 600 ml) were used which were incubated in a gyratory shaker at 200 rpm. The bottles contained 200 ml of medium, and the remaining volume was filled with oxygen-free gas (20% CO_2 plus 80% N_2 or H_2). Gasses were freed of oxygen by passage over hot copper filings.

In some growth experiments, fluid from the first reactor of the purification plant in De Krim was used. Before this fluid was autoclaved, particular material and heat-coagulable components were removed by heating the fluid, followed by filtration and centrifugation.

Enumeration and isolation. Enumerations of SRB were carried out by the three-tube, most-probable-number technique (1). The anaerobic fluid media contained the following substrates, in millimolar: sulfate, 15; lactate, 5; butyrate, 5; propionate, 5; and acetate, 5. A tube was positive only when sulfide production could be demonstrated.

Pure cultures were obtained by repeated application of the agar shake culture method (31).

Chemical analyses. Volatile and nonvolatile fatty acids (26), as well as alcohols (17), were analyzed by gas chromatography. For ammonium a colorimetric assay based on the Berthelot reaction was used (34). Formate (18), thiosulfate (38), and sulfite and sulfide (42) were also determined colorimetrically. In samples from media, sulfate was determined by the method described by Tabatabai (40); and in samples from the anaerobic wastewater treatment plant in De Krim, sulfate was determined as described by Howarth (11). Inorganic and organic carbon were determined with a total carbon analyzer (model 915A; Beckman Instruments, Inc., Fullerton, Calif.).

Other methods. Gram staining was carried out by a standard method (8), with *Pseudomonas oxalaticus* and *Streptococcus cremoris* used as controls.

Cultures that were used for electron microscopy were harvested in the late-exponential growth phase. Preparation of thin sections of cells for electron microscopy was performed as described by Stams et al. (39). Poly-β-hydroxybutyrate granules of thin sections were stained on golden grids with phosphotungstic acid (35).

The presence of cytochromes was investigated by recording air-oxidized versus dithionite-reduced spectra of whole cells with a spectrophotometer (type DW2a; Aminco). Desulfoviridin was determined as described by Postgate (32).

DNA was isolated by the method described by Marmur (22) and further purified by banding in a cesium chloride-ethidium bromide gradient (6). The DNA band was separated under UV light, and the ethidium bromide was removed from the DNA by isopropanol extraction. Subsequently, the DNA was dialyzed for 48 h against 10 mM Tris hydrochloride (pH 8) containing 1 mM EDTA. The mol%

G+C content was determined by thermal denaturation in 0.1× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (20).

Doubling times were determined by following the increase in the A_{660} in a colorimeter (UPS; Vitatron). In some cases in which accurate measurement of the optical density was not possible due to clumping of cells, the doubling times were calculated from substrate decrease, product formation rates, or both.

Anaerobic filter system. The filter system consisted of a cylindrical column (inner diameter, 5 cm; height; 30 cm). After sterilization the column was packed aseptically with autoclaved pumice particles (specific gravity, approx. 1.73 × 10^3 kg m^{-3}). A metal support present at 3 cm from the bottom ensured the presence of a small area that allowed proper mixing of the influent by means of a magnetic stirring bar. The diameter of the particles was 2 to 6 mm in the first experiment and 0.95 to 1.5 mm in the second experiment. Of the total liquid volume of the column (370 ml) approximately 65 to 70 ml was occupied by the pumice material. The headspace of the column was gassed permanently with sterile, oxygen-free gas (80% N_2 and 20% CO_2). The pH of the influent was 6.5 ± 0.2. Samples were taken with sterile syringes via sampling ports that were constructed in the column wall and sealed with a rubber septum.

RESULTS

Isolation of SRB. Enumerations of SRB were carried out in four different samples from the effluent of the first reactor. Most-probable-number determinations in liquid dilution series revealed the presence of 9.3×10^7 to 1.2×10^7 SRB/ml. Three of these enumerations indicated the predominance of morphologically very similar types of SRB. Four strains of these bacteria were isolated and subsequently shown to be physiologically very similar too. One representative strain (DK81) of these SRB was chosen for further characterization. Further enumerations were carried out on agar-containing medium in roll tubes (12) with sulfate (15 mM), acetate (5 mM), and formate (5 mM) and a gas phase of 80% H_2 and 20% CO_2. From the highest positive dilutions of these enumerations, bacteria were isolated that were also very similar to strain DK81. These results indicate that in the first reactor of the purification plant, strain DK81 is the predominant sulfate-reducing organism. In one of the liquid dilution series, two morphologically distinct SRB were detected in the highest positive dilutions. One of these bacteria was similar to strain DK81, but the other, strain DKlac11, was a clearly spirilloid, motile bacterium that will be further described below.

