# Detection, Phylogeny and Population Dynamics of Syntrophic Propionate-oxidizing Bacteria in Anaerobic Granular Sludge.

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# Detection, phylogeny and population dynamics of syntrophic propionate-oxidizing bacteria in anaerobic granular sludge.

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## Stellingen

1. Kalorie-inname gedurende de werktijd van een ingezetene van twee werkgroepen is twee maal zo hoog in vergelijking met een ingezetene van één werkgroep. Dit gezien de dubbele hoeveelheid gebak, ijs en drank die aangeboden wordt.

2. Het feit dat *Desulfobulbus*-achtige micro-organismen voorkomen in methanogeen slib betekent niet dat de *Desulfobulbus* syntroof propionaat kan oxyderen.

   Heppner, B., G. Zellner, and H. Diekmann. 1992. Start-up and operation of a propionate-degrading fluidized-bed reactor. Appl. Microbiol. Biotechnol. 36:810-816.

   Wu, W. M., R. F. Hickey, and J. G. Zeikus. 1991. Characterization of metabolic performance of methanogenic granules treating brewery wastewater: role of sulfate-reducing bacteria. Appl. Environ. Microbiol. 57:3438-3449.

3. "Man-made" ecosystemen bestaan niet. De mens legt groeicondities en selectiedruk op aan het systeem, de natuur maakt het.

4. Een ijsbaan op de Wageningse berg maakt een "hoogte" stage bereikbaar voor iedere schaatsminnende nederlander.

5. Er zijn in de wetenschap, net als in de gewone wereld, mensen die de middelen hebben en zij die dat niet hebben. Mensen met middelen doen, mensen zonder denken. Gelukkig kunnen ze ook in de wetenschap niet zonder elkaar.

6. Uit het feit dat molybdaat de syntrofe propionaat-oxydatie remt wordt afgeleid dat propionaat in het slib omgezet wordt door sulfaat-reducerende bacteriën (SRB). Maar dat daarom de SRB een ecologisch voordeel hebben boven obligaat syntrofe propionaat-oxydeerders gaat voorbij aan de gedachte dat de "obligaat" syntrofen zelf SRB zouden kunnen zijn.

   Wu, W. M., R. F. Hickey, and J. G. Zeikus. 1991. Characterization of metabolic performance of methanogenic granules treating brewery wastewater: role of sulfate-reducing bacteria. Appl. Environ. Microbiol. 57:3438-3449.

7. Informatici kunnen sneller nieuwe computerprogramma's maken dan de gebruikers kunnen leren.

8. Nederland is te groot voor de fiets en te klein voor de auto.

9. Het is uitermate vreemd dat er in de studie van MacLeod et al. (1990) de formiaatomzettings-aktiviteit van korrelslib gemeten wordt en niet de waterstofomzettings-aktiviteit, terwijl vervolgens alleen gesproken wordt over waterstof-producerende en -consumerende microorganismen.

MacLeod, F. A., S. R. Guiot, and J. W. Costerton. 1990. Layered structure of bacterial aggregates produced in an upflow anaerobic sludge bed and filter reactor. Appl. Environ. Microbiol. 56:1598-1607.

10. De interactie tussen korrelslibbacteriën is als tussen kippen en mensen, wat de één uitscheidt consumeert de ander. Aan beiden zit een luchtje.

11. Ruimte is een betrekkelijk begrip. Hoe kleiner het organisme hoe meer ruimte, totdat je als bacterie midden in een slibkorrel zit.

12. Het AIO-stelsel zorgt voor een overschot aan wetenschappers. Het PostDoc-stelsel stelt werkt dit overschot tijdelijk weg. De tijd is nu gekomen om een stelsel te ontwikkelen voor wetenschappers die uit"gePostDoct" zijn.

13. Een boom zonder wortels valt om, behalve als het een fylogenetische boom is.

Stellingen behorende bij het proefschrift "Detection, phylogeny and population dynamics of syntrophic propionate-oxidizing bacteria in anaerobic granular sludge".

Hermie J.M. Harmsen Wageningen, 10 januari 1996

## Contents

# Chapter 1

## General introduction

## General Introduction.

One of the challenges in microbial ecology is to study microbes directly in their natural habitat. The activity, spatial distribution, and interactions between the microbes, such as those in biofilms, can only be studied *in situ*. In most cases it is impossible to install a microscope in the ecosystem, and even that would not be a solution because usually the individual microbial members of such ecosystems can not be identified by their morphology. For a long time, microbial ecologists used selective enrichment and pure culture methods, but it is generally accepted that these techniques cause many biases. Plate counts and Most Probable Number (MPN) counts on selective media, do often not correlate with the numbers obtained by direct microscopic counting and the dominant microbes as seen under the microscope are often not the ones that are enriched. Furthermore, growth in pure culture does not reflect the way microbes interact in the ecosystem. Moreover, for a lot of microbes there is no suitable culture method yet or they are not culturable at all. It has been estimated that only 20 % of the microbes present in natural environments could obtained in pure culture (Ward et al., 1992).

These problems of cultivation and identification were also faced in studying the ecology of anaerobic microorganisms in methanogenic environments such as granular sludge, where different groups of microbes cooperate with each other in the mineralization of organic matter. Conventional culture and detection techniques are not sufficiently adequate to describe the complex relationships between different anaerobes in these environments (Grotenhuis et al., 1991a). Furthermore, syntrophic consortia of microorganisms were encountered, which are dependent on each other for the growth on certain substrates. Therefore, these microorganisms are hard to culture and until a few years ago could not be obtained in pure cultures. Hence, the ecology and dynamics of these syntrophic organisms were difficult to unravel using the quantification techniques and microscopic approaches available at that time. Therefore, sufficiently specific methods were needed to identify individual community members. Such methods would enable studies on a cellular level and preserve the ecosystem so it can be studied in the laboratory. In the last decade several methods have been developed that make this kind of studies feasible. These methods are not based on growth of the individual organisms but on the detection of specific molecular markers present in all

microbes (Amann et al., 1995).

This thesis describes the results of a project, which mainly focused on the syntrophic propionate oxidation in granular sludge in methanogenic bioreactors. The following introduction will first describe the ecosystem and its microbial interactions and then present an overview of the ribosomal RNA approach, which was used to study this ecosystem and its microbes. Finally, an outline of the thesis is given.

## 1. METHANOGENIC GRANULAR SLUDGE, THE "MAN-MADE" ECOSYSTEM

### Anaerobic wastewater treatment.

When the costs for energy raised at the beginning of the 1970's, anaerobic wastewater treatment started to gain interest, because biogas is produced and no energy for aeration is needed. This has led to the development of new types of reactors with immobilized biomass, such as the Fluidized Bed (FB), the Anaerobic Filter (AF) and the Upflow Anaerobic Sludge Bed (UASB) reactors (Hulshoff Pol, 1989). From these the UASB reactor has the widest application. Presently, more than 300 full-scale UASB reactors all over the world are operative for the treatment of domestic and industrial wastewaters (Lettinga, 1995). In an UASB reactor the wastewater is pumped from the bottom into the reactor, and is purified while passing a bed of methanogenic sludge (Fig. 1). The biomass is retained in the reactor by self-immobilisation of anaerobic microorganisms into densely packed granules.

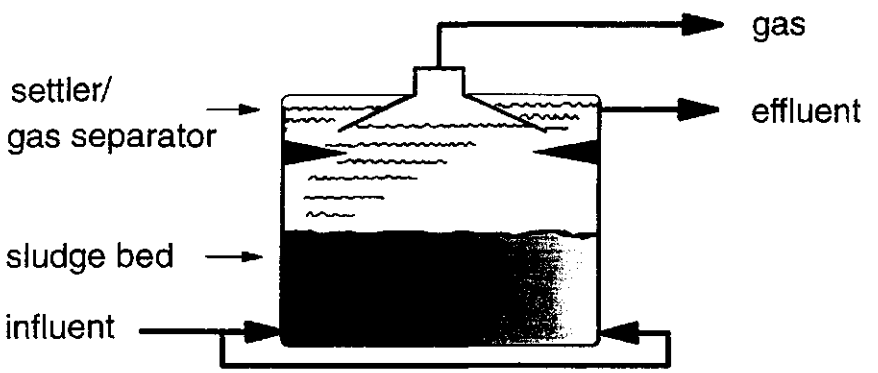


Fig. 1. Schematic diagram of a upflow anaerobic sludge bed (UASB) reactor. Adapted from Lettinga et al. (1980).

