Sulfate-reducing Bacteria in Anaerobic Bioreactors

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Stellingen

1. In hun lijst van mogelijke scenario's voor de anaërobe afbraak van propionaat onder sulfaatrijke condities vergeten Uberoi en Bhattacharya het scenario dat in de anaërobe waterzuiveringsreactor van de papierfabriek te Eerbeek lijkt op te treden, namelijk de afbraak van propionaat door syntrofen en sulfaatreduceerders en de afbraak van acetaat en waterstof door sulfaatreduceerders en methanogenen.

   Dit proefschrift, hoofdstuk 7
   Uberoi V, Bhattacharya SK (1995) Interactions among sulfate reducers, acetogens, and methanogens in anaerobic propionate systems.

2. De stelling van McCartney en Oleszkiewicz dat sulfaatreduceerders in anaërobe reactoren waarschijnlijk alleen competeren met methanogenen voor het aanwezige waterstof, omdat acetaatafbrekende sulfaatreduceerders nog nooit uit anaëroob slib waren geïsoleerd, was correct bij indiening, maar achterhaald bij publicatie.

   Oude Elferink SJWH, Maas RN, Stams AJM (1993) Characterization of a newly isolated *"Desulfobacterium"* species, enriched with acetate from an Upflow Anaerobic Sludge Bed (UASB) reactor. Bioengineer.1:48.
   McCartney DM, Oleszkiewicz JA (1993) Competition between methanogens and sulfate reducers: effect of COD:sulfate ratio and acclimation. Water Environ. Res. 65:655-664.

3. Identificatie van *Methanospirillum hungatei* in korrelslib met behulp van transmissie electronenmicroscopie (TEM) blijft twijfelachtig, ondanks de goed gedocumenteerde celkenmerken van *M. hungatei*.

   Fang HHP, Li YY, Chui HK (1995) Performance and sludge characteristics of UASB process treating propionate-rich wastewater. Wat. Res. 29:5-895-898.

4. Het ontdekken van een nieuwe bacteriesoort is geen kunst, het isoleren en karakteriseren wel.

   Amann R, Ludwig W, Schleifer K-H (1994) Identification of uncultured bacteria: A challenging task for molecular taxonomists. ASM News 60:360-365.

5. Het aantal sulfaatreduceerders in de mond hoeft, zeker gezien hun fermentatieve eigenschappen, niet gelimiteerd te worden door het beschikbare sulfaat.

   Van der Hoeven JS, Van den Kieboom CWA, Schaeken MJM (1995) Sulfate-reducing bacteria in the periodontal pocket. Oral Microbiol. Immunol. 10:280-290.

6. Zelfs de meest gecompliceerde methanogene reactor is slechts een vereenvoudigde versie van de pens.

   Van Soest PJ (1994) Nutritional Ecology of the ruminant. 2nd ed. Cornell University Press, Ithaca, USA.

7. Met geloof en vertrouwen is niets mis, zolang het maar niet blind is.

8. Leuk wetenschappelijk onderzoek voldoet aan dezelfde criteria als een leuk spel. Het bevat evolutie en tempo, het is een combinatie van kunde (of behendigheid) en toeval, succes kan worden toegeschreven aan eigen briljantie en mislukking aan domme pech.

9. De observatie van Prof. Dr. Andreasen dat succesvol schrijverschap is gerelateerd aan depressie, alcoholisme en manische depressiviteit, geeft geen positief beeld van AIO's die probleemloos een perfect proefschrift schrijven.
   N. Andreasen. In: De Volkskrant, 4 Oktober 1997.

10. Natuurreservaat "De Blauwe Kamer" is een mooi stukje groene ruimte.

11. Met de hondsdagen is het gewoonlijk geen hondeweer.

12. Vele handen maken wellicht werk.

13. Een nadeel van sluipverkeer is dat het, anders dan de naam doet vermoeden, vaak met grote snelheid voorbij raast.

14. Netwerken is net werken.

15. Een slimme meid houdt niet van voorkeursbeleid.

Stellingen behorend bij het proefschrift:
"Sulfate reducing bacteria in anaerobic bioreactors."
Stefanie J.W.H. Oude Elferink, Wageningen, 22 mei 1998.

Nature, to be commanded, must be obeyed.

Sir Francis Bacon

Aan mijn ouders

CONTENTS

CHAPTER 1

Introduction

Partly published as:

Oude Elferink SJWH, Visser A, Hulshoff Pol LW, Stams AJM (1994) Sulfate reduction in methanogenic bioreactors. FEMS Microbiol. Rev. 15, 119-136.

Stams AJM, Oude Elferink SJWH (1997) Understanding and advancing wastewater treatment. Curr. Opin. Biotechnol. 8, 328-334.

1. INTRODUCTION

Since the 1970's the full-scale application of anaerobic wastewater treatment has increased rapidly [108,110,134,254]. This interest in anaerobic wastewater treatment was partly due to the increasing energy prices and for the other part due to the more stringent legislation for the discharge of industrial wastewaters. Contrary to anaerobic wastewater treatment systems, conventional aerobic systems demand high energy inputs for aeration, and large amounts of surplus sludge are produced during the purification process [196]. A break-through in the application of anaerobic wastewater treatment systems came with the development of several new anaerobic reactor types with immobilized biomass, e.g. the Upflow Anaerobic Sludge Bed (UASB) reactor [109-111], the Fluidized Bed reactor [72,88] and the Anaerobic Filter [254,255]. These reactor types have been reviewed by others [71,88,108,196]. The system with the widest application is undoubtedly the UASB reactor [110]. At present more than 400 full-scale UASB reactors are in operation for the treatment of industrial wastewaters [223]. In the UASB reactor an uncoupling of the solids retention time and the hydraulic retention time is achieved by the immobilization of anaerobic bacteria into highly active, well-settleable sludge granules [80,109,111].

Municipal sewage and industrial wastewaters from e.g. sugar-refineries, beer-breweries, potato-processing factories and slaughterhouses, which contain sulfate concentrations of only about 50-200 mg/l, are treated satisfactorily in UASB reactors. However, problems may arise when wastewaters are treated which contain high concentrations of sulfate or sulfite. Some industrial wastewaters, e.g. wastewaters from edible oil industry, paper mills and patato-starch factories, may contain sulfate concentrations of several grams per liter [172,173,253], as a result of the use of sulfuric acid, a very concentrated and relatively cheap strong acid, or sulfite, a bleaching agent, in production processes.

During anaerobic treatment of these wastewaters sulfate reduction will interfere with methanogenesis. Occurrence of sulfate reduction has several disadvantages: 1) sulfate reducers and methanogens compete for substrates resulting in a decrease of methane production [172], 2) sulfide is inhibitory for several types of anaerobic bacteria [74,75,100,197], 3) H_2S gas is toxic and malodorous and can cause corrosion problems in boilers and internal combustion engines [172], and therefore it has to be removed from the biogas, 4) sulfide-containing effluents of anaerobic reactors can not be discharged at the surface water, because sulfide contributes to the oxygen demanding capacity of the water and because of malodor problems. Therefore, a post-treatment of the anaerobically purified wastewater will become essential.

In general occurrence of sulfate reduction in anaerobic bioreactors is undesirable due to the problems associated with the process. However, besides the disadvantages, there are also some advantages of the occurrence of sulfate reduction: 1) sulfate reduction can be used together with

sulfide removal techniques as a biotechnological method for the removal of sulfate [29,78,123,188,207], 2) metal-sulfides have extremely low solubilities, a property which can be used to precipitate toxic heavy metals, such as Co, Ni, Pb and Zn [46,123,135], 3) problems with the anaerobic treatment of sulfite-containing wastewater can be solved by the reduction of sulfite to sulfide [173,140].

The following introduction will first describe how microbiological processes in mesophilic bioreactors are affected by the presence of sulfate. Special attention will be given to the mutualistic and competitive interactions between the anaerobic bacteria involved in the degradation of short-chain fatty acids. Subsequently classical and more advanced techniques for the characterization of the microbial sludge biomass will be presented. Finally an outline of the thesis is given.

2. ANAEROBIC DEGRADATION OF ORGANIC MATERIAL

2.1 Methanogenic bioreactors

In methanogenic bioreactors the mineralization of organic material to methane and carbon dioxide is accomplished by the concerted action of various metabolic groups of bacteria, as is presented in Fig. 1 [61,183]. Biopolymers like carbohydrates, proteins, nucleic acids and lipids are first hydrolyzed to mono- and oligomers, and then fermented to products which can be used by methanogens directly (acetate, hydrogen, formate) and to reduced organic compounds like propionate, butyrate, long-chain fatty acids, alcohols, lactate and succinate. The higher fatty acids are anaerobically oxidized by acetogenic bacteria to acetate, CO_2, hydrogen and formate. Lactate and ethanol can be oxidized to acetate by acetogenic bacteria. However, these compounds can also be fermented to e.g. acetate and propionate [105,182]. Succinate can be decarboxylated to propionate [35,59,184]. In the last stage acetate, formate and hydrogen are converted by methanogenic bacteria [183,256,258].

Volatile fatty acids are known to be important intermediates in the degradation of organic matter under methanogenic conditions. About 70% of the reducing equivalents formed in the anaerobic digestion process is transferred via acetate to methane [61]. The importance of propionate and butyrate is dependent on the type of digester and the nature of the organic compounds. The complete oxidation of propionate and butyrate can account for 20 to 43% of the total methane formation [119,120].

Most of the reactions carried out by the acetogenic bacteria are highly endergonic at standard conditions (Table 1). However, these reactions become exergonic when the H_2-partial pressure is kept low, as is the case by syntrophic consortia of acetogens and hydrogenotrophic methanogens

[38,183]. In natural ecosystems, steady-state H_2-partial pressures between 6 and 400 Pa have been measured [259]. Syntrophic consortia of acetogenic and methanogenic bacteria grow slowly and often they are sensitive to changes in the environmental conditions. Syntrophic degradation and the role of interspecies hydrogen transfer in the oxidation of fatty acids has been reviewed [38,183,200]. It is possible that in the acetogenic reactions as given in Table 1 not hydrogen but formate is formed. In that case formate will be the compound via which reducing equivalents are shuttled from the acetogen to the methanogen. Evidence for a role of formate transfer in syntrophic degradation was reported [19,40,216]. It was calculated that in suspended cultures the distance between the bacteria is too large to explain the conversion rates by a diffusion of hydrogen [19,40,151]. However, there is also evidence that in reactors with dispersed or granular sludge the syntrophic partners are close enough together to explain the measured degradation rates by hydrogen transfer [55,57,58,83,192], and formate transfer is not essential for syntrophic degradation of propionate and butyrate [190]. In some cases, the occurrence of formate transfer even can be excluded, because methanogens were present which are able to use hydrogen but not formate [2,107,126,202]. Because relatively little is known about the growth characteristics of sulfate reducers on formate, this introduction is mainly focused on hydrogen.

Table 1. Acetogenic and methanogenic reactions, and sulfate-reducing reactions involved in the degradation of organic matter in methanogenic bioreactors, and sulfate-reducing bioreactors, respectively.

			$\Delta G^{0'a}$ [kJ/mol]
Syntrophic Acetogenic reactions			
$Propionate^- + 3\ H_2O$	→	$Acetate^- + HCO_3^- + H^+ + 3\ H_2$	+ 76.1
$Butyrate^- + 2\ H_2O$	→	$2\ Acetate^- + H^+ + 2\ H_2$	+ 48.3
$Lactate^- + 2\ H_2O$	→	$Acetate^- + HCO_3^- + H^+ + 2\ H_2$	- 4.2
$Ethanol + H_2O$	→	$Acetate^- + H^+ + 2\ H_2$	+ 9.6
Methanogenic reactions			
$4\ H_2 + HCO_3^- + H^+$	→	$CH_4 + 3\ H_2O$	- 33.9
$Acetate^- + H_2O$	→	$CH_4 + HCO_3^-$	- 31.0
Sulfate-reducing reactions			
$4\ H_2 + SO_4^{2-} + H^+$	→	$HS^- + 4\ H_2O$	- 38.1
$Acetate^- + SO_4^{2-}$	→	$2\ HCO_3^- + HS^-$	- 47.6
$Propionate^- + ¾\ SO_4^{2-}$	→	$Acetate^- + HCO_3^- + ¾\ HS^- + ¼\ H^+$	- 37.7
$Butyrate^- + ½\ SO_4^{2-}$	→	$2\ Acetate^- + ½\ HS^- + ½\ H^+$	- 27.8
$Lactate^- + ½\ SO_4^{2-}$	→	$Acetate^- + HCO_3^- + ½\ HS^- + ½\ H^+$	- 80.0
$Ethanol + ½\ SO_4^{2-}$	→	$Acetate^- + ½\ HS^- + ½\ H^+ + H_2O$	- 66.4

[a] $\Delta G^{0'}$-values are taken from Thauer et al. [215].

2.2 Sulfidogenic bioreactors, competition of sulfate reducers with methanogens and acetogens

When sulfate is present in the wastewater sulfate-reducing bacteria are able to couple the oxidation of organic compounds and hydrogen to sulfate reduction. Compared with methanogens, sulfate-reducing bacteria are very diverse in terms of their metabolic possibilities. The physiology of sulfate-reducing bacteria has been reviewed extensively [239-242]. Some of the reactions which can be carried out by sulfate-reducing bacteria in anaerobic bioreactors are presented in Table 1. Both autotrophic and heterotrophic growth on hydrogen or formate is possible. The classical *Desulfovibrio* sp. require acetate as a source of carbon, whereas e.g. *Desulfobacterium* sp. can use CO_2 as the sole source of carbon [241,242]. Many sulfate-reducing bacteria degrade propionate and butyrate; some oxidize these fatty acids completely to carbon dioxide, whereas others oxidize these compounds only to acetate. Other compounds which can be completely or incompletely degraded by sulfate reducers are branched and long-chain fatty acids, ethanol and other alcohols, organic acids (lactate, succinate, malate) and aromatic compounds like benzoate, catechol, phenol, indole, aniline [239,242]. Even compounds without a functional group such as toluene and alkanes can be degraded by sulfate reducers [1,16,161].

The anaerobic degradation process can become very complex in the presence of sulfate (Fig. 1), because sulfate reducers will compete with methanogens for compounds such as formate and hydrogen, and with acetogens for compounds like propionate and butyrate [32]. Kinetic properties of sulfate reducers, methanogens, and acetogens can be used to predict the outcome of the competition for these common substrates [101,113,176,186]. For bacteria growing in suspension, Monod kinetic parameters such as the half-saturation constant (K_s) and the specific growth rate (μ_{max}) can be used. When bacterial growth is negligible, as is often the case in reactors with a dense biomass concentration, Michaelis-Menten kinetics may be used to predict which type of organism has the most appropriate enzyme systems to degrade substrates. Therefore, both the V_{max}/K_m and the μ_{max}/K_s ratio gives an indication of the outcome of competition at low substrate concentrations [176]. Selected kinetic data of methanogens and sulfate reducers for hydrogen and acetate are listed in Tables 2 and 3. Based on these data the competition between sulfate reducers and methanogens for hydrogen and acetate, can partly be explained, as will be discussed in par. 2.2.1 and 2.2.2. Another important factor in the competition sulfaat reducers, methanogens and acetogens is the chemical oxygen demand (COD)/sulfate ratio of the wastewater [64], because this ratio determines which part of the organic material (based on g COD) can be maximally degraded via sulfate reduction. In theory all organic material can be degraded via sulfate reducion if the COD/sulfate ratio (g/g) is below 0.66 (mol COD/mol sulfate <0.5). If the COD/sulfate ratio (g/g) is above 0.66,

sulfate reducers will not only have to compete with methanogens and acetogens for the available substrate, but also with other sulfate reducers for the available sulfate.

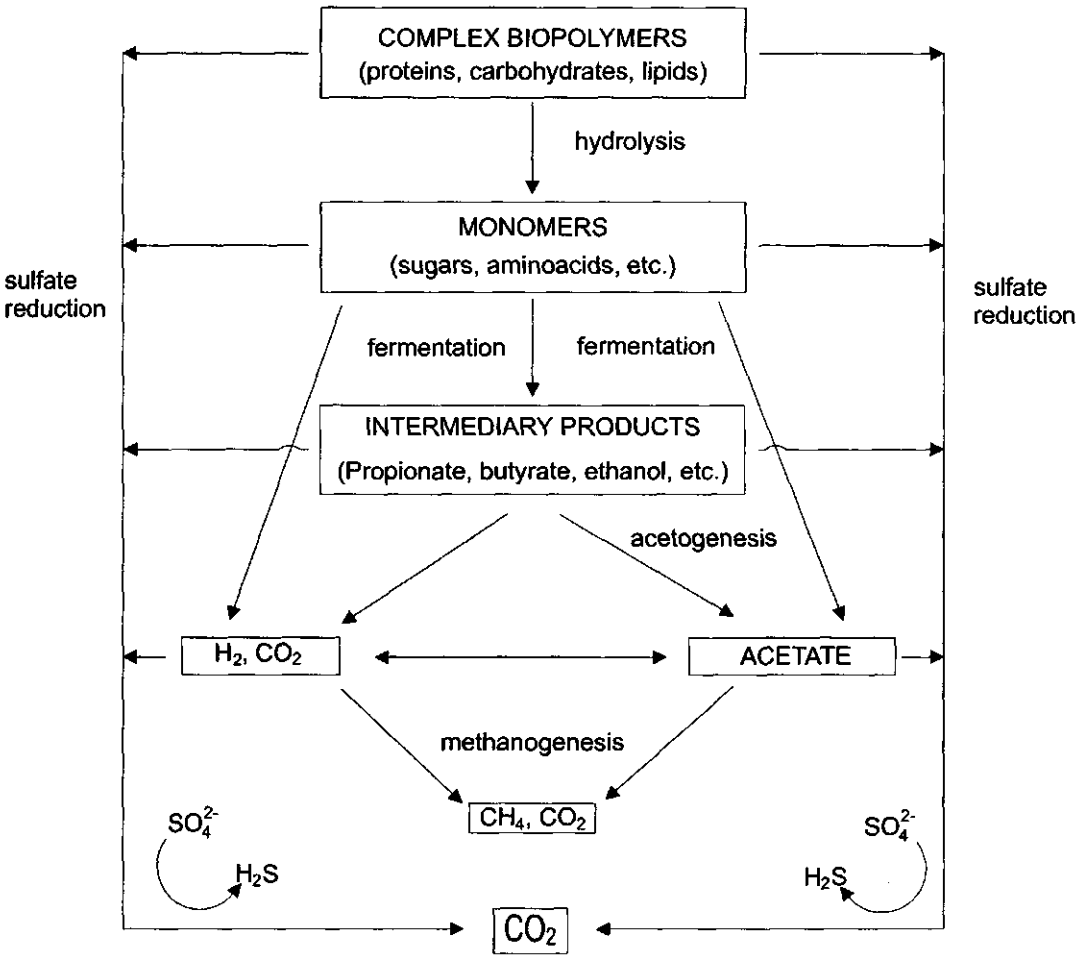

Figure 1. Anaerobic degradation of organic compounds in the presence of sulfate.

2.2.1 Competition for hydrogen.

Studies with sediments and sludge from bioreactors have indicated that at an excess of sulfate hydrogen is mainly consumed by sulfate reducers [3,13,77,86,87,113, 124,159,174,247]. In reactors with immobilized biomass the activity of hydrogenotrophic methanogens is completely suppressed

within a few weeks when sulfate is added [225]. As hydrogenotrophic methanogens are still present in high numbers in the sludge, this effect can not simply be explained by Michaelis-Menten or Monod kinetic data (Table 2). It has to be assumed that reactors operate at the threshold value for hydrogen of methanogens, and that hydrogenotrophic sulfate reducers have a lower threshold for hydrogen. In freshwater sediments a threshold hydrogen concentration of 1.1 Pa has been measured; this value was lowered to 0.2 Pa by the addition of sulfate [113]. The effect of threshold values is illustrated in Figure 2.

An alternative explanation for the rapid suppression of methanogenesis is that hydrogen formation is not important any more. In the absence of sulfate, acetogenic bacteria form hydrogen during the the oxidation of compounds such as lactate, alcohols, propionate and butyrate. In the presence of sulfate, all these compounds can be oxidized directly by sulfate reducers without the intermediate formation of hydrogen. This explanation can not be the only one because some hydrogen will always be formed by fermentative glucose- and amino acid-degrading bacteria.

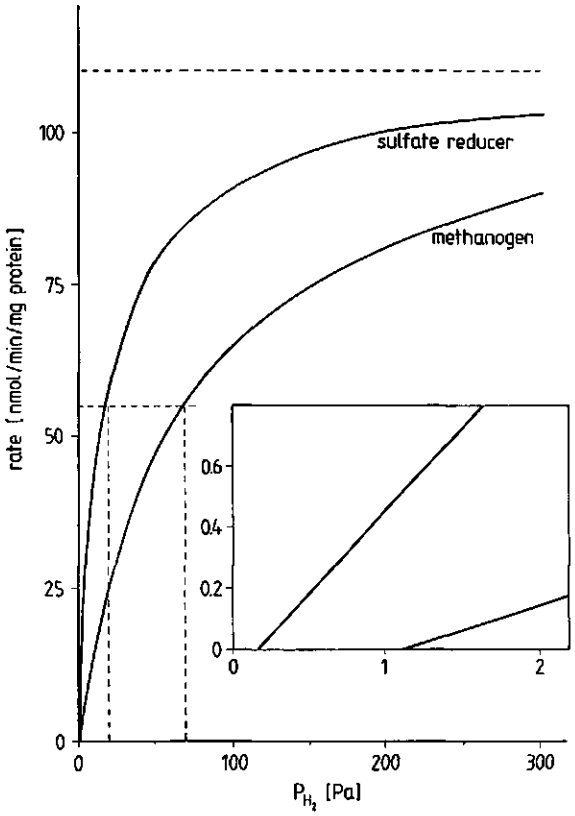

Figure 2. Kinetics of hydrogen oxidation by sulfate-reducing bacteria and methanogens. Insert: Treshold values for hydrogen oxidation. Kinetic data were taken from [176] and the threshold values from [113].

Table 2. Selected growth kinetic data of hydrogenotrophic sulfate-reducing bacteria and methanogenic bacteria.

Bacterial strain	K_s (μM)	μ_{max} (1/day)	Yield[a] (g/mol H_2)	K_m (μM)	V_{max} (μmol/min.g)	Reference
Sulfate reducers						
Desulfovibrio						
desulfuricans[b]		1.6-4.3	1.9	1.8-4.0	88	22,33,92,136,176
vulgaris[b]		0.7-5.5	0.6-3.1	1.3-4.0	30	11,12,22,114,139,176
strain DG2				1.4	23	176
strain G11	2.4-4.2	1.2-1.6	1.4-2.0	1.1	65	176
strain PS1				0.7	65	176
Desulfobacter						
hydrogenophilus		1.0				238
Desulfobacterium						
autotrophicum		0.7-1.1				27
Desulfobulbus						
propionicus[b]		0.2-1.7				136,246
Desufomicrobium						
escambium		1.4				187
Methanogens						
Methanobacterium						
bryantii		0.3-1.9	0.6			9,89,175
formicicum[b]		1.2-3.1	0.9	2		9,181,250
ivanovii		0.8-1.7	1.1	14		12,89
Methanobrevibacter						
arboriphilus[b]		0.7-3.4	0.6-1.3	6.6		9,10,101,260,261
smithii		4.1				9
Methanococcus						
vannielii		4.1				9
Methanospirillum						
hungatei	5.8-7.3	1.2-1.8	0.3-0.5	5.0	70	176,9
strain BD		2.4-2.8				250
strain PM1				2.5	90	176
strain PM2				4.1	6.5	176
Methanosarcina						
barkeri[b]		1.4-1.8	1.6-2.2	13	110	9,176,193,235
mazei		1.4-1.7				250

[a]The yield is given in gram cell dry weight per mol. When only $Y_{protein}$ was given, we assumed $Y_{protein} = 0.5 * Y_{cell\ dry\ weight}$.
[b]Several strains.

2.2.2 *Competition for acetate*

It has been shown that in marine and freshwater sediments acetate is mainly consumed by sulfate-reducers when sufficient sulfate is present [13,113,195,247]. However, for anaerobic digesters it is less clear how acetate is degraded. A complete conversion of acetate by methanogens, even at an excess of sulfate, has been reported [77,86,87,159,174,220,221,225,253]. However, in some studies a predominance of acetate-degrading sulfate reducers was found [3,62,172,224]. Some factors which may affect the competition between sulfate reducers and methanogens are discussed below.

Table 3. Selected growth kinetic data of acetotrophic sulfate-reducing bacteria and methanogenic bacteria

Bacterial strain	K_s (mM)	μ_{max} (1/day)	Yield[a] (g/mol ac.)	K_m (mM)	V_{max} (µmol/min.g)	Reference
Sulfate reducers						
Desulfobacter						
curvatus		0.79				238
hydrogenophilus		0.92				238
latus		0.79				238
postgatei[b]		0.72-1.11	4.3-4.8	0.07-0.23	53	23,85,186,244
strain AcKo		0.55				238
Desulfotomaculum						
acetoxidans		0.65-1.39	5.6			243
Methanogens						
Methanosarcina						
barkeri[b]	5.0	0.46-0.69	1.6-3.4	3.0		186,193,194,234,252
mazei[b]		0.49-0.53	1.9			250,252
Methanosaeta						
soehngenii[b]	0.5	0.08-0.29	1.1-1.4	0.39-0.7	38	81,145,217,257
concilii		0.21-0.69	1.1-1.2	0.84-1.2	16	145,154,217
strain MTAS		0.37		0.49	85	145,147
strain MTKO		0.38		1.17	49	145,147

[a]The yield is given in gram cell dry weight per mol. When only $Y_{protein}$ was given, we assumed $Y_{protein} = 0.5 * Y_{cell\ dry\ weight}$.
[b]Several strains.

Affinity for acetate

Work of Schönheit et al. [186] has indicated that the predominance of *Desulfobacter postgatei* in marine sediments could be explained by its higher affinity for acetate than *Methanosarcina barkeri*. The K_m values were 0.2 and 3.0 mM, respectively (Table 3). However, in bioreactors

Methanosarcina sp. are only present in high numbers when the reactors are operated at a high acetate concentration or operated at a low pH [57]. Generally, *Methanosaeta* (former *Methanothrix*, [155]) sp. are the most important aceticlastic methanogens in high-rate bioreactors [26,57,80,99, 118,131,144]. *Methanosaeta* sp. have a higher affinity for acetate than *Methanosarcina* sp.; their K_s is about 0.4 mM [91]. In addition, *D. postgatei* is a typical marine bacterium which has not yet been isolated from freshwater sources. The aceticlastic sulfate reducers that prefer freshwater conditions, such as *Desulfoarculus baarsii* [237], *Desulfabacterium catecholicum* [209], and *Desulfococcus biacutus* [156] show very poor growth with acetate. Only *Desulfobacterium* strain AcKo and *Desulfotomaculum acetoxidans* show good growth with acetate under mesophilic conditions (see Table 4). Unfortunately no K_s or K_m values are available for these bacteria.

Seed sludge

Another factor which should be taken into account is that for most reactor experiments methanogenic seed sludge was used. In such sludge aceticlastic methanogens are present in high numbers, whereas the initial numbers of aceticlastic sulfate reducers will be low. In UASB reactors the sludge retention time can be as high as 0.5-1 year [80]. Therefore competition experiments may be hampered by the long time which may be needed for one type of bacterium to outcompete others. This is especially the case if the initial numbers of the competing bacteria are low, or if the growth kinetic properties are only slighty better compared to others. Visser et al. [225] have simulated the competition between sulfate-reducing bacteria and methanogens using a biomass retention time in the reactor of 0.02 day^{-1}, a maximum specific growth rate of 0.055 and 0.07 per day for the methanogen and sulfate-reducing bacterium, respectively, a K_s value for acetate of 0.08 and 0.4 mM acetate, respectively, and different initial ratios of bacteria [225]. Starting with a ratio of methanogens/sulfate reducers of 10^4, it will take already one year before the numbers of acetate-degrading sulfate-reducing bacteria and acetate-degrading methanogens are equal.

Nevertheless, long-term UASB reactor experiments of Visser [223] showed that sulfate reducers are able to outcompete methanogens for acetate, even if the seed sludge initially only contains low numbers of aceticlastic sulfate reducers. In his acetate and sulfate fed UASB reactor it took 50 days before acetate degradation via sulfate reduction was observed, and another 50 days to increase it to 10%. The shift from 50 to 90% of acetate degradation via sulfate reduction took approximately 400 days.

Utilization of other substrates

Methanosaeta can only grow on acetate, whereas *Methanosarcina* can use a few other substrates besides acetate, like hydrogen, methanol and methylated amines [20,91]. Aceticlastic *Desulfobacter* sp. also use a limited range of substrates; solely hydrogen, acetate and ethanol provide good growth

[238]. However, *Desulfotomaculum acetoxidans* uses a wide range of substrates such as, formate, acetate, butyrate, isobutyrate, butanol, and ethanol [245]. It is not clear to which extent *D. acetoxidans* can grow mixotrophically. During growth on butyrate, isobutyrate, ethanol or butanol acetate is even excreted [245]. However, if at low concentrations acetate and other substrates are used at the same time the outcome of the competition between *Methanosaeta* and *D. acetoxidans* for acetate will be affected. Gottschal and Thingstad [56] described a model in which it is shown that during competition on mixtures of substrates in continuous cultures not only the specific growth rate determines the outcome of a competition, but also the yield on the different substrates.

The sulfate concentration

At low sulfate concentrations the growth of the sulfate-reducing bacteria will be sulfate limited. Also under conditions of high sulfate concentrations, sulfate limitation of the biomass in the anaerobic digester may occur due to mass transfer limitation of sulfate into the biofilm or the sludge granule. Thus far insufficient data are available to predict sulfate concentration profiles in biofilms. However, Nielsen [142] reported that sulfate limitation could already occur in a biofilm of a few hundred μm thick when the sulfate concentration in the bulk solution was below 0.5 mM.

Under sulfate-limiting conditions aceticlasic sulfate reducers will have to compete with other sulfate reducers for the available sulfate. Laanbroek et al. [106] experimented with three bacterial strains, *Desulfobacter postgatei*, *Desulfobulbus propionicus* and *Desulfomicrobium baculatum* in sulfate-limited chemostats. They found that *D. baculatum* was the most successful competitor for limiting amounts of sulfate, followed by *D. propionicus* and then by *D. postgatei*. The K_m for sulfate of *D. postgatei* is 200 μM [85], a value which is much higher than the reported K_s and K_m values for several *Desulfovibrio* strains (5-77 μM) [84,139,148]. The affinities for sulfate of *Desulfobacter* strain AcKo and *Desulfotomaculum acetoxidans* are not known. However, if these species sp. have a higher K_s value than other sulfate reducers, one might speculate that limiting amounts of sulfate would result in an oxidation of compounds like hydrogen, formate and butyrate by sulfate-reducing bacteria, while acetate is used by the aceticlastic methanogens.

Competition for sulfate between sulfate-reducing bacteria could explain the results obtained in studies with sulfate-limited reactors, where acetate seemed to be the least favoured substrate for sulfate reduction, compared to propionate, butyrate and hydrogen [130,219,225].

The effect of pH

Insufficient data are available to predict the effect of the pH on the competition for acetate between sulfate reducers and methanogens. Visser et al. [226] showed that at 55°C a high pH favors acetate degradation via sulfate reduction, whereas methanogenesis was favored at a low pH. The effect of the pH on the competition between the bacteria can also be indirect. An example is the pH

dependence of the toxicity of compounds such as sulfide (as discussed below) or ammonia.

Toxicity of sulfide

Sulfate reduction results in the accumulation of sulfide. Sulfide is most toxic in its undissociated form, because the neutral molecule can permeate the cell membrane [185]. The pK_a-value of the dissociation equilibrium of H_2S is about 7 at 18°C [233]. Consequently, small pH variations in the optimal pH range (6-8) for anaerobic digestion can cause great changes in the degree of inhibition. In addition, the concentration of free H_2S in the reactor fluid is largely affected by the rate of biogas production. A high rate will result in an increased transfer of H_2S to the gas phase. The effect of sulfide on methanogenesis has been studied extensively, but relatively little attention has been paid to the inhibition of sulfate reducers by sulfide. McCartney and Oleszkiewicz [124] found that sulfate-reducing bacteria are more sensitive to an increase in the total sulfide concentrations than methanogens. Values for the free H_2S concentration at which methanogenesis was inhibited for 50% vary between 50 mg to 270 mg H_2S/l [94,100,102,124,149]. For sulfate reduction a value of 85 mg H_2S/l was reported [124]. A linear correlation between the free H_2S concentration and the maximum specific aceticlastic methanogenic activity was found between pH 6.4 and 7.2, but between pH 7.8 and 8.0 the aceticlastic methanogenic activity correlated better with the total sulfide concentration [100]. Studies of Visser [223] showed that acetate-degrading sulfate reducers and acetate-degrading methanogens were equally inhibited by sulfide at pH values between 7 and 7.5. Fifty percent inhibition of the acetate degradation rates as well as the growth rates was obtained at ca. 500 ml/l total sulfide. At higher pH values (>8) the acetate-degrading methanogens were significantly more inhibited by the total sulfide concentration than the sulfate reducers. Besides a direct toxic effect of sulfide, the precipitation of essential metal ions could also lead to a decrease of the metabolic activity [168]. However, it was shown that sulfate-reducing bacteria are inhibited directly by H_2S [167,168]. A complete inhibition of the growth of a *Desulfovibrio* sp. was achieved at a free H_2S concentration of 550 mg/l at a pH of about 6.5.

The effect of temperature

Visser et al. [227] found that under mesophilic conditions acetate is rapidly degraded by sulfate reducers when the inoculum is pretreated at 65°C. This could indicate that methanogens are more sensitive to temperature increases than sulfate reducers.

Immobilisation of bacteria

The principle of modern high rate anaerobic reactors is based on the ability of anaerobic bacteria to immobilize into sludge granules or onto solid particles. Bacteria with poor adherence properties are washed out of the reactor. Therefore, the competition between the different bacteria will also be

determined by the immobilisation properties of the bacteria. Isa et al. [86,87] investigated the role of the attachment capacity of sulfate-reducing bacteria and methanogenic bacteria in an anaerobic filter reactor fed with acetate or acetate and ethanol, and operated at an excess of sulfate. They showed that sulfate-reducing bacteria were poor competitors of methanogenic bacteria for acetate, and ascribed this to the superior capability of methanogens to colonize the support material of the anaerobic filter. However, their experimentation was criticized [172] because the observed superior colonization of the methanogenic bacteria was based on viable cell counts which for immobilized bacteria has only a limited accuracy, and on a wrong interpretation of the activity tests. There is now even evidence that acetotrophic sulfate-reducing bacteria are well able to attach to solid particles and to grow in sludge granules [3,224].