Enrichment cultures were set up containing 10 mM sulfate and 10 mM of lactate, butyrate, propionate, ethanol, or acetate. These cultures were inoculated with samples from the first reactor and from the methane reactor. With lactate both fermentative bacteria and SRB became dominant. When the latter organisms were isolated, they appeared very similar to strain DK81. With butyrate a short motile rod was the dominant SRB; it was isolated and designated strain DKbu14. The use of propionate resulted in the isolation of motile, lemon-shaped cells (strain DKpr12). The enrichment with ethanol yielded a curved, nonmotile, rod-shaped SRB (strain EDK82). Acetate appeared to be metabolized by methanogens. Sulfide production could not be demonstrated in such cultures. This was also true for enrichments in which the concentrations of NaCl and $MgCl_2$ were raised to 120 and 6 mM, respectively.
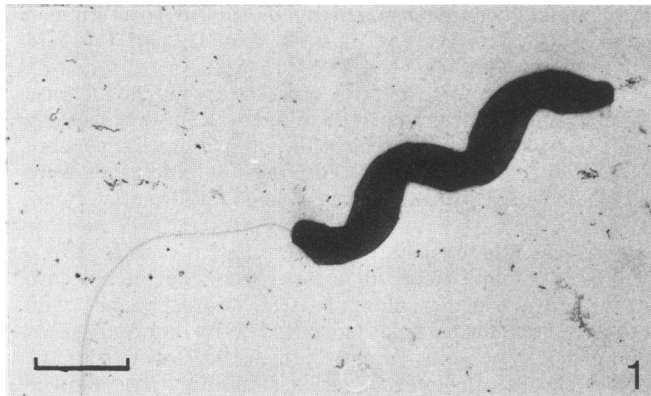
FIG. 1. Electron micrograph of strain DKlac11 negatively stained with uranyl acetate. Bar, 1 μm.

Characterization and identification of the isolated strains. All isolates were strictly anaerobic, non-spore forming, and gram negative. With the exception of strain DKlac11, none of the strains required yeast extract or Casamino Acids for growth. With all strains dithionite-reduced versus air-oxidized difference spectra of whole cells, which were grown on the substrates that were used in their isolation, exhibited absorption maxima at 420 to 421, 525 to 527, and 553 to 554 nm, which is indicative of the presence of c-type cytochromes. Cytochromes of the b-type were not detected, but it should be noted that absorption spectra of malate- or fumarate-grown cells have not yet been recorded.

In Tables 1 to 3 the properties of the isolated bacteria are summarized.

The cells of strain DKlac11 were motile, relatively slender, spiral-shaped rods (Fig. 1). This strain utilized only a very limited number of substrates (Tables 1 and 2). The morphology of this strain and the incomplete oxidation of lactate to acetate plus CO_2 are in accord with its assignment to the genus *Desulfovibrio* (46).

Based on its physiological characteristics strain DK81 most likely represents a strain of *Desulfovibrio desulfuricans* subsp. *desulfuricans* (46). The utilization of elemental sulfur as the electron acceptor is not unique within this species, because this has been demonstrated also for *D. desulfuricans* Norway 4 (3).