**Mineralization of organic material.**

Methanogenic environments, where complex organic matter is converted into carbon dioxide and methane, are widely distributed in nature. These include wetlands and paddy fields, digestive tracts of higher animals and insects, and freshwater sediments (Boone, 1991; Miller, 1991). This thesis however, will focus mainly on granular sludge in anaerobic bioreactors. In this sludge the conversion of organic matter is carried out by at least three different trophic groups of microorganisms (Fig. 2). The first group of hydrolyzing and fermenting bacteria degrades complex organic molecules into monomers like sugars, amino acids and long chain fatty acids, and subsequently ferments these to acetate, carbon dioxide, hydrogen and reduced products like alcohols, lactate and volatile fatty acids (e.g. propionate, butyrate) (Gujer and Zehnder, 1983; McInerney, 1988). The second group of hydrogen-producing acetogenic bacteria converts long chain fatty acids and the reduced products into hydrogen, formate, acetate and carbon dioxide (Mah et al., 1990, Stams and Zehnder, 1990). The third group of methanogenic microorganisms converts the hydrogen, formate and acetate into methane and carbon dioxide (Oremland, 1988,; Jetten et al., 1992). The different conversions are carried out by specialized microbes, and this leads to consortia of a large variety of microorganisms in the granular sludge (Fig. 3). The concerted action of the different groups of microorganisms leads to high rates of bioconversions in the granules (Thiele et al., 1988). A large amount of the organic matter is degraded via reduced intermediates, such as propionate and butyrate. Therefore, the oxidation of these intermediates is a key process in the mineralization of complex organic matter in granular sludge.

**Syntrophic consortia.**

The second step of the anaerobic mineralization of organic matter is the degradation of reduced organic compounds, like propionate, butyrate, longer chained fatty acids and some aromatic acids, into hydrogen or formate, acetate and carbon dioxide. The oxidation of these compounds under methanogenic conditions is energetically unfavourably (Table 1). These reactions can only proceed when they are coupled to the removal of the products, hydrogen or formate and to some extent acetate by methanogens (McInerney 1992). Therefore, acetogenic bacteria have to cooperate with methanogens. To such a cooperation the term "Syntrophism" was assigned (Pfennig and Biebl, 1976). This term was defined as "a cooperation where both partners depend

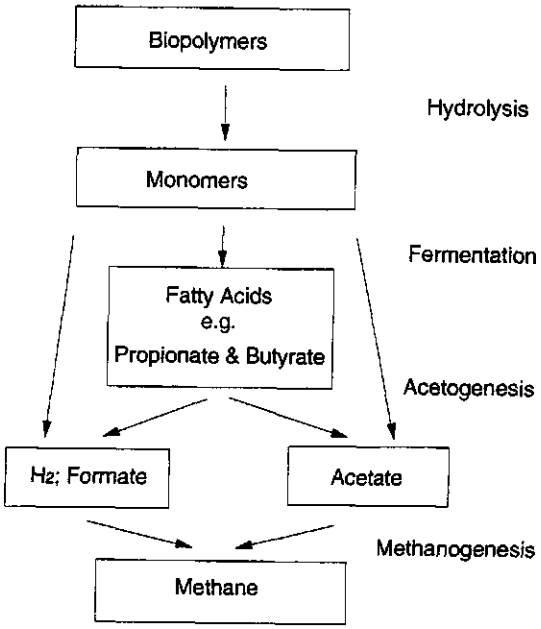


Fig. 2.
Degradation of organic matter under methanogenic conditions, involving hydrolyzing and fermenting, acetogenic, and methanogenic microorganisms.

Fig. 3.
Microbial diversity of granular sludge. Transmission electron microscopic micrograph from sludge fed with volatile fatty acids (also see Chapter 6).

Table 1. Some reactions involved in syntrophic degradation processes. $\Delta G°$-values are taken from Thauer et al. (1977).

| Reactions | | $\Delta G°$ (kJ/mol) |
|---|---|---|
| Acetogenic reactions: | | |
| $Propionate^- + 3H_2O$ | $--> acetate^- + HCO_3^- + 3H_2 + H^+$ | +76.1 |
| $Butyrate^- + 2H_2O$ | $--> 2\ acetate^- + 2H_2 + H^+$ | +48.1 |
| $Methanol + 2H_2O$ | $--> HCO_3^- + 4H_2 + H^+$ | +23.5 |
| $Ethanol + H_2O$ | $--> acetate^- + 2H_2 + H^+$ | +9.6 |
| $Lactate^- + 2H_2O$ | $--> acetate^- + HCO_3^- + 2H_2 + H^+$ | -4.2 |
| $Benzoate^- + 7H_2O$ | $--> 3\ acetate^- + HCO_3^- + 3H_2 + 3H^+$ | +58.9 |
| Methanogenic reactions: | | |
| $4H_2 + HCO_3^- + H^+$ | $--> CH_4 + 3H_2O$ | -33.9 |
| $4Formate^- + H_2O + H^+$ | $--> CH_4 + 3HCO_3^-$ | -32.6 |
| $Acetate^- + H_2O$ | $--> CH_4 + HCO_3^-$ | -31.0 |
| Sulfidogenic reaction: | | |
| $4H_2 + SO_4^{2-} + H^+$ | $--> HS^- + 4H_2O$ | -38.1 |

on each other to perform the metabolic activity observed" (Schink 1992) or more popularly: "Two organisms do something together that neither one can do separately" (Brock and Madigan, 1988).

The classic case of a syntrophic coculture is *Methanobacillus omelianskii*, described as a pure culture (Barker, 1940). It was later shown to consist of the S-organism which converted ethanol into acetate and hydrogen, and *Methanobacterium bryantii*, which utilized the hydrogen to convert it into methane (Bryant et al., 1967). A well-studied substrate that can only be degraded syntrophically, is butyrate (Stieb and Schink, 1985; Dong and Stams, 1995; Wallrabenstein and Schink, 1994; McInerney et al., 1981). It can be converted in coculture with methanogens by several different genera of acetogens: *Syntrophospora*, *Syntrophomonas* and *Syntrophus*

(McInerney, 1992). The first two genera are related to the Gram-positive bacteria with a low DNA G + C content (Woese 1987; Zhao et al., 1990), while the latter one recently was found to be related to the delta-subclass of Proteobacteria which includes many sulfate-reducing bacteria (Wallrabenstein et al., 1995a). Another intermediate in the degradation of organic matter is propionate. The methanogenic conversion of propionate to acetate is even more unfavourable than that of ethanol or butyrate (Table 1). Below, the syntrophic oxidation of this intermediate will be discussed in detail.

**Syntrophic propionate oxidation.**

The oxidation of propionate under methanogenic conditions can only proceed if the partial hydrogen pressure is kept below 10 Pa and the formate concentration is kept below 10 $\mu$M (Stams, 1994). At the moment there are three well-described cocultures that can perform this reaction. The first isolated defined coculture consisted of *Syntrophobacter wolinii* and *Desulfovibrio* G11 (Boone and Bryant, 1980). *Syntrophobacter wolinii* oxidizes propionate into acetate, hydrogen and carbon dioxide and/or formate, while *Desulfovibrio* G11 converts the hydrogen and/or formate by sulfate reduction. Recently, a pure culture of *S. wolinii*, growing on pyruvate, was obtained (Wallrabenstein et al., 1994). A second coculture was described by Stams et al. (1993) and contained a mesophilic propionate-oxidizing bacterium (MPOB) and *Methanospirillum* sp. isolated from granular sludge. Remarkably, MPOB was able to grow in the absence of methanogens on fumarate (Stams et al., 1993). A third well-described syntrophic propionate-oxidizing bacterium is *Syntrophobacter pfennigii,* formally indicated as KOPROP1, isolated from anaerobic sludge in coculture with *Methanosprillum* sp. (Dörner 1992; Wallrabenstein et al., 1995b). Furthermore, other cocultures have been enriched from various anaerobic ecosystems that have not yet been physiologically and taxonomically characterized (Koch et al., 1983; Boone and Xun, 1987; Mucha et al., 1988; Stams et al., 1992; Wu et al., 1992).

The biochemistry of syntrophic propionate oxidation has been studied relatively well. A propionate oxidation pathway similar to that found in *Desulfobulbus propionicus* was proposed based on experiments with $^{14}C$- and $^{13}C$- labeled propionate (Stams et al., 1984; Koch et al., 1983; Houwen et. al., 1987; Robbins, 1988). This methylmanonyl-CoA pathway as given in Fig. 4 was confirmed by enzyme measurements in cell extracts of *S. wolinii* (Houwen et al., 1990) and MPOB (Plugge et al., 1993). However, *S. wolinii* and MPOB differ in the way propionate is activated.