2.2.3 Competition for propionate and butyrate

In anaerobic reactors with high sulfate concentrations, sulfate-reducing bacteria compete with acetogenic bacteria for substrates like propionate and butyrate. Little is known about this competition. For wastewater with an excess of sulfate it is to be expected that sulfate-reducing bacteria become predominant, because of their better growth kinetic properties (Table 4). It is obvious that at high sulfate concentrations, sulfate-reducing bacteria grow much faster than the syntrophic consortia. Almost no K_s and K_m values for propionate and butyrate degradation have been reported. Therefore, a comparison of the growth of syntrophic cultures and sulfate reducers at low substrate concentrations is not possible. The existence of two subpopulations of propionate oxidizers in methanogenic sludge was reported [73], a fast-growing with a μ_{max} of 1.2 day^{-1} and a K_s of 4.5 mM, and a slow-growing with a higher affinity (μ_{max} of 0.13 day^{-1} and a K_s of 0.15 mM).
Several researchers investigated the competition for propionate and butyrate between sulfate reducers and acetogens in anaerobic reactors. However, since mass balances were used to determine this competition [3,159,224,225], no destinction can be made between a direct oxidation of propionate and butyrate by sulfate reducers or an indirect conversion, whereby the fatty acids are oxidized to acetate and hydrogen by the acetogenic bacteria followed by hydrogen conversion via sulfate reduction. In this respect it is interesting to mention that sulfate reducers keep the hydrogen partial pressure lower than methanogens, and that propionate- and butyrate-degrading acetogens grow much faster in coculture with hydrogen-consuming sulfate reducers than with hydrogen-consuming methanogens [18,126]. Therefore, the reported critical role of sulfate reducers in mediating propionate and butyrate degradation [3,64,121,124,130,159,224,225] may be that of a hydrogen consumer rather or that of a direct propionate or butyrate oxidizer.

Table 4. Specific growth rates (1/day) of sulfate-reducing bacteria and of acetogenic bacteria in cocultures with hydrogenotrophic methanogens/sulfate reducers, growing on butyrate or propionate.

	Sulfate-reducing culture	Syntrophic coculture -sulfate	+ sulfate	Reference
Butyrate-degrading strains				
Desulfoarculus baarsii	0.4			180
Desulfobacterium autotrophicum	0.67-1.11			27
Desulfococcus multivorans	0.17-0.23			206
Desulfotomaculum acetoxidans	1.11			245
Desulfotomaculum strain Gro111	1.2-1.3			103
Syntrophomonas sapovorans		0.6		178
Syntrophomonas wolfei		0.2	0.3	41,126,127
Syntrophospora (*Clostridium*) *bryantii*		0.25		205,250,268
sporeforming strain FMS2		0.31		269
sporeforming strain FSS7			0.34	269
non-sporeforming strain FM4		0.24		269
non-sporeforming strain B1		0.1		128
Propionate-degrading strains				
Desulfobulbus elongatus	1.39			179
Desulfobulbus propionicus[a]	0.89-2.64			136,204,246
Desulfococcus multivorans	0.17-0.23			206
Syntrophobacter strain MPOB	0.02	0.15-0.17		66,104,201
Syntrophobacter pfennigii	0.07	0.07		41,232
Syntrophobacter wolinii	0.06	0.02-0.10	0.18-0.21	18,41,231
culture PT		0.1		250
culture PW	0.23	0.14		250

[a]Several strains.

Recent findings of Harmsen et al. [67] seem to support the direct propionate oxidation by sulfate reducers. They followed the population dynamics of propionate-oxidizing bacteria in two UASB reactors, inoculated with methanogenic sludge, and fed with either propionate and sulfate, or propionate only. In the first reactor the number of *Desulfobulbus* sp. increased rapidly, and in the second reactor the number of syntrophic propionate oxidizers. It seems unlikely that *Desulfobulbus* acted as an hydrogen scavenger in the first reactor, although *Desulfobulbus* sp. are able to use H_2 as well as propionate [241], because no syntrophic propionate oxidizers were enriched in this reactor, and all *Desulfobulbus* cells were localized on the ouside of the granule, not intertwined with other bacteria.

2.3 Competition between sulfate reducers and acetogens in the absence of sulfate

The role of sulfate-reducing bacteria in the anaerobic digestion in the absence of sulfate has hardly been investigated. Yet, recent studies showed that sulfate reducing bacteria can be present in methanogenic sludge to upto 15% of the total biomass [164]. It is known that several types of sulfate-reducing bacteria have fermentative or syntrophic capacities. Widdel and Hansen [242] gave an overview of the fermentative and syntrophic growth of sulfate-reducing bacteria. Growth of sulfate reducers in the absence of sulfate could explain the fast response of methanogenic ecosystems to the addition of sulfate. Some substrates which can be fermented by sulfate reducers are pyruvate [44,82,157,203,246], lactate [205,246], ethanol [105,204], fumarate and malate [129,203,210], fructose [150], serine [203], choline [48], acetoin and S-1,2-propanediol [210] and propanol + acetate [211]. Sulfate reducers can also grow as acetogens in the absence of sulfate. *Desulfovibrio* sp. oxidize ethanol or lactate to acetate when co-cultured with methanogens [28,125,212,218,251]. It has been reported that *Desulfovibrio* sp. were the main lactate and ethanol-degrading bacteria in a reactor treating whey in the absence of sulfate [31,267]. However others reported that only in the presence of sulfate *Desulfovibrio* sp. were the dominant lactate degraders. In the absence of sulfate lactate was fermented according to the usual fermentation pattern of *Propionibacterium* [159]. Syntrophic formate degradation has been reported for *Desulfovibrio vulgaris* in association with *Methanobacterium bryantii* [63], and a *Desulfovibrio* like organism could syntrophically degrade alcohols like 1,3-butanediol, 1,4-butanediol, 1-butanol and 1-propanol in the presence of 10 mM acetate and *Methanospirillum hungatei* [210].

The role of sulfate-reducing bacteria in propionate degradation becomes more intricate by the recent work of Wu et al. [249,250]. They were the first to report that the syntrophic conversion of propionate was mainly performed by sulfate-reducing bacteria, and they were able to isolate such an organism [250]. This indicates that in the absence of sulfate certain propionate-degrading sulfate-reducing bacteria are able to oxidize propionate in syntrophic association with H_2-consuming anaerobes, while in the presence of sulfate they couple propionate oxidation to sulfate reduction. This represents a considerable ecological advantage of this type of sulfate-reducing bacteria over obligate syntrophic propionate degraders in ecosystems where sulfate is continuously or intermittently available.

Recently several other bacteria have been isolated which are also able to degrade propionate either via sulfate reduction or syntrophically, namely *Syntrophobacter pfennigii* KoProp [201,232], strain HP1.1 [262], and strain Syn7 [66]. Furthermore, two other strains, which already had been isolated and had been previously characterized as syntrophic propionate degraders, namely *Syntrophobacter* strain MPOB [66,104,201], and *Syntrophobacter wolinii* [18,41,231], were also able to grow in pure cultures with propionate and sulfate. For *S. wolinii* this finding is very remarkable because *S.*

wolinii grows as an acetogen in the presence of *Desulfovibrio* G11 [18,132]. Phylogenetic research, based on 16S rRNA sequences, showed that some of these "facultative syntrophic" propionate degraders (*Syntrophobacter* strain MPOB, *S. pfennigii*, *S. wolinii*, stain HP1.1, and strain Syn7) are belonging to the group of gram-negative sulfate reducers [66,68,70,232,262].
Thus far, growth of sulfate-reducers on butyrate in the absence of sulfate but in the presence of methanogens was not yet demonstrated. However, *Desulfovibrio* sp. were detected in a fixed bed reactor fed with butyrate without sulfate [264,265].

2.4 The microbial sludge composition

From the information presented above general conclusions about the effect of sulfate in bioreactors can be drawn. At an excess of sulfate the better growth kinetic properties of the sulfate reducers compared with methanogens and methanogenic consortia should enable the sulfate reducers to become predominant. However, specific environmental conditions in the bioreactors are of significant importance in this respect. A complete suppression of sulfate reduction at high COD/sulfate ratios is difficult to achieve because hydrogen oxidation by methanogens is easily taken over by sulfate reducers. However, a complete reduction of sulfate at low COD/sulfate ratios is difficult to achieve as well. This because acetate-degrading methanogens are not easily outcompeted by sulfate reducers. Futhermore the occurrence of sulfate reduction at limiting amounts of sulfate is intriguing because competition among the different types of sulfate reducers occurs as well, and several types of sulfate reducers have the ability to grow fermentatively or acetogenically.
Overall it is clear that, in order to get a more detailed insight in the role of sulfate reducers, acetogens and methanogens in anearobic bioreactor, it is essential to combine reactor competition studies with the characterization of the microbial sludge composition. Some tools which are nowadays available for scientists to study the microbial sludge composition will be presented in the following paragraph.

3. CHARACTERIZATION OF MICROBIAL BIOMASS

3.1 Conventional methods

Several methods exist to characterize (and quantify) the microbial sludge composition. Conventional sludge characterization methods are often based on selective growth media. The Most probable Number (MPN) technique for example is a technique in which serial sludge dilutions are

inoculated in selective liquid media. By assuming that single cells will grow in the highest dilutions, the number of a certain group of microorganisms in the sludge can be estimated [15]. This method can give very useful information on bacteria that are present in the sludge in high numbers, and are able to grow on artificial media. However, it should be kept in mind that many bacteria cannot be cultivated in artificial media. Futhermore this method will underestimate the number of microorganisms if the microorganisms are attached to solid substrates or are associated to each other, like threaded bacteria such as the methanogenic *Methanosaeta* sp. [236]. Another group of microorganisms which will probably be underestimated with this technique are bacteria which grow in syntrophic consortia, like propionate or butyrate-degrading acetogens, because their syntrophic partner might for example not be present in the same high numbers. Some researches have circumvented this problem with syntrophic propionate and butyrate degraders by adding a hydrogen-scavenging methanogen to the MPN-dilution medium [225,250].

Substrate conversion rates are also often used for microbial sludge characterization. In wastewater treatment plants these rates are usually related to overall sludge parameters such as amount of volatile suspended solids or dry weight. These calculated rates give valuable information on the maximum possible metabolic activity of the different microbial groups. Unfortunately, they cannot be used for the identification or quantification of the microorganisms in the sludge [45,250], and it is very questionable if the rates which have been otained with sludge from a certain reactor can be applied in other systems, with different reactor operating conditions (e.g. pH, temperature) [14].

Direct microscopical analyses have always played an important role in the characterization of sludge and biofilms. With some techniques a direct visualization of the sludge is possible, others require fixation and staining of the sludge prior to examination. The major drawback of most microscope techniques is the fact that the identification of microbes is usually based upon cell morphology only, which for most bacteria is not very distinctive. An exception are methanogens, since they can be identified with epifluorescence microscopy by detecting the factor F420-dependent autofluorescence [37]. However some methanogens, like *Methanosaeta* do not exhibit autofluorescence [39].

Recently, Surman and coworkers [208] have compared the applicability of several light, fluorescence, and electron microscopy techniques such as scanning and transmission electron microscopy (SEM and TEM), atomic force microscopy (AFM), and confocal laser scanning microscopy (CLSM) for the examination of microbial biofilms. Their conclusion was that no one technique was unequivocally better than another. All techniques had their special advantages and disadvantages, and the most accurate picture of the true biofilm structure and composition can be obtained by combining as many techniques as available. That such a "combined microscopic approach" can provide a lot of information has been shown by Zellner and coworkers [263], who used SEM, phase contrast microscopy and epifluorescence microscopy of methanogens to study

biofilm formation in anaerobic fixed bed reactors.

Despite their limitations, conventional microbial identification techniques based on isolation, cultivation and morphology, have been, and still are, useful for a rough characterization of the microbial sludge population. However, for detailed characterization studies direct identification methods for microorganisms are essential. Fortunately, in the past decades, such methods have become available. These new methods allow a direct identification of microorganisms in the sludge by using for example specific antibodies, biomarkers, or nucleic acid probes.

3.2 Immunodetection

Immunodetection techniques are based on the fact that bacterial surface cell wall polymers such as proteins and lipopolysaccharides have strong antigenic properties which can be used to raise antibodies. These antibodies, which are often highly specific, can be produces by injecting rabbits or other experimental animals with bacterial cells (polyclonal antibodies). If these antibodies are labelled with a fluorescent dye or gold, in combination with respectively fluorescence or electron microscopy, they can be used for the specific detection of bacteria. For detailed information about the technical apects see Harlow and Lane [65]. Many immunodetection studies have been focussing on methanogenic Archaea in anaerobic bioreactors [58,99,115-117,177]. Some other microorganisms which have also been succesfully identified in anaerobic sludge are *Pseudomonas stutzeri* [266], and *Clostridium aldrichii* [24]. Immunodetection techniques can also be applied for the identification of sulfate reducers [189,191]. Fluorescein-labelled antibodies were for example used to detect sulfate reducers in marine sediments [112]. However, to our knowledge, these immunoprobes for sulfate reducers have not been applied in extensive sludge characterization studies.

Quantification of microorganisms in multilayered biofilms has long been impossible, because it was difficult to ensure that the antibodies reacted with all antigens in the biofilm. Recently, Bauer-Kreisel and coworkers [14] were able to circumvent this difficulty by combining mechanical and chemical treatments with an enzyme-linked immunosorbent assay (ELISA) for the quantification of *Dehalospirillum multivorans* in a anaerobic reactor biofilm.

Immunodetection is certainly a very powerful tool for the identification of microorganisms in sludge, it is rather easy to use, inexpensive, and it can be very specific. Furthermore, fluorescently labelled antibodies can be used elegantly in combination with flow cytometry to sort viable cells from mixed microbial communities [34]. Nevertheless it should be taken into account that antibodies can cross-react with other non-related strains [116,191].

3.3 Membrane lipid analysis

Another approach for studying the microbial sludge composition is the identification of microorganisms by analysing bacterial components which are specific for individual species. Membrane lipids and their associated fatty acids have been used extensively in this respect [60,153]. Bacteria (eubacteria) can be characterized by the patterns of their methylated phospholipid ester-linked fatty acids, known as (PL)FAME-patterns or PLFA profiles. Methanogens are characterized via their phospholipid-derived ether lipids (PLEL). Although FAME-patterns are often very useful for the characterization of unknown bacterial isolates [21], they are not always suitable for the characterization and quantification of microbial sludge biomass. Especially microorganisms which lack signature lipid biomarkers will be difficult to distinguish in the sludge profile [170]. Furthermore, the detection method is not very sensitive, and therefore microbes which are present in lower numbers are beyond detection. Moreover, environmental conditions (e.g. growth substrate and temperature) can cause major changes in the microbial FAME-pattern [21,34], which makes an accurate quantification of the microbial sludge populations very difficult. Nevertheless, lipid analysis have been succesfully applied for the identification [143] and quantification [146] of methanogens in natural environments and wastewater digester sludges. Lipid compositions of sulfate reducers have also been studied extensively [43,47,98,170,213,222], and PLFA profiles have been used to identify (and quantify) sulfate reducers in (marine) sediments [170,214], and biofilms [42].

3.4 Ribosomal RNA based detection techniques

3.4.1 Ribosomal rRNA as biomarker

Without doubt ribosomal RNA (rRNA) based detection and identification methods have become extremely important in the unravelling of the microbial composition of sludge from wastewater treatment systems. Ribosomes are present in all living organisms, and are essential in protein systhesis. Prokaryotic ribosomes consist of three rRNA molecules and approximately 50 ribosomal proteins. The 5S rRNA (120 nucleotides) forms together with the 23S rRNA (2900 nucleotides) and ca. 30 proteins the 50S large-subunit of the ribosome. The 16S rRNA (1540 nucleotides) and approximately 20 proteins form the 30S small-subunit of the ribosome. The rRNAs are encoded by an operon, which was first characterized in *Escherichia coli* by Brosius et al. [25]. The number of operons per chromosome varies between microorganisms from 1 copy (e.g. mycoplasmas) upto 10 copies (bacilli) [8,90]. The number of ribosomes in the cell is proportional to the growth rate [52,95-97,158]. Starving or slowly growing cells contain only low numbers of ribosomes

[49,50,54]. Sequence analysis has revealed that ribosomal RNA and the corresponding genes are very suitable as (evolutionaty) biomarkers [152], because rRNA molecules are extremely conservative in overall structure. Their nucleotide sequence contains highly conserved as well as highly variable regions (Fig. 3). Furthermore, RNA molecules have a relative high copy number per cell (10^3-10^5), and lateral gene transfer of rRNA genes has never been reported. 16S and 23S rRNA molecules are especially suitable as biomarkers because they contain sufficient sequence information to perform statistically significant comparisons.

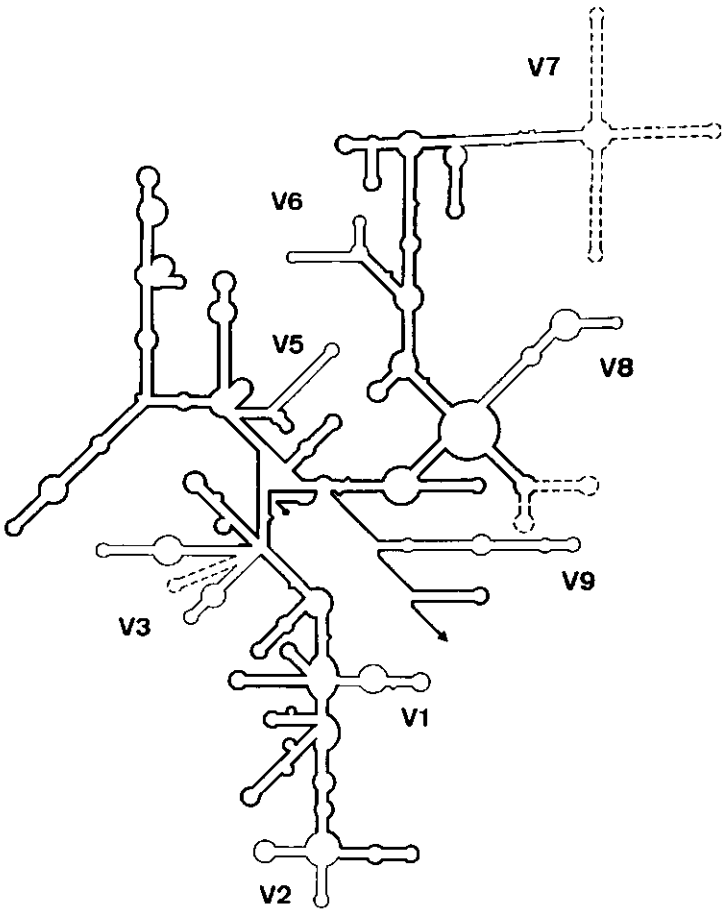

Figure 3. Secundary structure model of 16S rRNA. Adapted from Neefs et al. [137]. The variable areas are distinguised by V1 to V9. Conserved areas are indicated with bold lines, areas of variable sequence and/or length are indicated with thin lines. Helices drawn in broken lines are only present in few known sequences.

Currently, more than 5000 complete or partially complete 16S rRNA sequences are available [79] in the major databases (i.e. GenBank [17], EMBL [169], RDP [121]).
Ribosomal RNA sequence analysis has had a major impact on our current view of the microbial evolution, which nowadays relies for a large part on the comparative and statistical analysis of rRNA sequences [248]. Phylogenetic trees based on alignments of 16S rRNA's reveal the enormous biodiversity within the prokaryotes, and additionally they suggest that all living organisms can be devided into three domains, the Archaea, the Bacteria and the Eukarya (Fig. 4).

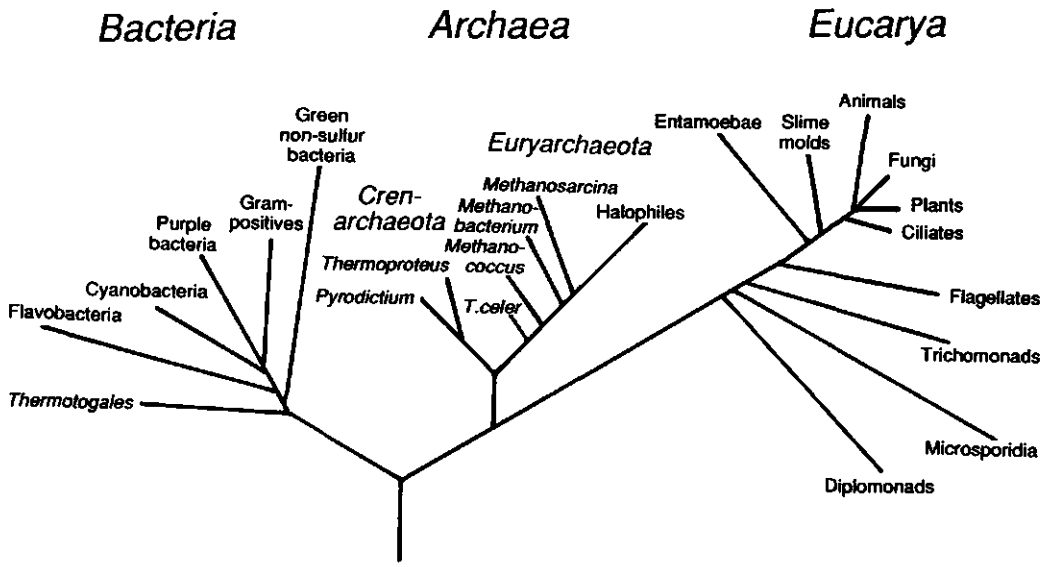

Figure 4. Rooted phylogenetic tree representing the three domains Bacteria, Archaea and Eukarya (taken from Woese [248]).

3.4.2 rRNA-targeted oligonucleotide probes

During recent years several identification methods based on rRNA sequences have been developed. Some of these methods which can be used for the analysis of the microbial sludge composition are depicted in Figure 5. One of these methods is hybridization with rRNA-targeted oligonucleotide probes [4,198]. Oligonucleotides are short single stranded oligomers of 15 to 40 nucleotides. They can be made chemically by a DNA synthesizer in any desired sequence. The oligonucleotide probes sequence has to be complementary to the target sequence (e.g. a 16S rRNA molecule) in order to hybridize with it.

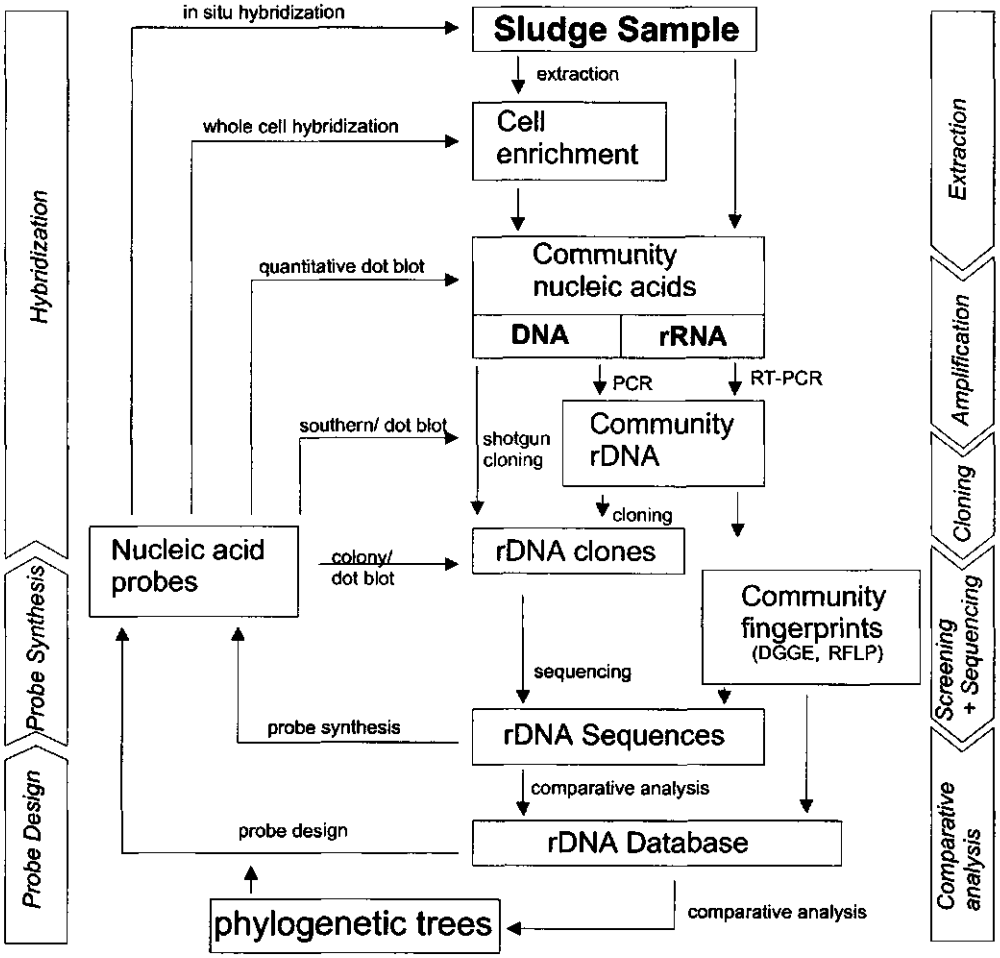

Figure 5. Strategies based on rRNA sequences for the characterization of sludge microbial communities, adapted from Amann et al. [5], and Hugenholz and Pace [79]. RT, reverse transcriptase; DGGE, denaturing gradient gel electrophoresis; RFLP, restriction fragment length polymorphism.

The 16S rRNA oligonucleotide hybridization method is most commonly used for sludge characterization. For this, oligonucleotide probes, which have been previously labelled (e.g. radioactive ^{32}P, fluorescent dye) are used for the specific detection of microorganisms. The best probe hybridization will be obtained when all nucleotides bind to the target. One or more

mismatches weaken the binding. Under optimal probe incubation conditions only probes which perfectly match and hybridize with the target can be made visible. The probes can be applied after extraction of the 16S rRNA from the sludge (dot blot hybridization), or can be used *in situ* in combination with fluorescent microscopy, or scanning confocal laser microscopy.

The major advantage of oligonucleotide hybridization methods over other hybridization methods (e.g. immunolabelling) is the fact that the probe specificity can be controlled. Species-specific probes can be designed by targeting the most variable regions of the 16S rRNA, while more general probes (group/genus) are complementary to the more conserved regions of the 16S rRNA [199]. Various groups of microorganisms have been detected (and quantified) with 16S rRNA hybridization probes in biofilms and sludge, such as sulfate reducers [7,67,69,93,162,164,166, methanogens [67,69,162-164,166] syntrophic propionate oxidizers [67,69], ammonia-oxidizing bacteria [230], *Acinetobacter* [229], and *Paracoccus* [139]. The most timeconsuming part of the 16S rRNA hybridization method is the development of probes and the evaluation of their specificity. This explains why detailed sludge characterization studies are still hampered by the lack of suitable probes. Fortunately, the number of probes suitable for sludge studies still increases. In Table 5 a collection of 16S and 23S rRNA probes are presented which can be used in research with anaerobic sludge.

3.4.3 Polymerase chain reaction (PCR) amplification of rRNA-genes

With the polymerase chain reaction (PCR) amplification method a few target 16S rDNA genes can be amplified to make them detectable and quantifiable [53]. The selection of the PCR primers determines which 16S rRNA genes and which part of the 16S rRNA gene will be amplified. By combining non-specific PCR primers with cloning and sequence analysis techniques it is possible to get information about the microbial sludge composition. For example Ng and coworkers [141] applied the PCR technique in combination with 16S rRNA sequencing to identify a whole range of anaerobic digester bacteria. If selective primers are used it is possible amplify 16S rRNA-genes from specific groups of microorganisms present in the sludge. Hiraishi and coworkers [76] used for example PCR amplification with methanogen-specific primers in combination with cloning, and restriction fragment length polymorphism analysis to identify the methanogenic population in anaerobic sludge, while Voytek and Ward [228] used PCR primers selective for ammonium-oxidizing bacteria.

Table 5. Selection of oligonucleotide probes useful for the microbial characterization of anaerobic sludge.

Probe	Target group	Reference
Univ1392	Virtually all known organisms	152
BACTERIA		
EUB338	*Bacteria*	6
ALF1b	Proteobacteria α-subclass[1]	122
ALF73a	Proteobacteria α-subclass[1]	122
BET42a	Proteobacteria ß-subclass	122
GAM42a	Proteobacteria τ-subclass	122
SRB385	Gram-neg. mesophilic sulfate reducers[2]	6
SRB385Db	*Desulfobacteriaceae*[2]	160
D687	*Desulfovibrio*[3]	36
D660	*Desulfobulbus*	36
D804	*Desulfobacterium, Desulfobacter, Desulfosarcina, Desulfococcus, Desulfobotulus*	36
MPOB1	*Syntrophobacter* strain MPOB, *Syntrophobacter pfennigii*	68
MPOB2	*Syntrophobacter* strain MPOB	68
KOP1	*Syntrophobacter pfennigii*	68
S223	*Syntrophobacter wolinii*	67
ARCHAEA		
ARC915	*Archaea*	199
EURY498	*Euryarchaeota*	30
MG1200	*Methanomicrobiaceae* and relatives	165
MS821	*Methanosarcina*	165
MX825	*Methanosaeta*	165
MC1109	*Methanococcales*	165
MB1174	*Methanobacteriaceae*	165

[1]Probe ALF73a only reacts with part of the alpha-subclass and probe ALFb is not specific only for the alpha-subclass [122].
[2]Several non-target organism share the target sequence of this probe [7,160], while some target organisms have one or more mismatches with the probe.
[3]This probe also hybridizes to a few organisms in the *Pelobacter/Geobacter/Desulfuromonas* lineage [164].

To circumvent the cloning technique it is also possible to separate the PCR products with denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electroporesis (TGGE). With these electrophoresis techniques DNA fragments of the same length, but with different nucleotide sequences can be separated. The separation of the amplified 16S rRNA genes is based on the difference in melting temperatures of the amplified genes and the slower mobility of partially melted amplified fragments in comparison to the intact fragments. By using these electrophoresis

techniques in combination with selective PCR amplification before electrophoresis, or specific DNA probes after electrophoresis, complex microbial populations can be studied [133]. The combination of DGGE and specific DNA probes has for example been used to reveal the presence of sulfate reducers in anaerobic sludge [133]. The PCR method is a powerful tool for identifying microorganisms in sludge. However, if PCR is used for the quantification of microorganisms in the sludge or for the elucidation of the microbial sludge composition, it should be taken into account that the results can be biased, because the PCR method can preferentially amplify certain 16S rDNA molecules. Therefore, 16S rDNA of microbes abundantly present in the sludge could remain unamplified [5].

3. OUTLINE OF THE THESIS

The aim of the research presented in this thesis was to investigate the effect of sulfate on the presence and activity of sulfate reducers and their acetogenic and methanogenic counterparts in sulfate-fed anaerobic bioreactors.

A key intermediate in the anaerobic degradation of organic material is acetate. In the presence of sulfate acetate degradation can proceed either via sulfidogenesis or via methanogenesis. However, based on current literature, it is difficult to predict the occurrence of methanogenesis or sulfidogenesis from acetate. Therefore, the first part of the thesis has been focused on the characterization of acetate-degrading sulfate reducers which are dominantly present in sulfidogenic granular sludge. The isolation and characterization of two thus far unknown acetate-degrading sulfate reducers, named *Desulforhabdus amnigenus* and *Desulfobacca acetoxidans*, is described (Chapter 2 and 3, respectively). The growth kinetic properties of these acetate-degrading sulfate reducers are compared with those of acetate-degrading methanogens (Chapter 4). While Chapter 5 describes the isomerisation of butyrate to isobutyrate by *Desulforhabdus amnigenus*, a property which was thus far not described for sulfate reducers.

The second part of the thesis concerns the microbial sludge composition in sulfate-fed bioreactors. Chapter 6 deals with the applicability of PCR amplification and dot blot hybridization for the detection and quantification of *D. amnigenus* in granular sludge. In order to get more insight in the effect of the COD/sulfate ratio, and composition of the reactor influent on the microbial sludge composition, the presence and role of sulfate reducers, acetogens and methanogens in several methanogenic and sulfidogenic sludges is investigated (Chapter 7 and 8). The techniques which are used for these sludge characterization studies are 16S rRNA dot blot hybridizations (Chapter 7 and 8), Most Probable Number (MPN) dilutions (Chapter 7), and phospholipid fatty acid (PLFA) analyses (Chapter 8).

The results presented in this thesis are summarized in Chapter 9, and the relevance and importance of this work for full-scale sulfidogenic reactors is discussed.

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CHAPTER 2

Desulforhabdus amnigenus gen. nov. sp. nov., a sulfate reducer isolated from anaerobic granular sludge.

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Desulforhabdus amnigenus gen. nov. sp. nov., a sulfate reducer isolated from anaerobic granular sludge

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Abstract From granular sludge of an upflow anaerobic sludge bed (UASB) reactor treating paper-mill wastewater, a sulfate-reducing bacterium (strain ASRB1) was isolated with acetate as sole carbon and energy source. The bacterium was rod-shaped, (1.4–1.9 × 2.5–3.4 μm), nonmotile, and gram-negative. Optimum growth with acetate occurred around 37°C in freshwater medium (doubling time: 3.5–5.0 days). The bacterium grew on a range of organic acids, such as acetate, propionate, and butyrate, and on alcohols, and grew autotrophically with H_2, CO_2, and sulfate. Fastest growth occurred with formate, propionate, and ethanol (doubling time: approx. 1.5 days). Strain ASRB1 clusters with the delta subdivision of Proteobacteria and is closely related to *Syntrophobacter wolinii*, a syntrophic propionate oxidizer. Strain ASRB1 was characterized as a new genus and species: *Desulforhabdus amnigenus*.

Key words *Desulforhabdus amnigenus* · 16S rRNA · Sulfate-reducing bacteria · Upflow anaerobic sludge bed (UASB) reactor · Acetate · Wastewater

Introduction

Acetate is an important intermediate in the anaerobic breakdown of organic matter in methanogenic bioreactors (Jeris and McCarty 1965; Smith and Mah 1966; Gujer and Zehnder 1983). The two genera of methanogenic Archaea known to use acetate as sole energy source are *Methanosarcina* and *Methanosaeta* ("*Methanothrix*") (Whitman et al. 1992). *Methanosaeta* is the dominant acetoclastic methanogen in anaerobic habitats with low acetate concentrations (e.g., anaerobic bioreactors), because of its high affinity and low-threshold value for acetate (Jetten et al. 1992). In anaerobic reactors treating sulfate-rich wastewaters, such as paper-mill wastewaters or food oil industry wastewaters, sulfate-reducing bacteria compete with methanogens for acetate. The outcome of this competition is not yet clear. In many studies with freshwater or low-salt systems, acetate conversion via methanogenesis is predominant, even with excess sulfate (Isa et al. 1986; McCartney and Oleszkiewicz 1991; Visser et al. 1993a). However, after long-term operation of the reactors, acetate degradation mainly by sulfate reducers has also been reported (Alphenaar et al. 1993; Visser et al. 1993b; Harada et al. 1994). Factors that affect the outcome of the competition between methanogens and sulfate reducers have been reviewed by us (Oude Elferink et al. 1994).