The cells of strain DKbu14 were also motile, vibroid rods (Fig. 2). Thin sections of cells grown on butyrate plus sulfate
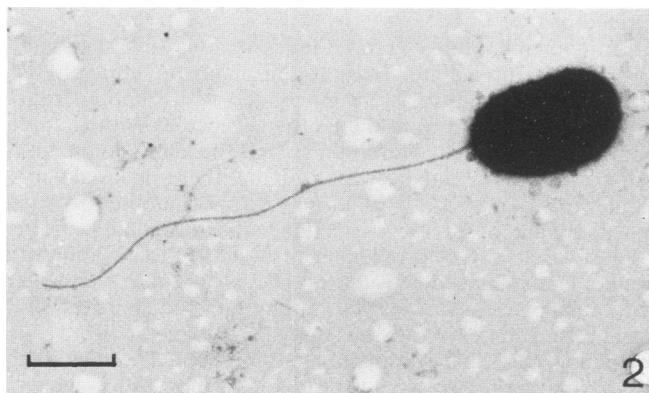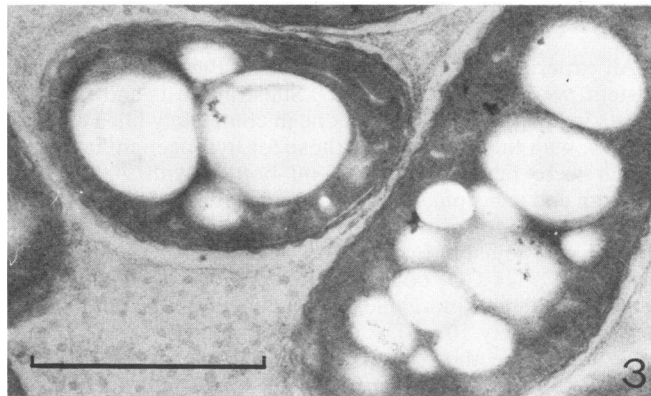

FIG. 3. Ultrathin sections of butyrate- and sulfate-grown cells of strain DKbu14. Note the large intracellular electron-transparent granules. Bar, 1 μm.

revealed the presence of intracellular granules (Fig. 3). When stained with phosphotungstic acid, these granules were darkly colored (Fig. 4), which is indicative of the presence of polyglucose or poly-β-hydroxybutyrate. Based on their actual shape, which is spherical rather than rosette-like, however, it is likely that these granules contain poly-β-hydroxybutyrate (39). Other features of strain DKbu14 cells were the presence of many fimbriae (not shown) and the formation of extracellular vesicles. These vesicles, or blebs (Fig. 5), which apparently originated from the cell surface (Fig. 6), could be observed also by light microscopy (stained with crystal violet; magnification, ×1,000). When the characteristics of strain DKbu14 are compared with those of *Desulfovibrio sapovorans* (46), many similarities are evident, e.g., with respect to the morphology, temperature and pH-range, mol% G+C content of the DNA, and the presence of desulfoviridin and poly-β-hydroxybutyrate storage granules. With the exception of formate, which was oxidized to CO_2, all electron donors utilized by strain DKbu14 were oxidized to acetate. Furthermore, during growth on fatty acids with odd numbers of carbon atoms, propionate was formed in addition to acetate. This property is another characteristic that is shared with *D. sapovorans*. However, the range of substrates utilized by the two organisms is not identical. In contrast to strain DKbu14, *D. sapovorans* is able to oxidize 2-methylbutyrate, lactate, and pyruvate (46). Moreover, pyruvate can be fermented by *D. sapovorans*. On
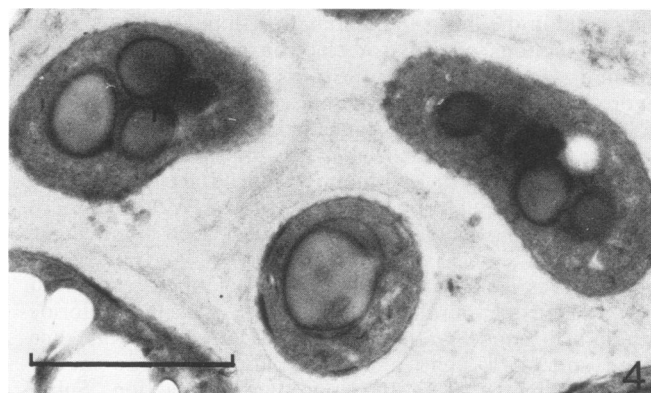

FIG. 2. Electron micrograph of strain DKbu14 negatively stained with uranyl acetate. Bar, 1 μm.