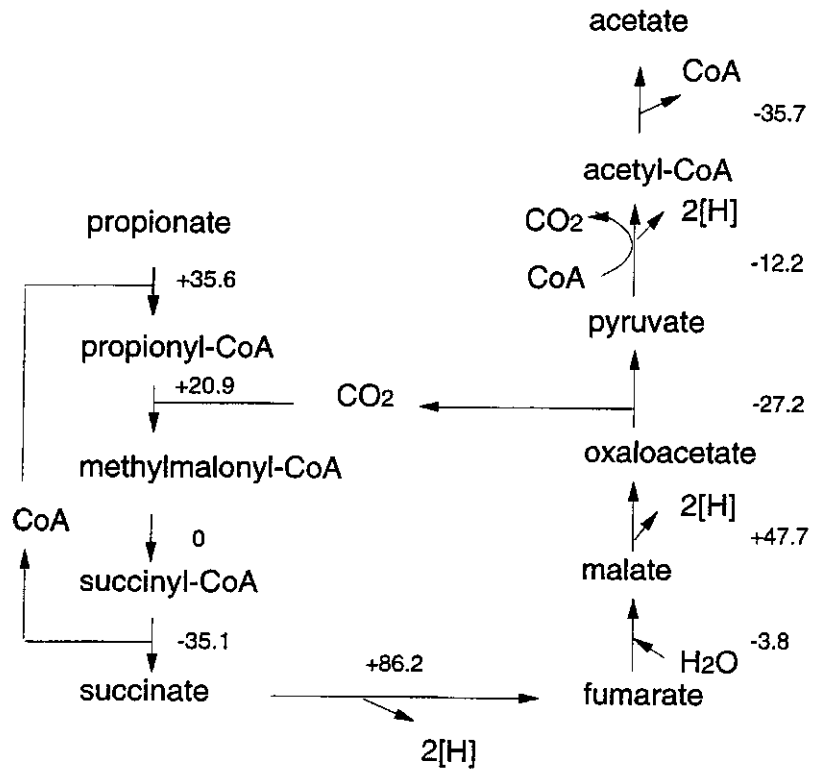


Fig. 4.Biochemical pathway of syntrophic propionate oxidation. The numbers refer to the $\Delta G^{0'}$ values of the different intermediate steps. The oxidation reactions are coupled to hydrogen formation. Adapted from Stams (1994).

In *S. wolinii* propionate is activated via a propionate kinase while in MPOB propionate is activated via propionate:acetyl-CoA HS-CoA transferase.

The most difficult step in the pathway of propionate oxidation is the oxidation of succinate to fumarate. The midpoint redox potential of the redox couple succinate/fumarate is +30 mV, while the redox potentials of $H^+/H_2$ and $HCO_3^-/HCOO^-$ are respectively -414 mV and -407 mV (Thauer et al., 1977). To couple the succinate oxidation to hydrogen or formate formation, hydrogen or formate levels of $10^{-9}$ and $10^{-8}$ $\mu$M are required. However, hydrogen/formate utilizing methanogens can not create

such low hydrogen or formate levels. Therefore, a reversed electron transport mechanism has to be proposed, which may be driven by the investment of ATP (Stams, 1994). Evidence for the existence of this mechanism was obtained with other syntrophic processes such as glycolate oxidation and butyrate oxidation (Friedrich and Schink, 1993; Wallrabenstein and Schink, 1994). However, its function in propionate oxidation remains to be established.

Syntrophic propionate-oxidizing bacteria currently investigated are not obligately syntrophic. They can grow in pure culture on propionate coupled to sulfate reduction, and in the case of *S. wolinii* and MPOB on pyruvate and fumarate, respectively (Stams et al., 1993; Wallrabenstein et al., 1994). These abilities were very important for the enrichment and isolation of these bacteria (Wallrabenstein et al., 1994, 1995b; this thesis) and simplified physiological and enzymatic studies (Dong, 1994).

**Interspecies hydrogen or formate transfer.**

Whether molecular hydrogen or formate is used for transfer of reducing equivalents from acetogens to methanogens is unclear. Formate is better soluble in water and may therefore create a steeper diffusion gradient (Stams, 1994). On the other hand, hydrogen might easier diffuse across lipophilic membranes (Schink, 1992). Recently, Dong et al., (1995) showed that MPOB could only degrade propionate in coculture with *Methanospirillum hungatei* or *Methanobacterium formicicum*, which utilize hydrogen and formate, and not with *Methanobrevibacter* strains, which only utilize hydrogen, unless a bacterium was present which was able to interconvert formate to hydrogen and carbon dioxide. Furthermore, experiments done with MPOB, growing on propionate with a limiting amount of fumarate as electron acceptor, showed that both hydrogen and formate were formed (Dong and Stams, 1995). These experiments show that both hydrogen transfer and formate transfer are important and that hydrogen transfer only occurred when the formate concentration was kept low aswell. However, in granular sludge the average distance between the microbes will be shorter than in suspended cultures, and hydrogen transfer might be more important because the easy diffusibility of hydrogen across the lipophilic membranes may compensate for the lesser solubility of hydrogen in water. This possibility is supported by the observation that in propionate-degrading granular sludge *Methanobrevibacter* was the most numerous methanogen in syntrophic microcolonies (Grotenhuis et al., 1991a).

**Ecology of granular sludge.**

Anaerobic granular sludge is a methanogenic environment, usually consisting of mainly biomass where microorganisms are aggregated to form a functional biological unit that mineralizes complex organic matter (Lettinga, 1995). The formation, stabilization, and microbial composition of the sludge has been subject of many studies over the last two decades. The formation and stability of the granules is influenced by environmental conditions like temperature, pH, type of wastewaters, and availability of nutrients (Hulshoff Pol, 1989). In addition, the adhesion properties of the microorganisms, such as hydrophobicity and the formation of extracelluar polymers are of importance (Grotenhuis et al., 1991b). Attempts to study the microbial composition of the granules have been made by conventional methods like the determination of the Most Probable Number (MPN), a technique based on the assumption that single cells will grow in high dilutions. Although there are major drawbacks of this technique which will be discussed below, it showed interesting differences in the microbial composition of various granules grown on different substrates (Stams et al., 1989).

The syntrophic degradation of intermediate compounds like ethanol, butyrate and most important propionate, are rate limiting in the degradation of organic matter because the degradation depends on the efficiency of interspecies hydrogen or formate transfer. However, in granular sludge high conversion rates of these intermediates have been obtained (Stams et al., 1989). To obtain these high conversion rates the microorganisms need to be organized into syntrophic consortia to minimize the distance between the syntrophic partners, so that an efficient interspecies transfer of hydrogen or formate is ensured (Thiele et al., 1988). Microscopic studies of granules revealed often an internal organization (MacLeod et al., 1990). In the case of syntrophic propionate oxidation a specific spatial distribution between acetogens and methanogens, a so called juxta-positioning, was observed (Prensier et al., 1988; Grotenhuis et al., 1991a; Wu et al., 1991). In one of these studies, an unidentified *Syntrophobacter*-like acetogen was surrounded by a *Methanobrevibacter* sp. which could be identified using specific antibodies. A specific antibody for *S. wolinii* was used, which did not react specifically with the microbes in the sludge, indicating that the acetogen was not serologically related to *S. wolinii* (Grotenhuis et al., 1991a). Immunogold labeling methods with specific antibodies against different methanogens were used for the identification of the methanogenic subpopulation in granular sludge (Macario and Conway de Macario, 1988; Prensier et al., 1988; Koornneef et al., 1990; Grotenhuis et

al., 1991a; Macario et al., 1991). In almost all types of sludge *Methanosaeta* (previously named *Methanothrix*) could be identified as the acetate utilizing methanogen, and only in the cases where high acetate concentrations were present *Methanosarcina* was detected, as in the case of ethanol-degrading sludge (Koornneef et al., 1990). The overall structure of the granules was investigated using transmission and scanning electron microscopy (Macleod et al., 1990). In the latter study, a layered structure of the granules was proposed, based on morphological identification of the microbes. The model proposed, consisted of an inner core of *Methanosaeta*-like aceticlastic bacteria, surrounded by a layer of syntrophic consortia, and an outer layer of fermenting bacteria, acetogens and hydrogen-consuming methanogens. Such a layered structure was also observed in granular sludge adapted to thermophilic conditions (Macario et al., 1991). In this study, fluorescently-labeled antibodies were used to identify the different subpopulation in the sludge. Although the methanogenic populations shifted from mesophilic to thermophilic, the basic architecture remained the same.