Most of the gram-negative acetate-degrading sulfate reducers known prefer marine growth conditions; therefore, it is unlikely that these bacteria play an important role in freshwater anaerobic bioreactors. The gram-negative sulfate reducers that prefer freshwater conditions, such as *Desulfoarculus baarsii* (Widdel 1980), *Desulfobacterium catecholicum* (Szewzyk and Pfennig 1987), and *Desulfococcus biacutus* (Platen et al. 1990) show very poor growth with acetate. Only the gram-negative *Desulfobacter* strain AcKo (Widdel 1987) and some gram-positive *Desulfotomaculum* species show good growth with acetate. However, most of the acetate-degrading *Desulfotomaculum* species prefer enhanced temperatures (Widdel 1992; Love et al. 1993). In this paper, we describe the isolation and characterization of a sulfate reducer from granular sludge of an upflow anaerobic sludge bed (UASB) reactor treating paper-mill wastewater. In this reactor, sulfate reduction partially supersedes methanogenesis. The sulfate-reducing bacterium, obtained by direct serial dilution of crushed granular sludge in media with acetate and sulfate, was present in higher numbers than acetoclastic methanogens.

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Materials and methods

Source of organisms

The sulfate-reducing bacterium, strain ASRB1, was isolated from the granular sludge of a pilot-scale UASB reactor treating paper-mill wastewater, in which both sulfate reduction and methanogenesis occurred (Paques Environmental Technology BV, Balk, The Netherlands). The wastewater had a chemical oxygen demand (COD)/sulfate ratio of 1.1 (g/g) and was treated at approximately 40° C. The COD (75%) was converted via sulfate reduction; the other 25% was converted via methanogenesis. The total COD removal efficiency was around 60% (C. J. N. Buisman, personal communication).

Methanospirillum hungatei JF-1 (DSM 864) was obtained from the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany).

Media and cultivation

A bicarbonate-buffered medium was prepared as described by Stams et al. (1993). Unless stated otherwise, bacteria were cultured at 37° C in 120-ml serum vials closed with butyl rubber stoppers and aluminum crimp seals. The vials contained 50 ml medium and a gas phase of 172.2 kPa N_2-CO_2 (80:20, v/v).. The inoculum size was 1%. Electron acceptors and donors were added separately by syringe from 0.5 or 2.0 M sterile, anoxic stock solutions. Except for some heat-labile substrates that were filter-sterilized, all substrates were sterilized by heat (20 min, 120° C). For growth with H_2 as electron donor, the N_2-CO_2 head space was replaced by H_2-CO_2 (80:20, v/v). To test syntrophic growth, exponential-phase cells grown on propionate and sulfate (5% inoculum size) were cultured together with exponential-phase H_2-grown *M. hungatei* cells (10% inoculum size).

Isolation

Reactor sludge (10 ml) was diluted with 90 ml basal medium. The sludge was disintegrated by pressing it repeatedly through a syringe needle (microlance 25G5/8 0.5 × 16, BBL-Becton Dickinson, Cockeysville, Md., USA). This crushed granular sludge was used to make serial dilutions in liquid media containing acetate and sulfate (20 mM each). For each dilution, 5 ml inoculum was added to 45 ml medium. The highest dilutions that showed growth were used for further isolation. Pure cultures were obtained by repeated application of the agar roll-tube-dilution method as described by Hungate (1969). To check purity, isolates were inoculated into media with 0.1% yeast extract (BBL-Becton Dickinson), fumarate, pyruvate, or glucose as substrates. Additionally, Wilkins-Chalgren anaerobe broth (Oxoid, Basingstroke, UK) was used as a test medium. After incubation, the cultures were examined microscopically.

Determination of growth parameters

Growth rates were tested in 1-l screw-cap bottles with butyl rubber stoppers containing 500 ml medium. In most cases, growth was followed by measuring substrate utilization and sulfide production. Salt tolerance was tested by addition of different amounts of a concentrated salt solution as described by Widdel and Bak (1992). For pH tests, the pH in the media was adjusted by varying the concentrations of KH_2PO_4-Na_2HPO_4 or $NaHCO_3$-Na_2CO_3 so that the total phosphate or inorganic carbon concentration amounted to approximately 50 mM. All tests were carried out at least in duplicate.

Analysis of cell compounds

Desulfoviridin was determined as described by Postgate (1959). Cytochromes were identified in cell extracts by recording reduced-minus-oxidized difference spectra with a Beckman DU7500 spectrophotometer. The G+C content of the DNA was determined at the DSM (Braunschweig, Germany) by HPLC (Tamaoka and Komagata 1984; Mesbah et al. 1989). DNA was isolated according to Cashion et al. (1977). Poly-hydroxybutyrate granules were stained with the fluorescent stain Nile blue A (Ostle and Holt 1982). Gram-staining was done according to standard procedures (Doetsch 1981).

Chemical analysis

Most substrates were measured by HPLC as described by Stams et al. (1993). Benzoate and indole were measured by HPLC with two Chromspher C8 columns (Chrompack, Bergen op Zoom, The Netherlands) connected in series. The mobile phase was 70% 0.01 N H_2SO_4/30% CH_3CN at a flow rate of 0.6 ml/min. The columns were used at room temperature. Samples were injected by using a Spectra System AS1000 autosampler, and the eluted compounds were quantified with an LKB 2158 Uvicord SD at 206 nm. Hydrogen, methane, and methanol were determined quantitatively by gas chromatography (Stams et al. 1993). Amino acids were analyzed as described by Kengen and Stams (1994). Sulfide was determined as described by Trüper and Schlegel (1964). The method of Bradford (1976) was used for protein determinations in cell-free extracts.

Enzyme measurements

All enzyme activities were measured anoxically with anoxically prepared cell extracts of cells grown with propionate and sulfate and harvested in the late exponential phase. Cell extracts were prepared as described by Jetten et al. (1990). Carbon monoxide dehydrogenase, formate dehydrogenase, and 2-oxoglutarate dehydrogenase activities were assayed according to Schauder et al. (1986).

Sequence analysis and phylogenetic tree

The 16S rRNA gene of strain ASRB1 was amplified selectively as described previously (Harmsen et al. 1993), using a set of primers corresponding to positions 8–27 (5′-CACGGATCCAGAGTTTG-AT(C/T)(A/C)TGGCTCAG-3′) and 1492–1513 (5′-GTGCTGC-AGTACGG(T/C)TACCTTGTTACGACTT-3′) of *Escherichia coli* 16S rRNA. Amplification products were cloned in M13mp18. DNA sequencing was carried out by the dideoxy chain-termination method (Sanger et al. 1977).

The phylogenetic tree was constructed by Dr. W. Ludwig (Technische Universität, Munich). The 16S rRNA gene sequence was compared with about 1900 homologous sequences of bacteria taken from the ribosomal database project RDP (De Rijk et al. 1992; Olsen et al. 1992). The position of strain ASRB1 within the phylogenetic tree was determined using the maximum parsimony approach implemented in the program package ARB (W. Ludwig et al., unpublished). The partial tree was based on the result of a maximum likelihood analysis (fastDNAml; Larsen et al. 1993) of all of the delta subclass, including reference sequences from all other bacterial phyla. The topology of this tree was evaluated applying distance matrix, maximum-parsimony, and maximum-likelihood methods by using the programs NEIGHBOR and DNAPARS implemented in PHYLIP (Felsenstein 1982), ARB, and fastDNAml. The data sets were changed by successively deleting highly variable alignment positions (SEDIS, part of ARB).

Results

Isolation and morphological characterization

Strain ASRB1 was obtained from 1×10^8-fold diluted, crushed granular sludge inoculated in medium with acetate and sulfate, and was isolated by repeated application of the agar roll-tube-dilution method. In agar, the strain grew as brownish, lens-shaped colonies. No formation of methane was detected in the highest sludge dilutions (1×10^7 and 1×10^8) that showed growth.

Cells of the isolate were non-motile, rod-shaped to ellipsoidal (1.4–1.9 µm wide and 2.5–3.4 µm long), and appeared singly or in pairs. After growth with ethanol, long chains of up to 15 cells were observed (Fig. 1). Cells stained gram-negative. Spores were never observed.

Growth and substrate utilization

The optimum growth temperature for strain ASRB1 on acetate was around 37°C. Little growth was observed below 25°C or above 45°C. The optimum pH for growth was 7.2–7.6; growth was possible between pH 6.6 and 8.5. Growth in brackish medium was slow (the doubling time increased four- to sixfold), and almost no growth was observed in marine medium. When vitamins were omitted from the media, cultures could be transferred (1% inoculum size) at least five times without any growth retardation.

In the presence of acetate, strain ASRB1 used sulfate (20 mM), thiosulfate (20 mM), sulfite (10 mM), or dithionite (1 mM) as electron acceptor. High concentrations of sulfite (20 mM) or dithionite (5 mM) inhibited growth. Sulfur, nitrate, or fumarate (5 mM each) could not be used as electron acceptor with acetate or propionate as electron donor.

The isolate grew chemolithoautotrophically with H_2 and sulfate as energy substrate and CO_2 as sole carbon source and grew chemoorganotrophically with a large number of organic compounds (Table 1). All substrates were oxidized completely to CO_2. Complete oxidation of acetate (16 mM) led to concomitant formation of 16 mM sulfide. With sulfate, an average growth yield of 1.6 g cell protein was obtained per mol of acetate oxidized. With some substrates, the intermediate formation of acetate and propionate was observed (Table 1). During growth on butyrate (20 mM), an intermediate formation of isobutyrate (2 mM) and acetate (18 mM) was observed. Fastest growth occurred with formate, propionate, and ethanol with a minimum doubling time of about 1.5 days. With acetate, doubling times of 3.5–5 days were measured. No significant substrate utilization and no methane production occurred in co-cultures of strain ASRB1 with *M. hungatei* and propionate or ethanol as substrate.

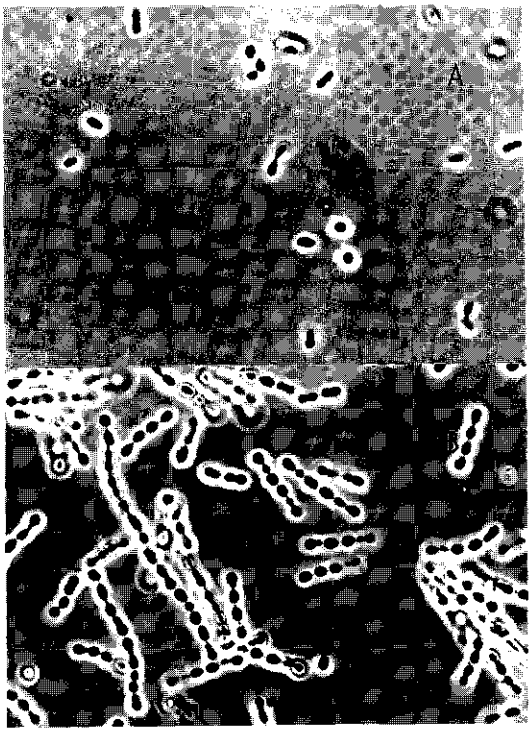

Fig. 1 Phase contrast photomicrograph of strain ASRB1, grown on **A** acetate or **B** ethanol. *Bar* 15 µm

Table 1 Organic compounds tested as electron donors and carbon sources in the presence of 20 mM sulfate. The substrate concentrations are given in mM in *parentheses*

Utilized:
H_2-CO_2 (80:20, v/v), formate (20), acetate (20), propionate[a] (20), butyrate[b] (20), isobutyrate (5), ethanol[a] (20), propanol[c] (20), butanol[a] (5), pyruvate (5), lactate[c] (10)

Tested, but not utilized:
Glucose (20), fructose (20), xylose (20), valerate (5), 2-methylbutyrate (10), 3-methylbutyrate (10), succinate (5), fumarate (5), malate (5), oxaloacetate (10), methanol (5), 2,3-butanediol (5), glycerol (10), acetone (5), mannitol (10), indole (0.5), catechol (2), benzoate (0.5), phenol (0.4), aspartate (5), glutamate (5), glycine (5), alanine (5), cysteine (5), leucine (5)

Tested, but not utilized in the absence of sulfate:
Ethanol (10), pyruvate (10)

Tested, but not utilized in co-culture with Methanospirillum hungatei:
Propionate (10), ethanol (10)

[a] Intermediate formation of acetate
[b] Intermediate formation of acetate and isobutyrate
[c] Intermediate formation of acetate and propionate

Pigments and other cell compounds

Dithionite-reduced versus air-oxidized spectra of cell extracts of ASRB1 exhibited absorption bands with maxima at 420, 523, and 552 nm, indicating the presence of *c*-type cytochromes. Desulfoviridin was not detected. The G+C content of the DNA was 52.5 (± 0.3) mol%. Large polyhydroxybutyrate granules were observed in late-exponential-phase cells grown on acetate, propionate, butyrate, or ethanol.

Key enzymes of acetyl-CoA oxidation

The specific activities of carbon monoxide dehydrogenase and formate dehydrogenase were 0.42 and 0.44 μmol min^{-1} (mg protein)$^{-1}$, respectively. 2-Oxoglutarate dehydrogenase activity was not detected.

Phylogenetic analysis

The phylogenetic relationships of strain ASRB1 derived from 16S rRNA sequence analysis are depicted in Fig. 2. The major groups shown in the tree were supported by the vast majority of the analyses. However, a relative branching order for the group with ASRB1 versus the *Pelobacter* group and *Desulfobulbus* could not be unambiguously determined. This is indicated by the (near) multifurcation of these lines in the tree. The deeper branching of *Myxobacterium* and *Desulfovibrio* is supported by most of the analyses, but not all. *Syntrophobacter wolinii* is one of the closest relatives of ASRB1 (level of similarity, 93.1%).

The 16S rRNA gene sequence of strain ASRB1 is deposited under EMBL accession number X83274.

Discussion

Physiology and ecology of the isolated sulfate reducer

Strain ASRB1 grew on a variety of organic compounds that are formed during anaerobic digestion, such as acetate, volatile fatty acids, alcohols, formate, and H_2/CO_2 (Jeris and McCarty 1965; Gujer and Zehnder 1983). The isolation of strain ASRB1 from crushed granular sludge using the highest positive dilution of a serial dilution range on acetate and sulfate strongly indicates that strain ASRB1 is the most abundant acetate-degrading sulfate reducer in this sludge. It is possible that acetate degradation is not the only activity of strain ASRB1 in the sludge because growth on several other compounds, such as propionate and formate, was much faster than growth on acetate. Growth on compounds such as propionate and ethanol even led to an intermediate formation of acetate. The specific growth rate of ASRB1 (μ_{max} = 0.14–0.20 day^{-1}) is comparable to that of most *Methanosaeta* species (μ_{max} = 0.08–0.29 day^{-1}). *Methanosaeta* species are generally the most important methanogenic acetate degraders in anaerobic bioreactors (Jetten et al. 1992; Oude Elferink et al. 1994). The high enzyme activity of carbon monoxide dehydrogenase and formate dehydrogenase (two key enzymes of the CO-dehydrogenase pathway), together with the absence of 2-oxoglutarate dehydrogenase activity (a key enzyme of the citric acid cycle), indicates that strain ASRB1 degrades acetate via the CO-dehydrogenase pathway (Schauder et al. 1986).

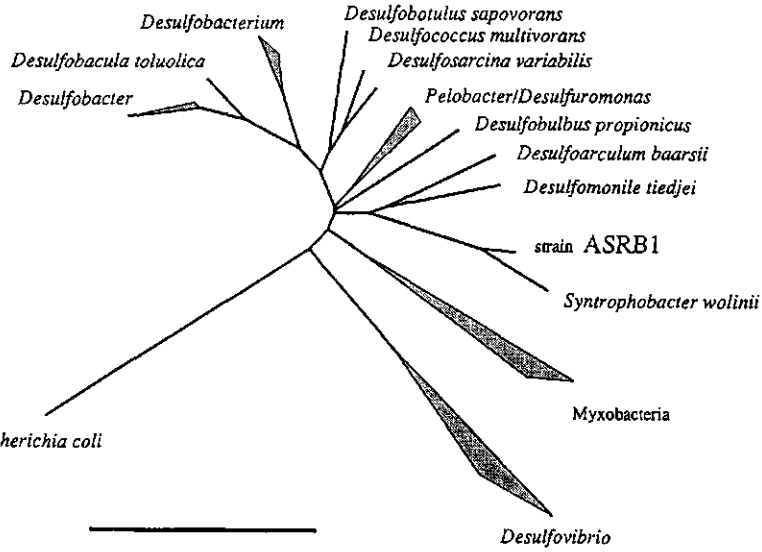

Fig. 2 Distance matrix tree reflecting the phylogenetic relationships of strain ASRB1 with other sulfate reducers. Only alignment positions were used that shared identical residues in at least 50% of the sequences from the representatives of the delta subclass of proteobacteria. *Bar* 0.10 K_{nuc}

Taxonomy

On the basis of the 16S rRNA sequence analysis, strain ASRB1 clusters with the family Desulfobacteriaceae [proposed by Widdel and Bak (1992)]. Although strain ASRB1 is phylogenetically closely related to *Syntrophobacter wolinii*, syntrophic propionate oxidation by strain ASRB1 in co-culture with *M. hungatei* was not observed. Recent studies revealed that *S. wolinii* is actually a sulfate reducer that couples propionate oxidation to sulfate reduction and to proton reduction, with the latter process being preferred (H. J. M. Harmsen et al., unpublished results; Wallrabenstein et al. 1994). Two other syntrophic propionate-oxidizing bacteria, strain MPOB and strain KoProp, are also closely related to strain ASRB1 and *S. wolinii* (H. J. M. Harmsen et al., unpublished results).

Although strain ASRB1 is phylogenetically closely related to *S. wolinii* and two other syntrophic propionate oxidizers, its physiology appears to be significantly different. Therefore, we propose that strain ASRB1 represents a new species of a new genus. We propose *Desulforhabdus amnigenus* gen. nov. sp. nov.

Genus *Desulforhabdus* gen. nov.

De.sul.fo.rhab'dus. L. pref. *de* from; L. neut. n. *sulfur* sulfur; Gr. fem. n. *rhabdus* rod; M. L. masc. n. *Desulforhabdus* rod-shaped sulfate reducer.

Non-motile, rod-shaped-to-ellipsoidal cells. Sulfate or other sulfur compounds, but not elemental sulfur, serve as terminal electron acceptor and are reduced to H_2S. Simple organic substrates, such as acetate, volatile fatty acids, and low-molecular-weight alcohols are completely oxidized to CO_2 via the CO-dehydrogenase pathway. Syntrophic oxidation of propionate or ethanol was not observed. *Desulforhabdus* belongs to the delta subclass of the Proteobacteria; the closest relatives are the syntrophic propionate oxidizers *Syntrophobacter wolinii*, strain MPOB, and strain KoProp.

Desulforhabdus amnigenus sp. nov.

Am.ni'ge.nus. L. masc. n. *amnis* water; L. neut. n. *genus* origin; L. adj. *amnigenus* coming from water. Cells are rod-shaped-to-ellipsoidal, 1.4–1.9 × 2.5–3.4 μm, occurring singly, in pairs, or in long chains. No spore formation. Gram-negative. Cells grow autotrophically with H_2, CO_2, and sulfate. Acetate and other compounds are completely oxidized to CO_2. Sulfate, sulfite, thiosulfate, and dithionite serve as electron acceptors. The optimum pH is 7.2–7.6; the optimum temperature is 37°C. Growth is optimal in freshwater medium. The G+C content of the DNA is 52.5 mol%. Habitat is granular sludge from an upflow anaerobic sludge bed (UASB) reactor treating sulfate-rich wastewater.

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CHAPTER 3

Desulfobacca acetoxidans gen. nov. sp. nov., a novel acetate-degrading sulfate reducer isolated from sulfidogenic granular sludge.

In press as:

Oude Elferink SJWH, Akkermans-van Vliet WM, Bogte JJ, Stams AJM. *Desulfobacca acetoxidans* gen. nov. sp. nov., a novel acetate-degrading sulfate reducer isolated from sulfidogenic granular sludge. Int. J. Syst. Bacteriol.

A mesophilic sulfate reducer, strain ASRB2, was isolated with acetate as sole carbon and energy source from granular sludge of a lab-scale upflow anaerobic sludge bed (UASB) reactor fed with acetate and sulfate. The bacterium was oval-shaped, 1.3 by 1.9 to 2.2 μm, non-motile and Gram negative. Optimum growth with acetate occurred around 37°C in freshwater medium (doubling time: 1.7-2.2 days). Enzyme studies indicated that acetate was oxidized via the carbon monoxide dehydrogenase pathway. Other organic acids, such as propionate, butyrate and lactate, alcohols like ethanol and propanol, and hydrogen or formate did not support growth. Sulfite and thiosulfate were also used as electron acceptors, but sulfur and nitrate were not reduced. Phylogenetically, strain ASRB2 clustered with the delta subdivision of the *Proteobacteria*. Its closest relatives were *Desulfosarcina variabilis*, *Desulfacinum infernum* and *Syntrophus buswellii*. Strain ASRB2 is described as a new genus and species, *Desulfobacca acetoxidans*.

INTRODUCTION

Sulfate-reducing bacteria play an important role in the degradation of organic matter in anaerobic bioreactors treating sulfate-rich wastewaters, such as those from paper mills, tanneries, or food oil industry (1,2). If sufficient sulfate is available, sulfate reducers can easily outcompete hydrogenotrophic methanogens and syntrophic consortia for substrates like hydrogen and propionate (1-3). However, the outcome of the competition for acetate between sulfate reducers and methanogens in anaerobic wastewater treatment systems is less clear. In some studies with freshwater or low-salt systems, acetate conversion via methanogenesis was predominant, even at an excess of sulfate (4-6). Other studies report the predominance of acetate degradation via sulfate reduction (7-9). Factors which could affect the outcome of the competition between methanogens and sulfate reducers are for example the kinetic properties of the bacteria involved, the pH and temperature of the reactor, and the chemical oxygen demand (COD)/sulfate ratio of the wastewater (1-3). In wastewater with a COD/sulfate ratio below 0.67 (g/g) there will be excess of sulfate allowing the degradation of all organic material via sulfate reduction.

Acetate is one of the major intermediates in the breakdown of organic matter in anaerobic bioreactors (10,11). Therefore, it is important to know which sulfate reducers can compete with the acetoclastic methanogens present in the sludge. Although many mesophilic sulfate reducers can grow with acetate as sole electron donor and carbon source (12,13), only a few show good growth with acetate under freshwater conditions. Among them are the Gram-positive *Desulfotomaculum acetoxidans* (14), the Gram-negative *Desulfobacter* strain AcKo (15), and the Gram-negative *Desulforhabdus amnigenus*, which was recently isolated from granular sludge of an upflow anaerobic sludge bed (UASB) reactor treating papermill

wastewater (16). In this paper we describe the isolation and characterization of a sulfate reducer from granular sludge of a lab-scale UASB reactor fed with acetate at an excess of sulfate. In this reactor sulfate reduction had completely superseded methanogenesis after one year of reactor operation.

MATERIALS AND METHODS

Origin of strain ASRB2. The sulfate-reducing bacterium, strain ASRB2, was isolated from the granular sludge of a pilot-scale UASB reactor (1.7 l) fed with acetate and an excess of sulfate. Initially, the reactor was seeded with sludge from a 10 l UASB reactor that had been fed with acetate and sulfate for more than two years. Detailed characteristics of this seed-sludge have been described elsewhere (3). The reactor influent had a COD/sulfate ratio of 0.6 (g/g) and was treated at a temperature of 30°C. Sludge samples were taken after 6 months and 1 year of reactor operation. During this period acetate degradation via sulfate reduction increased from approximately 80% to 100%, while degradation via methanogenesis decreased from 20% to 0%.

Media and cultivation. Unless stated otherwise, bacteria were cultured at 37°C in 120-ml serum vials containing 50 ml of bicarbonate-buffered medium, and a gas phase of 172.2 kPa N_2-CO_2(80:20, v/v) as described before (16). The inoculum size was 1%.

Isolation. Granular sludge samples (10 ml), taken from the reactor after 6 months and after 1 year of operation, were 10-fold diluted and disintegrated immediately after sampling as described previously (16). This crushed granular sludge was used to make 10-fold serial dilutions in liquid media containing acetate and sulfate (20 mM each). For each dilution, 5 ml inoculum was added to 45 ml medium. The cultures were incubated at 30°C, and the highest dilutions which showed growth were used for further isolation. Pure cultures were obtained by repeated application of the agar roll tube dilution method as described by Hungate (17). Purity of the isolates was checked by microscopic observations and by testing anaerobic growth on pyruvate and glucose with 0.1% yeast extract (BBL, Becton Dickinson, Cockeysville, MD, USA), and on Wilkins-Chalgren anaerobe broth (Oxoid, Basingstroke, UK).

Growth experiments. Utilization of carbon sources, electron donors, and electron acceptors were tested in basal bicarbonate-buffered medium as described before (16). In most cases growth was followed by measuring substrate utilization and sulfide production, and by visual examination of culture turbidity. All tests were performed at a predetermined temperature, pH, and salinity allowing optimal growth of the isolate (16).

Analysis of cell compounds. Desulfoviridin was detected according to Postgate (18).

Cytochromes were identified in cell extracts by recording reduced-minus-oxidized difference spectra with a Beckman DU7500 spectrophotometer, and on sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE gel) according to Thomas et al. (19). The G + C content of the DNA was determined by HPLC at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM, Braunschweig, Germany). Gram staining was done according to standard procedures (20). The presence of gas vacuoles was determined by microscopic examination of late-exponential phase cultures before and after a pressure shock treatment in a hypodermic syringe.

Chemical analysis and enzyme measurements. Substrates were measured by HPLC or gas chromatography as described by Oude Elferink et al. (16). Sulfide was determined as described by Trüper and Schlegel (21), and protein was measured according to the method of Bradford (22). The enzyme activities of carbon monoxide dehydrogenase, formate dehydrogenase and 2-oxoglutarate dehydrogenase activities were assayed according to Schauder et al. (23), using anoxically prepared cell extracts (24) of cells grown with acetate and sulfate and harvested in the late-exponential phase.

Sequence analysis and phylogenetic tree. The 16S rRNA-gene of strain ASRB2 was selectively amplified as described previously (25), using a set of universal 16S rRNA-based primers: forward primer (5'CACGGATCCAG-AGTTTGAT(C/T)(A/C)TGGCTCAG) corresponded to positions 8 to 27 of *Escherichia coli* 16S rRNA, and the reverse primer (5'GTGCTGCAGGGTTACCTTGTTAC-GACT) corresponded to positions 1493 to 1510. Amplification products were cloned in the pGEM^R-T vector according to the manufacturers protocol (pGEM^R-T Vector Systems, Promega, Madison, WI, USA). Plasmids of the clones were isolated by Wizard Plus Minipreps DNA Purifica-tion System according to the manufacturers instruction (Promega). The inserts were amplified using primerset T7 (5'AATACGACTCACTATAG) and Sp6 (5'ATTTAGGTGACACTATA). The PCR products were sequenced with a LICOR 4000L sequencer, by using Thermo Sequenase fluorescent labelled primer cycle sequencing with 7-deaza-dGTP according to the manufacturers protocols (Amersham, Buckinghamshire, UK).

The total 16S rRNA gene sequence was determined and aligned to those of other bacterial sequences, taking into account sequence similarity and higher order structure, using the alignment tool of the ARB program package (26).

Nucleotide sequence accession number. The 16S rRNA gene sequence of strain ASRB2 was deposited under GenBank accession number AF002671.

RESULTS

Isolation and morphological characterization. Strain ASRB2 was the dominant acetoclastic sulfate reducer in sludge samples taken from the reactor after 6 months and 1 year of operation. The highest sludge dilutions ($1x10^8$ and $1x10^9$) showing growth on acetate and sulfate were used for the isolation of strain ASRB2 by a repeated application of the agar roll tube dilution method.

In agar the strain grew in greyish colonies with an irregular shape. Cells of the isolate were non-motile oval to rod-shaped (1.3 µm wide and 1.9 to 2.2 µm long), and appeared singly or in pairs (Fig. 1). Cells stained Gram negative. Spores were never observed. Late-exponential phase or stationary phase cells often contained light reflecting inclusions that could be destroyed by pressure shock treatment, indicating that the inclusions were gas-vacuoles.

Growth conditions and substrate utilization. The optimum growth temperature for strain ASRB2 on acetate and sulfate was between 36 and 40°C. Little growth was observed below 27°C or above 47° C. The optimum pH for growth was 7.1-7.5; growth was possible between pH 6.5 and 8.3. The shortest doubling time on acetate was 1.7 to 2.2 days.

Growth in brackish medium was slow (the doubling time increased 4 to 8 times), and no growth was observed in marine medium. When vitamins were omitted from the media cultures could be transferred (1% inoculum size) at least 4 times without any growth retardation.

In the presence of acetate, strain ASRB2 could use sulfate (20 mM), thiosulfate (20 mM) or sulfite (5 mM) as electron acceptor; Sulfur (5 mM), nitrate (5 mM) or fumarate (10 mM) were not used. Strain ASRB2 was specialized in the degradation of acetate, and complete oxidation of 10 mM acetate led to a concomitant formation of 9.6 mM sulfide. The threshold for acetate was below 15 µM, the detection threshold of our gas chromatograph. Compounds tested but not utilized as electron donors by strain ASRB2 were: propionate (20 mM), butyrate (20 mM), lactate (20 mM), H_2-CO_2 (80:20, v/v) with or without acetate (2 mM), formate (10 mM) with or without acetate (2 mM), ethanol (20 mM), propanol (10 mM), butanol (10 mM), pyruvate (20 mM), fumarate (20 mM), glucose (20 mM), crotonate (5 mM), benzoate (1 mM), phenol (0.5 mM), aspartate (5 mM), and glutamate (5 mM).

The pathway of acetate oxidation was studied by enzyme measurements of key enzymes in cell-free extracts. The specific activities of carbon monoxide dehydrogenase and formate dehydrogenase were 0.63 and 0.84 $mol.min^{-1}.(mg\ protein)^{-1}$ respectively. 2-Oxoglutarate dehydrogenase activity could not be detected.

Pigments and other cell compounds. Dithionite-reduced versus air-oxidized spectra of cell extracts of ASRB2 revealed absorption maxima at 422, 527 and 557 nm, indicating the

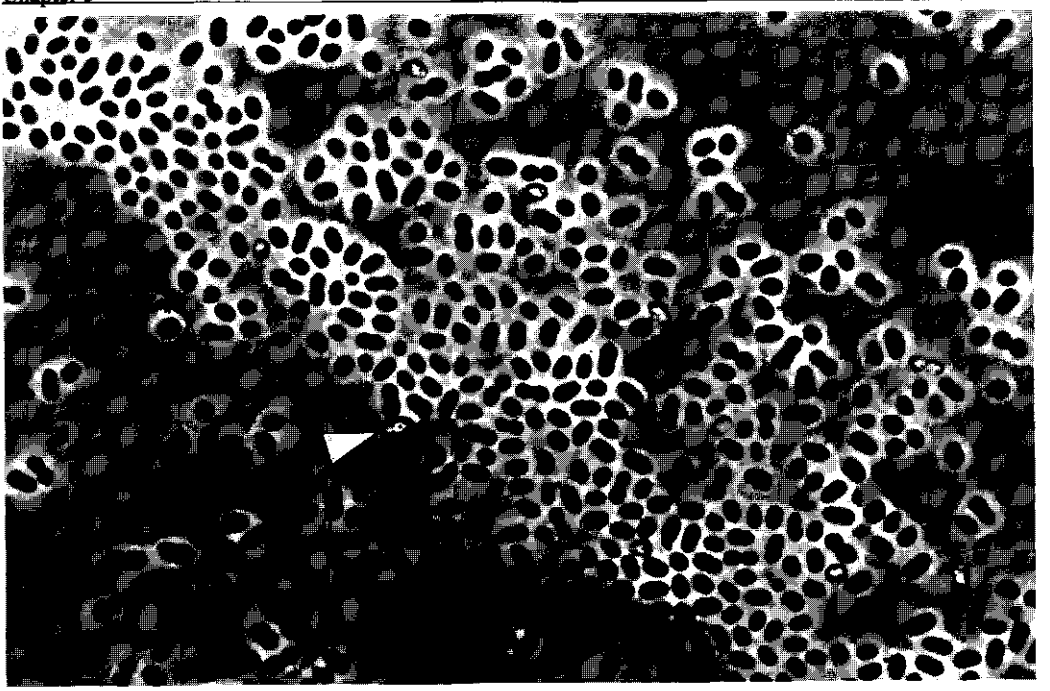

FIG. 1.
Phase contrast photomicrograph of strain ASRB2. The white arrow points out a cell with a gas-vacuole. The marker bar represents 10 μm.

presence of c-type cytochromes (27). The presence of c-type cytochromes was confirmed with the staining procedure on SDS-gel (19) (results not shown). Desulfoviridin could not be detected. The G+C content of the DNA was 51.1 (± 0.2) mol%.

Phylogenetic analysis. The phylogenetic relationships of strain ASRB2 derived from 16S rRNA sequence analysis are depicted in Fig. 2. The 16S rRNA sequence shows that strain ASRB2 is a member of the delta sub-division of the *Proteobacteria*. A 16S rRNA sequence highly similar to that of ASRB2 was not available in the database. *Desulfosarcina variabilis*, *Desulfacinum infernum* and *Syntrophus buswellii* were the closest relatives of ASRB2; the level of sequence similarity was, 86.9%, 85.6% and 85.5%, respectively. The acetate-degrading sulfate reducer *Desulforhabdus amnigenus* was only moderately related to strain ASRB2 (sequence similarity 85.1%).