FIG. 4. Ultrathin sections of butyrate- and sulfate-grown cells of strain DKbu14 stained with phosphotungstic acid. Bar, 1 μm.
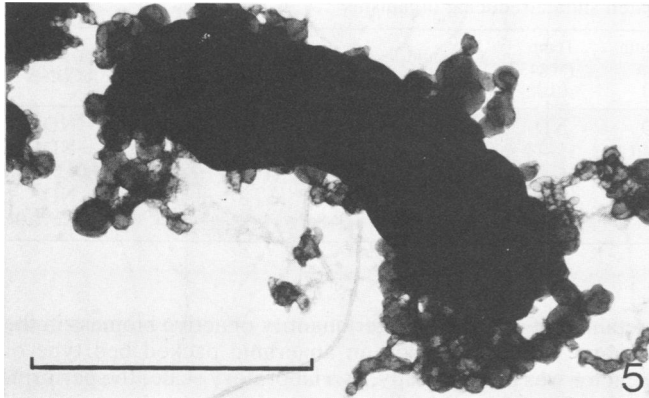
FIG. 5. Electron micrograph of strain DKbu14 negatively stained with uranyl acetate suggesting the presence of vesicles. Bar, 1 μm.

the other hand, unlike *D. sapovorans*, strain DKbu14 does utilize formate and butanol as electron donors and thiosulfate and elemental sulfur as electron acceptors (Tables 1 and 2). In spite of these differences, strain DKbu14 shares sufficient critical properties with *D. sapovorans* to assign it to the same species.

Strain DKpr12 is a motile, lemon-shaped bacterium that strongly resembles *Desulfobulbus propionicus* (16, 45) with respect to almost all characteristics tested (Tables 1 to 3). The only difference with the *D. propionicus* type strain, but not with some other strains (45), appears to be its inability to use nitrate as an electron acceptor. Therefore, we consider strain DKpr12 as a strain of *D. propionicus*.

Two striking features of strain EDK82 were its ability to oxidize methanol and to ferment glycerol (Tables 1 and 2). Details concerning the growth of strain EDK82 with these and other substrates have been reported previously (29). In addition to these results, growth of strain EDK82 was tested in the presence of sulfate (10 mM) and one of the following alcohols, in millimolar: 1,2-propanediol, 13; 1,3-propanediol, 10; 2,3-butanediol, 10; 1-pentanol, 9; and 1-hexanol, 7.5. Of these alcohols 1,3-propanediol and 1-pentanol were stoichiometrically oxidized to 3-hydroxypropionate and valerate, respectively. 1-Hexanol, 1,2-propanediol, and 2,3-butanediol were not metabolized. Another characteristic property of this strain was the formation of intracellular membranes, which have been observed in electron micro-
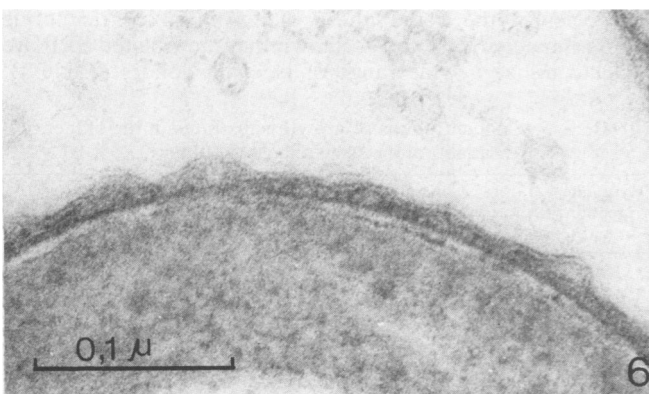

FIG. 6. Ultrathin sections of strain DKbu14 showing the cell wall structure. Bar, 0.1 μm.