**Problems in studying the ecology of granular sludge.**

Above, some techniques were described to study the microbial diversity of granular sludge and the population dynamics of microbial subpopulations. However, there are some principle drawbacks that relate to the nature of these methods and their application. The MPN technique is based on the assumption that each single cell will grow in high dilutions. However, microorganisms in ecosystems do not always grow in this ideal way. A number of microorganisms will be underestimated, such as slow growers, microorganisms associated to each other or to solid substrates, and microbes growing in syntrophic associations, while other microorganisms can not yet be cultivated in artificial media. The quantification of propionate-oxidizing bacteria, depends not only on the growth of the acetogen but also on the growth of the hydrogen-consuming bacteria. This could mean that when the acetogen has overcome the lagphase and starts to grow, the methanogen has already died, because of lack of substrate. Moreover, syntrophic consortia grow faster in microbial aggregates (Thiele et al., 1988). Therefore, growth in suspended and diluted form might be another reason for the underestimation of syntrophic consortia using MPN counts (Visser et al., 1993). The determination of substrate conversion rates gives valuable information on the mass fluxes through the granules and on the maximum possible metabolic activity

of the different microbial groups. However, it does not give information on which microorganisms perform these conversions and on the numbers of these microorganisms (Dolfing, 1985; Dubourguier et al., 1988; Wu et al., 1992).

Microscopic investigations with light, fluorescence, and electron microscopy have strong limitations because usually the identification of the microorganisms based on their morphology is very doubtful if not impossible at all (Heppner et al., 1992). This could be partially overcome by the use of specific monoclonal and polyclonal antibodies combined with fluorescence and electron microscopy (Macario et al., 1991). The topography of methanogenic subpopulations in granular sludge has been studied successfully using this approach, although the cross reactions of specific antibodies remains problematic (Grotenhuis et al., 1991a; Macario et al., 1991). However, the generation of specific antibodies for syntrophic propionate-oxidizing bacteria was difficult, mainly because of the lack of defined pure cocultures. Prensier et al. (1988) managed to make specific polyclonal antibodies against *S. wolinii* by adsorption of antibodies from the rabbit antisera to cells of *Desulfovibrio* G11. These antibodies labeled only large rods within syntrophic microcolonies, which were probably the syntrophic acetogens. However, in retrospect it can be argued that the antibodies also detected other cells than that of *Syntrophobacter* by cross-reaction to other bacteria.

It is evident that there is a need for new techniques that are not based on morphology, isolation and cultivation, and allow a direct labeling and identification of the microorganisms. The next part will discuss the development of such new approaches and some of their applications.

## 2. RIBOSOMAL RNA AS A TOOL IN PHYLOGENY AND MICROBIAL ECOLOGY.

### The Ribosomal RNA Molecule.

About two decades ago the progress in molecular biology enabled genetic information to be studied on a molecular level and provided many new tools to study the biological and medical problems. Sequence analysis of DNA and RNA has become extremely important as a tool in phylogeny and microbial ecology. The chemical method of Maxam and Gilbert (1977), and the more efficient enzymatic method of Sanger (1977) enabled the use of the genetic information of the microbes to reveal their phylogenetic relationships and to detect them.

One of the first genes encoding for 16S rRNA which was sequenced was that of *Escherichia coli* (Brosius et al., 1978). Since then, the ribosomal RNA or rRNA genes, of many Eucarya, Bacteria and Archaea were sequenced. Comparative studies using all these ribosomal sequences revealed that this molecule contained information which could be used for both microbial taxonomy and microbial identification. Studies on the secondary structure of the 16S rRNA of *E. coli* showed that this molecule consists of a basic skeleton of base-pairing nucleotides, varied by non-pairing nucleotides and different hairpin loops (Fig. 5). The structure of the *E. coli* 16S rRNA is basically the same for all organisms, although individual nucleotides and several hairpin structures may differ (De Rijk et al., 1992). There are stretches of nucleotides in the rRNA that have evolved faster than other regions. This resulted in regions that are more or less conserved among all organisms, and regions that are more variable (Woese, 1987).

The prokaryotic ribosomes consist of three rRNA molecules and about 50 ribosomal proteins. The 16S rRNA of 1540 nucleotides forms together with approximately 20 proteins the 30S small-subunit and the 23S rRNA of 2900 nucleotides forms with the 5S rRNA of 120 nucleotides and approximately 30 proteins the 50S large-subunit. These two subunits assembled together form the 70S functional unit which catalyzes protein synthesis. The rRNA molecules are transcribed from the rRNA operons located on the chromosome. The number of operons varies between organisms from 1 copy (e.g. mycoplasmas) upto 10 copies (bacilli) per chromosome (Amikam et al.,1984; Jarvis et al., 1988). *Escherichia coli* has seven operons located at different chromosomal positions, which are organized in almost the same way, the rRNA-genes itself are identical (Wagner, 1994). This organization of the ribosomal operons found

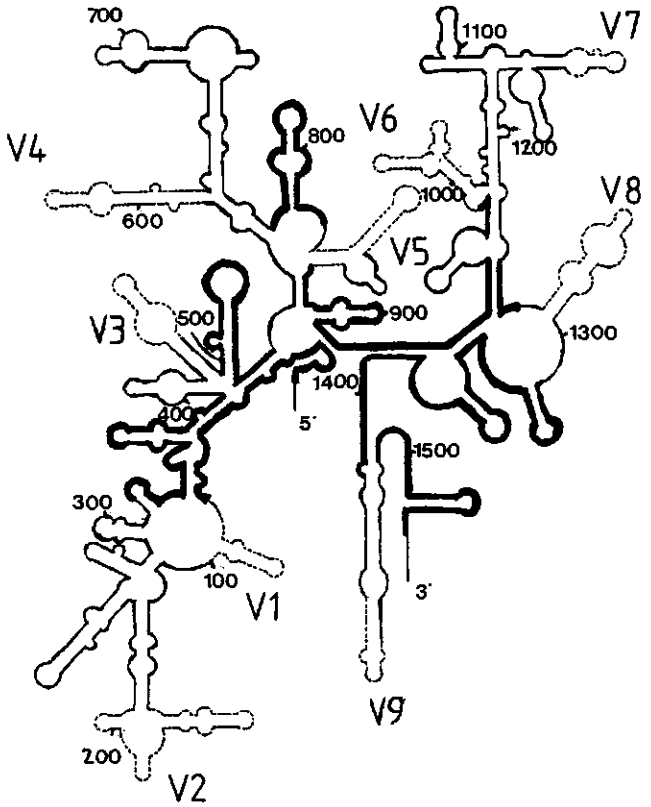


Fig. 5. Secondary structure of 16S rRNA of *Escherichia coli*. Adapted from Ward et al. (1992), with variable region-numbering according to Van de Peer et al. (1994). The schematic secundary structure highlights sequence domains that are of nearly universal conservation (thick lines), intermediate conservation (normal lines), or hypervariable domains (dashed lines).

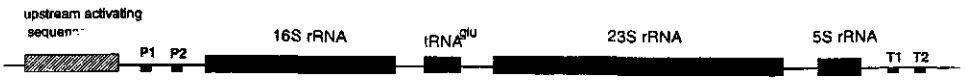


Fig. 6. Schematic arrangement of the *rrn*B operon of *Escherichia coli* according to Wagner (1994). P1 and P2 are the tandem promoters, T1 and T2 are terminators.

in *E. coli* (Fig. 6) is generally present in all procaryotes. However, the number and identity of the tRNA between the 16S and 23S rRNA-gene is not always the same and the spacer regions may vary in size (Fig. 6). The operons are transcribed as a whole into a primary transcript, which forms typical stemloops. These stemloops are cleaved by ribonucleases into individual pre-rRNA species, which are matured by ribonucleases into rRNA (Srivastava and Schlessinger, 1990).

The number of ribosomes is assumed to be proportional to the growth rate of the organism (Nomura et al., 1984). At a high rate of biosynthesis the formation of polypeptides by a single ribosome becomes rate limiting (ca. 20 amino acids per second) and thus the number of ribosomes needs to increase. The number of ribosomes can vary from $10^3$ to $10^5$ copies per cell. The number of ribosomes is mainly regulated on a transcriptional level of the rRNA operon. The two strong promoters of the *E. coli* rRNA operons are strictly controlled by several positive and negative regulation mechanisms (reviewed in Wagner, 1994).