DISCUSSION

Physiology, ecology, and taxonomy of strain ASRB2. Strain ASRB2 was isolated from granular sludge of a lab-scale UASB reactor fed with acetate at an excess of sulfate. Cells resembling those of strain ASRB2 were isolated from the sludge before and after

sulfate reduction had superseded methanogenesis, by using the highest positive dilutions of two serial dilution ranges on acetate and sulfate. This strongly indicates that strain ASRB2 is the most abundant acetate-degrading sulfate reducer in this sludge and is able to outcompete acetate-degrading methanogens. The only two genera of acetate-degrading methanogenic archaea known are *Methanosarcina* and *Methanosaeta* (formerly "*Methanothrix*") (28). *Methanosaeta* species generally are the most important methanogenic acetate degraders in anaerobic bioreactors, because of their high affinity and low threshold (7-69 M) for acetate (1,29). The threshold of strain ASRB2 (<15 μM) is in the same range as that of *Methanosaeta* sp. However, strain ASRB2 has a higher specific growth rate (μ_{max}= 0.32-0.41 day^{-1}) than *Methanosaeta* sp. (μ_{max}= 0.08-0.29 day^{-1}). This could be one of

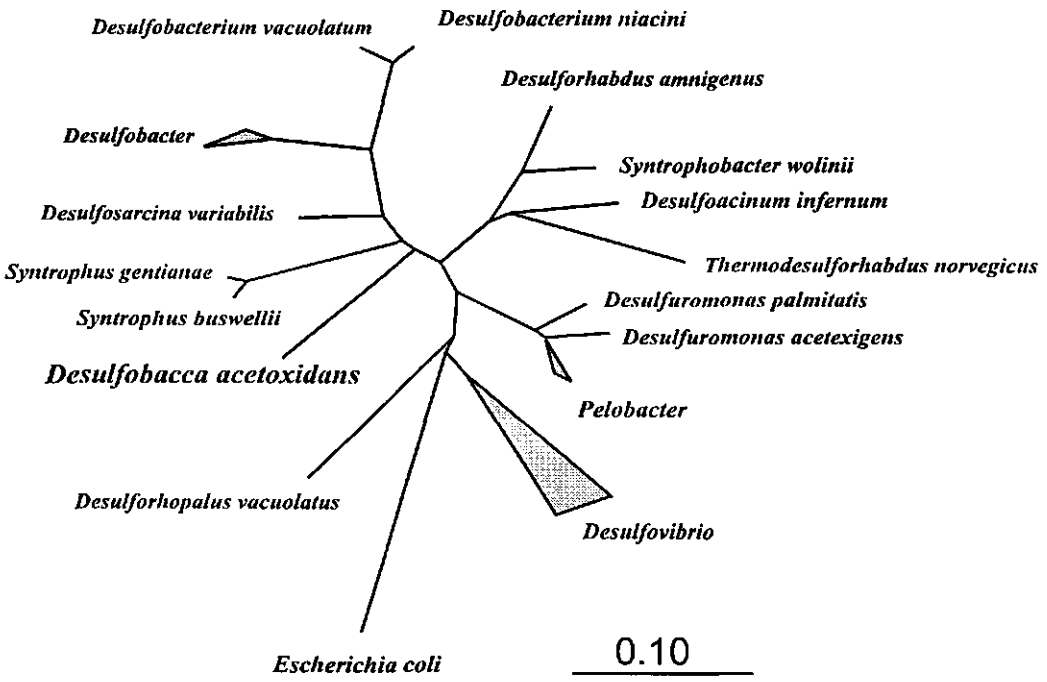

FIG. 2.
Distance matrix tree reflecting the phylogenetic relationships of strain ASRB2 with other sulfate reducers and relatives belonging to the delta subdivision of the *Proteobacteria*. Marker bar = 0.10 K_{nuc}.

the reasons why strain ASRB2 is able to outcompete the acetate-degrading methanogens in the reactor. Strain ASRB2 seems to be specialized in acetate consumption, this in contrast to the nutritionally versatile acetate-degrading *Desulforhabdus amnigenus*, which was recently isolated from a pilot-scale UASB reactor treating papermill wastewater using the same isolation procedures as described for strain ASRB2 (16). Apparently, the acetate-degrading sulfate-reducing population in the lab-scale reactor differs significantly from the population in the pilot-scale reactor. This is probably due to the different conditions in the pilot-scale reactor, such as the limiting sulfate concentration (COD/sulfate= 1.1 g/g) and the more complex wastewater. The nutritional specialization of strain ASRB2 is comparable to that of *Desulfobacter* sp. (15), although some *Desulfobacter* species can use hydrogen and ethanol as well. However, the average specific growth rate of *Desulfobacter* sp. (μ_{max}= 0.8-1.1 day^{-1}) (1) is approximately twice as high as that of strain ASRB2. The oxidation of acetate in *Desulfobacter* sp. and in strain ASRB2 occurs via different pathways. *Desulfobacter* sp. use the citric acid cycle (15), while strain ASRB2 degrades acetate via the CO-dehydrogenase pathway. This is indicated by the high activity in cell-free extracts of strain ASRB2, of carbon monoxide dehydrogenase and formate dehydrogenase, two key enzymes of the CO-dehydrogenase pathway, together with the absence of 2-oxoglutarate dehydrogenase activity, a key enzyme of the citric acid cycle (23).

Phylogenetically, stain ASRB2 clusters with the delta sub-division of the *Proteobacteria*. *Desulfobacter* sp. is only distantly related to strain ASRB2. The closest relatives are *Desulfosarcina variabilis* (level of similarity 86.9%), *Desulfacinum infernum* (level of similarity 85.6%), and *Syntrophus buswellii* (level of similarity 85.5%). Physiologically and phylogenetically strain ASRB2 differs significantly from the syntrophically benzoate-oxidizing *S. buswellii* (30), the thermophilic *Desulfacinum infernum* (31), and the nutritionally versatile *D. variabilis* (12). Therefore, we propose that strain ASRB2 represents a new species of a new genus. We propose the name *Desulfobacca acetoxidans* gen. nov. sp. nov. for this organism.

Description of *Desulfobacca* gen. nov. *Desulfobacca* (de.sul.fo.bac'ca. L. pref. *de*, from; L. n. *sulfur*, sulfur; M.L. pref. *Desulfo-*, desulfuricating, used to characterize a dissimilatory sulfate-reducing prokaryote; L. fem. n. *baca* or *bacca*, berry, especially olive; M.L. fem. n. *Desulfobacca*, a sulfate-reducing, olive-shaped bacterium). Non-motile, oval to rod-shaped cells. Sulfate or other inorganic sulfur compounds, but not elemental sulfur, serve as terminal electron acceptor and are reduced to H_2S. Acetate is the common electron donor and carbon source, and is completely oxidized to CO_2 via the CO-dehydrogenase pathway.

Desulfobacca belongs to the delta subclass of the *Proteobacteria*; the closest relatives are

Desulfosarcina variabilis, *Desulfacinum infernum* and *Syntrophus buswellii*.

Description of *Desulfobacca acetoxidans* sp. nov. *Desulfobacca acetoxidans* (a.cet. o'xi.dans. L. n. *acetum*, vinegar; M.L. part. pres. *oxidans*, oxidizing, M.L. part. adj. *acetoxidans*, oxidizing acetate. Cells are oval to rod-shaped, 1.3 x 1.9-2.2 µm, singly or in pairs. Cells do not form spores and are Gram negative. Acetate is the only electron donor and carbon source used. Sulfate, sulfite, and thiosulfate can serve as electron acceptors. The optimum pH is 7.7-7.5, the optimum temperature is 37°C. Growth is optimal in freshwater medium. The G + C content of the DNA is 51.1 (± 0.2) mol%. Habitat is granular sludge from an upflow anaerobic sludge bed (UASB) reactor fed with acetate and sulfate.

The type strain is ASRB2, which has been deposited in the Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany, under accession number DSM 11109.

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CHAPTER 4

Kinetics of acetate oxidation by two sulfate reducers isolated from anaerobic granular sludge.

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ABSTRACT

To examine the competition for acetate between sulfate reducers and methanogens in anaerobic bioreactors, kinetic parameters of acetate oxidation were determined for the generalist *Desulforhabdus amnigenus* and the specialist *Desulfobacca acetoxidans*, and compared with those of acetate degrading methanogens. The Michaelis-Menten parameters K_m and V_{max}, were 0.6 mM and 28 μmol min^{-1} g $protein^{-1}$ for *Drb. amnigenus*, and 0.6 mM, and 43 μmol min^{-1} g $protein^{-1}$ for *Dbc. acetoxidans*, respectively. Compared to *Methanosaeta* sp. (K_m= 0.8 ± 0.3 mM, V_{max} = 94 ± 58 μmol min^{-1} g $protein^{-1}$), the K_m values of *Drb. amnigenus* and *Dbc. acetoxidans* were slightly better than that of most *Methanosaeta* sp., while the V_{max} values were slightly lower. The maximum specific growth rate (μ_{max}) and the acetate-degradation threshold were 0.14-0.20 day^{-1}, and below 15 μM for *Drb. amnigenus*, and 0.31-0.41 day^{-1}, and below 15 μM for *Dbc. acetoxidans*, respectively. Compared to *Methanosaeta* sp. (μ_{max}= 0.35 ± 0.11 day^{-1}, threshold 7-69 μM), the μ_{max} and threshold value of *Dbc. acetoxidans* were better than those of most *Methanosaeta* sp. The same applied for the threshold value of *Drb. amnigenus*. Mixed substrate studies showed that *Drb. amnigenus* degraded acetate and hydrogen simultaneously, but also indicated that this bacterium preferred lactate, propionate and ethanol over acetate.

Based on the acetate kinetic parameters *Dbc. acetoxidans* and *Drb. amnigenus* seem to be able outcompete *Methanosaeta* sp. for acetate in acetate-fed anaerobic bioreactors. However, it should be taken into account that the presence of additional substrates, such as lactate, propionate, or ethanol, could have a negative effect on the rate of acetate degradation of *Drb. amnigenus*.

INTRODUCTION

Acetate and hydrogen are the key intermediates in the breakdown of organic matter in anaerobic bioreactors (5, 15). In anaerobic reactors treating sulfate-rich wastewaters, such as paper mill or food oil industry wastewaters, sulfate reducers will compete for these compounds with methanogens (3, 11). In order to be able to steer the reactor process in the direction of either sulfidogenesis or methanogenesis, it is essential to have a clear insight in this competition. Reactor studies have indicated that with excess sulfate, hydrogen is mainly consumed by sulfate reducers (1, 17, 18), but the outcome of the competition for acetate is not yet clear. Generally, acetate is utilized by methanogens (6, 18), but in some reactors it is mainly utilized by sulfate reducers (1, 17). To explain the apparent competitive advantage of acetate-degrading methanogens over sulfate reducers, various theories have been put forward, including a higher growth rate of methanogens compared to sulfate reducers (21), a too short duration of reactor studies, i.e. not enough time allowed

for acetate-degrading sulfate reducers to outcompete the methanogens (18, 19).

Comparison of acetate degradation kinetics of methanogens and sulfate reducers can give more insight in the competition for acetate. For the two genera of methanogens known to use acetate as sole energy source, *Methanosarcina* and *Methanosaeta* ("*Methanothrix*") (20)), acetate utilization kinetics have been studied extensively (7, 10).

Unfortunately, kinetic data for acetate utilization by freshwater sulfate reducers are hardly available. Most researchers studied acetate oxidation by marine sulfate reducers, as reviewed by Oude Elferink et al. (11). It is unlikely that these marine sulfate reducers, such as *Desulfobacter* sp., play an important role in freshwater anaerobic bioreactors.

The aim of the present study was to investigate the oxidation acetate by freshwater sulfate reducers. For our study we used the specialist *Desulfobacca acetoxidans* and the generalist *Desulforhabdus amnigenus*, i.e. *Dbc. acetoxidans* only utilizes acetate (13), whereas *Drb. amnigenus* can use a wide variety of substrates such as acetate, propionate, hydrogen, and ethanol (12). Both sulfate reducers have been isolated from anaerobic granular sludge obtained from reactors in which acetate was mainly converted via sulfate reduction.

In our study we determined the Michaelis-Menten parameters (V_{max} and K_m) for acetate of both sulfate reducers. Furthermore we investigated if mixtures of substrates, including mixtures of acetate and propionate or hydrogen, led to sequential substrate utilization in batch cultures of *Drb. amnigenus*.

MATERIALS AND METHODS

Organisms, media and cultivation

Desulfobacca acetoxidans strain ASRB2 (DSM 11109) and *Desulforhabdus amnigenus* strain ASRB1 (DSM 10338) were isolated previously (12, 13). The bacteria were cultured in 1.2-l glass bottles closed with butyl rubber stoppers and aluminum caps, and incubated at 37°C. The bottles contained 500ml of a bicarbonate-buffered medium as described before (16), and a gas phase of 172.2 kPa N_2-CO_2 (80:20, vol/vol). The inoculum size was 1%. Electron acceptors and electron donors were added separately by syringe from 0.5 or 2.0 M autoclaved (20 min., 120°C) anaerobic stock solutions.

Acetate consumption in resting cell suspensions

The Michaelis-Menten kinetic parameters V_{max} and K_m were estimated from acetate depletion curves (acetate consumption versus time) obtained with concentrated cell-suspensions. The depletion data were fitted to an integrated solution of the Michaelis-Menten equation $V_{max}*t = S_0 - S + K_m \ln(S_0/S)$ using nonlinear regression analyses, as described by Robinson and Characklis (14). In this equation S_0 is the initial substrate concentration, S is the substrate concentration at time t, V_{max} the maximum consumption rate, and K_m the half-saturation constant.

Table 1. The effect of mixed substrates on the rate of acetate consumption/production by cells of *Drb. amnigenus* cells, tested in batch cultures with high initial substrate concentrations, starting with a single substrate and adding a pulse of second substrate at logarithmic growth phase of the culture.

Start substrate (mM)		Pulse substrate (mM)		Acetate Consumption (C)/ Production (P)	
				before pulse	after pulse
Acetate	(18)	Propionate	(17)	C 100%	C 0%
Acetate	(22)	Hydrogen	(10)	C 100%	C 100%
Ethanol	(16)	Propionate	(17)	P 100%	P 100% (85% via ethanol)
Propionate	(17)	Ethanol	(11)	P 100%	P 100% (70% via propionate)
Propionate	(17)	Lactate	(11)	P 100%	P 100% (80% via propionate)
Lactate	(20)	Propionate	(14)	P 100%	P 100% (50% via lactate)

To obtain concentrated cell suspensions, cells were harvested anaerobically by centrifugation in the late exponential phase of growth. The cells were resuspended and washed twice with the bicarbonate-buffered medium, and were then transferred to 120-ml serum vials in an anaerobic glove box, and sealed with butyl rubber stoppers and aluminum crimp seals.

Cells of *Dbc. acetoxidans* were concentrated approximately 20-fold, and cells of *Drb. amnigenus* 50-fold, to eliminate the interference of growth.

The vials were preincubated at 37°C for one hour, in the presence of 10 mM sulfate, for the removal of intracellular acetate. For both bacteria four independent acetate depletion experiments were carried out, starting with respectively 1, 2.5, 4 or 6 mM of acetate as initial acetate concentration. The protein content of the cell suspensions was determined at the end of each experiment.

Mixed substrate utilization

Drb. amnigenus was adapted to acetate, propionate, ethanol or lactate by subculturing the organism on one of these substrates for at least three times. Substrate preferences were tested in batch cultures, by growing the cells on a single substrate and adding a puls of a different substrate as soon as the culture reached log phase, according to the scheme presented in Table 1.

For hydrogen pulse experiments the cultures were incubated on a rotary shaker (125 rpm), and sterile hydrogen gas was added by syringe.

Chemical analyses

Hydrogen and other substrates, including acetate, were measured by gas chromatography and high-performance liquid chromatography HPLC, respectively (16). For the substrate depletion curves, acetate was analyzed by gas chromatography as described by Stams et al.

(16), but using a Chrompack CP9001 instead of a CP9000 gas chromatograph. Total cell protein was estimated using the method of Bradford (2), after disruption of the cells by sonification (5 times 20 s with an intermittent cooling for 20 s).

RESULTS

Acetate consumption by concentrated cell suspensions of *Dbc. acetoxidans* and *Drb. amnigenus* followed Michaelis-Menten kinetics. Measured acetate depletion data and the constructed Michaelis-Menten plot are illustrated in Fig. 1. for a cell suspension of *Drb. amnigenus* and a cell suspension *Dbc. acetoxidans*. Similar fits were obtained with the other acetate depletion data. In Table 2 the V_{max}, K_m for acetate degradation, and several other physiological parameters of *Dbc. acetoxidans* and *Drb. amnigenus* are compared with those of acetate-degrading methanogens.

Thresholds for acetate consumption by *Dbc. acetoxidans* and *Drb. amnigenus* were not determined, but both strains could reach acetate concentrations below the detection limit of our GC analysis (15 μM). In mixed substrate experiments with *Drb. amnigenus* a simultaneous utilization of substrates was observed with combinations of propionate and lactate or ethanol (Table 1). In these experiments propionate, lactate and ethanol were incompletely oxidized by *Drb. amnigenus*, i.e. for each mole of propionate, lactate or ethanol used one mole of acetate was formed (Fig. 2). The rate of acetate production in propionate, lactate or ethanol grown cultures was hardly influenced by addition of a second substrate. Furthermore, cultures growing on acetate and pulsed with propionate immediately switched to incomplete propionate oxidation (Fig. 3a). Acetate was only simultaneously consumed in combination with hydrogen (Fig. 3b).

Table 2. Selected acetate kinetic parameters of *Desulfobacca acetoxidans*, *Desulforhabdus amnigenus*, and the two genera of acetate-degrading methanogens.

Strain	Substrate utilization	μ_{max} ($days^{-1}$)	V_{max} (μmol min^{-1} gram $prot.^{-1}$)	K_m (mM)	Threshold (μM)	Reference
Dbc. acetoxidans	specialist	0.31-0.41	43 ± 14[a]	0.6 ± 0.4[a]	< 15	This study, 13
Drb. amnigenus	generalist	0.14-0.20	28 ± 7[a]	0.6 ± 0.4[a]	< 15	This study, 12
Methanosarcina sp.	generalist	0.46-0.69	-[b]	3.0	190-1180	7, 11
Methanosaeta						
soehngenii	specialist	0.08-0.29	76	0.4-0.7	7-69	7, 10, 11
concilii	specialist	0.21-0.69	32	0.8-1.2	-[b]	10, 11
strain MTAS	specialist	0.37	170	0.5	-[b]	9, 10
strain MTKO	specialist	0.38	98	1.17	-[b]	9, 10

[a]Mean of four independent experiments (± SD)
[b]Not determined

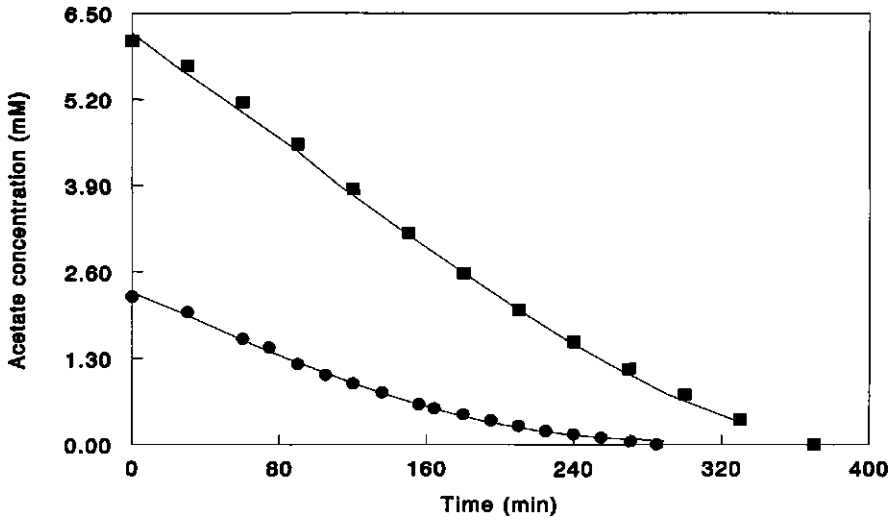

Figure 1. Acetate depletion curve for a concentrated suspension of *Drb. amnigenus* cells (■) and of *Dbc. acetoxidans* cells (●). The markers represent the measured acetate concentrations, while the solid line is the best-fit curve, calculated from the estimates of K_m, V_{max}, and the initial acetate concentration (S_0) via nonlinear regression analysis.

DISCUSSION

Drb. amnigenus and *Dbc. acetoxidans* were both isolated from sulfidogenic bioreactors in which acetate was mainly degraded via sulfate reduction. For *Drb. amnigenus* this was an upflow anaerobic sludge bed (UASB) reactor treating papermill wastewater, while *Dbc. acetoxidans* was isolated from a UASB reactor inoculated with methanogenic seed sludge and fed with a synthetic medium with acetate and sulfate. *Drb. amnigenus* and *Dbc. acetoxidans* were present in higher numbers than acetate-degrading methanogens and other acetate-degrading sulfate reducers in these UASB reactors (12, 13) indicating that they successfully competed with these microorganisms for the available acetate.

In most methanogenic bioreactors *Methanosaeta* sp. are the dominant acetate-degrading methanogens, because of their high affinity and low threshold value for acetate, compared to *Methanosarcina* sp. (8). In sulfate reducing reactors acetate-degrading sulfate reducers have to compete with these *Methanosaeta* sp. for the available acetate.

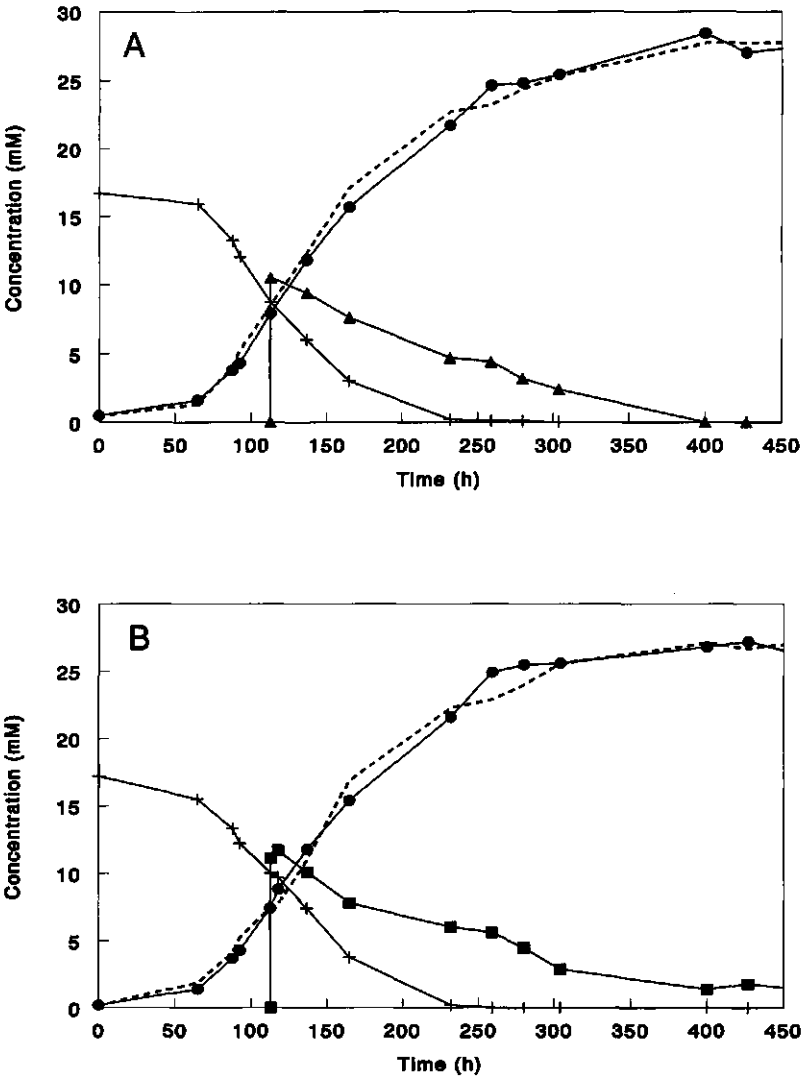

Figure 2. Acetate (●) production of *Drb. amnigenus* growing in batch cultures, with propionate (+), and pulsed with **A**: Ethanol (▲); **B**: Lactate (■). The dotted line represents the calculated acetate concentration, assuming an incomplete oxidation of propionate, lactate and ethanol.

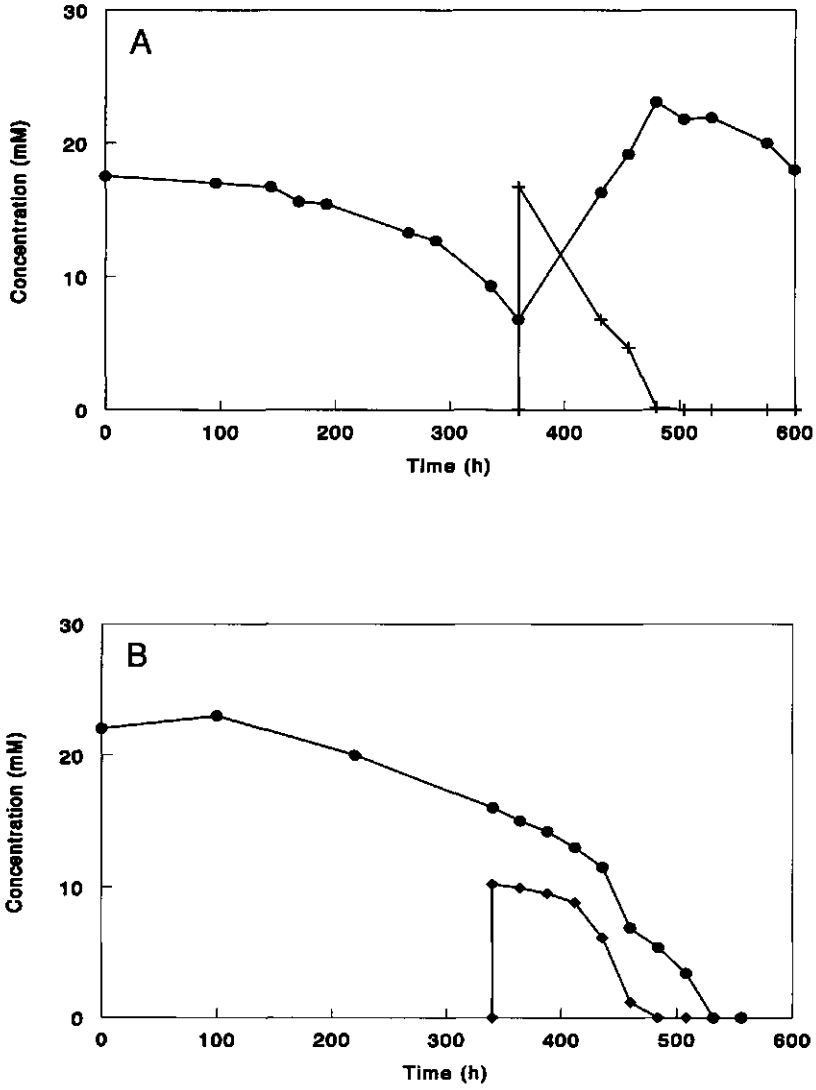

Figure 3. Substrate consumption of *Drb. amnigenus* growing in batch cultures with acetate (●), and pulsed with **A**: Propionate (+); **B**: Hydrogen (♦).

The theory that methanogens can outcompete sulfate reducers for acetate, because of their higher growth rate (21) is clearly not valid for all reactors, since *Dbc. acetoxidans* had a higher growth rate than most *Methanosaeta* sp., and the growth rate of *Drb. amnigenus* was in the same range as that of *Methanosaeta soehngenii* (Table 2). However, if several kinetic properties are compared (i.e. μ_{max}, V_{max}, K_m, threshold) acetate-degrading sulfate reducers from bioreactors only seem to have a slight kinetic advantage over *Methanosaeta* sp. Therefore, in some reactor studies, acetate-degrading methanogens may have predominated over sulfate reducers due to the fact that the duration of the competition study was not long enough to allow sulfate reducers to become dominant (6, 17). How long it can take before sulfidogens have outcompeted methanogens was shown in reactor studies of Visser (19). He experimented with UASB reactors inoculated with methanogenic sludge and fed with sulfate and a mixture of volatile fatty acids (VFA) or acetate. In the reactor fed with acetate and sulfate, from which *Dbc. acetoxidans* was isolated, it took about 100 days to increase the amount of acetate degraded via sulfate reduction from 0 to 10 %, and 400 days to shift it from 50 % to 90 %. In the VFA-fed reactor this shift from 50% to 90% was faster, but it still took about 250 days. The very slow shift from a methanogenic to a sulfidogenic acetate degrading reactor was probably due to the relative small competitive advantage of *Dbc. acetoxidans* over *Methanosaeta* sp. (Table 2), and the high ratio between the number of *Methanosaeta* and acetate-degrading sulfate reducers in the seed sludge.

In full-scale anaerobic bioreactors acetate is not the only organic compound available for microorganisms. For *Drb. amnigenus* the presence of compounds such as propionate, hydrogen, or lactate will influence the competitive advantage of this bacterium over acetate-degrading methanogens. From the mixed substrate studies it is clear that the presence of hydrogen can increase the competitive advantage of *Drb. amnigenus* over *Methanosaeta*, because *Drb. amnigenus* can use acetate and hydrogen simultaneously, while *Methanosaeta* sp. can only use acetate. How the presence of propionate, lactate or ethanol will influence the competition is less clear. The mixed substrate experiments showed that acetate consumption stopped if these other substrates were present in excess. In fact, *Drb. amnigenus* even started to produce acetate and the rate of lactate, propionate or ethanol degradation seemed to be limited by the rate of acetate production. On the other hand it is known that carbon substrates which usually lead to diauxic growth under batch conditions are used simultaneously under carbon limited conditions (4). Which condition *Drb. amnigenus* will encounter in the sludge is not clear, because substrate availability is not only related to the concentrations in the reactor, but also to diffusion of the substrate into the granule, and the location of the *Drb. amnigenus* cells in the granule. Since *Drb.*

amnigenus outcompeted the acetate-degrading methanogens in a bioreactor treating complex wastewater, and the kinetic properties of this bacterium are similar to those of *Methanosaeta* sp., one could speculate that the ability to use other substrates besides acetate gives *Drb. amnigenus* a competitive advantage over *Methanosaeta* sp.

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CHAPTER 5

Isomerization of butyrate to isobutyrate by *Desulforhabdus amnigenus.*

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Isomerization of butyrate to isobutyrate by *Desulforhabdus amnigenus*

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Abstract

The isomerization of butyrate and isobutyrate was investigated for the sulfate reducer *Desulforhabdus amnigenus*. Nuclear magnetic resonance (NMR) studies with ^{13}C-labelled butyrate showed that isobutyrate was formed by migration of the carboxyl group, in conformity with the butyrate isomerization reaction reported for methanogenic consortia. In addition to *D. amnigenus*, several other butyrate-degrading sulfate reducers (*Desulfobacterium vacuolatum*, *Desulfoarculus baarsii* and *Desulfotomaculum* sp.) were capable of butyrate isomerization.

Keywords: Butyrate degradation; Volatile fatty acid interconversion; ^{13}C-NMR; *Desulforhabdus amnigenus*

1. Introduction

Butyrate and isobutyrate are both intermediates in the anaerobic degradation of complex organic matter. Butyrate can be formed during the degradation of carbohydrates, proteins and lipids [1], while isobutyrate is mainly produced during valine fermentation [2]. Butyrate and isobutyrate concentrations are normally low in well balanced anaerobic bioreactors; high concentrations of these compounds in a reactor are indicative of process imbalance [3].

In methanogenic bioreactors butyrate is degraded via β-oxidation to acetate and hydrogen. This reaction is energetically feasible only if the hydrogen partial pressure is kept low. Therefore, syntrophic consortia of butyrate-degrading acetogens and hydrogenotrophic methanogens are essential [4]. In anaerobic bioreactors treating sulfate-containing waste water, sulfate reducers are able to couple the oxidation of butyrate to sulfate reduction [5]. It is not completely clear how isobutyrate is degraded in methanogenic and sulfidogenic environments. Because isobutyrate cannot be directly degraded via β-oxidation, it has been suggested that under methanogenic conditions, isobutyrate is first isomerized to butyrate and then degraded further [6,7]. Under sulfate-reducing conditions, isobutyrate can be degraded via propionyl-CoA, as was reported for the

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sulfate reducer *Desulfococcus multivorans* [7]. However, the importance of this degradation pathway in sulfidogenic bioreactors is not known.

It is well known that a reversible isomerization between butyrate and isobutyrate can occur under methanogenic conditions [6,8–11]. Nuclear magnetic resonance (NMR) studies with ^{13}C-labelled butyrate in a methanogenic enrichment culture [12], and a pure culture of a glutarate-fermenting strict anaerobe [13] demonstrated that this isomerization was due to the migration of the carboxyl group. Recently, we reported for the first time that a sulfate reducer, *Desulforhabdus amnigenus*, is able to convert butyrate to isobutyrate [14]. In this paper, the isomerization of butyrate by *D. amnigenus* is investigated by ^{13}C-NMR. In addition, we show that other pure cultures of butyrate-degrading sulfate reducers are also able to isomerize butyrate.

2. Materials and methods

2.1. Organisms and growth conditions

Four butyrate-degrading sulfate reducers were selected for butyrate isomerization studies. *D. amnigenus* (DSM 10338) was isolated from mesophilic anaerobic granular sludge [14]. *Desulfotomaculum* sp. was isolated at our laboratory from thermophilic anaerobic granular sludge, and nutritionally resembled *Desulfotomaculum kuznetsovii* [15] (unpublished results). *Desulfoarculus baarsii* (DSM 2075), and *Desulfobacterium vacuolatum* (DSM 3385) were obtained from the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany).

All strains were cultured in 580-ml serum bottles containing 250 ml of a bicarbonate-buffered medium as described before [14]. The bottles were inoculated

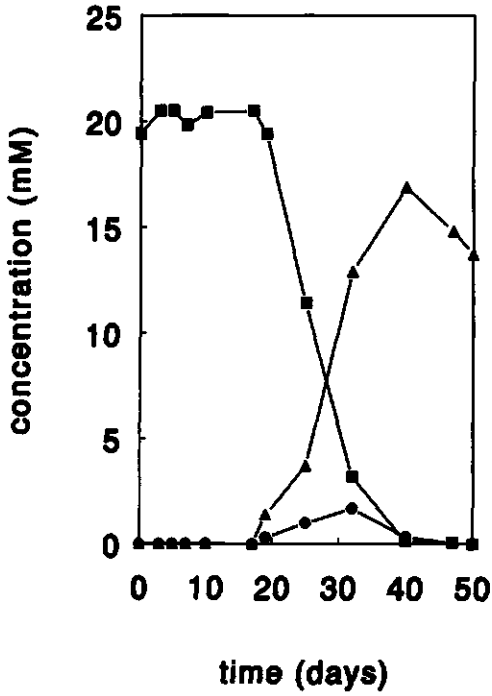

Fig. 1. Time course of butyrate degradation by *Desulforhabdus amnigenus*. Butyrate (■), isobutyrate (●), acetate (▲).

Table 1
Stoichiometry of butyrate conversion and sulfide formation by several butyrate degrading sulfate reducers

Strain	Butyrate added (mM)	Butyrate consumed (mM)	Acetate formed (mM)	Isobutyrate formed (mM)	Propionate formed (mM)	Sulfide formed (mM)
Desulforhabdus amnigenus	20.0	20.0	16.9	1.7	nd[a]	32
Desulfoarculus baarsii	20.0	20.0	0.19	0.01	nd[a]	48
Desulfobacterium vacuolatum	30.5	17.2	15.0	0.06	0.05	27
Desulfotomaculum sp.	37.5	11.5	2.7	0.26	0.03	24

The acetate, isobutyrate and propionate concentrations reported are the maximum concentrations and not the final concentrations measured during butyrate degradation.
[a]nd, not detected.

with 10% of a butyrate-grown stationary-phase culture. The electron donors butyrate or isobutyrate and the electron acceptor sulfate were added separately by syringe from 2 M sterile, anoxic stock solutions in a molar ratio of 1:2.5. *D. amnigenus* and *Desulfotomaculum* sp. were cultured at 37 and 55°C, respectively. *Da. baarsii* and *Db. vacuolatum* were both cultured at 30°C; for *Db. vacuolatum* the basal medium was amended with NaCl (20 g/l), $MgCl_2 \cdot 6H_2O$ (3 g/l), and $CaCl_2 \cdot 2H_2O$ (0.15 g/l) to achieve marine conditions.