TABLE 1. Growth substrates utilized by the isolated sulfate-reducing microorganisms[a]

Substrate (initial concn [mM])[b]	DKlac11	DK81	DKbu14	DKpr12	EDK82
Hydrogen (excess)	+	+	−	+	+
Formate (10)	−	+	+	−	(+)[c]
Propionate (10)	−	−	−	+	−
Butyrate (10)	−	−	+	−	−
Valerate (5)	−	−	+	−	−
Caproate (5)	NT[d]	NT	+	NT	−
Heptanoate (2.5)	NT	NT	+	NT	−
Caprylate (2.5)	NT	NT	+	NT	−
Pelargonate (2.5)	NT	NT	+	NT	−
Methanol (15)	−	−	−	−	+
Ethanol (10)	−	+	−	+	+
Propanol-1 (10)	−	+	−	+	+
Butanol-1 (10)	NT	NT	+	−	+
Glycerol (10)	−	−	−	−	+
Lactate (10)	+	+	−	+	+
Pyruvate (10)	+	+	−	+	+
Succinate (10)	−	(+)	−	−	(+)
Fumarate (10)	−	+	−	−	+
Malate (10)	−	+	−	−	+
Oxaloacetate (10)	NT	NT	−	+	+
Choline (10)	−	+	−	−	−

[a] The growth medium contained 15 mM sulfate. Hydrogen, formate, and methanol were tested in the presence of 5 mM acetate.

[b] Substrates tested but not utilized by any of the strains were as follows, with initial concentrations in millimolar: acetate, 10; isobutyrate, 10; 2-methylbutyrate, 5; 3-methylbutyrate, 5; propanol-2, 10; 2-ketoglutarate, 10; 3-hydroxybenzoate, 2.5; benzoate, 2; glucose, 10; fructose, 10; glycine, 10; and the L-forms of the amino acids alanine, 10; aspartate, 10; cysteine, 10; glutamate, 10; histidine, 10; leucine, 10; serine, 10; threonine, 10; and valine 10.

[c] (+), Incomplete conversion after an incubation period of 3 weeks.

[d] NT, Not tested.

graphs of thin sections (29). The properties of this strain are sufficiently different from those of all species described thus far to warrant the proposal of a new species (see below).

Doubling times with various substrates. Minimum doubling

TABLE 2. Substrates fermented and electron acceptors utilized by the isolated sulfate-reducing microorganisms

Substrates fermented and electron acceptors utilized (initial concn [mM])	DKlac11	DK81	DKbu14	DKpr12	EDK82
Substrates fermented					
Lactate (10)	−	−	−	+	−
Pyruvate (10)	−	+	−	+	+
Fumarate (10)	−	+	−	+	+
Malate (10)	−	−	−	−	+
Ethanol (10)	−	−	−	+	−
Glycerol (10)	−	−	−	−	+
Choline (10)	−	+	−	−	−
Electron acceptors utilized					
Sulfate (10)	+	+	+	+	+
Sulfite (5)	NT[a]	+	+	+	+
Thiosulfate (10)	NT	+	+	+	+
Sulfur (4)	−	+	+	−	(+)[b]
Nitrate (10)	−	−	−	−	−
Nitrite (5)	−	−	−	−	−
Fumarate (10)	−	+	−	−	−

[a] NT, Not tested.

[b] (+), Incomplete conversion after an incubation period of 3 weeks.

TABLE 3. Characteristics of the isolated sulfate-reducing organisms[a]

Strain	Cell diam (μm)	Cell length (μm)	Flagella	Desulfoviridin	Optimum temp (°C)	Temp range (°C)	Optimum pH	pH range	NaCl requirement	mol% G+C of DNA
DKlac11	0.3–0.6	1.4–4.0	1 polar	+	ND	ND	ND	ND	–	ND
DK81	0.5–0.9	1.5–3.0	1 polar	+	38–40	15–44	6.5–7.0	5.0–8.5	–	ND
DKbu14	0.7–1.6	1.2–4.0	1 polar	–	30–33	15–42	7.0–7.5	6.5–8.7	–	54.0 ± 1.0
DKpr12	0.9–1.4	1.2–2.0	ND	–	35–38	15–44	7.0–7.3	5.3–8.7	–	ND
EDK82	0.6–1.1	1.5–5.0	–	+	37–38	5–44	7.0–7.3	5.3–8.7	–	65.0 ± 1.0

[a] Abbreviations and symbols: ND, not determined; +, present; –, absent.

times of the isolated sulfate-reducing strains were determined for various substrates (Table 4). Differences between the values presented in Table 4 and the doubling times found in medium with 0.01% yeast extract were less than 10%. Only with strain DKpr12, which was grown with 0.01% yeast extract plus 5 mM acetate and H_2, the difference was much larger, i.e., a doubling time of 28 versus 70 h in the absence of extra yeast extract.