**The use of ribosomal RNA sequences in phylogeny.**

Until approximately one decade ago, microbial phylogeny, that describes evolutionary relatedness of microorganisms, was mainly based on their morphology, physiology and biochemistry. But microbes posses a simple morphology and many microbes share a similar physiology or biochemistry. These characteristics, although useful for classification, might not always be good evolutionary markers. In hindsight this often led to misclassifications of several groups of microorganisms. Therefore, there was a need for more reliable biomarkers. Nucleic acid sequencing techniques made genetic information accessible to serve as biomarkers. Ribosomal RNA and the corresponding genes are now widely used as evolutionary biomarkers for several reasons (Olsen et al., 1986):

(i) rRNAs, as part of the protein-synthesizing machinery, are present and have the same function in all organisms;

(ii) The overall structure of rRNAs is extremely conserved;

(iii) The nucleotide sequences are highly conserved. The mutation rate during evolution is relatively low as compared to other genes. This makes the rRNA a kind of evolutionary clock, which provides reliable phylogenic information;

(iv) The rRNAs and their genes are easy accessible, mainly because of modern PCR and sequencing technology;

(v) 16S and 23S rRNAs contain sufficient sequence information to perform statistically significant comparisons;

(vi) There seems to be no rRNA-gene transfer between organisms, so relationships between rRNAs reflect evolutionary relationships.

Although 16S rRNA sequences are mostly used, both 16S and 23S rRNA basically contain the same phylogenetic information.

Usually only nucleotides are included in the analysis that are invariant in 50 % of all sequences (the more conserved regions), hypervariable regions are not included because so many mutations have occurred in these regions that they do not contain significant evolutionary information. Comparative and statistical analysis of the rRNA molecules enabled taxonomists to construct trees which indicate the phylogenetic relation between the organisms. Until the sequence information of rRNA came available, the living organisms were divided into Eucaryotes, the ones that have a nucleus, and Procaryotes, the ones that do not have a nucleus. Surprisingly, trees based on partial 16S rRNA sequences (oligonucleotide catalogues) revealed that the procaryotes should be divided into two distinct groups that were first termed Eubacteria and Archaebacteria (Woese, 1987). When more evidence for this prokaryotic dichotomy became available, mainly by comparing more and more full-length 16S rRNA sequences, the three kingdoms were renamed into the domains Eucarya, Archaea and Bacteria (Woese et al., 1990; Winker and Woese, 1991). The universal phylogenetic tree presented in the first reference, is a rooted tree that suggests that all organisms diverged from a common ancestor (Fig. 7). This is in contrast to the unrooted trees used in other studies. In such a phylogenetic tree, the distance between the endpoints of the lines is directly related to the amount of nucleotides that differ between the individual sequences. This evolutionary distance can be calculated mainly via three different statistical algorithms:

(i) Cluster analysis, that is based on assigning taxa to groups on the basis of similarities. This method is only valid when the rates of sequences divergence are sufficiently similar in all lineages of the tree. However, in evolution this is not always the case.

(ii) Maximum parsimony, that is based on the assumption that the evolution always occurs taking the shortest possible pathway. This method seeks the tree branching order (topology) that requires the fewest mutational events. Also this approach is quite

susceptible to variations in evolution rate.

(iii) Distance matrix approach, that use the differences between pairs of sequences to estimate the "evolutionary distance" separating the sequence pairs. The evolutionary distance is usually corrected for multiple mutations on a single sequence position, which might have occurred during evolution. Because of this correction, this approach is the most reliable one, with respect to the variations in evolution rate. In practice, the distance matrix approach is now most widely used in phylogeny and the topology of the outcoming tree is subsequently checked by preforming parsimony analysis.

At the present, more than 3000 bacterial and archaeal 16S rRNA sequences are available in various databases. The Ribosomal Database Project (RDP) is the most important one, and contains most of these sequences in an aligned and easy accessible form (Maidak et al., 1994). Many detailed phylogenetic analysis have been performed, focusing on specific groups of microorganisms (Devereux et al., 1989; Rouvière et al., 1992, Collins et al., 1994).

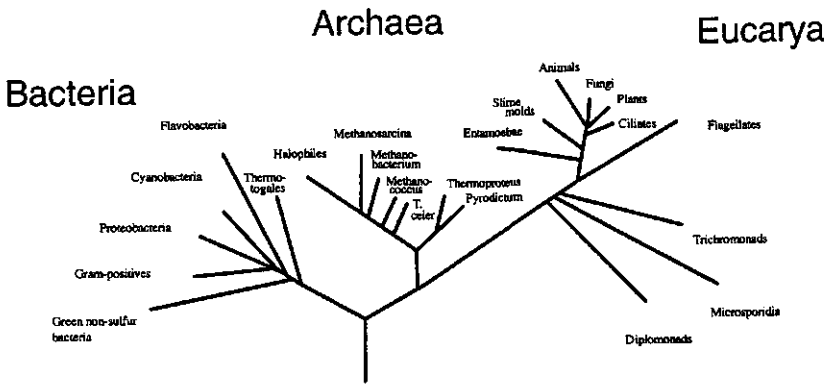


Fig. 7. Phylogenetic tree representing the three domains Bacteria, Archaea and Eucarya adapted from Woese et al. (1990).

**The use of rRNA in ecology: The ribosomal approach**

About one decade ago microbial ecologists started to use the ribosomal RNA molecules to study microbial communities. 5S rRNA molecules were isolated from relatively small communities, like hot springs and hydrothermal vents (Stahl et al., 1985). The molecules were separated by electrophoresis and the 5S rRNA molecules were sequenced and subsequently compared. Although these experiments gave new insight in the complexity of such small ecosystems, it turned out that the 120 nucleotides long 5S rRNA molecule contained only limited information. Olsen et al. (1986) started to develop strategies to analyze larger rRNA molecules. The 16S rRNA and the 23S rRNA molecules are sufficiently long to obtain reliable phylogenetic information and contain enough variable regions to discriminate between the different molecules. For reasons of convenience they focused on the shorter 16S rRNA. These strategies involved extraction of total community DNA, preparation of a shot-gun DNA library in bacteriophage Lambda, screening by hybridization with a 16S rRNA specific probe, sequencing of the 16S rRNA-gene containing clones, and comparative analysis of the retrieved sequences. A few years later the PCR reaction was introduced into molecular biology by Mullis and coworkers (Saiki et al., 1985). This has simplified the methods, because now the 16S rRNA-genes could be specifically amplified using 16S rRNA-based primers. Another approach was extraction of the 16S rRNA from the ecosystem followed by its transcription into c-DNA using reverse transcriptase, and subsequent cloning and sequencing (Ward et al., 1990). Studies using this approach revealed that a lot of the sequences retrieved from ecosystems were until then unknown sequences, which showed only limited homology with the 16S rRNA sequences obtained from type strains in the culture collections, such as the two new clusters of sequences obtained from the Sargasso Sea bacterioplankton (Giovannoni et al., 1990). This surprising finding was an indication for the enormous diversity in the microbial world. Furthermore, this approach circumvented culture techniques and allowed for the identification and detection (see below) of unculturable organisms, which was at that time almost impossible.

**Advantages and drawbacks of using sequence information rRNA obtained from ecosystems.**

Retrieving rRNA sequences directly from ecosystems has an important advantage over other methods, because it allows for the identification and detection (see below) of

organisms without culturing. In one experiment, information about different members of the ecosystem can be obtained, even if they can not yet be cultured. The obtained sequences contain phylogenetic information and may give hints on how to culture these organisms in order to study their physiology.

Although the ribosomal approach has now been proven to be a powerful tool to study complex ecosystems, there are some drawbacks that need to be considered. The constructed 16S rDNA libraries do not always reflect the total microbial community and biases might occur during its construction. Five most important problems can occur. (i) The most crucial step in construction of a library is the extraction of nucleic acids from the ecosystem. Microbial cells do not lyse equally well. Although mechanical lysis methods are used nowadays, it can not be excluded that some microorganisms do not lyse. (ii) The PCR reaction can preferentially amplify certain 16S rDNA molecules, and therefore the 16S rDNA of microbes abundantly present in the ecosystem could remain unamplified (Amann 1995). (iii) The formation of chimeric molecules that consist of parts of the 16S rDNA of two different microorganisms, is reported during the construction of 16S rRNA libraries (Liesack et al., 1991). (iv) Cloning efficiencies of the 16S rRNA molecules might be different. (v) The copy number of the 16S rRNA-genes can range from 1 to 10 per chromosome. All these problems make that feedback methods are needed to check if the found sequences really occur in the ecosystem and what the abundance of the organisms is from which the sequences were derived. Hybridization with specific probes is often used for this purpose. A flow chart showing the principle steps in the ribosomal approach as discussed in the previous section combined with feedback and detection methods involving specific probes is shown in Fig. 8.