2.2. NMR experiments

The fate of [2-^{13}C]butyrate was studied in batch experiments with *D. amnigenus*, incubated in 30-ml vials containing 10 ml medium, under the conditions as described above, except that ^{13}C-enriched butyrate was used as a substrate. The ^{13}C-labelled compounds (>99 atom% ^{13}C) were obtained from Isotec Inc. (Pixie Corp. B.V., Tjuchem, The Netherlands). At time zero and after 3 weeks of incubation 2 ml of the supernatant was sampled, centrifuged, and stored at −20°C until analysis with the NMR spectrometer. The ^{13}C-labelled compounds were analyzed at 75.47 MHz with a Bruker AMX-300 spectrometer, equipped with a 10 mm ^{13}C probe as described previously [16], except that spectra were acquired at 20°C during 2 h (14400 scans). The ^{13}C chemical shifts were referenced to the C_2 of butyrate (40.5 ppm).

2.3. Analytical methods

The degradation of butyrate and isobutyrate was analyzed by gas chromatography [17], sulfide formation being determined as described by Trüper and Schlegel [18].

3. Results and discussion

3.1. Butyrate degradation

Butyrate degradation and isomerization was investigated with pure cultures of *D. amnigenus*, *Da. baarsii*, *Db. vacuolatum*, and *Desulfotomaculum* sp. During growth on butyrate, *D. amnigenus* was the only sulfate reducer which excreted large amounts of isobutyrate (1.7–2.0 mM) into the medium (Fig. 1); the other three strains (Table 1) only produced small amounts of isobutyrate (0.06–0.26 mM). Thus far, *D. amnigenus* was the only sulfate reducer for which butyrate isomerization had been reported [14], but the results presented above indicate that butyrate isomerization is a more widespread phenomenon among butyrate-degrading sulfate reducers. In most studies low concentrations of butyrate (1–10 mM) are used to study butyrate degradation [7,19–22]. This might be the reason why butyrate isomerization by sulfate reducers was not observed before. The four cultures used in this study did not produce detectable amounts of isobutyrate when grown with 5 mM of butyrate.

D. amnigenus, *Db. vacuolatum* and *Desulfotomaculum* sp. all formed large amounts of acetate during butyrate oxidation (Fig. 1, Table 1). The intermediate formation of acetate during butyrate degradation has been reported for spore-forming [22] as well as for non-spore-forming [19–21] completely oxidizing sulfate reducers. According to Schauder et al. [20]

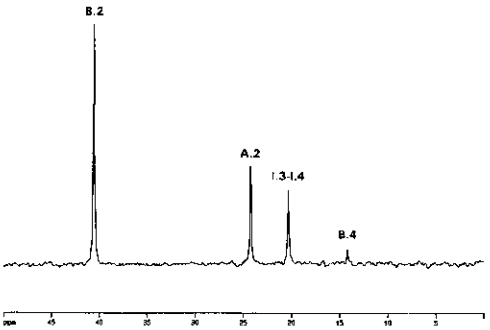

Fig. 2. High-resolution ^{1}H-decoupled ^{13}C-NMR spectrum of culture supernatant from a *D. amnigenus* culture, recorded after 3 weeks incubation with [2-^{13}C]butyrate and sulfate. The letters indicated refer to: A, acetate; B, butyrate; I, isobutyrate. The numbers following the one-letter abbreviations give the positions of the labelled carbon in the molecule.

acetate accumulates during β-oxidation of activated butyrate.

The small amount of propionate in cultures of *Db. vacuolatum* and *Desulfotomaculum* sp. is probably formed from acetyl-CoA via the reverse pathway of propionate degradation [23].

3.2. Isobutyrate conversion

Isobutyrate degradation by cultures of *D. amnigenus* was studied in batch experiments with 30 mM isobutyrate and 75 mM sulfate. No intermediate formation of butyrate could be detected in these cultures and intermediate acetate concentrations were maximally 5 mM. Intermediate acetate formation during isobutyrate degradation has also been reported for *Dc. multivorans* [7].

3.3. NMR studies

The chemistry of the butyrate-isobutyrate isomerization reaction by sulfate reducers was studied in batch experiments containing 4 mM [2-^{13}C]butyrate, 16 mM unlabelled butyrate and 50 mM sulfate. *D. amnigenus* was chosen as a model organism, because it showed the highest isobutyrate accumulation during growth on butyrate. The NMR spectrum of the supernatant sample, taken after 3 weeks of incubation (Fig. 2), shows peaks of [2-^{13}C]acetate (24.2 ppm), [4-^{13}C]butyrate (14.2 ppm), [3-^{13}C]- or [4-^{13}C]isobutyrate (20.3 ppm), and [2-^{13}C]butyrate (40.5 ppm). Proton-coupled ^{13}C spectra showed that the peak at 20.3 ppm originated from a ^{13}C-labelled carbon-atom surrounded by 3 protons, which confirmed that the observed peak was indeed [3-^{13}C]- or [4-^{13}C]isobutyrate (20.3 ppm) and not [3-^{13}C]butyrate (20.2 ppm). These results clearly indicate that butyrate was degraded via β-oxidation [24] and that isobutyrate was formed by migration of the carboxyl group (Fig. 3), in analogy to butyrate isomerization in methanogenic or fermenting cultures [12,13]. Studies with the strict anaerobic glutarate fermenting bacterium strain WoG13 [13] and the aerobic bacterium *Streptomyces cinnamonensis* [25] showed that the reversible conversion of butyrate to isobutyrate required activation of these fatty acids to their CoA derivatives. The migration of the carboxyl group to the adjacent carbon atom was catalyzed by a coenzyme B_{12}-dependent isobutyryl-CoA mutase. Stereochemical studies with this isobutyryl-

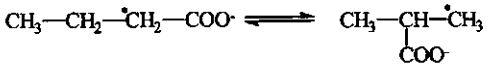

Fig. 3. Scheme of the rearrangement of [2-^{13}C]butyrate to [3-^{13}C]- and [4-^{13}C]isobutyrate by *D. amnigenus* cells. The labelled C atom is marked by a dot.

CoA mutase showed that it was not completely stereospecific. (2*S*)-[3-^{13}C]Isobutyryl-CoA was transformed predominantly into [2-^{13}C]butyryl-CoA, and also at a lower rate into [4-^{13}C]butyryl-CoA [25]. The formation of [4-^{13}C]butyrate in a *D. amnigenus* culture incubated with [2-^{13}C]butyrate (Fig. 2) could indicate that a similar isobutyryl-CoA mutase was active in this bacterium.

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CHAPTER 6

Detection and quantification of *Desulforhabdus amnigenus* in anaerobic granular sludge by dot blot hybridization and PCR amplification.

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Detection and quantification of *Desulforhabdus amnigenus* in anaerobic granular sludge by dot blot hybridization and PCR amplification

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S.J.W.H. OUDE ELFERINK, H.A. RINIA, M.E. BRUINS, W.M. DE VOS AND A.J.M. STAMS. 1997. A species-specific 16S rRNA oligonucleotide probe (ASRB1) was developed for the detection of *Desulforhabdus amnigenus* in anaerobic granular sludge. The presence of nucleic acids from cells of *D. amnigenus* in granular sludge was determined using ASRB1 as a specific primer for polymerase chain reaction (PCR) amplification or as a probe for dot blot hybridizations. The detection threshold and the reproducibility of these two methods were determined with sludge amended with 10^4–10^{10} *D. amnigenus* cells per gram of volatile suspended solids (VSS). For *D. amnigenus* cells with a ribosomal RNA content of 15 fg cell^{-1}, the lowest number of target cells detected by hybridization was 1×10^8 cells g^{-1} VSS. With the PCR amplification method the lowest number of target cells which could be detected was 1×10^7 g^{-1} VSS. This corresponds to a threshold level for hybridization of 0·1–0·001‰ of the total bacterial sludge population, while the threshold level obtained with the PCR approach amounted to 0·01–0·0001‰. The rRNA content of *D. amnigenus* was found to be affected by the growth rate and the growth phase, and it ranged from 19 fg cell^{-1} in slow-growing cultures to 90 fg cell^{-1} in fast-growing cultures. Therefore, the detection threshold of the dot blot hybridization method for fast-growing cells is lower than for slow-growing cells.

INTRODUCTION

Anaerobic wastewater treatment systems, based on the upflow sludge bed principle, represent a proven sustainable technology for a wide range of industrial effluents. The applied wastewater treatment systems (e.g. the UASB-reactor) function optimally when the active (granular) biomass consists of a well-balanced methanogenic consortium (Lettinga 1995). At present, our insight in the microbial composition of this anaerobic granular sludge is far from complete. This is partly due to the limitations of traditional identification techniques, which are often laborious, time consuming or give only limited information about the micro-organisms present in the sludge. In past decades, various methodologies have been developed that allow a rapid identification and quantification of bacteria in anaerobic bioreactors. Examples are detection methods based on direct visualization using transmission electron microscopy (Howgrave-Graham and Wallis 1993), use of specific antibodies against whole cells (Koornneef *et al.* 1990) or analysis of ether-lipids (Ohtsubo *et al.* 1993; Nishihara *et al.* 1995). Other well-established techniques which specifically detect microbes are based on 16S rDNA or rRNA sequences, and include the polymerase chain reaction (PCR) and 16S rRNA hybridization approaches (Amann *et al.* 1995). With the PCR approach a few target 16S rDNA genes can be amplified to make them detectable and quantifiable (Giovannoni 1991). Hiraishi and co-workers (1995) used the PCR technique in combination with restriction fragment length polymorphism analysis to identify the methanogenic population in anaerobic sludge, while Ng and co-workers (1994) applied the PCR technique in combination with 16S rRNA sequencing to identify several anaerobic digester bacteria. The 16S rRNA hybridization technique is directed against ribosomal RNA. In active cells, rRNA molecules have a relatively high copy number per cell (10^3–10^5), and can be detected without amplification (Amann *et al.* 1995). The 16S

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rRNA hybridization technique has therefore been used in many studies, and allowed for the detection and quantification of sulphate-reducing bacteria (Amann *et al.* 1992; Kane *et al.* 1993; Raskin *et al.* 1995a,b) and methanogens (Raskin *et al.* 1994, 1995a,b) in anaerobic biofilms and sludge.

There are several advantages of using 16S rDNA or rRNA as target material to detect micro-organisms. Currently, a huge database of sequence information is available (Benson *et al.* 1993; Rice *et al.* 1993; Maidak *et al.* 1994). This sequence information makes it possible to design primers and probes which allow for a species-specific or a more general identification (at genus or family level) of a bacterium. Furthermore, 16S rDNA primers and 16S rRNA probes can be designed and used for the detection of non-culturable bacteria (Amann *et al.* 1995). An additional advantage of 16S rRNA hybridization techniques is their potential to monitor changes in microbial activity. It has been demonstrated that there is a tight correlation between RNA content and growth rate for fast-growing bacteria (doubling time of 25 min to a few hours) like *Escherichia coli* (Gausing 1977) and *Salmonella typhimurium* (Kjelgaard and Kurland 1963). This correlation between RNA content and growth rate is also observed for slow-growing bacteria, like *Pseudomonas stutzeri* (Kerkhof and Ward 1993), several marine isolates (Kemp *et al.* 1993) and the sulphate reducer strain PT2 (Poulsen *et al.* 1993), which all could grow with doubling times of a few hours to a few days. Starving or slowly growing cells contain only low numbers of ribosomes (Flärdh *et al.* 1992; Givskov *et al.* 1994; Fukui *et al.* 1996). This correlation between RNA content and cell activity makes it possible to monitor changes in microbial activity rather than cell numbers, using 16S rRNA probing techniques.

In anaerobic systems treating sulphate-containing wastewater, sulphate reducers compete with methanogens for substrates like hydrogen and acetate. Mostly, sulphate reduction is an unwanted process, but sometimes maximal sulphate reduction is desired, e.g. for the removal of oxidized sulphur compounds or heavy metals (Oude Elferink *et al.* 1994). Insight into the microbial sludge composition is desired to steer a process, either in the direction of methanogenesis or in the direction of sulphate reduction. Acetate is an important intermediate in the anaerobic degradation process. The outcome of the competition for acetate is not always the same. In some reactors acetate is mainly degraded via methanogenesis, even at high sulphate concentrations, while in other reactors acetate is predominantly degraded via sulphate reduction (Oude Elferink *et al.* 1994). Therefore, the detection and enumeration of acetate-degrading sulphate reducers in granular sludge had our special interest. Little is known about these bacteria in freshwater anaerobic bioreactors. Recently, we were able to isolate a novel acetate-degrading sulphate reducer from anaerobic granular sludge of an upflow anaerobic sludge blanket (UASB) reactor treating papermill wastewater. This bacterium, designated *Desulforhabdus amnigenus* strain ASRB1, was present in the sludge in higher numbers than aceticlastic methanogens (Oude Elferink *et al.* 1995a). Here, we describe the development of dot blot hybridization and PCR amplification, for the detection and enumeration of *D. amnigenus* in anaerobic granular sludge, and evaluate the effect of growth rate and growth phase on the cellular rRNA content of *D. amnigenus*. Furthermore we analyse the presence of *D. amnigenus* in some methanogenic and sulphidogenic sludges.

MATERIALS AND METHODS

Organisms and growth conditions

Desulforhabdus amnigenus strain ASRB1 was cultured at 37°C in 120-ml serum vials containing 50 ml of a bicarbonate-buffered medium as described before (Oude Elferink *et al.* 1995a). Growth was followed by measuring substrate utilization and sulphide production. *Syntrophobacter pfennigii* (DSM 10092) was kindly provided by B. Schink (Universität Konstanz, Germany). *Syntrophobacter fumaroxidans* strain MPOB (DSM 10017) (Harmsen 1996), *Clostridium granularum* EE121 (Grotenhuis 1992), a thermophilic *Desulfotomaculum* sp. and a mesophilic acetate-degrading sulphate-reducing strain ASRB2, were all isolated from anaerobic granular sludge and were obtained from this laboratory collection. All other organisms were obtained from the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany).

Sludge types

For enumeration of *D. amnigenus* in anaerobic granular sludge, mesophilic sludge was obtained from a full-scale UASB reactor (reactor 1, Table 1), treating papermill wastewater (Eerbeek, The Netherlands). In this sludge, acetate was mainly degraded via methanogenesis. The number of acetate-degrading methanogens and sulphate reducers in this sludge, estimated via the most probable number method, was 1×10^8 and 1×10^5 cells g^{-1} volatile suspended solids (VSS), respectively (Oude Elferink *et al.* 1995b).

The presence of *D. amnigenus* in mesophilic anaerobic granular sludges was tested using sludges from 15 methanogenic and five sulphidogenic full-scale and laboratory-scale UASB reactors, treating complex industrial wastewaters or (mixtures of) volatile fatty acids (VFA). One of the sulphidogenic sludges was analysed twice, with a 2 year interval. During these 2 years the chemical oxygen demand (COD)/sulphate ratio had increased from 2·4 (reactor 3a, Table 1) to 4 (reactor 3b, Table 1). Granular sludge from the pilot-scale UASB reactor from which *D. amnigenus* originally was isolated, was used as a control (reactor 2, Table 1). The

Reactor	Reactor scale	COD/sulphate ratio	Influent	Hybridization signal
1	Full-scale	9·5	Papermill waste	−
2	Pilot-scale	1·1	Papermill waste	+
3a	Full-scale	2·4	Papermill waste	+
3b		4		−
4	Lab-scale	0·5	VFA*	−
5	Lab-scale	0·5	Acetate	−

Table 1 Summary of the nucleic acid extracts of sulphidogenic sludges used in this study, and the results of dot blot hybridizations with the ASRB1 probe

* Acetate:propionate:butyrate = 1:2:2.

most probable number of *D. amnigenus* in this sludge was $\approx 10^9$ cells g^{-1} VSS (Oude Elferink *et al.* 1995a).

Analysis

All organic compounds were measured by HPLC as described by Stams *et al.* (1993). Sulphide was determined as described by Trüper and Schlegel (1964). Volatile suspended solids were determined according to standard methods (Anon. 1985).

Isolation of nucleic acids

Nucleic acids were extracted from cultures by the following procedure: 10 ml of a culture were centrifuged at 17 000 *g* for 15 min, the pellet was resuspended in 400 µl of autoclaved TE buffer (10 mmol l^{-1} Tris/HCl, 1 mmol l^{-1} EDTA, pH 8·0) and transferred to a 1·5 ml Eppendorf tube. Two hundred µl of Tris/HCl buffered phenol (pH 8·0) were added together with *ca* 300 µl of glass beads (diam. 0·11 mm). The cells were disrupted by treatment for 5 min in a cell homogenizer model MSK (Braun, Melsungen, Germany) under CO_2 cooling. The aqueous phase of the supernatant fluid, obtained after 10 min of centrifugation (15 000 *g*), was extracted with phenol/chloroform/isoamyl alcohol 25:24:1 (v:v:v), followed by chloroform/isoamyl alcohol 24:1 (v:v). Subsequently, the volume of the sample was adjusted to 0·5 ml, and nucleic acids were precipitated with 1 ml of 96% ethanol or 0·5 ml of isopropanol, and 40 µl of sodium acetate (3 mol l^{-1}, pH 5·2) at −70°C for 30 min. After 15 min centrifugation (15 000 *g*) the nucleic acid pellet was washed with 70% ethanol, dried under vacuum and resuspended in 100 µl of TE buffer. This mixture of DNA and rRNA was used for PCR and dot blot hybridization after judging the quality of the extract by agarose gel electrophoresis and ethidium bromide staining. Nucleic acids from granular sludge were extracted in essentially the same way; 10 ml of each sludge sample were disintegrated using a mortar and pestle, 100 µl (0·01 g VSS) of this crushed granular sludge were used for nucleic acids extraction. Besides analysing the sludge nucleic acid extracts on agarose gel, the presence of bacterial rRNA in the sludge samples was determined by dot blot hybridizations using the EUB338 probe, as described below.

16S rRNA targeted oligonucleotide probes

An oligonucleotide hybridization probe for *Desulforhabdus amnigenus* strain ASRB1 was designed using a reference collection of 16S rRNA sequences of sulphate reducers taken from the ribosomal database project RDP (Maidak *et al.* 1994), and the EMBL and GenBank data libraries (Benson *et al.* 1993; Rice *et al.* 1993). The sequences for the ASRB1 probe and the other probes used in this study, are listed in Table 2. All oligonucleotides were synthesized by Pharmacia (Uppsala, Sweden).

The probe specificity of the ASRB1 probe was examined

Table 2 The oligonucleotide probes used in this study (the number in brackets in the target column refers to the start of the target-site (*Escherichia coli* numbering))

Name oligo	Target	Sequence	T_d (°C)	Reference
ASRB1	*Desulforhabdus amnigenus* (454)	5′-GGCCTATTCGACCCCCAATC	58	This study
MPOB1	*D. amnigenus*, (222) *S. fumaroxidans*, *S. pfennigii*	5′-ACGCAGGCCCATCCCCGAA*	63	Harmsen *et al.* 1995, 1996
EUB338	Bacteria (338)	5′-GCTGCCTCCCGTAGGAGT	52	Amann *et al.* 1992

* The underlined C matches with the G of the two *Syntrophobacter* 16S rRNA but mismatches with an A of the *D. amnigenus* 16S rRNA (Harmsen *et al.* 1996).

with the dot blot hybridization method described below, using membranes blotted with nucleic acids from various sulphate reducers and from other micro-organisms isolated from anaerobic bioreactors. The dissociation temperature (T_d) of the ASRB1 probe was determined as described by Devereux *et al.* (1992).

Dot blot hybridizations

Dot blot hybridization experiments were performed on Hybond N+ filters (Amersham, Little Chalfont, UK). Nucleic acid samples (10 μl) were applied to the membrane with a Convertible Filtration manifold (Gibco BRL, Life Science Technologies, Gaithersburg, MD, USA) and immobilized by u.v.-light (4 min). Prior to hybridization, the membranes were pretreated with 10 ml of hybridization buffer (0·5 mol l^{-1} phosphate buffer, 7% sodium dodecyl sulphate (SDS), 1% bovine serum albumin and 1 mmol l^{-1} EDTA, pH 7·2) for 30–45 min. The oligonucleotide probes were 5′-end labelled with ^{32}P by using polynucleotide kinase and [γ-^{32}P]ATP. One hundred ng (1 μl) of the probe were mixed with 2 μl of 10 × kinase buffer (Sambrook *et al.* 1989), 1 μl (10 U) of T4 polynucleotide kinase (Gibco BRL), 1·5 μl of [γ-^{32}P]ATP (3000 Ci mmol^{-1}, Amersham) and water to obtain a total volume of 20 μl. This mixture was incubated at 37°C for 30 min.

The membranes were hybridized overnight at a temperature of 10°C below the dissociation temperature T_d of the probe, rinsed once with 1 mmol l^{-1} EDTA, 5 × SSC (1 × SSC = 0·15 mol l^{-1} NaCl, 0·015 mol l^{-1} sodium citrate, pH 7·0), and washed in 1% SDS, 1 × SSC at 5°C below the T_d. The membranes were dried and exposed to a phospho storage screen for 2 h, and the screen was scanned for radioactive response on a Phosphor Imager (Molecular Dynamics, Sunnyvale, USA). The digital signals were processed and quantified by the manufacturer's software (ImageQuant). Blots were stripped for reprobing by incubating the membranes in 0·1% SDS, for 30 min at 80°C.

PCR amplification

Part of the 16S rRNA gene of *D. amnigenus* was amplified with the PCR technique, using forward primer 8 (5′-AGAGTTTGATC(C/A)TGGCTCAG) (Lane 1991) or forward primer MPOB1 (5′-TTCGGGGATGGGCCT-GCGT) (Harmsen *et al.* 1995) and reverse primer ASRB1 (see Table 2). These primer sets amplify nucleotides 8 to 474 and nucleotides 222 to 474, respectively. Unless stated otherwise, the reactions were carried out in sterile 0·5 ml tubes containing 50 μl of the following buffer: 75 mmol l^{-1} Tris–HCl (pH 9), 0·1‰ (w/v) Tween 20, 20 mmol l^{-1} $(NH_4)_2SO_4$, 1·25 mmol l^{-1} $MgCl_2$, 0·1% (v/v) glycerol, 20 μmol l^{-1} K_3PO_4, 0·2 mmol l^{-1} NaCl, 0·2 μmol l^{-1} EDTA, 4 μmol l^{-1} DTT, 0·5 U of Thermostrong DNA polymerase (Integro, Zaandam, The Netherlands), 0·1 mmol l^{-1} (each) deoxynucleoside triphosphates, and 50 ng (each) of primers ASRB1 and 8 or MPOB1. One μl of template DNA was added. PCR amplification was performed by using a DNA thermal cycler (Perkin Elmer Cetus, Gouda, The Netherlands). Amplification was done in 30 cycles of melting DNA at 93°C for 1 min, annealing at 54°C for 1 min and elongation at 72°C for 2 min.

Enumeration of *D. amnigenus* in anaerobic granular sludge

Varying numbers (1×10^2–1×10^8) of *D. amnigenus* cells from a propionate-grown, stationary phase culture, were mixed with 100 μl of crushed granular sludge before nucleic acids were extracted. The experiment was performed in triplicate. Appropriate dilutions of nucleic acids extracted from 1×10^8 cells of the same *D. amnigenus* culture served as a control.

RNA content per cell

To obtain different growth rates, batch cultures of *D. amnigenus* cells were grown with acetate, propionate or ethanol as a substrate. Growth rates were determined by measuring substrate conversion and sulphide production. Nucleic acids were extracted from $1{\cdot}1 \times 10^8$–$2{\cdot}3 \times 10^8$ cells from mid-logarithmic and stationary phase cultures (1 to 2 weeks after substrate depletion). A 10 μl aliquot of each sample was applied on an agarose gel. The gel image was visualized on a monitor with a CCD camera, and the signal was captured and digitalized using the software packages ColorVision and PhotoStyler. The 16S rRNA band signal intensity was quantified (ImageQuant). All mid-logarithmic and some stationary phase cultures were also analysed via dot blot hybridization with the EUB338 probe. An RNA standard curve was made with purified *Escherichia coli* 16S and 23S rRNA (Boehringer Mannheim Biochemicals, Almere, The Netherlands), the integrity of this commercial preparation was evaluated on an agarose gel. The RNA content of the standard samples was measured spectrophotometrically (Beckman DU640 spectrophotometer), assuming that an optical density at 260 nm of 1 was equivalent to 40 μg ml^{-1}. The ribosomal RNA content of the *D. amnigenus* cells was estimated using the RNA standard curve, assuming that the molar ratio between 5S rRNA (120 nucleotides), 16S rRNA ($\approx$1600 nucleotides) and 23S rRNA ($\approx$3500 nucleotides) was 1:1:1 in the nucleic acid extracts.

RESULTS

Probe design and characterization

The 16S rRNA gene sequence of *D. amnigenus* was used to develop a specific oligonucleotide probe (Table 2). The

ASRB1 probe was developed against a region which had more than three mismatches with the sequences present in the database. The specificity of the ASRB1 probe for *D. amnigenus* was tested by dot blot hybridization. The only pure culture nucleic acid extracts which hybridized with the ASRB1 probe were from a pure culture of *D. amnigenus* (Fig. 1). This strongly indicates that the ASRB1 probe is highly specific for *D. amnigenus*.

Quantification of *D. amnigenus* via dot blot hybridization

To investigate the accuracy and detection threshold of the dot blot hybridization method for the detection of *D. amnigenus* in anaerobic granular sludge, different numbers of *D. amnigenus* cells were mixed with crushed granular sludge. The unamended sludge did not react with the ASRB1 probe, but gave a reaction with the MPOB1 probe. The intensity of the hybridization signal with the ASRB1 probe increased with increasing amounts of target sequences present in nucleic acids extracts from mixed sludge samples as well as from pure cultures of *D. amnigenus* (Fig. 2). As a control, the same blot was also hybridized with the EUB338 probe. The intensity

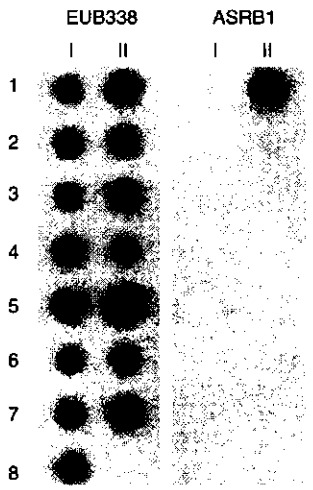

Fig. 1 Dot blot analysis of the ASRB1 probe specificity. Nucleic acids of the reference organisms were immobilized on the membranes as follows (column, row): column I – 1, *Desulfovibrio vulgaris* DSM 644; 2, *Desulfobacterium autotrophicum* DSM 3382; 3, *Desulfobacterium vacuolatum* DSM 3385; 4, *Desulfoarculus baarsii* DSM 2075; 5, *Desulfobulbus propionicus* DSM 2032; 6, *Desulfobacter latus* DSM 3381; 7, thermophilic *Desulfotomaculum* sp.; 8, mesophilic acetate-degrading sulphate reducer strain ASRB2; column II – 1, *Desulforhabdus amnigenus*; 2, *Syntrophobacter fumaroxidans* DSM 10017; 3, *Syntrophobacter wolinii* DSM 2805; 4, *Syntrophobacter pfennigii* DSM 10092; 5, *Bacteroides xylanolyticus* X5-1 DSM 3808; 6, *Clostridium granularum* EE121; 7, *Syntrophospora bryantii* DSM 3014B; 8, *Methanosaeta* (*Methanothrix*) *soehngenii* DSM 2139

(a)

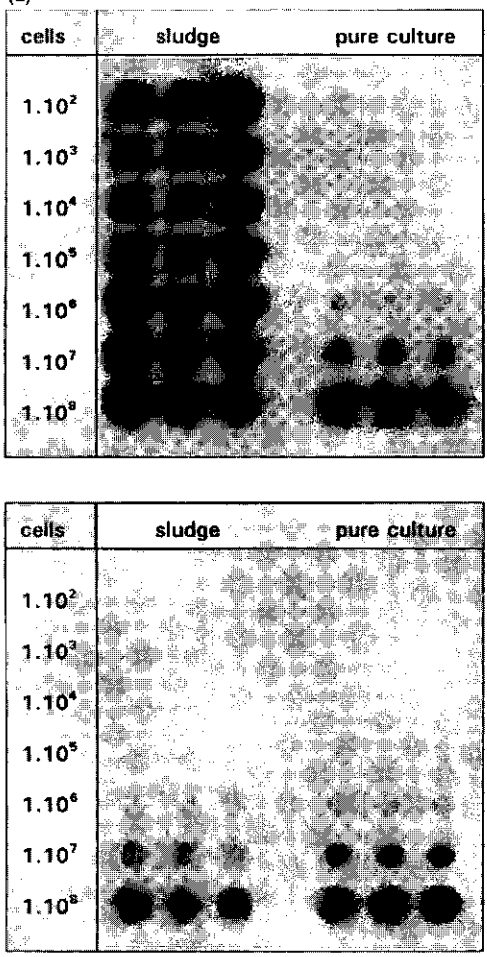

Fig. 2 Dot blot hybridization of (a) EUB338 and (b) ASRB1 probes to nucleic acids extracted from anaerobic sludge mixed with *Desulforhabdus amnigenus* (first three lanes), and from pure cultures of *D. amnigenus* (last three lanes). On the left, the cell number of *D. amnigenus* in the original sample is shown. Each dot from the sludge series represents one extraction. The three pure culture dilution series are originating from 10-fold dilutions of nucleic acids extracted from three samples of 1×10^8 *D. amnigenus* cells

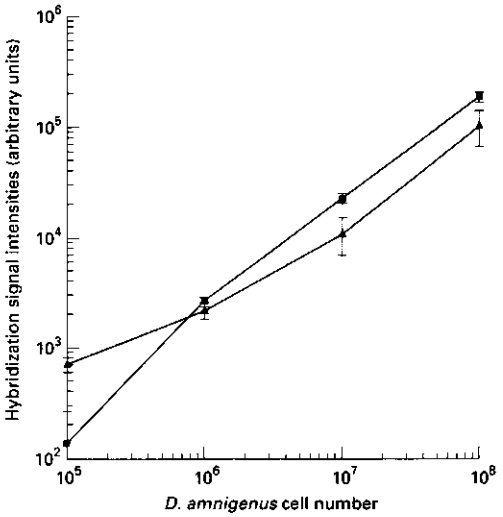

Fig. 3 Relationship between hybridization signal intensities (arbitrary units), determined from the phosphor screen, and *Desulforhabdus amnigenus* cell numbers. The squares correspond to *D. amnigenus* in pure culture, the triangles correspond to *D. amnigenus* in sludge. The error bars represent the standard deviations. The cells contained 15 fg rRNA $cell^{-1}$

of the hybridization signal from the ASRB1 probe with pure culture extracts of *D. amnigenus* was comparable to that obtained with the EUB338 probe (Fig. 2). The *D. amnigenus* cells used in this experiment contained 15 fg of rRNA per cell, as was determined with the rRNA standard curve of *E. coli* rRNA (results not shown).

The hybridization signals obtained with the ASRB1 probe were quantified and showed that the signal obtained with 1×10^2–1×10^4 cells of *D. amnigenus* in pure culture or in sludge was very weak and fell within the range of the background signal intensities. A linear relationship was observed between the *D. amnigenus* cell number and the signal intensity, if 1×10^5 or more cells of *D. amnigenus* were present in pure culture or in sludge (Fig. 3). The signal intensity variation from the triplicate sludge samples was higher than for the pure culture samples as is indicated by the error bars. Furthermore, the signal intensities from the sludge samples were lower than that from pure culture samples with the same number of *D. amnigenus* cells. This suggests that the recovery of *D. amnigenus* rRNA from sludge was not as good as from pure cultures.

The presence of *D. amnigenus* in anaerobic sludges

The number of *D. amnigenus* in different methanogenic and sulphidogenic sludges was estimated using the dot blot hybridization method and the calibration curve presented in Fig. 3. Although all nucleic acid extracts hybridized with the EUB338 probe (results not shown), none of the extracts from the 15 methanogenic sludges tested, hybridized with the ASRB1 probe (results not shown). However, two of the nucleic acid extracts from five sulphidogenic sludges, did react with the ASRB1 probe (Table 1). One of these extracts was obtained from the sludge from which *D. amnigenus* was originally isolated (reactor 2). The number of *D. amnigenus* cells in this sludge was $\approx 2 \times 10^9$ g^{-1} VSS. In the sludge from reactor 3a, the number of *D. amnigenus* cells was just above the detection threshold of the dot blot hybridization method, $\approx 3 \times 10^7$ cells g^{-1} VSS.

Quantification of *D. amnigenus* via PCR amplification

To investigate the accuracy and detection threshold of the PCR amplification for the detection of *D. amnigenus* in anaerobic sludge, the same samples were used as for the dot blot hybridizations (Fig. 2). PCR amplification with primers 8 and ASRB1, using one dilution series of *D. amnigenus* in pure culture and one in sludge, gave a specific PCR product which showed the expected size of 470 bp (Fig. 4). The two other dilution series yielded similar results. The detection threshold of the PCR amplification was $\approx 1 \times 10^5$ *D. amnigenus* cells for pure culture samples as well as for sludge samples. With pure culture samples the detection threshold for *D. amnigenus* could be lowered to 1×10^3 cells, if the annealing temperature was lowered to 51°C and the number of cycles was increased to 35. However, under these conditions many unspecific PCR products were obtained with the sludge samples.

Primer set MPOB1 and ASRB1 was not suitable for the detection of *D. amnigenus* in sludge, because even under stringent conditions, unspecific PCR products were visible. With pure cultures of *D. amnigenus* this problem was not observed. From dot blot hybridization experiments with the

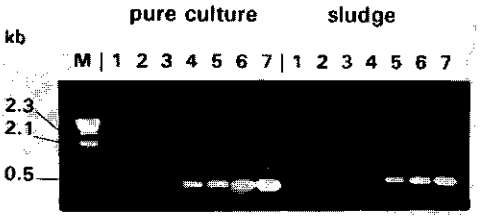

Fig. 4 Agarose gel electrophoresis of PCR products obtained with primer set 8 and ASRB1. Pure culture lanes 1–7 correspond to 1×10^2 to 1×10^8 *D. amnigenus* cells; sludge lanes 1–7 correspond to 1×10^2 to 1×10^8 *D. amnigenus* cells mixed with sludge. Lane M is marker lambda *Hin*dIII, size markers are shown on the left

MPOB1 and the EUB338 probe it was calculated that ≈0·5% of the bacterial rRNA hybridized with the MPOB1 probe.