With liquid obtained from the first reactor of the purification plant, additional growth experiments were performed with strain DK81, which was the predominant sulfate-reducing organism in this reactor. After sterilization of the liquid (see above); the growth of strain DK81 was monitored in this reactor liquid that had been supplemented as follows: (i) with sulfate (15 mM), (ii) with sulfate (15 mM) and lactate (15 mM), or (iii) with sulfate (15 mM) and H_2 (headspace/volume ratio of 2:1; the headspace consisted of 60% H_2 and 40% CO_2). The initial pH was 6.5 ± 0.2. In medium (i) hardly any growth was detected, whereas in media (ii) and (iii) doubling times of 3.0 and 3.9 h, respectively, were recorded. These doubling times were shorter than those obtained with artificial medium (Table 4). In artificial medium supplemented with sterilized reactor fluid (50 ml/liter), a similar shortening of the doubling times was found for this strain.

Sulfate reduction in an anaerobic filter system. At a hydraulic retention time of approximately 9.5 h, only 60% of the sulfate that was fed to the first reactor of the purification plant was reduced to sulfide (27). The conversion was improved considerably at lower feed rates; e.g., at a hydraulic retention time of 17 h more than 80% of the sulfate supplied was reduced to sulfide. Lowering of the feed rate would be an economically unattractive solution, as it would require much larger reactor volumes to treat the same volume of wastewater. An alternative solution could be the

retainment of a much larger quantity of active biomass in the reactor. For this reason an anaerobic packed bed type of reactor was used to study, at a laboratory scale, the performance of an anaerobic filter system based on the activity of sulfate-reducing bacteria. The reactor was inoculated with the isolated strains DK81, DKpr12, EDK82, and DKbu14. A medium of pH 6.5 was used that contained ethanol, lactate, propionate, butyrate, and sufficient sulfate to allow the complete oxidation of all electron donors. At different feed rates the reactor was allowed to reach a stable situation in which the effluent concentrations of the growth substrates remained unchanged. This was done with columns packed with porous material of different sizes. No significant differences were detected with particles of 2 to 6 mm and 0.95 to 1.5 mm in diameter. The results (Table 5) show that ethanol and lactate were metabolized completely, even at a hydraulic retention time as short as 0.5 h. The conversion of propionate was complete only up to a hydraulic retention time of approximately 2 h. Butyrate, on the other hand, was metabolized poorly. This might have been caused by the pH of the medium, as the butyrate-oxidizing strain DKbu14 does not grown well at pH values near 6.5 (Table 3). The amounts of sulfate reduced were in accordance with the conversion of ethanol and butyrate into acetate and of lactate and propionate into acetate and CO_2.

DISCUSSION

Before the wastewater enters the methane reactor, sulfate can be removed very efficiently if it is converted quantitatively into sulfide. Therefore, in the first reactor the presence of both SRB and suitable electron donors are required. In this reactor there was no appreciable sludge retention. This implies that a stable population of sulfate-reducing organisms would be possible only if they multiply fast enough to compensate for the washout of cells. At a hydraulic retention time of 9.5 h, the doubling time necessary for maintenance of a species in the liquid phase of a well-mixed reactor is approximately 6.6 h. It was shown that the isolated SRB are able to utilize a wide range of electron donors (Table 1).

TABLE 4. Doubling times of the isolated strains during growth with various substrates in batch cultures[a]

Electron donor (initial concn [mM])	Doubling times (h) of the following strains:			
	DK81	DKbu14	DKpr12	EDK82
H_2 (excess)	5.9		70.0	5.4
Formate (20)	19.8	49.0		68.0
Propionate (15)			6.3	
Butyrate (15)		10.5		
Lactate (15)	5.2		3.7	15.8
Malate (15)	19.8			13.9
Fumarate (15)	9.0			10.2
Succinate (15)	>140			>140
Methanol (20)				75
Ethanol (15)	41.0		6.3	5.3

[a] The media contained 15 mM sulfate. When H_2, methanol, or formate served as an electron donor, the medium was supplied with 5 mM acetate as well.