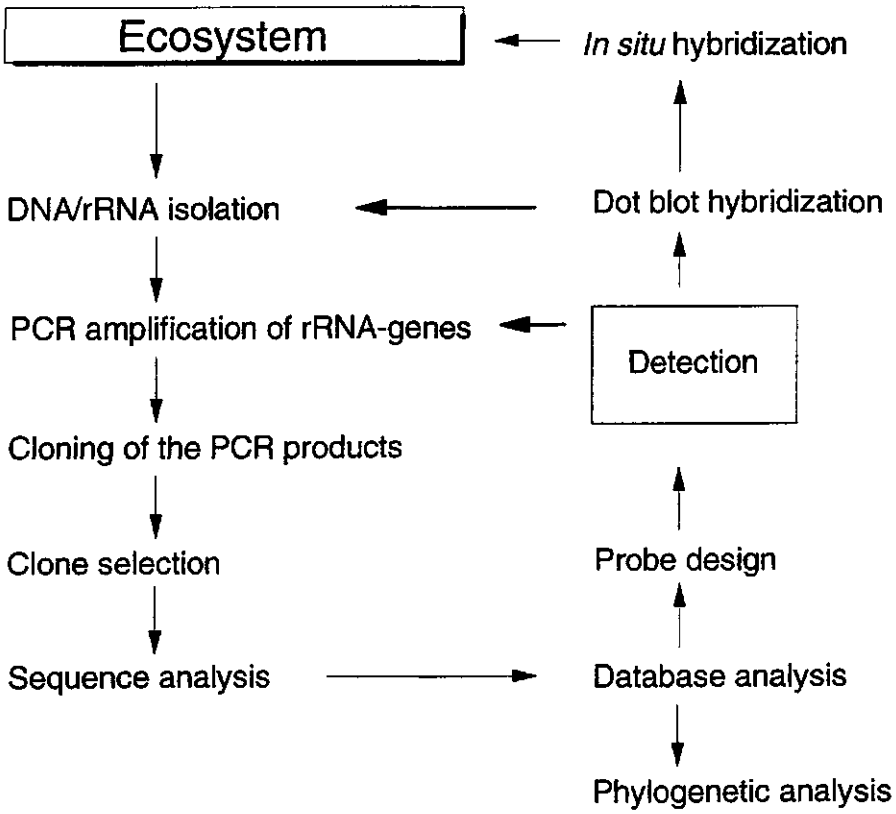


Fig. 8. Flow chart showing the principal phases of the ribosomal approach, adapted from Amann et al. (1995).

**The application of oligonucleotide probes based on rRNA sequences.**

Oligonucleotides are short single stranded oligomers of 15 to 40 DNA nucleotides, which can be made chemically by a DNA synthesizer in any desired sequence. They can be used as primers to initiate polymerase reactions, such as PCR, or they can be labeled and used as a probe to detect DNA or RNA sequences to which they are directed. In both cases they form duplexes with complementary sequences of the target

molecule. The amount of binding and specificity of the oligonucleotide-target duplex depend on the temperature and the ionic strength of the buffer in which this duplex in formed, and on its length. Experimentally, the binding is usually determined by keeping the ionic strength constant and varying the temperature. The temperature at which half of the oligonucleotides are melted from the targets is called dissociation temperature (Td). An oligonucleotide probe which is hybridized below the Td can also bind to target sequences which are not completely homologous. In practice, an oligonucleotide probe can distinguish a homologous sequence from a sequence with one or two mismatches (Manz et al., 1992). Because of this discriminative potential, oligonucleotide probes of 15 to 30 nucleotides are more sensitive than long DNA probes (more than 100 nucleotides). However, a disadvantage may be that oligonucleotide probes can be labeled with a limited number of labels, usually one or two, and therefore have a lower detection limit than longer probes.

Oligonucleotide probes are very useful to detect rRNA molecules. Because of their short length and high specificity they can be directed against conserved regions to detect all rRNA or domains of organisms, or against hypervariable regions to specifically detect one organism. If directed against regions specific for groups of microorganisms, the probes can detect different phyla or groups of organisms (Stahl and Amann, 1991).

**Design of rRNA-based oligonucleotide probes.**

To identify and quantify subpopulations and specific microbes universal, group-, genus- and species-specific oliogonucleotide probes can be made. There are four principal steps involved (Amann et al., 1995); (i) The alignment of the rRNA (or their gene) sequences. (ii) Identification of the target sequence, which is usually unique for a single sequence or a group of sequences. (iii) Synthesis and labeling of complementary nucleic acids probes. (iv) Experimental evaluation and optimization of the probe specificities, and determination of the dissociation temperature. Computer software has recently been developed to assist in the first two steps (Strunk et al., 1995). The third step involves chemical DNA synthesis, that can be performed automatically, and labeling of the oligonucleotides by either enzymatic coupling with radionucleotides or dioxigenin, or by chemical coupling with dioxigenin or fluorescent dye (Stahl and Amann, 1991; Zarda et al., 1991). So the last step is the most tedious one and may not always give the desired result, for instance as a consequence of steric hinderance by

secondary structure or a too low melting temperature.

Although the database may contain many sequences and probes can be checked thoroughly, it can never be ruled out that more than one bacterium in the ecosystem is detected by the "specific" probe. During the last decade, many universal, group-, genus- and species-specific 16S and 23S rRNA probes have been designed and tested. These can now be used for the identification of specific microbes, although the probes should regularly be reevaluated for new sequences (Manz et al., 1992).

**Detection and quantification of microbes with oligonucleotide probes.**

To apply oligonucleotide probes for the detection and quantification of microorganisms, three different methods can be used: specific PCR amplification, dot blot and quantitative dot blot hybridization, and finally *in situ* hybridization.

PCR assays are used to specifically amplify parts of the 16S or 23S rDNA, or the intergenic spacer between the two genes. As templates nucleic acids isolated from the studied ecosystem are used. By the use of specific primers, sensitive assays can be designed to detect low amounts of a single species or groups of microorganisms. It is generally accepted that these methods only detect microbes and do not provide quantitative information, although quantitative PCR assays are now being developed and evaluated (Blok et al., 1995).

Dot blot hybridization is used to quantify the amount of a specific 16S rRNA relative to the total amount of rRNA. Nucleic acids mixtures are extracted from ecosystems by mechanical and chemical lysis of the bacteria. The DNA and RNA in these extracts are then purified and bound to a filter using a special manifold to obtain dots or slots. These filters are hybridized with radioactively labeled oligonucleotide probes. The amount of radioactivity bound to the filter is a measure for the amount of rRNA targets to which the probes bind. The relative amount of rRNA is calculated by dividing the radioactive signals from the bound specific probes by that of a similarly hybridized and labeled universal probe. The relative abundance of the rRNA can not be extrapolated to the relative abundance of microbes because the amount of ribosomes per cell is dependent on the growth phase. This approach was first successfully used in studying different *Fibrobacter* species in the rumen, where pronounced population changes could be observed, depending on the animals diet (Stahl et al., 1988). A combination of these methods with *in situ* hybridization (see below) was used in an elaborate study of the microbial composition of different anaerobic wastewater and

solid waste digestion tanks (Raskin et al., 1994b).

From the three detection techniques discussed here, *in situ* hybridization is the most direct one. *In situ* hybridization is the hybrid formation between specific nucleotide sequences and labeled DNA probes inside the intact cell, hence *in situ*. When this technique is applied to detect microbes, this usually means hybrid formation between 16S rRNA target and labeled oligonucleotide probes in the intact microbial cell (DeLong et al., 1989; Amann et al., 1990a). For this purpose, the morphology of the microbial cell needs to be fixed and the cell membrane needs to be made permeable to allow penetration of the probe. This is usually done by crosslinking the cell proteins with paraformaldehyde, which is sufficient for most microorganisms. For microbes with rigid cell walls, such as Gram-positive bacteria, alternative fixation procedures have been developed, that involve treatment with lytic enzymes, and organic solvents (Beimfohr et al., 1993; Hahn et al., 1993). The fixed cells are usually bound to a glass slide and hybridized with the oligonucleotide probe inside a moist chamber. The probes can be labeled with radioactive nucleotides, biotin or dioxigenin combined with immuno-detection using labeled antibodies against these molecules, but usually probes are labeled with fluorescent dye like fluorescein (green) or rhodamine (red). The fluorescent signals are detected by epifluorescent microscopy or, when more details are needed, by confocal laser scanning microscopy.