RNA content per cell

The RNA content of *D. amnigenus* cells was determined for mid-logarithmic and stationary phase cultures (Table 3). Growth rate and rRNA content were related to each other. The growth rates on propionate and ethanol are approximately three times higher than the growth rate on acetate. Mid-logarithmic propionate and ethanol-grown cells contained approximately four times more rRNA than acetate-grown cells. Stationary phase cultures had a much lower rRNA cell content than mid-logarithmic cultures; for propionate-grown cells, an eightfold decrease was observed, but acetate-grown cells only lost half of their original rRNA content. Depending on the growth rate and growth phase, the rRNA content of *D. amnigenus* cells varied by a factor 10.

DISCUSSION

Dot blot hybridization as well as PCR amplification, using primer set 8 and ASRB1, are suitable for the detection of *D. amnigenus* in anaerobic granular sludge. Why PCR amplification with primer set MPOB1 and ASRB1 could be used for the quantification of *D. amnigenus* cells in pure cultures, but was not suitable for the detection of *D. amnigenus* in sludge, is not clear. Maybe the presence of MPOB1 target sequences in the sludge nucleic acid extract (≈0·5% of the bacterial rRNA hybridized with the MPOB1 probe) influenced the PCR amplification results.

It is well known that the rRNA content of cells is not constant, but depends on the growth rate (Gausing 1977; Kemp *et al.* 1993; Kerkhof and Ward 1993; Givskov *et al.* 1994). Therefore, it is difficult to link a 16S rRNA hybridization signal accurately to a cell number. To estimate the effect of the growth rate on our detection and quantification results, rRNA contents of mid-logarithmic and stationary phase cultures of *D. amnigenus* were determined. The rRNA cell content between slow-growing (acetate) and fast-growing (ethanol, propionate) cultures ranged from 19 to 67–90 fg cell^{-1}. This corresponds to the rRNA range of ≈10–60 fg cell^{-1} that Kemp *et al.* (1993) reported for several marine bacteria, although these bacteria reached much higher specific growth rates (0·15 h^{-1}). In stationary phase (1–2 weeks after substrate depletion), cellular rRNA levels decreased to 50% (acetate) or 13% (propionate) of the original level. Similar results were reported for a marine *Vibrio* sp. that showed a decrease in cellular rRNA levels of 85% after 9 d of glucose starvation (Flärdh *et al.* 1992) and *Pseudomonas putida* that showed a decrease of 78% after 30 d of starvation. Recently, Fukui *et al.* (1996) demonstrated that the cellular 16S rRNA content of *Desulfobacter latus*, a marine acetate-degrading sulphate reducer, decreased exponentially during the first 48 h of starvation, but then remained stable at 30% of the 16S rRNA level obtained for exponentially growing cells.

Table 3 The rRNA content per cell

Substrate	Maximum specific growth rate μ_{max} (h^{-1})	rRNA content (fg cell^{-1}) mid-logarithmic phase	rRNA content (fg cell^{-1}) stationary phase
Acetate	$0{\cdot}007 \pm 0{\cdot}8 \times 10^{-3}$	19 ± 2	9 ± 3
Propionate	$0{\cdot}019 \pm 1{\cdot}3 \times 10^{-3}$	90 ± 5	12 ± 3
Ethanol	$0{\cdot}018 \pm 1{\cdot}3 \times 10^{-3}$	67 ± 5	25 ± 10

Detection thresholds presented below were calculated for stationary phase propionate-grown cultures, containing 15 fg rRNA cell^{-1}. Thresholds for *D. amnigenus* in sludge were almost the same for dot blot hybridization and PCR amplification detection, and were 1×10^8 and 1×10^7 cells g^{-1} VSS, respectively. For dot blot hybridization, this detection threshold corresponds to a *D. amnigenus* rRNA concentration of 1500 ng g^{-1} VSS. A detection threshold concentration of 1×10^7–1×10^8 cells g^{-1} VSS is quite satisfying for the detection of dominant microbes in sludge, especially if the total number of micro-organisms per g VSS of anaerobic granular sludge is taken into account. Viable cell numbers of 1×10^{12} to 1×10^{14} g^{-1} VSS are reported (Dubourguier *et al.* 1988). This indicates that even with the dot blot method, *D. amnigenus* can still be detected if only one out of 1×10^4–1×10^6 sludge bacteria is a *D. amnigenus* cell, provided that the total number of *D. amnigenus* cells in the sample is at least 1×10^6. For fast-growing cells, this detection threshold will probably be lower, because of the higher rRNA content of these cells; for mid-logarithmic phase propionate-grown cells (90 fg cell^{-1}), the detection threshold can even be up to sixfold lower.

The described PCR amplification method, using primer set 8 and ASRB1, was only 10 times more sensitive than the dot blot hybridization method, but this threshold can probably be lowered further, if two *D. amnigenus*-specific primers are used, instead of only one.

The quantification of *D. amnigenus* cells in sludge, via the dot blot hybridization method or the PCR amplification method, is not affected by the presence of acetate-degrading methanogens. This gives both methods an advantage over the MPN technique. The MPN technique is based on the assumption that each single cell will grow in high dilutions. However, if the acetate-degrading sulphate reducers are outnumbered by the acetate-degrading methanogens in the sludge, this will hamper an accurate estimation of the total number of acetate-degrading sulphate reducers present, and

the characterization of the dominant acetate-degrading sulphate reducers.

For quantification of *D. amnigenus* in sludge, both dot blot and PCR approaches could be used. The dot blot method showed a linear correlation between cell number and hybridization signal. The calibration curve presented in Fig. 3 can be used to estimate the number of *D. amnigenus* cells in a sludge sample. However, the accuracy of the enumeration will be influenced by the rRNA content of the cells. The calibration curve was determined with cells containing 15 fg rRNA $cell^{-1}$. An overestimation of the cell number is possible, if cells contain more rRNA per cell.

With PCR amplification, no linear correlation between cell number and amount of PCR product was measured. To use PCR for quantification of *D. amnigenus* in sludge, serial (10-fold) dilutions of the purified nucleic acids from the sludge sample should be amplified, in order to calculate the number of cells in the original sample.

The dot blot hybridization method was used to determine the presence of *D. amnigenus* in anaerobic sludges. *Desulforhabdus amnigenus* could not be detected in the methanogenic sludges. This was expected, because very little sulphate was present in these reactors, and to our knowledge *D. amnigenus* has no fermentative or syntrophic capacities (Oude Elferink *et al.* 1995a). *Desulforhabdus amnigenus* could be detected in two of the sulphidogenic sludges. One of these sludges was the sludge from which *D. amnigenus* was originally isolated (reactor 2). The number of *D. amnigenus* cells in this sludge, estimated with the dot blot hybridization method (2×10^9 g^{-1} VSS), was in the same range as the number of *D. amnigenus* cells estimated by serial dilutions (10^9 g^{-1} VSS) (Oude Elferink *et al.* 1995a). The other sludge in which *D. amnigenus* could be detected was also from a reactor treating papermill wastewater (reactor 3a). Two years later the same reactor was analysed again (reactor 3b), but *D. amnigenus* could no longer be detected in the sludge. Maybe this decrease of the *D. amnigenus* population was due to the increase of the COD/sulphate ratio in this reactor. Under sulphate-limited conditions, *D. amnigenus* has to compete with methanogens for acetate, but also with other sulphate reducers for the available sulphate. The fact that *D. amnigenus* could not be detected in the sludge from reactors 4 and 5, although these reactors operated at an excess of sulphate, and part of the acetate in these reactors was degraded via sulphidogenesis (unpublished results), strongly indicates that *D. amnigenus* is not the only acetate-degrading sulphate reducer which can play a role in sulphidogenic reactors.

In conclusion, both dot blot hybridization and PCR amplification can be used for the detection and quantification of *D. amnigenus* in anaerobic sludge. The PCR amplification technique showed a lower detection threshold for *D. amnigenus* cells, but was less quantitative than the dot blot hybridization method. The dot blot hybridization method might also be suitable for determination of major activity changes of *D. amnigenus* cells in sludge, because the method is sufficiently sensitive to detect the variation in the cellular rRNA content of fast-growing, slow-growing or starving *D. amnigenus* cells. This offers interesting possibilities to monitor microbial activity of a reactor rather than cell numbers. However, the method does not allow the detection of small changes in rRNA concentrations. This may be realized with an rRNA-based PCR amplification approach, using reverse transcriptase to make double-stranded rRNA (RT-PCR) (Amann *et al.* 1995).

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CHAPTER 7

Characterization of the sulfate-reducing and syntrophic population in granular sludge from a full-scale anaerobic reactor treating papermill wastewater.

Submitted as:

Oude Elferink SJWH, Vorstman WJC, Sopjes A, Stams AJM. Characterization of the sulfate-reducing and syntrophic population in granular sludge from a full-scale anaerobic reactor treating papermill wastewater.

Abstract

To gain more insight in the competitive and syntrophic interactions between sulfate-reducing and syntrophic methanogenic consortia in sulfate-limited anaerobic reactors, the microbial population in granular sludge from a full-scale upflow anaerobic sludge blanket (UASB) reactor was studied, by using the 16S rRNA dot-blot hybridization method in combination with most probable number (MPN) estimates. The wastewater which was treated in the UASB-reactor contained mainly starch, acetate, propionate, butyrate and formate, and had a chemical oxygen demand (COD)/sulfate ratio of 9.5. Evidence was obtained that acetate was mainly degraded by *Methanosaeta*-like bacteria, while propionate was the preferred substrate for sulfate reduction. The *Desulfobulbus*-like propionate-degrading sulfate reducers in the sludge competed with *Syntrophobacter*-like bacteria for the available propionate. Hydrogen and formate were probably mainly degraded via methanogenesis by members of the order of *Methanobacteriales*. Hydrogen, formate and butyrate-degrading sulfate reducers could not be characterized with the 16S rRNA probes available to date. The same was true for syntrophic butyrate degraders.

1 Introduction

It has long been recognized that the degradation pathway of complex organic compounds in anaerobic wastewater treatment systems is strongly influenced by the presence of sulfate in the wastewater. The first steps in the anaerobic degradation process are the hydrolysis and fermentation of biopolymers like carbohydrates and proteins to intermediates such as propionate, butyrate, formate and $H_2 + CO_2$ by fermenting bacteria. In the absence of sulfate, propionate and butyrate are degraded by acetogens to acetate, formate, and hydrogen, which are then converted by methanogens [1,2] according to the equations shown in Table 1. Because the conversion of propionate and butyrate by acetogens is thermodynamically only favourable at a low H_2 partial pressure and a low formate concentration, acetogens degrade these compounds in syntrophy with hydrogenotrophic methanogens [2]. In the presence of sulfate the anaerobic degradation process becomes more complex, because sulfate reducers will compete with acetogens and methanogens for propionate, butyrate, acetate, hydrogen and formate (Table 1), by coupling the oxidation of these compounds to sulfate reduction [5,6]. The chemical oxygen demand (COD)/sulfate ratio of the wastewater determines which part of the COD can be degraded via sulfate reduction. In theory all COD can be degraded via sulfate reduction if the COD/sulfate (g/g) ratio is below 0.66 (mole COD/ mole sulfate <0.5). Studies on the competition between

Table 1. Stoichiometry of some anaerobic degradation reactions

Reaction		Reaction number
Syntrophic acetogenic reactions		
propionate$^-$ + 3 H_2O	→ acetate$^-$ + HCO_3^- + H^+ + 3 H_2	1
butyrate$^-$ + 2 H_2O	→ 2 acetate$^-$ + H^+ + 2 H_2	2
propionate$^-$ + 2 HCO_3^-	→ acetate$^-$ + 3 formate$^-$ + H^+	3
butyrate$^-$ + 2 HCO_3^-	→ 2 acetate$^-$ + 2 formate$^-$ + H^+	4
Methanogenic reactions		
acetate$^-$ + H_2O	→ CH_4 + HCO_3^-	5
H_2 + 0.25 HCO_3^- + 0.25 H^+	→ 0.25 CH_4 + 0.75 H_2O	6
formate$^-$ + 0.25 H_2O + 0.25 H^+	→ 0.25 CH_4 + 0.75 HCO_3^-	7
Sulfidogenic reactions		
propionate$^-$ + 0.75 SO_4^{2-}	→ acetate$^-$ + HCO_3^- + 0.75 HS^- + 0.25 H^+	8
butyrate$^-$ + 0.5 SO_4^{2-}	→ 2 acetate$^-$ + 0.5 HS^- + 0.5 H^+	9
butyrate$^-$ + 1.5 SO_4^{2-}	→ acetate$^-$ + 2 HCO_3^- + 1.5 HS^- + 0.5 H^+	10*
acetate$^-$ + SO_4^{2-}	→ 2 HCO_3^- + HS^-	11
H_2 + 0.25 SO_4^{2-} + 0.25 H^+	→ 0.25 HS^- + H_20	12
formate$^-$ + 0.25 SO_4^{2-} + 0.25 H^+	→ 0.25 HS^- + HCO_3^-	13

* Several completely oxidizing sulfate reducers form (some) acetate during butyrate degradation [3,4].

sulfate reducers and methanogens for hydrogen, showed that in anaerobic reactors operated with excess sulfate, hydrogen was completely used by the sulfate reducers [7,8]. For acetate the outcome of the competition is less clear. In some reactors a predominance of sulfidogenic acetate conversion has been found [8-10], whereas in other reactors acetate was mainly converted by methanogens, even at an excess of sulfate [7,10-12].

Little is known about the competition between acetogens and sulfate reducers for propionate and butyrate, because in most reactor studies mass balances were used to determine the competition between sulfate reducers and syntrophic consortia [7,8,10-12]. Therefore, no distinction could be made between a direct propionate and butyrate conversion via sulfate reduction (reaction 8 and 9, Table 1), or an indirect conversion, whereby propionate and butyrate are first degraded to acetate and H_2 or formate (reaction 1 and 2, or 3 and 4) by acetogens, followed by H_2 or formate conversion via sulfate reduction (reaction 12 or 13). However, many reactor studies indicated that the, direct or indirect, conversion of propionate and butyrate via sulfate reduction is very important in sulfidogenic bioreactors with low COD/sulfate ratios [8,10, 12-14].

In sulfate-limited reactors the degradation pathway of organic compounds can become very complex. Besides the "normal" competition between sulfate reducers and syn-

trophic consortia as described above, sulfate reducers will compete with each other for the available sulfate. In competition studies with sulfate-limited anaerobic reactors, acetate seemed to be the least favoured substrate for sulfate reduction. Unfortunately, a preference for propionate, butyrate or hydrogen was not determined [12,14, 15]. Furthermore, some sulfate reducers have fermentative or syntrophic capacities, and can grow in the absence of sulfate on compounds such as propionate, lactate, and ethanol. These fermentative and/or syntrophic sulfate reducers have been shown to play an important role in sulfate-depleted reactors [16-18].

In general, our knowledge about the competition between sulfate reducers and methanogenic consortia, especially in sulfate-limited reactors, is still very limited, and only fragmentary information is available on the species which are involved. This lack of knowledge hampers the development of practical procedures to control sulfate reduction and methanogenesis in the bioreactor. The aim of this study was to gain more insight in the competitive and syntrophic interactions between sulfidogenic and syntrophic consortia in sulfate-limited reactors. For this purpose, the microbial population in granular sludge from a full-scale anaerobic bioreactor, which had been fed with papermill wastewater (COD/sulfate= 9.5) for more than 5 years, was examined by using the 16S rRNA hybridization technique in combination with the conventional most probable number (MPN) enumeration technique, and microscopical examinations.

2 Materials and methods

2.1 Granular sludge origin

Granular sludge was obtained from a full-scale (2500 m^3) upflow anaerobic sludge blanket (UASB) reactor treating sulfate-rich papermill wastewater. The COD/SO_4^{2-} ratio of the wastewater was 9.5, the total COD load of the wastewater was 1.7 g/l, and the pH of the effluent was approximately 6.9. The reactor was maintained at a constant hydraulic retention time (HRT) of 4.6 hours, and a temperature of 30°C (winter) to 37°C (summer). The COD and sulfate removal efficiencies were approximately 70% and 95%, respectively. The major constituents of the papermill wastewater fed to the UASB reactor were (mg COD l^{-1}): carbohydrates (mainly starch) (850); acetate (500); propionate (300); butyrate (45); formate (20). The composition of the reactor effluent was as follows (mg COD l^{-1}): carbohydrates (350); acetate (30); propionate (80); butyrate (1).

2.2 Organisms and growth conditions

Desulforhabdus amnigenus (DSM 10338) and *Syntrophobacter* strain MPOB (DSM 10017) [19] were from our laboratory collection, and cultured at 37°C in 120-ml serum vials, containing 50 ml of a basal bicarbonate-buffered medium as de-scribed before [20]. This medium was supplemented with 20 mM propionate and 20 mM sulfate for *D.*

amnigenus, and with 40 mM fumarate for *S. fumaroxidans*. All other strains were obtained from the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany).

2.3 Most probable number (MPN) estimates

The different physiological types of bacteria were enumerated using the MPN technique (n=3). For this purpose reactor sludge (10 ml) was diluted with 90 ml basal bicarbonate-buffered medium and disintegrated by pressing it repeatedly through a syringe needle as described before [20]. This crushed granular sludge was used to make serial dilutions in 120-ml serum vials containing 45 ml bicarbonate-buffered medium, with 20 mM acetate, formate, propionate or butyrate as substrate, and with or without 20 mM sulfate. The gas phase consisted of N_2/CO_2 (4:1). Hydrogenotrophic bacteria were quantified using a H_2/CO_2 (4:1) gas phase. In the series with propionate or butyrate without sulfate, 5 ml of a H_2-grown culture of *Methanobacterium formicicum* (DSM 2639) was added to allow steady H_2 and formate consumption. After 3 months of incubation (37°C) methane production, substrate depletion, and sulfide formation were determined. The most probable number of methanogens (determined by methane production) and sulfate reducers (determined by sulfide production) in the sludge, was estimated according to Hurley and Roscoe [21]. Furthermore, the cultures were examined microscopically, and nucleic acids were extracted for dot blot hybridization analyses.

2.4 Nucleic acids isolation and dot blot hybridizations

Nucleic acids were extracted from the MPN dilution series (10 ml samples) and from homogenized inoculum sludge (0.1 ml), using glass beads and a cell homogenizer as described previously [25]. The extracts were purified by phenol-chloroform-isoamylalcohol extraction and ethanol precipitation. The purified extracts were transferred to membranes with a dot-blot manifold, and hybridized overnight with radiolabelled 16S rRNA hybridization probes (Table 2). A detailed description of the nucleic acid isolation and dot-blot hybridization procedure was published previously [25]. The hybridization temperature was 10°C below the reported probe dissociation temperature (Td) (see references in Table 2). The final washing temperature of each hybridization experiment equalled the Td of the probe used. Hybridization responses were quantified by using a Phosphor screen and a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA). The total amount of bacterial rRNA in the sludge was quantified with the EUB338 probe in combination with *Escherichia coli* rRNA standards (Boehringer Mannheim Biochemicals, Almere, NL). The total amount of archaeal rRNA was quantified with the ARC915 probe in combination with *Methanospirillum hungatei* (DSM 864) standards. The rRNA content of the *E. coli* RNA standards was measured spectrophotometrically, and the RNA content of the *M. hungatei* standards was determined by quantifying the signal intensity of the 16S rRNA

band on an agarose gel as described before [25]. The relative abundance of 16S rRNA from sulfate reducers, acetogens, and methanogens was expressed as a percentage of the total bacterial 16S rRNA, in the case of sulfate reducers and acetogens, and as a percentage of the total archaeal 16S rRNA in the case of methanogens. Hybridization results obtained with dilution series of nucleic acid extracts from reference organisms were used as standards as described before [30]. Besides extracts from *E. coli* and *M. hungatei*, nucleic acids extracts from the following reference organisms were used: *Desulforhabdus amnigenus* (DSM 10338), *Desulfobacterium vacuolatum* (DSM 3385), *Desulfobacterium phenolicum* (DSM 3384), *Desulfovibrio vulgaris* (DSM 644), *Desulfobulbus propionicus* (DSM 2032), *Desulfobacter latus* (DSM 3381), *Desulfotomaculum acetoxidans* (DSM 771), *Syntrophobacter wolinii* (DSM 2805), *Syntrophobacter* strain MPOB (DSM 10017), *Syntrophobacter pfennigii* (DSM 10092), *Methanobacterium formicicum* (DSM 2639), *Methanococcus jannaschii* (DSM 2661), *Methanosarcina barkeri* (DSM 800), and *Methanosaeta* (*Methanothrix*) *soehngenii* (DSM 2139).

2.5 Chemical analyses

Volatile fatty acids and sulfate were analyzed by HPLC as described previously [10,31]. Hydrogen and methane were determined quantitatively by gas chromatography [31]. The volatile suspended solids (VSS) content of the sludge, and the COD of the wastewater were analyzed according to the Dutch Standard Methods [32]. Sulfide was measured colorimetrically as described by Trüper and Schlegel [33].

3 Results and discussion

3.1 Enumeration of the sulfate reducers, acetogens and methanogens in the sludge.

The different trophic groups of microorganisms in the sludge were estimated with the MPN method (Table 3). With this method sulfate reducers and methanogens could be easily discriminated. Hydrogen-, formate-, and acetate-utilizing methanogens clearly outnumbered their sulfate-reducing counterparts. In the MPN dilution series with sulfate and propionate or butyrate, it was not possible to make a distinction between a direct propionate or butyrate degradation by sulfate reducers (reaction 8 and 9, Table 1), or an indirect degradation through "interspecies electron transfer" (reaction 1, 2, 3, 4, and 12 and/or 13). The exact number of acetogens in the MPN series with propionate and sulfate could not be determined, but it was maximally 1.4×10^8-1.2×10^9 cells/g VSS (Table 3). This number of acetogens is thousand times lower than the number estimated via the MPN series with only propionate. A similar (500-fold) difference was found between the number of butyrate-degrading acetogens in the MPN series with butyrate and sulfate, and in the series with butyrate only (Table 3). These results indicate that the MPN method underestimates the number of acetogens in the sludge, when the dilution series are not supplemented with

hydrogenotrophic methanogens. Apparently, this occurs even if the total number of hydrogenotrophic methanogens in the sludge exceeds the number of acetogens. Similar observations were made before [12].

3.2 The sulfate-reducing potential of the sludge

The sulfate-reducing potential of sludge under non-sulfate-limiting conditions (Table 4), was calculated using the substrate consumption, sulfide production, and methane production in the lowest dilutions of the MPN series (100-fold diluted sludge), and the conversion stoichiometries of Table 1. The maxi-mum percentage of acetate, hydrogen and formate degradation via sulfate reduction could be calculated directly from the measured substrate consumption, sulfide production, and methane production.

Table 2. Summary of the oligonucleotide probes used in this study

Probe	Target group	Reference
	kingdoms	
EUB338	*Bacteria*	22
ARC915	*Archaea*	23
	sulfate reducers	
SRB385	Gram-neg. sulfate reducing bacteria (SRB)[1]	22
D687	*Desulfovibrio*	24
D660	*Desulfobulbus*	24
D221	*Desulfobacterium*	24
D129	*Desulfobacter*	24
ASRB1	*Desulforhabdus amnigenus*	25
D804	*Desulfobacterium, Desulfobacter, Desulfosarcina, Desulfococcus, Desulfobotulus*	24
	acetogens	
MPOB1	*Syntrophobacter* strain MOPB, *S. pfennigii, D. amnigenus*	26
KOP1	*S. pfennigii*	26
S223	*Syntrophobacter wolinii*	27
117	Syntrophic propionate oxidizer SYN7	27
	methanogens	
MB1174	*Methanobacteriales*[2]	28
MC1109	*Methanococcales*[2]	28
MG1200	*Methanomicrobiales*	28
MS821	*Methanosarcina*	28
MX825	*Methanosaeta* (*Methanothrix*)	28

[1]Several non-target organisms share the target sequence of this probe [22], while some target organisms (e.g. *D. amnigenus*) have several mismatches with the probe.
[2]Probes MB1174 and MC1109 could be considered as family-specific probes for *Methanomicrobiaceae* and *Methanococcaceae*, when used in mesophilic environments [29].

Table 3. Most probable number of sulfidogens, acetogens and methanogens present in the granular sludge and the prevalent *Bacteria* and *Archaea* in the higher dilutions of the MPN series, based on 16S rRNA dot blot hybridizations.

Substrate	Sulfidogens	Acetogens	Methanogens	Prevalent bacterial groups[1]
	cells/g VSS 95% confidence interval[2]			
Acetate	-	-	$2.3x10^8$ - $1.7x10^9$	ARC, MX
Acetate + SO_4^{2-}	$4.1x10^4$ - $4.6x10^5$	-	$2.3x10^6$ - $1.7x10^7$	EUB[3], ARC, MX
Formate	-	-	$1.5x10^{11}$ - $1.0x10^{12}$	ARC, MB
Formate + SO_4^{2-}	$2.9x10^9$ - $1.8x10^{10}$	-	$7.9x10^{10}$ - $9.0x10^{11}$	EUB[3], ARC, MB
H_2/CO_2	-	-	$1.5x10^{12}$ - $1.3x10^{13}$	ARC, MB
H_2/CO_2 + SO_4^{2-}	$2.3x10^{10}$ - $3.4x10^{11}$	-	$7.4x10^{11}$ - $7.4x10^{12}$	EUB[3], SRB[3], ARC, MB
Propionate	-	$4.3x10^{11}$ - $5.3x10^{12}$	-[4]	EUB, SF, ARC, MB, MG[3], MX[3]
Propionate + SO_4^{2-}	$1.4x10^8$ - $1.2x10^9$	ND[5]	$1.4x10^7$ - $1.2x10^8$	EUB, SRB, SF, DB[3], ARC[3], MB[3], MX[3]
Butyrate	-	$6.2x10^8$ - $6.2x10^9$	-[4]	EUB, ARC, MB, MX
Butyrate + SO_4^{2-}	$7.3x10^5$ - $8.4x10^6$	$1.2x10^6$ - $1.1x10^7$	$1.2x10^6$ - $1.1x10^7$	EUB, ARC, MB, MX

[1] Bacterial groups were only listed if their relative abundance, in the three highest dilutions of the MPN series, was at least 10%. The bacterial groups were identified with the probes presented in Table 2, the names were abbreviated as follows: EUB, *Bacteria*; SRB, Gram-negative sulfate reducers; DB, *Desulfobulbus*; SF, *Syntrophobacter fumaroxidans*; ARC, *Archaea*; MB, *Methanobacteriales*; MG, *Methanomicrobiales*; MX, *Methanosaeta*.

[2] Estimated according to Hurley and Roscoe [21].

[3] This group was not present in the highest dilutions of these MPN series.

[4] *Methanobacterium formicicum* was added to all dilutions.

[5] Not determinable.

However, to calculate the percentage of propionate and butyrate-degradation via sulfate-reduction, the following assumptions were made: All propionate and butyrate is first degraded to acetate, via (a combination of) reaction 1, 3 and 8, or reaction 2, 4 and 9, respectively. Furthermore, acetate, hydrogen and formate, which are formed in the MPN vials during the degradation of propionate and butyrate, are further degraded via sulfate reduction and methanogenesis in the same way as in the vials with only acetate, hydrogen or formate, i.e. respectively 3%, 28% and 19% via sulfate reduction and the rest via methanogenesis.

The maximum amount of sulfate reduced in the reactor was calculated to be 2.2 mM, based on the results of Table 4 and the substrate consumption in the reactor. It should be mentioned that the percentage of butyrate conversion via sulfate reduction as presented in Table 4, would decrease drastically (from 30-38% to 8-11%) if it is assumed that butyrate-conversion mainly proceeds via reaction 10, and not via reaction 9 (Table 1). However, this would not change the calculated sulfate reduction largely, because butyrate was only present in the reactor in small amounts.

Surprisingly, the calculated maximum amount of sulfate reduced (2.2 mM), was only slightly higher than the actual amount of sulfate reduced in the reactor, which was 1.9 mM. It was expected that the maximum sulfate reducing potential of the sludge would be much higher than the actual sulfate reduction in the sludge. Apparently, the sludge is well adapted to the available sulfate, and does not have a large overcapacity of sulfate reduction.

It is clear that acetotrophic sulfate reducers endure a strong competition from acetotrophic methanogens. In the reactor, acetate seems to be mainly degraded via methanogenesis. This corresponds with the acetate-degradation pattern found by Mulder and by others [7,11,12]. Interestingly, hydrogen also seemed to be mainly degraded by methanogens. This differs from the hydrogen oxidation in sulfidogenic bioreactors, operated with an excess of sulfate, where hydrogen is mainly degraded via sulfate reduction [7,8]. The only substrate which appeared to be mainly degraded via sulfate reduction was propionate. These results could indicate that propionate, rather than hydrogen is the preferred substrate for sulfate reduction in this sulfate-limited reactors.

3.3 Characterization of the microbial sludge population

In order to characterize the different groups of microorganisms present in the sludge, and to obtain more insight in their physiological role, the 16S rRNA compositions of the sludge and the MPN dilution series (Table 3) were analyzed with the 16S rRNA dot blot hybridization method. The total bacterial and archaeal rRNA in the sludge nucleic acid extract was 1.6 ± 0.1 μg ml^{-1} and 11.1 ± 2.8 μg ml^{-1}, respectively. This corresponds to a ratio of 0.14 between bacterial and archaeal rRNA in the sludge. The low ratio between bacterial and archaeal rRNA

Table 4. Partitioning of substrate degradation by sulfate reducers, acetogens and methanogens, under non-sulfate-limiting conditions.

Substrate	% degraded via[1]: sulfate reduction	methanogenesis	acetogenesis
Hydrogen	28	72	-
Formate	19	81	-
Acetate	3	97	-
Propionate	60-65[2,3]	-	35-40[2,3]
Butyrate	30-38[2]	-	62-70[2]

[1] Average of three independent experiments, the SD is always below 2%, unless indicated otherwise.
[2] Percentage range; first percentage: assuming only interspecies hydrogen transfer; second percentage: assuming only formate transfer.
[3] SD ± 13%

could indicate that methanogens are the largest group of microorganisms in the sludge, which seems to correspond with the results of the MPN estimates (Table 3).

Methanosaeta sp. were the dominant acetate-degrading methanogens in the sludge. They could be detected in all MPN series where acetate had been degraded. In the sludge 80 ± 3% of the total archaeal 16S rRNA was from *Methanosaeta*. Nucleic acid extracts from the sludge and the MPN dilutions did not hybridize with the MS825 probe, which indicated that acetate-degrading *Methanosarcina* sp. were not present or present only in low numbers. The dominance of *Methanosaeta* in the sludge, could be due to the low acetate concentration (0.4 mM) in the reactor. In anaerobic bioreactors with low acetate concentrations, *Methanosaeta* sp. are often the dominant acetate-degrading methanogens. According to Jetten et al. [34] the competitive advantage of *Methanosaeta* over *Methanosarcina* can be explained by their higher affinity and lower threshold for acetate.

Acetate-degrading sulfate reducers were only present in the sludge in low numbers. To which group these sulfate reducers belong, could not be determined with the available probes, because none of the probes for sulfate reducers (Table 2) hybridized with the RNA extracts from the MPN series with acetate and sulfate. At present probes for acetate-degrading sulfate reducers belonging to the Gram positive, sporeforming *Desulfotomaculum* sp. [35] are not available. However, the presence of sporeforming bacteria could not be confirmed by microscopic examinations. Instead small coccus-shaped cells and thick vibrio-shaped cells were observed in the MPN bottles with acetate and sulfate, in addition to the long filaments typical for *Methanosaeta* [36].

Based on hybridization studies the dominant hydrogen and formate-consuming methanogens in the sludge belonged to the order of the *Methanobacteriales*. Under the microscope, mainly thin, rod-shaped cells, resembling *Methanobacterium* sp. were observed in the MPN cultures with hydrogen or formate. Based on MPN estimates *Methanobacteriales* cells outnumbered *Methanosaeta* cells by far, but only a low amount of *Methanobacteriales* rRNA (2% relative

abundance) was found in the sludge, compared to *Methanosaeta* rRNA (80% relative abundance). It is possible that the *Methanobacteriales* cells were not very active in the sludge, and therefore only contained a low amount of rRNA. However, it is known that MPN tests underestimate the number of *Methanosaeta* cells present, due to incomplete dispersion of the *Methanosaeta* filaments into single cells [37]. In addition, due to the slow growth of *Methanosaeta* sp., an incubation period of three months might not be long enough to determine the true numbers in the MPN tests. This underestimation of *Methanosaeta* cell-numbers by MPN tests seems to be a more likely explanation for the difference between cell-numbers and RNA abundance of *Methanobacteriales* and *Methanosaeta*.

Methanogens of the order *Methanomicrobiales* or *Methanococcales* could not be detected in hybridization studies with the sludge extract. Nevertheless, the *Methanomicrobiales* probe MG1200 did react with some of the lower dilutions of the MPN series with propionate, indicating that *Methanomicrobiales* were present in the sludge in low numbers. It remains unclear why *Methanobacteriales* sp. and not *Methanomicrobiales* sp. seem to be the dominant H_2 and formate consuming methanogens in the sludge. Up to now, nothing is known about the competition between *Methanobacteriales* and *Methanomicrobiales* species for H_2 and formate in anaerobic sludge. In some studies *Methanobacteriales*, such as *Methanobacterium* and *Methanobrevibacter* are reported to be the dominant hydrogen and formate consuming methanogens [16-18,30], while other studies show that *Methanomicrobiales* can also be present in the sludge in high numbers [38] and can even be more abundant than *Methanobacteriales* [29,39].

Hydrogen and/or formate-consuming sulfate reducers could not be characterized. This is partly due to the fact that only a small percentage of hydrogen (<7%) and formate (<6%) was converted via sulfate reduction in the higher dilutions of the MPN series with sulfate, and hydrogen or formate. The bacterial 16S rRNA in these higher dilutions, represented only a small fraction (5-14%) of the total 16S rRNA. In some vials, up to 50% of this bacterial 16S rRNA could be attributed to Gram-negative sulfate reducers. In most vials small, vibrio-shaped cells, and short, thin, rod-shaped cells were observed, in addition to *Methanobacterium*-like cells. The *Desulfovibrio* probe (D687) did not hybridize with the sludge extract nor with any of the MPN-extracts. Recently, we have reported that the *Desulfovibrio* probe D687 is not very sensitive for the detection of *Desulfovibrio* sp. [40], and a relative *Desulfovibrio* rRNA abundance of at least 1-1.5% is required for a positive hybridization signal. Nevertheless, the hybridization results with the *Desulfovibrio* probe indicate that *Desulfovibrio* sp. are not present in the sludge in high numbers.