TABLE 5. Concentrations of growth substrates in the effluent of a laboratory-scale anaerobic filter[a]

Hydraulic retention time (h)	Concn (mM) of the following growth substrates:				
	Butyrate	Propionate	Ethanol	Lactate	Sulfate
10	3–4	<0.1	<0.1	<0.1	5
2	4.9	<0.1	<0.1	<0.1	6
0.9	5.0	2–3	<0.1	<0.1	7
0.5	5.0	3–4	<0.1	<0.1	7

[a] Concentrations were determined after a stable situation was obtained at four different hydraulic retention times. The influent contained ethanol (5 mM), lactate (5 mM), propionate (5 mM), butyrate (5 mM), and sulfate (10 mM). The diameter of the packing material was 2 to 6 mm.

Apparently, H_2, lactate, ethanol, and propionate were the only substrates that allowed a doubling time of less than 6.6 h (Table 4). The predominant sulfate-reducing organism in the first reactor, *Desulfovibrio desulfuricans* DK81, is capable of rapid growth with H_2 or lactate. The influent of the first reactor did not contain lactate, however; and it is unlikely that considerable amounts of lactate are formed in the first reactor itself (27). Moreover, results of experiments with samples taken from the first reactor to which lactate-sulfate or lactate-sulfate-molybdate were added (molybdate is a specific inhibitor of sulfate reduction [32]) indicate that lactate is metabolized by fermentative bacteria (25). This leaves H_2 as the principal electron donor for strain DK81 in the first reactor. Of course, other substrates such as ethanol and formate may contribute to the growth of strain DK81. However, during operation of the first reactor with the usually employed feeding rate (hydraulic retention time ± 9.5 h) sulfate conversion to sulfide was incomplete, which could indicate a limiting availability of H_2. This raises the question of why other strains like EDK82 and DKpr12, both of which are capable of sufficiently rapid growth with ethanol or propionate-ethanol, respectively, did not consume the remaining sulfate. In particular, propionate was found to be present in fairly large amounts, i.e., 10 to 15 mM (27). An explanation may be the relatively low pH in the first reactor (6.0 to 6.3), which is far off the optimum pH (7.0 to 7.3) for the growth of these two strains. Although in principle pH regulation of the content of the first reactor could improve the extent of the reduction of sulfate, in practice this would not be a convenient solution. Apart from the additional costs, it is also undesirable to raise the pH in the first reactor, as this would interfere with the efficiency of the sulfide removal in the sulfide stripper. Moreover, an increased pH in the methane reactor might hamper methanogenesis, particularly because of the increased toxicity of ammonium (27, 28). A more realistic solution may be the application of an anaerobic filter (Table 5). On a semitechnical scale, the influence of biomass retention on sulfate reduction has been examined by Heynen (9) in a two-stage process. In the first reactor, which was an anaerobic fluidized bed system operated at a hydraulic retention time of 1.4 to 2.8 h and a pH of 5.8 to 6.6, all sulfate (approx. 6 mM) was reduced to sulfide. Thus, the application of biomass retention, e.g., in a filter or fluidized bed system, can be useful for achieving a complete conversion of sulfate into sulfide in the first (acidification) reactor of a purification plant. Additional experiments along these lines are required, however, before scaling up of this type of process can be considered.

Taxonomy. Strains DK81, DKbu14, and DKpr12 were classified as strains of *Desulfovibrio desulfuricans* subsp. *desulfuricans*, *Desulfovibrio sapovorans*, and *Desulfobulbus propionicus*, respectively. Strain DKlac11, which was not characterized completely, could be ascribed to the genus *Desulfovibrio*.

The identification of strain EDK82, however, was less straightforward. Strain EDK82 is a strictly anaerobic, gram-negative, sulfate-reducing bacterium. Important distinctive properties of this strain are the cell shape (curved rods); the inability to form spores; the inability to oxidize acetate, propionate, or butyrate; and the incomplete oxidation of pyruvate and lactate to acetate plus CO_2. Based on these properties, strain EDK82 can be affiliated with genus *Desulfovibrio* (32, 46), although strain EDK82 is nonmotile, whereas almost all *Desulfovibrio* strains exhibit motility. However, motility is not a decisive characteristic in classi-