*In situ* hybridization has shown to be very useful to detect unculturable microorganisms such as (endo)symbionts of protozoa, insects and animals (Amann et al., 1991; Distel et al., 1991; Springer et al. 1993, Snel et al., 1993) and magnetotactic bacteria (Spring et al., 1992). Furthermore, this method has been used for the identification of bacteria in complex ecosystems such as soil, biofilms and activated sludge (Hahn et al., 1992, Amann et al., 1992, Wagner et al., 1993). In all these cases the microbes were identified and their spatial distribution was investigated. The use of multiple fluorescent labels allows to study different subpopulations at the same time (Wagner et al., 1994). The quantification of the fluorescent signal of single cells can also give information about the *in situ* growth rate of the individual cell (Poulsen et al., 1993). Quantification of microbes can be automated by flow-cytometry, analogous to immunostained flow-cytometry (Amann et al., 1990b), and computer-assisted image analysis (Caldwell et al., 1992). Presently new applications of these techniques are developed, especially since the procedures become more sensitive and can be automated.

**New techniques in molecular microbial ecology.**

Additional techniques become available now that extend the toolbox of molecular microbial ecology. Denaturing Gradient Gel Electrophoresis (DGGE) is a molecular technique which has been adapted to separate different rDNA molecules based on their denaturation point (Muyzer et al., 1993; Muyzer and de Waal, 1994). With this technique PCR-amplified 16S rDNA molecules of the same length obtained from a mixed community, can be separated. Separated 16S rDNA molecules can be hybridized with group-specific probes or extracted from the gel and sequenced directly. By this method information about the diversity of the ecosystem can be obtained without constructing clone-libraries and the phylogeny of uncultured microbes can be studied. A drawback is that this method involves PCR-amplification, which might cause biases as discussed before and make quantification of the products doubtful.

The newest technique is the combination of phylogenetic staining with *in situ* hybridization and isolation of microbes with 'optical tweezers', consisting of laserbeams that are used to separate individual cells from the rest of the population in a complex ecosystem (Huber et al., 1995) .

## 3. OUTLINE OF THE THESIS.

The aim of the research presented in this thesis was to study the diversity and phylogeny of syntrophic propionate-oxidizing bacteria, to detect these bacteria, and to study their population dynamics in various types of granular sludge. The detection and population dynamics were to be done quantitatively and *in situ*, using 16S rRNA-based oligonucleotide probes. Following introduction into granular sludge ecology and physiology, and the use of the 16S rRNA approach (Chapter 1), Chapter 2 deals with the phylogenetic analysis of *Syntrophobacter wolinii*. For this purpose, the rRNA-genes of *S. wolinii* had to be retrieved from a defined coculture. In the research described in Chapter 3 the complexity of the phylogenetic analysis increased when the highly enriched, but hence not pure syntrophic propionate-oxidizing cultures MPOB and KOPROP1 were analyzed. The phylogenetic status of sporeforming syntrophic propionate-oxidizing bacteria from granular sludge, enriched by repeated pasteurization, is described in Chapter 4. The phylogenetic analysis of these bacteria revealed that syntrophic propionate oxidation can also be performed by Gram-positive bacteria. The detection of syntrophic MPOB-like bacteria in granular sludge fed with different substrates is discussed in Chapter 5. In this study the spatial distribution of MPOB-like cells, other bacteria and methanogens is investigated by *in situ* hybridization on sections of the sludge, using species- and group-specific oligonucleotide probes that were fluorescently labeled. The ribosomal approach was also used in Chapter 6 to monitor the enrichment of strain SYN7, a new type of syntrophic propionate-oxidizing bacterium, from granular sludge originating from a UASB reactor treating potato wastewater. In Chapter 7 the same sludge was used to study the population dynamics of propionate-oxidizing bacteria when fed with either propionate or propionate and sulfate. The microbial subpopulations were quantified using dot blot hybridizations with various oligonucleotide probes and the changes in the microbial architecture of the sludge was investigated using *in situ* hybridizations on sections of the sludge. In Chapter 8 the isolation and substrate use of strain MPOB is described and the taxonomic position of this bacterium is investigated using DNA-DNA hybridization in order to name the bacterium. Finally, results as presented in this thesis is summarized and discussed.

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# Chapter 2

## Phylogenetic analysis of *Syntrophobacter wolinii* reveals a relationship with sulfate-reducing bacteria.

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# Phylogenetic analysis of *Syntrophobacter wolinii* reveals a relationship with sulfate-reducing bacteria

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**Abstract.** A 16S rRNA sequence analysis of *Syntrophobacter wolinii* was done by using PCR amplification of the 16S rRNA-genes from DNA isolated from the *S. wolinii-Desulfovibrio* sp. coculture. Phylogenetic analysis using the obtained sequence revealed that *S. wolinii* was not related to bacteria growing syntrophically on other fatty acids than propionate, but was related to sulfate-reducing bacteria. The closest related bacteria are *Desulfomonile tiedjei* and *Desulfoarculus baarsii*.

**Key words:** *Syntrophobacter wolinii* – Syntrophic bacteria – Sulfate-reducing bacteria – PCR – 16S rRNA – Phylogeny

Propionate is an important intermediate in the anaerobic breakdown of organic matter in methanogenic ecosystems (Schink 1992). Complete mineralisation of propionate to methane and carbon dioxide is carried out by syntrophic consortia of bacteria. The first step, the oxidation to acetate, carbon dioxide and hydrogen is a thermodynamically unfavourable reaction, and bacteria can only gain energy from this conversion if the hydrogen partial pressure is kept below $10^{-5}$ atm. This can be achieved by hydrogen consumption by sulfate reducers or methanogens (McInerney 1992). The first propionate-degrading syntrophic consortium was reported by Boone and Bryant (1980). They described *Syntrophobacter wolinii* as a new genus and species of obligately anaerobic, gram-negative rods which degrade propionate in coculture with *Desulfovibrio* sp. Since then other syntrophic propionate-oxidizing cocultures have been enriched (Koch et al. 1983; Boone and Xun 1987; Mucha et al. 1988; Stams et al. 1992; Dörner 1992).

The classification of *S. wolinii* was solely based on morphology and its ability to grow syntrophically on propionate. Syntrophs growing on butyrate or long chain fatty acids have been isolated, such as *Syntrophomonas wolfei* (McInerney et al. 1981) and the sporeforming bacterium *Syntrophospora (Clostridium) bryantii* (Stieb and Schink 1985; Zhao et al. 1990) but these are not able to grow on propionate. 16S rRNA analyses of these two butyrate-degrading bacteria show that they are phylogenetically related, and that they belong to the group of gram-positives, although *S. wolfei* has a gram-negative cell wall (Zhao et al. 1990). Based on differences in morphology and physiology *S. wolinii* seems not to be related to these bacteria (Houwen et al. 1990). Recently, *S. wolinii* was obtained as a defined methanogenic biculture (Dörner 1992). This culture contained no *Desulfovibrio* sp. anymore. From this culture *S. wolinii* could be further enriched in media with propionate and sulfate, which might indicate that *S. wolinii* itself is able to reduce sulfate (Dörner 1992).

The research described in this article was undertaken to determine the phylogenetical position of *S. wolinii*. We analyzed the sequence of the 16S rRNA-gene of this bacterium. At the time this research was started only a defined coculture with *Desulfovibrio* sp. existed. Therefore, the 16S rRNA analyses were done by using PCR amplification of the 16S rRNA-genes of the *S. wolinii-Desulfovibrio* sp. coculture, cloning in M13-phages and subsequent sequencing. To discriminate between the two types of sequences obtained, a partial sequence analysis of the 16S rRNA from a pure culture of the *Desulfovibrio* sp. was also performed. Later on, we could confirm our findings with a highly purified culture of *S. wolinii*.

## Materials and methods

### *Bacterial strains*

The defined sulfate-reducing coculture of *Syntrophobacter wolinii* and *Desulfovibrio* G11 (DSM 2805) (Boone and Bryant 1980) was obtained from the German Collection of Microorganisms (DSM; Braunschweig, Germany). It was cultured as described before (Houwen et al. 1990). *Desulfovibrio* G11 was cultured in the same medium as the coculture except that propionate was replaced by 20 mM lactate and the yeast extract was omitted. A highly purified culture of *S. wolinii* was kindly provided by C. Dörner (Universität Konstanz, Germany). This culture was free of the *Desulfovibrio* sp., but still contained a few cells of *Methanospirillum hungatei*. This culture was grown on pyruvate as described (Dörner 1992).