Propionate could be degraded in the sludge via three different pathways, as was demonstrated in the MPN series with propionate and with or without sulfate. In the absence of sulfate, propionate was degraded by syntrophic propionate degrading bacteria and hydrogen/formate-scavenging *Methanobacteriales* and *Methanomicrobiales*. These syntrophic propionate oxidizers could be *Syntrophobacter* strain MPOB, because the nucleic acid

extracts of the MPN cultures hybridized only with the MPOB1 probe, and not with the KOP1 or ASRB1 probe. In the lower dilutions of the MPN series with propionate and sulfate, propionate was mainly degraded by *Desulfobulbus* sp, the acetate which was formed as an intermediate was degraded by *Methanosaeta*. This was in accordance with earlier findings of Harmsen et al. [27]. In the higher dilutions no methane production was observed, thus propionate had been directly or indirectly degraded via sulfate reduction. The latter seemed to be the case, because *Desulfobulbus* 16S rRNA was absent, but 16S rRNA of *S. fumaroxidans* and Gram-negative sulfate reducers was present in high amounts. These Gram-negative sulfate reducers, which were vibrio-shaped, were probably the hydrogen or formate-consuming sulfate reducers in these cultures. However, it cannot be ruled out that these vibrio-shaped sulfate reducers were propionate degraders, because *S. fumaroxidans* is not restricted to syntrophic propionate degradation, but can oxidize propionate with sulfate as electron acceptor [41]. In the sludge approximately 5.3 ± 0.1% of the bacterial 16S rRNA could be attributed to *Desulfobulbus*, 0.5 ± 0.2% to *S. fumaroxidans*, and 18 ± 1% to Gram negative sulfate reducers.

The most obscure process in the sludge was butyrate conversion. Without doubt a major part of the butyrate in the sludge was degraded by syntrophic butyrate degraders (Table 4). Unfortunately, probes to characterize syntrophic butyrate degraders, such as *Syntrophospora* and *Syntrophomonas* [2] are not available. In the MPN cultures with butyrate, sporeforming rods, resembling *Syntrophospora* could be observed, but small, bulbus-shaped bacteria were present as well. Because none of the probes for sulfate-reducers hybridized with the extracts from the MPN series with butyrate and sulfate, it cannot be concluded if butyrate is directly or indirectly degraded via sulfate reduction. In addition to the bacteria observed in the MPN series without sulfate, thick vibrios and thick short rods were observed. These bacteria could have been the sulfate-reducing bacteria.

A small percentage (4 ± 1%) of the bacterial 16S rRNA from the sludge hybridized with the D804 probe, for *Desulfobacterium* and relatives. The role of these bacteria in the sludge is unclear, especially because they were not detected in any of the MPN extracts.

3.4 Concluding remarks.

The aim of this study was to gain more insight in the competitive and syntrophic interactions between sulfate-reducing, methanogenic and acetogenic bacteria in sulfate-limited reactors. From the results presented in this paper it is clear that the bacterial interactions in such sulfate-limited reactors are very complex. No substrate seemed to be completely degraded by sulfate reducers, methanogens or acetogens only. However, there was evidence that in this reactor acetate was mainly degraded by methanogens and propionate was the preferred substrate for sulfate reducers. Further studies are needed to reveal if these results can be extrapolated to other sulfate-limited reactors. The characterization of the bacteria in the MPN series, helped to get more insight in the role of the different bacterial groups in the sludge. The methanogenic population could be characte-

rized relatively well with the available probes. Our results indicated that *Methanosaeta* sp. were the dominant acetate degraders, while *Methanobacterium* sp. were the dominant hydrogen and formate-consuming methanogens. *Desulfobulbus* sp. and *Syntrophobacter* sp. were important for propionate degradation. However, new probes have to be developed to get a better picture of the sulfate-reducing and acetogenic sludge populations. For example, probes for Gram positive sulfate reducers and syntrophic butyrate degraders would be very useful in this respect.

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CHAPTER 8

Identification of sulfate reducers and *Syntrophobacter* sp. in anaerobic granular sludge by fatty-acid biomarkers and 16S rRNA probing.

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The sulfate-reducing bacterial sludge population in anaerobic bioreactors, treating different types of wastewater in the presence or absence of sulfate, was evaluated by polar-lipid fatty acid (PLFA) analyses, and by 16S rRNA dot blot hybridizations using specific 16S rRNA-targeted oligonucleotide probes for sulfate reducers and *Syntrophobacter* sp. The 16S rRNA dot blot hybridizations were useful for estimating the relative amount of sulfate reducers in the sludge. The PLFA profiles of the sludge were useful to obtain a quick general impression of the total bacterial sludge composition, but were less suitable for an accurate characterization and quantification of the sulfate-reducing population in the sludge. This was due to the lack of selective biomarkers for these bacteria.

The combined results of the PLFA analysis and 16S rRNA dot blot hybridizations showed that presence of sulfate reducers in the sludge was not dependent on the presence of sulfate in the wastewater. This may be explained by the syntrophic and/or fermentative capacities of some sulfate reducers in the absence of sulfate. *Desulfobulbus* sp. were important in reactors with carbohydrates and/or volatile fatty acids containing wastewater. These bacteria could play a role in propionate degradation in these reactors. *Desulfobacter* sp. did not seem to be important for acetate degradation in any of the sulfate-fed reactors. In the acetate and sulfate-fed reactor, *Desulfotomaculum acetoxidans*-like bacteria seemed to play a role in acetate degradation.

Introduction

Anaerobic wastewater treatment systems are suitable for the treatment of a wide range of industrial wastewaters (Lettinga, 1995). In the anaerobic digestion process organic material is mineralized to methane and CO_2 by the concerted action of various metabolic groups of bacteria. Important inter-mediates of the anaerobic digestion process are hydrogen, formate, acetate, and volatile fatty acids (Gujer and Zehnder, 1983). Dependent on the type of anaerobic bioreactor the bacteria are aggregated in flocs, granules or biofilms. The microbial composition of these aggregates is influenced by the type of carbon sources and electron acceptors present in the wastewater. In methanogenic bioreactors, propionate and butyrate conversion is only feasible at a low H_2-partial pressure and a low formate concentration. Therefore, propionate- and butyrate-oxidizing acetogens can degrade these compounds only in syntrophy with H_2 and/or formate scavenging methanogens (Stams, 1994). Anaerobic bioreactors are also used for the treatment of sulfate-rich waste streams. The presence of sulfate in the reactor leads to a competition between sulfate-reducing bacteria and syntrophic bacteria for compounds such as propionate and butyrate, and a competition between sulfate reducers and methanogens for acetate, hydrogen and formate (Oude Elferink et al., 1994).

Recently, it was found that syntrophic

propionate oxidizers, belonging to the genus *Syntrophobacter*, are able to grow without a syntrophic partner, with propionate plus sulfate as electron acceptor (Wallrabenstein et al., 1994; 1995; van Kuijk and Stams, 1995; Zellner et al., 1996). Based on 16S rRNA analyses *Syntrophobacter* species belong, together with Gram-negative sulfate reducers, to the delta subclass of the proteobacteria (Harmsen et al., 1995; Wallrabenstein et al., 1995). A close relative of the genus *Syntrophobacter* is *Desulforhabdus amnigenus*, a sulfate reducer which is not able to grow on propionate syntrophically (Oude Elferink et al., 1995). *Syntrophobacter* sp. can be present in methanogenic bioreactors in high numbers (Harmsen et al., 1996b), but their presence and role in sulfate-reducing bioreactors is not clear.

The aim of this study was to gain more insight in the sulfate-reducing bacterial sludge population in anaerobic bioreactors treating different types of wastewater in the presence or absence of sulfate. Several sludge-types were analysed with the 16S rRNA dot blot hybridization method and their polar-lipid fatty acid (PLFA) profile was determined. Both characterization methods have been successfully applied for the identification of sulfate reducers in complex ecosystems (Taylor and Parkes, 1985; Parkes et al., 1993; Raskin et al. 1995; 1996). The lipid composition of sulfate-reducing bacteria has been studied extensively (e.g. Taylor and Parkes, 1983; Dowling et al., 1986; Vainshtein et al., 1992; Kohring et al., 1994), but PLFA compositions of *Syntrophobacter* sp. are not known. We determined PLFA profiles of three *Syntrophobacter* sp. (i.e. *S. wolinii, S. pfennigii*, and strain MPOB) (Boone and Bryant, 1980; Stams et al., 1993; Wallrabenstein et al., 1995; Harmsen, 1996) and also of *D. amnigenus*. Species-specific 16S rRNA probes already available for the detection of these organisms were also used to analyse the sludges (Harmsen et al., 1995; Harmsen et al., 1996a; Oude Elferink et al., 1997).

Materials and Methods

Granular sludge types

Granular sludge samples were taken from one full-scale upflow anaerobic sludge blanket (UASB) reactor, fed with papermill wastewater, and several lab-scale UASB reactors, fed with synthetic wastewater (Table 1). The lab-scale reactors fed with sulfate had been running for more than 3 months before the sludge samples were taken. The other lab-scale reactors had been running for more than one year, and the full-scale reactor for more than five years. A part of each sludge sample was homogenized with a mortar and pestle and used immediately for nucleic acids extraction, the remainder was stored at -20°C for PLFA-analysis.

Organisms and growth conditions

Desulforhabdus amnigenus strain ASRB1 (DSM 10338) and *Syntrophobacter* sp. strain MPOB (DSM 10017) (Stams et al., 1993; Harmsen, 1996) were from our

Table 1. Summary of relevant reactor-characteristics.

Reactor	Scale (m^3)	Feed	COD[a]-load ($kg/m^3.d$)	Total COD[2]-removal	COD[a]-removal via sulfate	Temperature (°C)	COD^a/SO_4 ratio
C+S	2500	Complex[b] + Sulfate	8.5	70%	10%	37	9.5
VFA+S	$5.5x10^{-3}$	Volatile Fatty Acids[c] + Sulfate	11.3	80%	60%	30	0.5
Ac+S	$6.5x10^{-3}$	Acetate + Sulfate	2.5	80%	40%	30	0.5
C	0.01	Complex[d]	5	90%	-	30	-[f]
VFA	0.01	Volatile Fatty Acids[e]	5	90%	-	30	-[f]

[a]Chemical Oxygen Demand (COD)
[b]papermill wastewater, containing carbohydrates (mainly starch), acetate, propionate, butyrate, and formate.
[c]COD ratio acetate: propionate: butyrate= 1:2:2
[d]Sucrose
[e]COD ratio acetate: propionate: butyrate= 19:34:44
[f]No significant amount of sulfate present in the feed.

laboratory collection. *Syntrophobacter wolinii* (DSM 2805), and *Syntrophobacter pfennigii* (DSM 10092) were obtained from the Deutsche Sammlung von Mikroorganismen (Braun-schweig, Germany). All strains were grown at 37°C, in 120-ml serum vials or 1-l screw-cap bottles, containing 50 and 500 ml, respectively, of a bicarbonate-buffered medium as described previously (Oude Elferink et al., 1995). *D. amnigenus* was grown with either 20 mM acetate, propionate, lactate or ethanol as electron donor and sulfate (40 mM) as electron acceptor. The *Syntrophobacter* sp. were cultured syntrophically on propionate (20 mM) with *Methanospirillum hungatei* (DSM 864) as hydrogen scavenger.

PLFA analysis

Lipids were extracted by a modified Bligh and Dyer extraction using 50-ml screw-cap centrifuge tubes (Guckert et al., 1985). Bacterial cultures (100 to 500 ml) were harvested by centrifugation (20,000 *g*, 20 min., 4°C) and pellets were directly extracted. UASB sludges were homogenised with a mortar and pestle, and approximately 1 g (wet) was extracted. The total lipid extract was fractionated on silicic acid, and mild alkaline transmethylation was used to yield fatty acid methyl esters from the polar lipid fraction (Guckert et al., 1985). The fatty acid methyl esters (FAME) were determined by capillary GC-FID using both an nonpolar column (Hewlett-Packard Ultra-2, 50 m x 0.32 mm x 0.17 mm) and a polar column (Scientific Glass Engineering BPX-70, 50 m x 0.32 mm x 0.25 mm). Internal FAME standards of both 19:0 and 12:0 were used. Identification of PLFA was based on comparison of retention time data from both analytical columns with known standards or previously characterised extracts from bacterial cultures. Additional identification was done by GC-MS using a Hewlett-Packard Mass Selective Detector (HP 5970).

PLFA nomenclature

Fatty acids are designated as A:BωC, where A is the number of carbon atoms, B the number of double bonds, and C the position of the double bond from the aliphatic (ω) end. Unsaturated bonds may occur in either *cis* (c) or *trans* (t) configurations. The prefixes "i" and "a", refer to iso- and anteiso-methyl branching, respectively. Mid-chain methyl branches are designated by "Me", preceded by the position of the branch from the acid end. A cyclopropyl ring is indicated as "cy". The roman numbers indicate a mono-unsaturated PLFA with unknown double bond position, or a PLFA with an unknown position of the cyclopropyl ring. No appropriate standards were available for these compounds.

Nucleic acids isolation and dot blot hybridizations

Nucleic acids were extracted from 10 ml of a late logarithmic culture or 0.1 ml of homogenized granular sludge, using glass beads and a cell homogenizer in the presence of phenol. The extracts were purified by phenol-chloroform-isoamylalcohol extraction

and ethanol precipitation as described previously (Harmsen et al., 1995). The 16S rRNA composition of the (duplicate) sludge samples was analysed via dot blot hybridization, using ^{32}P-labelled 16S rRNA probes (Table 4), according to Oude Elferink et al. (1997). The dot blots were hybridized overnight at 10°C below the probe dissociation temperature (Td). The final wash temperature of each hybridization experiment equalled the Td of the probe used. Hybridization responses were quantified by use of a Phosphor screen and a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA). Total bacterial 16S rRNA was quantified by hybridization of extracted nucleic acids and dilution series of the rRNA of the reference organisms, with the general bacterial probe EUB338 (Amann et al., 1990). The resulting standard curves were used to calculate the concentration of 16S rRNA for the different groups of microorganisms as described before (Raskin et al., 1994). Nucleic acid extracts from the following reference organisms were used:

Escherichia coli (Boehringer Mannheim Biochemicals, Almere, NL), *Desulforhabdus amnigenus*, *Desulfobacterium vacuolatum* (DSM 3385), *Desulfobacterium phenolicum* (DSM 3384), *Desulfovibrio vulgaris* (DSM 644), *Desulfobulbus propionicus* (DSM 2032), *Desulfobacter latus* (DSM 3381), *Desulfotomaculum acetoxidans* (DSM 771), *Syntrophobacter wolinii*, *Syntrophobacter pfennigii* and *Syntrophobacter* sp. strain MPOB.

Results and discussion

PLFA composition of bacterial cultures

The PLFA compositions of the three *Syntrophobacter* species, and of the sulfate reducer *D. amnigenus*, were analysed to find possible biomarkers for these phylogenetically closely related organisms. Although belonging to the same genus, the PLFA pattern of *Syntrophobacter* sp. strain MPOB was very different from that of *S. wolinii* and *S. pfennigii* (Table 2). Major PLFA in strain MPOB were 15:1 (III) and 15:0, whereas patterns of the other two strains were dominated by i15:0, 16:0 and i17:1ω7. The patterns of *S. wolinii* and *S. pfennigii* were similar to that generally found in *Desulfovibrio* sp. (Vainshtein et al., 1992). As a result, specific detection of these two *Syntrophobacter* strains will be difficult and i17:1ω7, an uncommon compound which was considered as specific marker for *Desulfovibrio* sp. (Taylor and Parkes, 1983; Vainshtein et al., 1992), can only be used as a combined marker for *Desulfovibrio* sp. and the two *Syntrophobacter* strains. Strain MPOB contained 20% of 15:1 (III), which was not further characterized for double bond position, because no appropriate standards were available. Since the exact identity of this monounsaturated PLFA is not known, it is unclear if it can be used as a specific marker for strain MPOB. On the other hand, since this compound is not a major component in the PLFA patterns of UASB sludges analysed (see

Table 2. PLFA composition of several recently isolated propionate-oxidizing bacteria, the major PLFA's are in bold.

PLFA	*D. amnigenus*	*Syntrophobacter* strain MPOB	*S. wolinii*	*S. pfennigii*
	Mol %			
13:0		0.9		
14:1		1.5		
14:0	1.6	8.4	3.1	1.5
i15:0	4.2	2.2	**37.8**	**27.9**
a15:0			1.6	0.6
15:1 (III)		**19.5**		
15:1 (IV)	1.3			
15:0	**17.4**	**22.0**	8.7	**11.3**
16:1ω7c		2.4	1.6	
16:1ω5	2.9	2.3		
16:0	**11.7**	6.7	**12.2**	7.8
i17:1ω7		1.3	**15.7**	**25.6**
10Me16:0				2.3
i17:0			2.4	1.9
a17:0		0.5		
17:1ω8	0.5	7.5		
17:1ω6	**12.0**	1.2		
cy17:0 (II)	**11.0**			
17:0	**11.8**	9.9	3.1	8.4
i18:1	4.9			
18:1ω9c		2.9	4.3	1.6
18:1ω9t		1.5	2.8	
18:1ω7c	0.3			
18:1 (II)	0.7			
18:1 (III)	**14.5**			
18:0	2.5	6.3	3.5	9.7
Total	97.3	97.0	96.8	98.6

Figure 1), it might be used as a marker for determining the maximum amount of strain MPOB present.

The PLFA pattern of *Desulforhabdus amnigenus* grown on acetate, was specific (Table 2). Especially the equal amounts of 17:1ω6, cy17:0 (II), and a not further characterised 18:1 (III) were not found in any other PLFA pattern of sulfate-reducing bacteria (Taylor and Parkes, 1983; Dowling et al., 1986; Vainshtein et al., 1992; Kohring et al., 1994). PLFA analysis of *D. amnigenus* cultures grown with propionate, lactate or ethanol as electron donor resulted in similar PLFA patterns (results not shown). 17:1ω6 was proposed as a marker for the genus *Desulfobulbus* (Taylor and Parkes, 1983) and it is found in much higher amounts in UASB

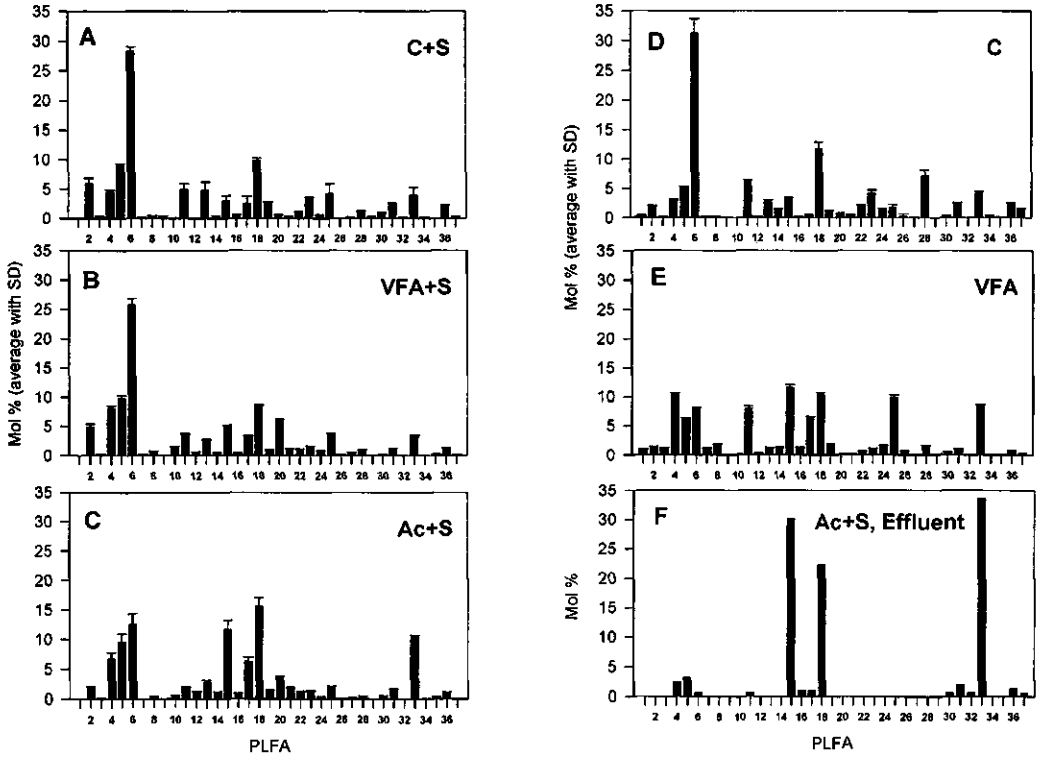

Figure 1. PLFA compositions of granular UASB sludge from several anaerobic reactors ((A) C+S, (B) VFA+S, (C) Ac+S, (D) C and (E) VFA) and UASB effluent from reactor Ac+S (F).
PLFA legend: **1**, 13:0; **2**, i14:0; **3**, 14:1; **4**, 14:0; **5**, i15:0; **6**, a15:0; **7**, 15:1 (I); **8**, 15:1(II); **9**, 15:1 (III); **10**, 15:1 (IV); **11**, 15:0; **12**, i16:1; **13**, i16:0; **14**, 16:1ω9c; **15**, 16:1ω7c; **16**, 16:1ω7t; **17**, 16:1ω5; **18**, 16:0; **19**, i17:1ω7; **20**, 10Me16:0; **21**, a17:1; **22**, i17:0; **23**, 17:0; **24**, 17:1ω8; **25**, 17:1ω6; **26**, cy17:0 (I); **27**, cy17:0(II); **28**, 17:0; **29**, i18:1; **30**, 18:1 (I); **31**, 18:1ω9c; **32**, 18:1ω9t; **33**, 18:1ω7c; **34**, 18:1 (II); **35**, 18:1 (III); **36**, 18:0; **37**, cy19:0.

sludges than the other two characteristic PLFA's (see Figure 1). This suggested that 17:1ω6 cannot be used as a marker for *Desulforhabdus amnigenus*. We therefore used cy17:0 (II) as a marker to determine the maximum abundance of this organism present in sludge.

A comparison of PLFA analysis and 16S rRNA dot blot hybridizations for the detection of sulfate reducers and Syntrophobacter *sp. in UASB sludge*

The sulfate-reducing and syntrophic populations in granular sludge from several UASB reactors, fed with complex or defined wastewaters, were characterized by PLFA analysis and 16S rRNA dot blot hybridizations. For the PLFA analysis several compounds were selected as possible marker molecules for sulfate-reducing bacteria in reactor sludge (Table 3). The selection was based on literature data (Taylor and Parkes, 1983; Vainshtein et al., 1992; Kohring et al., 1994) and on our own analysis of bacterial cultures.

The results of the PLFA analysis and dot blot hybridizations (Table 3 + 4) showed large differences in the estimated relative amounts of the sulfate-reducing and syntrophic populations. The relative amount of sulfate reducers in sludge, estimated from sludge PLFA patterns, using specific biomarkers, does not seem very accurate. For example with the PLFA analysis, an unlikely high amount of *Desulfobacter* sp. was estimated when 10Me-16:0 was used as a biomarker (Table 3). When the more general cy17:0 (I) was used as a marker for this genus, this resulted in a much lower apparent abundance, especially for the sulfidogenic reactors VFA+S and Ac+S. These results suggested that 10Me16:0 overestimated the true numbers of *Desulfobacter* present in the sludge. This could have been caused by the presence of this compound in other types of bacteria (e.g. *Desulfobacterium autotrophicum*, Vainshtein at al., 1992). However, it could also be that the *Desulfobacter* sp. present in sludge, contain higher levels of this fatty acid than reported in literature. Parkes et al. (1993) reported that 10Me16:0 was not a suitable biomarker for *Desulfobacter* in estuarine sediments. Our results suggest the same for the use of 10Me16:0 as biomarker for *Desulfobacter* in sludge.

The amounts of different groups of sulfate reducers in sludge, estimated with the dot blot hybridization method, probably are more accurate than the amounts estimated from sludge PLFA patterns. This in particular because of the lack of specificity of the fatty acid biomarkers, and the possible variation of the biomarker content in the target organisms. However, estimation errors with the dot blot hybridization method occur as well. Although the 16S rRNA probes have been tested for their specificity, it cannot be excluded that (thus far unknown) non-target organisms share the target sequence of a certain 16S rRNA probe, or that target organisms have mismatches with the target sequence of the probe.

Table 3. Abundance of sulfate reducing and syntrophic bacterial genera expressed as a percentage of the total bacterial sludge population, calculated from concentrations of marker PLFA in UASB sludges. Numbers between brackets show the range of molar percentages in which a marker PLFA is found within a genus, which are based on data from this study and from Kohring et al. (1994). Abundance of genera were calculated as: % abundance = (Mol % $PLFA_{sludge}$ / Mol % $PLFA_{genus}$)x100

Marker	Genus	% Abundance[a]				
		C+S	VFA+S	Ac+S	C	VFA
17:1ω6	*Desulfobulbus* (42%)	10 ± 4	9	5	4 ± 1	24 ± 1
10Me16:0	*Desulfobacter* (2-16%)	4-28 ± 1-5	39-310 ± 1-3	23-180 ± 1-8	4-39 ± 1-5	1-6
cy17:0 (I)	*Desulfobacter* (2-39%)	0	0-4	0	1-17	1-17
cy17:0 (II)	*D. amnigenus* (11%)	1	5	2	1	1
i17:1ω7	*Desulfovibrio* (5-33%)	8-50 ± 1-6	3-18 ± 1	4-27 ± 1-3	4-25	5-35 ± 2
	S. wolinii (16%)	10 ± 1	4	5 ± 1	5	7
	S. pfennigii (26%)	15 ± 2	6	9 ± 1	8	11 ± 1
15:1 (III)	str. MPOB (20%)	2 ± 1	0	0	1	0

[a]Standard Deviations below 1 are not presented in this table

Presence of sulfate reducers and Syntrophobacter *sp. in different sludge types*

Although the PLFA biomarker analysis did not seem very useful to estimate the relative amount of sulfate reducers in sludge, the complete PLFA patterns (Fig. 1) were useful for an overall impression of the bacterial sludge composition, and a quick comparison of the different sludge types. For example the presence or absence of sulfate in reactors with a complex influent did not seem to have a large effect on the PLFA patterns (compare Fig. 1A and 1D), which suggested that the bacterial populations in these reactors were rather similar. Dot blot hybridization results (Table 4) confirmed that the relative amounts of the sulfate-reducing populations in reactor C and C+S were in the same range.

The presence or absence of sulfate in VFA-fed reactors had a large effect on the PLFA patterns (compare Fig. 1B and 1E), which suggested that the bacterial populations in these reactors differed. However, the concentration of the biomarker PLFA's (except the 10Me16:0 concentration), and the results of the dot blot hybridizations were both rather similar for the two sludge types (Table 3 + 4). This indicated that the bacterial groups which were mainly influenced by the presence or absence of sulfate, were not the groups of sulfate reducers which hybridized with our probes. Whether these groups were sulfate reducers or other bacteria could not be deduced from our results.

In the reactor with only acetate and sulfate in the influent (Reactor Ac+S, Fig. 1C) the PLFA pattern showed a relatively high amount of certain 16-carbon and 18-carbon PLFA, and a low amount of 17-carbon PLFA characteristic for Gram-negative sulfate-reducers. Interestingly, the effluent of the Ac+S reactor had a rather simple PLFA pattern (Figure 1F); it contained mainly PLFA's that also were present in high levels in the sludge of this reactor (Figure 1C). This suggested that only a part of the sludge population was also dominantly present in the effluent. A large part of the effluent population consisted of spindle-like, polarly flagellated bacterial cells with spores, resembling the Gram-positive, acetate-degrading sulfate reducer *Desulfotomaculum acetoxidans* (Widdel, 1992). Although the PLFA pattern of the effluent contained mainly very common compounds (16:1ω7c, 16:0 and 18:1ω7c), this pattern indeed resembled that of *Desulfotomaculum acetoxidans* (Dowling et al. 1986). Spindle-like cells similar to those observed in the Ac+S effluent were also observed in the Ac+S sludge, but they were not the dominant cell type. Unfortunately, no 16S rRNA probe is available for *Desulfotomaculum* sp. to confirm our tentative identification.

It cannot be excluded that these *Desulfotomaculum acetoxidans*-like bacteria were present in the effluent in high numbers, because they did not play a role in the Ac+S reactor and were therefore washed out of the reactor sludge. However, if this is true, it is difficult to explain why this occurred more than three months after reactor start-up. Therefore it seems more likely that these bacteria do

Table 4. Estimation of the sulfate-reducing and syntrophic (propionate-degrading) population in granular sludge from five different UASB-reactors, based on 16S rRNA hybridization results. The radioactive signals of the specific probes are expressed as a percentage of that obtained with the bacterial probe (EUB338).

Probe (Ref)	Genus	% Total bacterial population				
		C+S	VFA+S	Ac+S	C	VFA
SRB385 (1)	Gram. negative SRB[a]	17-19	47-49	4-7	19-21	35-39
D660 (2)	*Desulfobulbus*	5.2-5.4	37-43	0	9-11	22-26
D804 (2)	*Desulfobacter* and relatives	3-5	8-10	3-5	0	0
ASRB1 (3)	*D. amnigenus*	0	0	0	0	0
D678 (2)	*Desulfovibrio*[b]	<1.5	<0.5	0	<1.5	<0.5
S223 (4)	*S. wolinii*	0	0	0	0	0
KOP1 (5)	*S. pfennigii*	0	0	0	0	0
MPOB1 (5,6)	*S. pfennigii* str. MPOB and *D. amnigenus*	0.3-0.7	0	0	1.5-2.5	1.5-2.5

References: 1. Amann et al., 1990; 2. Devereux et al., 1992; 3. Oude Elferink et al., 1997; 4. Harmsen et al., 1996a; 5. Harmsen et al., 1995; 6. Harmsen et al., 1996b.

[a]Several non-target organisms share the target sequence of this probe for Gram-negative sulfate-reducing bacteria (Amann et al., 1992), while some target organisms (e.g. *D. amnigenus*) have several mismatches with the probe.
[b]A few organisms in the *Pelobacter/Geobacter/Desulfuromonas* lineage share the target sequence of this probe.

play a role in the Ac+S reactor. Maybe their outflow from the reactor is compensated by a higher biomass production compared to the other species in the sludge. Our dot blot hybridization results also seem to indicate Gram-positive sulfate reducers, such as *Desulfotomaculum acetoxidans*-like bacteria, could indeed be playing a role in the Ac+S reactor, because the relative amount of 16S rRNA of Gram-negative sulfate reducers was very low (i.e. 4-7% in the sludge and 2-4% in the effluent), while the COD conversion via sulfate reduction was high (40%).

In marine sediments *Desulfobacter* sp. have been reported to be the main acetate-utilizing sulfate reducers (Taylor and Parkes, 1985), but in our Ac+S reactor *Desulfobacter* sp. did not play an important role if we assumed that cy17:0 (I) and not 10Me16:0 was a the most reliable biomarker. Our results agree with the results of Raskin et al. (1996) who observed that *Desulfobacter* sp. were not important for the acetate degradation in their anaerobic reactors. Since most *Desulfobacter* sp. prefer marine conditions for growth (Widdel and Bak, 1992), they suggested that the growth of *Desulfobacter* sp. was hampered due to the lack of high levels of sodium and magnesium chloride in the reactors.

The PLFA patterns (Table 3) as well as the dot blot hybridization results (Table 4) showed that mesophilic Gram-negative sulfate-reducing bacteria were present in all sludges, even if the reactor influent did not contain sulfate. In the absence of sulfate, several SRB can ferment compounds such as ethanol and pyruvate (Widdel, 1988), or grow syntrophically with a methanogenic partner (e.g. *Syntrophobacter* sp., and the Gram-positive, spore-forming strain PT (Wu et al., 1992)). This could explain the high levels of SRB in the reactors without sulfate in the influent. Our results partly corroborate the findings of Raskin et al. (1996), who reported that the presence of SRB in anaerobic fixed-bed reactors was independent of the presence of sulfate in the reactor. However, in the presence or absence of sulfate in the influent, they mainly found *Desulfovibrio* and *Desulfobacterium* sp. In our reactors *Desulfobulbus* sp. were the dominant Gram-negative SRB in most sludges (based on hybridization results), and *Desulfobacterium* sp. and relatives (D804) could only be detected in the reactors with sulfate in the influent.

The maximum relative amount of *Desulfobulbus* 16S rRNA was found for VFA+S sludge, where ± 80% of the SRB385 signal could be attributed to *Desulfobulbus* (D660). No *Desulfobulbus* 16S rRNA was detected in the reactor fed with acetate and sulfate (Ac+S). Since *Desulfobulbus* sp. does not utilize acetate or butyrate (Widdel and Bak, 1992), the substrate for *Desulfobulbus* sp. in the VFA+S reactor was probably propionate. It is known that *Desulfobulbus* sp. can be the dominant propionate-degrading organism in anaerobic bioreactors (Harmsen et al., 1996a).

The 16S rRNA probes KOP1 and ASRB1 did not hybridize with any of the sludge samples. This indicated that the hybridization results obtained with the MPOB1

probe are originating from strain MPOB 16S rRNA and not from *D. amnigenus* or *S. pfennigii* 16S rRNA. *S. wolinii* (probe S223) was not detected in any of the sludge samples.

Based on dot blot hybridization results *Desulfovibrio* sp. was not very abundant in the different sludge types. However, the presence of the marker PLFA i17:1ω7 in the sludge PLFA patterns, and the absence of detectable amounts of *S. wolinii* and *S. pfennigii* 16S rRNA, seems to contradict the results of the dot blot hybridizations. What causes this discrepancy between the two methods is not exactly clear. It is possible that the marker i17:1ω7 is not only present in *Desulfovibrio*, *S. wolinii* and S. *pfennigii*, but also in other bacteria. It is also possible that the *Desulfovibrio* 16S rRNA probe does not hybridize with all *Desulfovibrio* species. Furthermore, we cannot exclude that the relative amount of *Desulfovibrio* sp. in the sludge was underestimated, because we found that the *Desulfovibrio* probe D687 was not very sensitive for the detection of *Desulfovibrio* sp. The hybridization signal intensities we obtained with the D687 probe, and 16S rRNA from pure cultures of *Desulfovibrio vulgaris* or *D. desulfuricans*, were always lower (approx. 5-fold) than the signal intensities obtained with the EUB338 probe. This was surprising, because the hybridization signal intensities obtained with the other 16S rRNA probes and pure cultures of their target organisms were always comparable to the EUB338 probe hybridization signal intensity. The reason for this discrepancy between the D687 and EUB338 hybridization signal is unclear, but we can exclude the hypothesis that it was caused by impurities of the *Desulfovibrio* cultures or a wrong nucleotide sequence of our D687 probe.

Concluding remarks

In this study insight was obtained into the sulfate-reducing population in anaerobic bioreactors, by using the 16S rRNA dot blot hybridization method and by characterizing the PLFA profile of different sludge types. The dot blot hybridization method seemed to be the most useful method for estimating abundances of sulfate reducers in sludge. The PLFA profiles were useful for a quick general impression of the total bacterial sludge composition, but our results indicated that PLFA biomarkers for characterization and quantification of the sulfate-reducing sludge population should be used with care, and preferentially in combination with other quantification data.