fication of SRB (32). Several characteristics of strain EDK82, such as the curved shape, its temperature range for growth, the lack of a NaCl requirement, the presence of desulfoviridin, and the incomplete oxidation to lactate or pyruvate to acetate and CO_2, have been attributed to the species *D. desulfuricans*, *D. vulgaris*, *D. africanus*, and *D. gigas* (46). Like strain EDK82, some strains of *D. gigas* have been isolated from enrichments supplied with sulfate-ethanol (37). However, several characteristics of strain EDK82 do not fit those of *D. gigas* and other *Desulfovibrio* species (cf. Widdel and Pfennig [46]). The ability of *D. desulfuricans* to metabolize choline was not found with strain EDK82. In contrast with *D. vulgaris*, strain EDK82 is able to ferment malate. Furthermore, strain EDK82 is able to ferment pyruvate, whereas *D. africanus* and *D. gigas* do not possess this ability. Another characteristic that distinguishes strain EDK82 from *D. africanus* and *D. gigas* is that the cells of strain EDK82 are shorter (1.5 to 5 μm versus 5 to 10 μm diameter). In addition, strain EDK82 possesses three rather peculiar characteristics. Growth with methanol as the energy source has not been demonstrated for other non-spore-forming SRB (29). Likewise, fermentation of glycerol by sulfidogens has not been reported before. Moreover, in the presence of sulfate, strain EDK82 converted glycerol stoichiometrically into 3-hydroxypropionate (29). In contrast with strain EDK82, *D. desulfuricans* and *D. vulgaris* oxidize glycerol to acetate and CO_2 (D. R. Kremer and T. A. Hansen, manuscript in preparation), whereas *D. gigas* (D. R. Kremer and T. A. Hansen, personal communication) and *D. africanus* (5) are unable to metabolize glycerol. Finally, the cells of strain EDK82 contain stacked intracellular membranes (29). Among the SRB, this property has been reported only for *Desulfobacter postgatei* (41) and two *Desulfonema* species (43). However, these species are distinctly different from strain EDK82.

Thus, strain EDK82 does not seem to fit the description of any of the described sulfate-reducing species. Therefore, this strain is proposed as the type strain of a new species, *Desulfovibrio carbinolicus*.

Desulfovibrio carbinolicus sp. nov. car. bi. no'li. cus M.L. adj. referring to carbinols syn. alcohols; *carbinolicus* metabolizing alcohols.

The cells are rod shaped cells, curved, 0.6 to 1.1 by 1.5 to 5.0 μm, and immotile. The cells contain stacked intracellular membranes.

Sulfate, sulfite, thiosulfate, and elemental sulfur serve as electron acceptors and are reduced to H_2S. Nitrate, nitrite, and fumarate are not reduced. Molecular hydrogen, formate, methanol, ethanol, glycerol, 1,3-propanediol, propanol-1, butanol-1, pentanol-1, lactate, pyruvate, succinate, fumarate, malate, and oxaloacetate are utilized as electron donors. Growth with molecular hydrogen, formate, and methanol requires acetate as carbon source in the presence of CO_2, and growth with methanol requires yeast extract as well. Formate and methanol are oxidized to CO_2. Glycerol and 1,3-propanediol are stoichiometrically oxidized to 3-hydroxypropionate; propanol-1, butanol-1, and pentanol-1 are oxidized to propionate, butyrate, and valerate, respectively. The other electron donors yield acetate plus (depending on the electron donor) CO_2. Succinate may accumulate as an intermediate product. In the absence of an electron acceptor, fumarate and malate are fermented to succinate plus acetate, pyruvate is fermented mainly to acetate, and glycerol is fermented to 1,3-propanediol plus 3-hydroxypropionate. Choline, glucose, fructose, amino acids, acetate, and higher fatty acids are not utilized.

The organism grows in a pH range from 5.3 to 8.7 but optimally at pH 7.0 to 7.3. The temperature range for growth is 5 to 44°C; the optimum is at 37 to 38°C.

Desulfoviridin and c-type cytochromes are present. The DNA base ratio is 65 mol% G+C (thermal denaturation).

The type strain is strain EDK82, which has been deposited in the Deutsche Sammlung von Mikroorganismen, Göttingen, (accession number Federal Republic of Germany DSM 3852).

ACKNOWLEDGMENTS

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