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CHAPTER 9

Summary and concluding remarks

SUMMARY AND CONCLUDING REMARKS

The treatment of industrial wastewaters containing high amounts of easily degradable organic compounds in anaerobic bioreactors is a well-established process. Similarly, wastewaters which in addition to organic compounds also contain sulfate can be treated in this way. For a long time, the occurrence of sulfate reduction was considered to be undesired. However, there are some recent developments in which sulfate reduction is optimized for the removal of sulfur compounds from waste streams. In the treatment of wastewaters which contain sulfate and organic compounds, sulfate reduction interferes with methanogenesis. Both mutualistic and competitive interactions between sulfate-reducing bacteria and methanogenic bacteria have been observed. Sulfate reducers can compete with methanogens for substrates such as hydrogen, formate and acetate, and with acetogens for substrates such as propionate and butyrate. On the other hand sulfate reducers can also assist propionate- and butyrate-degrading acetogens by acting as hydrogen scavenger, and in the absence of sulfate some sulfate reducers are even able to grow fermentatively or in syntrophic association with methanogens.

Thus far it has been difficult to steer the wastewater treatment process in sulfate-fed bioreactors in the desired direction (i.e. in the direction of sulfidogenesis or of methanogenesis). Therefore, the aim of the research presented in this thesis was to investigate the effect of sulfate on the presence and activity of sulfate reducers and their acetogenic and methanogenic counterparts in sulfate-fed anaerobic bioreactors, in order to get a better grip on the treatment process.

Acetate-degrading sulfate reducers

Acetate is a key intermediate in the anaerobic degradation of organic material. Thus far, information about the competition between sulfate reducers and methanogens for acetate in anaerobic bioreactors has been scarce, and contradictory. Furthermore, information on the type of acetate-degrading sulfate reducers in reactor sludge was not available, which made predictions over the outcome of competition between sulfate reducers and methanogens difficult.

Therefore, the research first focused on the characterization of acetate-degrading sulfate reducers which are dominantly present in sulfidogenic granular sludge. This led to the isolation and characterization of two thus far unknown acetate-degrading sulfate reducers, now named *Desulforhabdus amnigenus* strain ASRB1 and *Desulfobacca acetoxidans* strain ASRB2 (Chapters 2 and 3, respectively). *Desulforhabdus amnigenus* was isolated from granular sludge of a pilot-scale upflow anaerobic sludge bed (UASB) reactor treating papermill wastewater, while *Desulfobacca acetoxidans* was isolated from a lab-scale UASB reactor fed with acetate and sulfate. In the pilot-scale reactor the COD/sulfate ratio (g/g) was approx. 1.1, and 75% of the degraded COD was degraded via sulfate reduction. The lab-scale reactor was operating at an excess of sulfate

(COD/sulfate ratio (g/g)= 0.6), and all acetate was degraded via sulfate reduction. Both acetate-degrading sulfate reducers were isolated from the highest serial dilutions of sludge which showed growth on acetate and sulfate. This indicated that these bacteria are the dominant acetate-degrading sulfate reducers in the two respective sludge-types. Based on 16S rRNA analyses both sulfate reducers phylogenetically cluster with the delta subdivision of the *Proteobacteria*, but they are not closely related to each other (Fig. 1). There are large differences between the physiological characteristics of *D. amnigenus* and *D. acetoxidans*. *D. amnigenus* is a substrate generalist, which besides acetate, can use a wide variety of other substrates, such as propionate, butyrate, lactate, H_2+CO_2, and alcohols, while *D. acetoxidans* is a substrate specialist, which only utilizes acetate as a carbon and energy source.

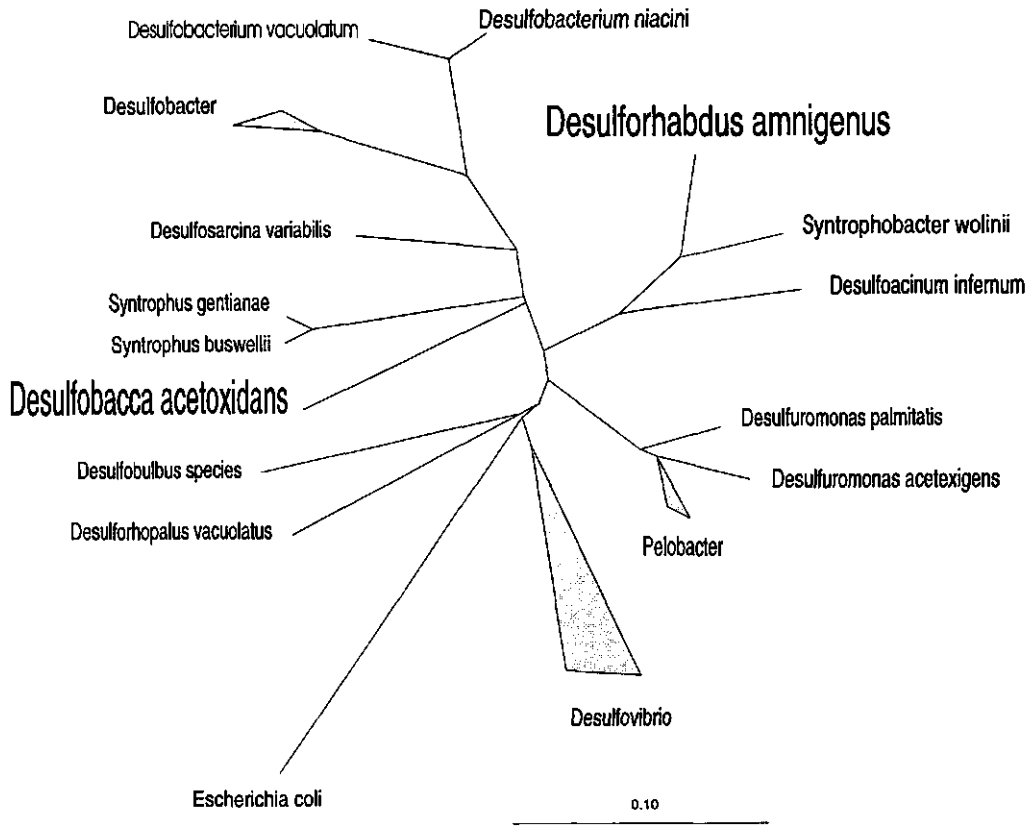

Figure 1. A 16S rRNA based phylogenetic tree reflecting the relationships of *Desulforhabus amnigenus* and *Desulfobacca acetoxidans* with other sulfate reducers and relatives belonging to the delta subdivision of the *Proteobacteria*. Marker bar = 0.10 K_{nuc}.

A special characteristic of *D. amnigenus* is its ability to isomerize butyrate to isobutyrate (Chapter 5), a property which was thus far not described for sulfate reducers. Nuclear Magnetic Resonance (NMR) studies with ^{13}C-labelled butyrate showed that isobutyrate was formed by a migration of the carboxyl group, conform the butyrate isomerization reaction reported for methanogenic consortia. Further investigations showed that the capacity of *D. amnigenus* to isomerize butyrate was not unique among sulfate reducers. Several other butyrate-degrading sulfate reducers, including *Desulfobacterium vacuolatum*, and *Desulfoarculus baarsii*, were to a lesser extent also capable of butyrate isomerization.

Competition for acetate

Important factors for the outcome of the competition for acetate between methanogens and sulfate reducers are the acetate degradation properties of the bacteria involved.

To examine the competition for acetate between *D. amnigenus*, *D. acetoxidans*, and acetate-degrading methanogens, the kinetics of acetate degradation was studies for both sulfate reducers and the kinetic properties were compared with those of acetate-degrading methanogens (Chapter 4). The Michaelis-Menten parameters for *D. amnigenus* (K_m= 0.2-1 mM, V_{max} = 21-35 μmol min^{-1} g protein^{-1}), and *D. acetoxidans* (K_m= 0.2-1 mM, V_{max}= 29-57 μmol min^{-1} g protein^{-1}) were in the same range or slightly better than those of most *Methanosaeta* sp. (K_m= 0.4-1.2 mM, V_{max}= 32-170 μmol min^{-1} g protein^{-1}). The same applied for the Monod kinetic parameter μ_{max} and the acetate-degradation threshold, which were 0.14-0.20 day^{-1} and <15 μM for *D. amnigenus*, 0.31-0.41 day^{-1} and <15 μM for *D. acetoxidans*, and 0.08-0.69 day^{-1} and 7-69 μM for *Methanosaeta*, respectively. Compared with *Methanosarcina* sp. (K_m= 3.0 mM, threshold= 0.19-1.2 mM, μ_{max}=0.46-0.69 day^{-1}), *D. amnigenus* and *D. acetoxidans* had much better K_m and threshold values, but only *D. acetoxidans* had a comparable μ_{max} value. Based on the acetate kinetic parameters of *D. amnigenus* and *D. acetoxidans* compared to those of *Methanosaeta* sp. and *Methanosarcina* sp., it can be predicted that *D. amnigenus* can slowly outcompete *Methanosaeta* sp., while *D. acetoxidans* can even outcompete *Methanosarcina* sp.

Generally, *Methanosaeta* sp. are the dominant acetate-degrading methanogens in methanogenic bioreactors under acetate-limiting conditions. Such acetate-limiting conditions also prevailed in the papermill UASB-reactor. This could explain why *D. amnigenus* became the dominant acetate-degrading sulfate reducer in the papermill UASB-reactor, and also why a long adaptation time was needed before sulfate-reduction became the dominant process in this reactor. Unfortunately, the fact that *D. amnigenus* is a substrate generalist makes it difficult to draw clear conclusion about the exact role of *D. amnigenus* in the sludge, because batch studies with mixed substrates indicated that acetate was one of the least preferred substrates of *D. amnigenus* (Chapter 4). Although it is known that carbon substrates which provoke diauxy under batch conditions are used simultaneously under

carbon limited conditions, it cannot be excluded that the presence of substrates, such as lactate, propionate, or ethanol, could have a negative effect on the acetate degradation rate of *D. amnigenus* in the sludge.

The microbial composition of sulfidogenic granular sludge

From the characterization studies presented above it became clear that reactors treating different wastewaters also contained different sulfate-reducing populations. Two factors which play an important in the microbial composition of sludge are the composition of the organic components in the wastewater and the COD/sulfate ratio of the wastewater. In order to investigate the effect of these factors on the presence and activity of sulfate reducers and their acetogenic and methanogenic counterparts in sludge, good sludge characterization methods are indispensable. Fortunately, to date several sludge characterization methods are available, including conventional methods such as Most Probable Number (MPN) methods, but also more modern methods such as polar-lipid fatty acid (PLFA) analyses, specific PCR amplifications, and 16S rRNA dot blot hybridizations. In Chapter 6, 7 and 8 the sludge from various methanogenic and sulfidogenic reactors was characterized using the different sludge characterization methods. This research did not only improve our knowledge on the effect of wastewater composition and COD/sulfate ratio on the microbial sludge composition, but it also showed the advantages and disadvantages of the different characterization methods.

Very useful methods for the species, genus or group-specific detection of microorganisms in sludge are the PCR amplification method, and the dot blot hybridization method. This is shown in Chapter 6 describing the development of a 16S rRNA oligonucleotide probe, probe ASRB1, for the species-specific detection of *D. amnigenus* in sludge. If this probe was used in dot blot hybridization studies, *D. amnigenus* could still be detected if approx. 0.1 to 0.001 ‰ of the total bacterial sludge population was *D. amnigenus*. If the probe was used as a PCR primer the sensitivity was even 10 times higher. It is not possible to use the dot blot hybridization method for the exact quantification of the number of bacterial cells in the sludge, because it is based on the rRNA content in the cell. For *D. amnigenus* as shown that this rRNA content was affected by the growth rate and the growth phase, and that it ranged from 19 fg cell^{-1} in slow-growing cultures to 90 fg cell^{-1} in fast-growing cultures. This also indicates that the detection threshold of the dot blot hybridization method for fast-growing cells is approximately 5-fold lower than for slow-growing cells.

Many sludge-types were analyzed for the presence of *D. amnigenus*. Unfortunately this bacterium could only be detected in the sludge from the pilot-scale reactor from which is was originally isolated, and in the sludge which was used as seed-sludge for this pilot-scale reactor. This seems to indicate that *D. amnigenus* plays no important role in other sulfidogenic bioreactors. This could be due to the absence of *D. amnigenus* in the seed sludge of the sulfidogenic bioreactors.

In Chapter 7 granular sludge from a full-scale UASB reactor was studied, by using the 16S rRNA

dot blot hybridization method in combination with MPN estimates. The wastewater which was treated in the UASB-reactor contained mainly starch, acetate, propionate, butyrate and formate, and had a COD/sulfate ratio of 9.5. Under these conditions acetate seemed to be mainly degraded by *Methanosaeta*-like bacteria, while propionate was the preferred substrate for sulfate reduction. However, the *Desulfobulbus*-like propionate-degrading sulfate reducers in the sludge, competed with *Syntrophobacter*-like bacteria, for the available propionate. Hydrogen and formate were probably mainly degraded via methanogenesis by members of the order of *Methanobacteriales*. Dot blot hybridization studies of the MPN enrichments showed that the hydrogen, formate and butyrate-degrading sulfate reducers in the sludge could not be characterized with the 16S rRNA probes available to date. The same was true for syntrophic butyrate degraders. This clearly demonstrates that, although the dot blot hybridization method is very useful for sludge characterization studies, it does not (yet) give a complete picture of the total sludge composition, and it should be used in combination with other methods to avoid missing important groups of microorganisms in the sludge.

A method which also gives insight in the sludge composition is the PLFA method. In Chapter 8 this method was combined with the dot blot hybridization method to study the sulfate-reducing and acetogenic population of several methanogenic and sulfidogenic sludges. The results show that PLFA analyses of the sludge were useful to obtain a quick general impression of the total bacterial sludge composition, but were less suitable for an accurate characterization and quantification of the sulfate-reducing population in the sludge. This was due to the lack of selective biomarkers for these bacteria. The combined results of the PLFA analysis and 16S rRNA dot blot hybridizations showed that presence of sulfate reducers in the sludge was not dependent on the presence of sulfate in the wastewater. This may be explained by the syntrophic and/or fermentative capacities of some sulfate reducers in the absence of sulfate. *Desulfobulbus* sp. seemed to be important in reactors with carbohydrates and/or volatile fatty acids containing wastewater. In the presence of sulfate these bacteria could play a role in propionate degradation, while in the absence of sulfate they might play a role in lactate and ethanol fermentation. *Desulfobacter* sp. did not seem to be important for acetate degradation in any of the sulfate-fed reactors. Apparently, most *Desulfobacter* sp. are typical marine organisms. In the acetate and sulfate-fed reactor, *Desulfotomaculum acetoxidans*-like bacteria seemed to play a role in acetate degradation.

Conclusions

The results which were presented in this thesis improved our knowledge of the effect sulfate on the microbial sludge population in anaerobic reactors. As already mentioned it has been, and still is, difficult to steer the wastewater treatment process in sulfate-fed bioreactors in the direction of sulfate reduction or methanogenesis. However, from this thesis a few conclusions can be drawn

which are useful for optimization of the reactor process, and for future sludge research.

1. There appears to be a thight competition between acetate-degrading sulfate reducers and acetate-degrading methanogens (mainly *Methanosaeta* sp.) in anaerobic reactor sludge. It will therefore take a long time before acetate-degrading sulfate reducers have outgrown acetate-degrading methanogens, even if there is an excess of sulfate.
2. In sulfate-limited bioreactors sulfate reducers compete with each other for the available sulfate. Under sulfate limitation acetate seems to be one of the least, and propionate one of the most favoured substrates for sulfate reduction.
3. Based on 16S rRNA analyses *Desulfobulbus*-like bacteria can be found in high numbers in many reactors, even in methanogenic reactors.
4. There is a large variation in the microbial composition of granular sludge. The choice of a seed sludge for a new reactor will probably not only affect the initial, but also the final purification efficiency of the reactor.
5. All sludge characterization methods have their advantages and disadvantages. The best picture of the microbial sludge composition can be obtained by combining as many of them as possible.

SAMENVATTING EN CONCLUSIES

Het in anaërobe bioreactoren zuiveren van industrieel afvalwater met hoge gehaltes aan snel afbreekbare organische stoffen is een gebruikelijke methode. Ook afvalwater dat naast organische stoffen tevens sulfaat bevat kan zo worden behandeld. Het optreden van sulfaatreductie was lange tijd ongewenst. Maar tegenwoordig zijn er ook enkele methoden waarbij sulfaatreductie juist is geoptimaliseerd om zo zwavelverbindingen uit het afvalwater te kunnen verwijderen. Bij het zuiveringsproces van afvalwater dat zowel sulfaat als organische stoffen bevat, beïnvloedt sulfaatreductie de methaanproductie. De interacties tussen sulfaatreduceerders en methanogene consortia kunnen zowel competitief als mutualistisch zijn. Sulfaatreduceerders kunnen competeren met methanogenen voor substraten als waterstof, formiaat en acetaat en met acetogenen voor stoffen als propionaat en butyraat. Sulfaatreduceerders kunnen echter ook optreden als syntrofe partner van propionaat en butyraat oxiderende acetogenen. In de afwezigheid van sulfaat kunnen sommige sulfaatreduceerders fermentatief of, in combinatie met methanogenen, zelfs syntroof groeien.

Tot nu toe was het moeilijk om het afvalwaterzuiveringsproces van sulfaat bevattend afvalwater in de gewenste richting te sturen (d.w.z. in de richting van sulfaatreductie of methanogenese). Het doel van het in dit proefschrift gepresenteerde onderzoek was om na te gaan hoe de aanwezigheid van sulfaat in afvalwater de aanwezigheid en de activiteit van sulfaatreduceerders en methanogene consortia beïnvloedt, om zo meer grip te krijgen op het zuiveringsproces.

Acetaat-afbrekende sulfaatreduceerders

Acetaat is één van de belangrijkste intermediairen in het afbraakproces van organische stoffen. Tot dusver was er weinig bekend over de competitie tussen sulfaat-reduceerders en methanogenen voor acetaat en studies over dit onderwerp spraken elkaar tegen. Verder was onbekend welke acetaat-afbrekende sulfaatreduceerders in reactorslib kunnen voorkomen. Hierdoor was het erg moeilijk om te voorspellen hoe de competitie voor acetaat tussen sulfaatreduceerders en methanogenen zou verlopen.

Het onderzoek heeft zich daarom eerst gericht op het karakteriseren van acetaat-afbrekende sulfaatreduceerders die dominant aanwezig zijn in sulfaatreducerend slib. Dit heeft geleid tot de isolatie en karakterisatie van twee tot dusver onbekende acetaat-afbrekende sulfaatreduceerders, nu *Desulforhabdus amnigenus* stam ASRB1 en *Desulfobacca acetoxidans* stam ASRB2 genaamd (Hoofdstuk 2 en 3, respectievelijk). Beide bacteriestammen werden geïsoleerd uit korrelslib van een "opstroom anaëroob slibbed" reactor, beter bekend als een upflow anaerobic sludge bed (UASB) reactor. *Desulforhabdus amnigenus* werd geïsoleerd uit slib van een pilot-schaal UASB reactor die afvalwater uit een papierfabriek zuiverde, terwijl *Desulfobacca acetoxidans* werd

geïsoleerd uit slib van een lab-schaal UASB reactor die werd gevoed met acetaat en sulfaat. De verhouding tussen chemisch zuurstof verbruik (CZV) en sulfaat was ongeveer 1,1 (g/g) in de pilot-schaal reactor en 75 % van het CZV werd afgebroken via sulfaatreductie. In de lab-schaal reactor was sulfaat in overmaat aanwezig (CZV/sulfaat ratio= 0.6 (g/g)) en werd alle acetaat via sulfaatreductie afgebroken. Om de acetaat-afbrekende sulfaatreduceerders uit het korrelslib te isoleren, waren seriële slibverdunningen gemaakt. Beide sulfaatreduceerders werden geïsoleerd van de hoogste verdunning die groei vertoonde met acetaat en sulfaat, wat aangaf dat deze bacteriën de dominante acetaat-afbrekende sulfaatreduceerders in de respectievelijke reactoren waren. Gebaseerd op 16S rRNA analyses behoren beide sulfaatreduceerders tot de delta subdivisie van de *Proteobacteria*, maar zij zijn niet nauw verwant aan elkaar (Fig. 1). Er zijn grote verschillen tussen de fysiologische eigenschappen van *D. amnigenus* en *D. acetoxidans*. *D. amnigenus* kan naast acetaat nog een scala van andere substraten gebruiken, zoals propionaat, butyraat, lactaat, H_2+CO_2 en alcoholen. *D. acetoxidans* daarentegen is gespecialiseerd in acetaat-afbraak en kan voor zo ver kon worden onderzocht geen andere substraten gebruiken. Een speciale eigenschap van *D. amnigenus* is de capaciteit om butyraat te isomeriseren (Hoofdstuk 5). Deze eigenschap was tot dusver nog niet beschreven voor sulfaatreduceerders. Nucleaire Magnetische Resonantie (NMR) studies met ^{13}C-gelabelde butyraat lieten zien dat isobutyraat, net als bij methanogene consortia, werd gevormd door de migratie van de carboxylgroep. Vervolgonderzoek liet zien dat niet alleen *D. amnigenus*, maar ook andere sulfaatreduceerders, waaronder *Desulfobacterium vacuolatum* en *Desulfoarculus baarsii* in staat waren butyraat te isomeriseren, zij het in mindere mate.

Competitie voor acetaat

Belangrijke factoren in de competitie voor acetaat tussen methanogenen en sulfaatreduceerders zijn de kinetische parameters voor acetaat-afbraak van de betrokken bacteriën. Om de competitie voor acetaat tussen *D. amnigenus*, *D. acetoxidans* en acetaat-afbrekende methanogenen te onderzoeken werden van beide sulfaatreduceerders de kinetische parameters voor acetaat-afbraak bepaald en vergeleken met die van acetaat-afbrekende methanogenen (Hoofdstuk 4). De Michaelis-Menten kinetische parameters voor *D. amnigenus* (K_m= 0.2-1 mM, V_{max}= 21-35 µmol min^{-1} g eiwit^{-1}), en *D. acetoxidans* (K_m= 0.2-1 mM, V_{max}= 29-57 µmol min^{-1} g eiwit $^{-1}$) lagen in de zelfde orde van grootte of waren iets beter dan die van *Methanosaeta* sp. (K_m= 0.4-1.2 mM, V_{max} = 32-170 µmol min^{-1} g eiwit^{-1}). Dit gold ook voor de Monod kinetische parameter μ_{max} en de drempel concentratie voor acetaat-afbraak, deze waren respectievelijk 0.14-0.20 dag^{-1} and <15 µM voor *D. amnigenus*, 0.31-0.41 dag^{-1} en <15 µM voor *D. acetoxidans*, en 0.08-0.69 dag $^{-1}$ en 7-69 µM voor *Methanosaeta*. Vergeleken met *Methanosarcina* sp. (K_m= 3.0 mM, drempel concentratie= 0.19-1.2 mM, μ_{max}=0.46-0.69 dag^{-1}), hadden *D. amnigenus* en *D. acetoxidans* veel betere K_m en drempel waarden, maar alleen *D. acetoxidans* had een vergelijkbare μ_{max} waarde. Op basis van de acetaat kinetische

parameters van *D. amnigenus* en *D. acetoxidans* in vergelijking met die van *Methanosaeta* sp. en *Methanosarcina* sp., kan worden voorspeld dat *D. amnigenus Methanosaeta* sp. kan verdringen uit het slib, terwijl *D. acetoxidans* waarschijnlijk zelfs *Methanosarcina* sp. kan verdringen.
Normaal gesproken zijn, onder acetaat-limiterende condities, *Methanosaeta* sp. de dominante acetaat-afbrekende methanogenen in methanogeen korrelslib. Dergelijke acetaat-limiterende condities waren ook aanwezig in de pilot-schaal reactor die papierfabriek afvalwater zuiverde. Mogelijk kan dit verklaren waarom *D. amnigenus* na een lange adaptatieperiode van het slib, uiteindelijk de dominante acetaat-afbrekende sulfaatreduceerder werd in deze reactor. Omdat *D. amnigenus* naast acetaat ook andere substraten kan gebruiken is het moeilijk om precies vast te stellen welke rol *D. amnigenus* speelt in het zuiveringsproces, zeker gezien de resultaten van de gemengde substraatstudies, die aangaven dat *D. amnigenus* andere substraten zoals propionaat en ethanol prefereerde boven acetaat (Hoofdstuk 4). Hoewel het bekend is dat substraten, die in batch cultures leiden tot diauxie, in koolstof gelimiteerde continue cultures vaak wel simultaan worden afgebroken, kan niet worden uitgesloten dat de aanwezigheid van substraten zoals lactaat, propionaat of ethanol in de reactor een negatief effect heeft op de acetaat afbraaksnelheid van *D. amnigenus*.

De microbiële compositie van sulfidogeen korrelslib.
Uit het hierboven beschreven onderzoek naar acetaat-afbrekende sulfaatreduceerders werd duidelijk dat reactoren die verschillende typen afvalwater zuiveren, ook verschillende sulfaatreducerende populaties bevatten. Twee factoren die een belangrijke rol spelen bij de microbiële compositie van slib zijn de samenstelling van organische verbindingen in het afvalwater en de CZV/sulfaat ratio van het afvalwater. Goede slibkarakterisatie is onontbeerlijk om te kunnen onderzoeken welk effect genoemde factoren nou precies hebben op de aanwezigheid en de activiteit van sulfaatreduceerders, methanogenen en acetogenen in het slib. Een onderzoeker heeft tegenwoordig een aantal verschillende methoden beschikbaar voor slibkarakterisatie, waaronder conventionele methoden zoals de "Most Probable Number" (MPN) test, maar ook wat moderne methoden zoals fosfolipide-vetzuur (PLFA) analyses, specifieke "Polymerase Chain Reaction" (PCR) technieken en 16S rRNA dot-blot hybridisatie technieken. In hoofdstuk 6,7, en 8 is slib van verschillende sulfidogene en methanogene reactoren onderzocht met behulp van combinaties van slibkarakterisatie methoden. Dit onderzoek gaf niet alleen informatie over het effect van de afvalwatersamenstelling en de CZV/sulfaat ratio op de microbiële samenstelling van slib, maar liet ook de voor- en nadelen van de verschillende slibkarakterisatie methoden zien.
Hele bruikbare methoden voor de species-, groep- of genus-specifieke detectie van micro-organismen zijn de PCR en de dot blot hybridisatie methode. Dit blijkt bijvoorbeeld uit de resultaten van hoofdstuk 6, waarin de ontwikkeling van een 16S rRNA probe (probe ASRB1) wordt

beschreven voor de species-specifieke detectie van *D. amnigenus* in slib. Wanneer de probe werd gebruik in dot blot hybridisatie studies kon *D. amnigenus* zelfs nog in slib gedetecteerd worden als deze slechts 0.1-0.001 ‰ van de totale bacteriepopulatie uitmaakte. Als de probe werd gebruikt als PCR primer was de gevoeligheid zelfs nog 10 keer beter. Het is niet mogelijk om de dot blot hybridisatie methode te gebruiken voor het exact kwantificeren van celaantallen, omdat de methode is gebaseerd op het rRNA van cellen en het rRNA gehalte van cellen is niet constant. Het rRNA gehalte van *D. amnigenus* cellen bleek beïnvloed te worden door de groeisnelheid en de groeifase van de cellen en het varieerde van 19 fg cel^{-1} in langzaam groeiende cellen tot 90 fg cel^{-1} in snel groeiende cellen. Dit geeft ook aan dat de dot blot hybridisatie methode ongeveer 5 keer gevoeliger is voor snelgroeiende dan voor langzaam groeiende cellen.

Alhoewel er veel slibtypen zijn onderzocht op de aanwezigheid van *D. amnigenus*, kon deze bacterie toch alleen maar aangetoond worden in het slib van de reactor waaruit zij oorspronkelijk was geïsoleerd en in het slib dat was gebruikt om deze pilot-schaal reactor mee op te starten. Dit geeft aan dat *D. amnigenus* waarschijnlijk geen rol speelt in andere sulfidogene bioreactoren. Mogelijk was *D. amnigenus* ook al niet aanwezig in het startslib van deze reactoren.

In hoofdstuk 7 is onderzoek beschreven met korrelslib uit een praktijk-schaal UASB reactor. De microbiële compositie van dit korrelslib werd geanalyseerd via 16S rRNA dot blot hybridisaties en MPN schattingen. Het afvalwater van de UASB reactor was afkomstig van een papierfabriek, het bevatte voornamelijk zetmeel, acetaat, propionaat, butyraat en formiaat en had een CZV/sulfaat ratio van 9.5 (g/g). In deze reactor werd acetaat voornamelijk afgebroken via methanogenese door *Methanosaeta*-achtige bacteriën. Propionaat werd vooral geoxideerd via sulfaatreductie, alhoewel de *Desulfobulbus*-achtige propionaat-afbrekende sulfaatreduceerders moesten competeren met *Syntrophobacter*-achtige syntrofe propionaat afbrekers. Waterstof en formiaat werden vooral afgebroken door methanogenen behorende tot de orde *Methanobacteriales*. Dot blot hybridisatie studies met de MPN ophopingscultures toonden aan dat de waterstof-, formiaat- en butyraat-afbrekende sulfaatreduceerders niet konden worden gekarakteriseerd met de momenteel beschikbare 16S rRNA probes. Dit gold ook voor de syntrofe butyraat oxideerders. Dit onderzoek maakte duidelijk dat de dot blot hybridisatie techniek goed bruikbaar is voor slibkarakterisatie, naar tot nu toe nog geen compleet beeld geeft van de totale slibpopulatie. Deze techniek moet dus gebruikt worden in combinatie met andere karakterisatie technieken, om zo te voorkomen dat belangrijke slibpopulaties niet worden opgemerkt.

Een andere methode die ook inzicht geeft in de slib compositie is PLFA analyse. In hoofdstuk 8 is deze methode gecombineerd met dot blot hybridisaties om sulfaatreduceerders en acetogenen in sulfidogeen en methanogeen slib te detecteren. De resultaten toonden aan dat de PLFA analyses bruikbaar zijn om snel een algemene indruk te krijgen van de totale microbiële slibsamenstelling, maar niet zo geschikt zijn voor nauwkeurige karakterisatie en kwantificatie van de

slibsamenstelling. De belangrijkste oorzaak hiervoor is het gebrek aan selectieve biomarkers voor de verschillende sulfaatreduceerders en acetogenen. Uit de gecombineerde resultaten van de PLFA analyses en de dot blot hybridisaties bleek dat de aanwezigheid van sulfaatreduceerders in slib niet afhankelijk was van de aanwezigheid van sulfaat in het afvalwater. Het is bekend dat sommige sulfaatreduceerders fermentatief of syntroof kunnen groeien, dit zou de aanwezigheid van sulfaatreduceerders in de afwezigheid van sulfaat kunnen verklaren. *Desulfobulbus* sp. leek een van de belangrijkste sulfaatreduceerders te zijn in reactoren waar koolhydraten of vluchtige vetzuren in het afvalwater aanwezig waren. In de aanwezigheid van sulfaat zou deze bacterie een rol kunnen spelen bij de afbraak van propionaat, terwijl zij in de afwezigheid van sulfaat ethanol en lactaat zou kunnen fermenteren. *Desulfobacter* sp. leek in geen enkele sulfidogene reactor een rol te spelen bij de afbraak van acetaat. Klaarblijkelijk zijn de meeste *Desulfobacter* sp. marine organismen. *Desulfotomaculum acetoxidans*-achtige bacteriën leken wel een rol te kunnen spelen bij de afbraak van acetaat in sulfidogene reactoren. Deze bacteriën werden namelijk aangetroffen in slib van een met acetaat en sulfaat gevoede reactor.

Conclusies

De resultaten die in dit proefschrift werden gepresenteerd hebben geholpen ons inzicht te vergroten in het effect van sulfaat of de microbiële slibsamenstelling van anaërobe reactoren. Zoals reeds is genoemd, was het en is het nog steeds moeilijk om het afvalwaterzuiveringsproces in sulfaat gevoede bioreactoren te sturen in de richting van sulfaatreductie of methanogenese. Echter, uit dit proefschrift kunnen enkele conclusies worden getrokken die gebruikt kunnen worden om het reactorproces te optimaliseren en het slibonderzoek te verbeteren.

1. Acetaat-afbrekende sulfaatreduceerders en acetaat-afbrekende methanogenen (voornamelijk *Methanosaeta* sp.) competeren met elkaar voor het in anaërobe bioreactoren aanwezige acetaat. Aangezien de competitieve eigenschappen van de acetaat-afbrekende sulfaatreduceerders nauwelijks beter lijken te zijn dan die van acetaat-afbrekende methanogenen, duurt het lang voordat deze sulfaatreduceerders de methanogenen uit het slib hebben verdrongen, zelfs indien er een overmaat aan sulfaat beschikbaar is.
2. In sulfaat gelimiteerde bioreactoren is er onderlinge competitie tussen de verschillende soorten sulfaatreduceerders voor het beschikbare sulfaat. Onder sulfaat gelimiteerde condities lijkt propionaat als substraat de meeste voorkeur te genieten en acetaat de minste.
3. *Desulfobulbus*-achtige bacteriën kunnen, gebaseerd op 16S rRNA analyses, zowel in sulfidogene als in methanogene bioreactoren in grote hoeveelheden voorkomen.
4. Er is een aanzienlijke variatie in de microbiële samenstelling van korrelslib. De keuze van het startslib voor een nieuwe reactor zal waarschijnlijk niet alleen het initiële, maar ook het

uiteindelijke zuiveringsrendement van de reactor beïnvloeden.

5. Elke techniek voor slibkarakterisatie heeft zijn voor- en nadelen. Het beste beeld van de microbiële slibsamenstelling wordt verkregen door zoveel mogelijk technieken te combineren.

CURRICULUM VITAE

Stefanie Johanna Wilhelma Helena Oude Elferink werd op 14 mei 1969 geboren te Oldenzaal. Na het behalen van het VWO diploma aan de Christelijke Scholengemeenschap te Assen in 1987, begon zij haar studie Moleculaire Wetenschappen aan de Landbouwuniversiteit te Wageningen. Het doctoraal pakket bestond uit de afstudeervakken Microbiologie en Waterzuivering en een stage in de Verenigde Staten bij de vakgroep Microbiologie aan de Universiteit van Cornell "Cornell University", Ithaca, New York. Na het afstuderen in januari 1992, startte zij als Assistent-in-Opleiding bij de vakgroep Microbiologie van de Landbouwuniversiteit Wageningen. Het promotieonderzoek, waarvan de resultaten staan beschreven in dit proefschrift, richtte zich op de fysiologie en ecologie van sulfaatreducerende bacteriën in anaërobe waterzuiveringsreactoren. Sinds september 1996 is de auteur werkzaam als wetenschappelijk medewerkster bij het Instituut voor Dierhouderij en Diergezondheid van de Dienst Landbouwkundig Onderzoek (ID-DLO). Daar werkt zij momenteel bij de afdeling Voedingsfysiologie Herkauwers aan de microbiële ecologie van gras- en snijmaïssilage.

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