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### *DNA techniques, PCR amplification and sequence analysis*

All enzymes were obtained from Life Science Technologies (Gaithersburg, Md., USA). All oligonucleotides were obtained from Pharmacia (Uppsala, Sweden). DNA isolation, ligation and transformation as well as other DNA manipulations were done by established procedures (Sambrook et al. 1989). The 16S rRNA-gene was selectively amplified by the polymerase chain reaction (PCR) using two sets of oligodeoxynucleotide primers which all contained restriction sites to facilitate cloning. The first set of primers corresponding to positions 107 to 124 (5'CACGGATCCG-GACGGGTGAGTAACACG) and 1493 to 1510 (5'GTGCTGC-AGGGTTACCTTGTTACGACT) of *Escherichia coli* 16S rRNA was used for the amplification of the 16S rRNA genes of the coculture. The second set corresponding to *E. coli* positions 8 to 27 (5'CACGGATCCAGAGTTTGAT(C/T)(A/C)TGGCTCAG) and 1100 to 1114 (5'GTGAAGCTTAGGGTTGCGCTCGTTG) was used for the amplification of the 16S rRNA-gene of the highly purified culture of *S. wolinii* to confirm the found sequence and to complete the missing 5' end (Embley et al. 1988; Lane 1991). PCR was performed in a total volume of 100 µl containing 10 µl 10 × PCR buffer (500 mM KCl, 25 mM $MgCl_2$, 200 mM Tris/HCl pH 8.4, 1 mg/ml gelatine, 0.001% v/v Nonidet-P40), 1 µl dNTPs (each 10 mM in 10 mM Tris/HCl, pH 7.5), 0.2 µl Taq polymerase (5 U/µl), 1 µl of each primer (100 ng/µl) and 1 µg DNA preparation; 35 cycles of the following temperature cycle were performed: 94 °C for 60 s, 48 °C for 120 s, and 72 °C for 240 s. This was followed by a final 7-min incubation at 72 °C. Amplification products were cloned in M13mp18 and M13mp19. DNA sequencing was carried out by the dideoxy-chain termination method (Sanger et al. 1977).

### *Sequence alignment and phylogenetic tree*

The partial nucleotide sequence was aligned to those of other bacterial sequences taking into account sequence similarity and higher order structure. The reference sequences were taken from the ribosomal database project RDP (Olsen et al. 1992; De Rijk et al. 1992). Evolutionary distance values (Knuc) were calculated according to Hori and Osawa (1979). The unrooted phylogenetic tree was reconstructed using the neighbour joining method of Saitou and Nei (1987) as implemented in the program "NEIGHBOR" (PHYLIP version 3.4) developed by Felsenstein (1982) including only sequence positions which are invariant in at least 50% of the entire sequence set. The topology of the tree was evaluated performing parsimony and bootstrapped parsimony analysis using the programmes DNAPARS and DNABOOT of Felsenstein (1982).

## Results

### *PCR-amplification, cloning and selection of the clones*

The PCR-amplification of the 16S rRNA-genes of the coculture resulted in a product of about 1.4 kb. This product was cloned in M13mp18 resulting in about 100 recombinant phages. The phages were screened for a *Syntrophobacter wolinii* insert by sequencing the variable region of the 16S rRNA gene with the internal primer complementary to the region at position 1100 to 1114 of *Escherichia coli*. Only one clone was found to have an insert that was different from those that were obtained from the *Desulfovibrio* sp. The complete insert of this clone was sequenced using internal universal primers (Lane 1991) and primers hybridizing to the M13 phage. This resulted in a sequence of 1391 basepairs long. The sequence itself has been deposited with the EMBL database under accession number X70905. PCR amplification of the 16S rRNA-gene with DNA isolated from a highly purified culture of *S. wolinii* and direct sequencing of the PCR-products confirmed that the sequence obtained from the coculture was indeed the 16S rRNA-gene of *S. wolinii*. This sequence was 1088 bp long and has also been deposited with the EMBL database under accession number X70906. There were no differences in the 1 kb overlap of the two sequences. Therefore the sequences were joined together resulting in a 1485 basepairs partial 16S rRNA sequence. This sequence was used for phylogenetic analysis.

### *Phylogenetic analysis*

The sequence of the 16S rRNA gene of *S. wolinii* was aligned to other sequences available from the databases (Olsen et al. 1992; De Rijk et al. 1992). Comparative sequence analyses revealed that *S. wolinii* phylogenetically clusters with the sulfate-reducing bacteria (SRB) A consensus tree based on distance matrix and parsimony

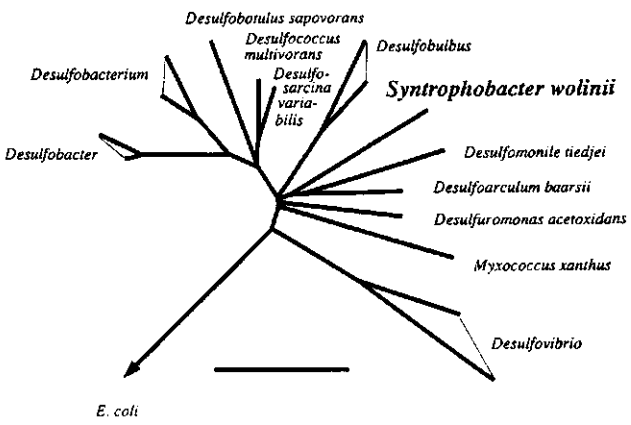


Fig. 1. Unrooted phylogenetic tree from 16S rRNA sequence divergence of *Syntrophobacter wolinii*, sulfate reducing bacteria and closely related organisms with *Escherichia coli* as an outgroup. Only positions were used that had 50% invariance in all sequences. The bar represents 0.05 Knuc

analyses is depicted in Fig. 1 for selected members of the delta subclass of proteobacteria. *Escherichia coli* was included as an outgroup. *S. wolinii* obviously shares a common ancestor with *Desulfomonile tiedjei* and *Desulfoarculus baarsii* (former *Desulfovibrio baarsii*), but it is only moderately related to these species.

## Discussion

From the tree presented in Fig. 1. it is clear that *Syntrophobacter wolinii* is related to sulfate reducing bacteria. The closest related bacteria *Desulfomonile tiedjei* and *Desulfoarculum baarsii* belong according to the grouping of Devereux et al. (1989), to group 7. This is a phylogenetically distinct group of the line of completely oxidizing sulfate-reducing bacteria. Recently, evidence was provided that *S. wolinii* is able to oxidize propionate to acetate with sulfate as electron acceptor (Dörner 1992). This is remarkable because in the presence of a *Desulfovibrio* sp. this strain grows syntrophically as an acetogen (Boone and Bryant 1980). The physiological explanation for this phenomenon is not yet clear. Some recent studies with mixed communities of anaerobic bacteria reported the ability of sulfate reducers to grow syntrophically on propionate (Wu et al. 1991; Heppner et al. 1992). This suggests that the ability of syntrophic propionate-oxidizing bacteria to reduce sulfate is not restricted to *S. wolinii*.

The phylogenetical and physiological findings show that *S. wolinii* is a sulfate reducer. As a consequence of this *Syntrophobacter wolinii* should be renamed to indicate its relatedness with sulfate reducers. However, it might well be that in future syntrophic propionate oxidizing bacteria are isolated which are unable to reduce sulfate. In order to judge the (taxonomic) status of the genus *Syntrophobacter* properly, more syntrophic propionate-oxidizing bacteria should be isolated and studied phylogenetically and physiologically.

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# Chapter 3

## Phylogenetic analysis of two syntrophic propionate-oxidizing bacteria in enrichments cultures.

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# Phylogenetic Analysis of two Syntrophic Propionate-oxidizing Bacteria in Enrichments Cultures

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## Summary

A 16S rDNA sequence analysis of two syntrophic propionate-oxidizing bacteria was done using PCR amplification of the 16S rRNA-genes from DNA isolated from two enrichment cultures. The two mesophilic propionate-oxidizing bacteria MPOB and KOPROP1 are closely related to *Syntrophobacter wolinii* and form one cluster within the sulfate-reducing bacteria. Specific oligonucleotides were developed and used in dot blot and *in situ* hybridization studies. These experiments showed that the obtained sequences were derived from the dominant propionate-oxidizing bacteria in the enrichment cultures.

Key words: Syntrophic bacteria – Propionate degradation – Sulfate-reducing bacteria – PCR – 16S rRNA – Phylogeny

## Introduction

Complete breakdown of propionate to methane and carbon dioxide is carried out by syntrophic consortia of anaerobic bacteria (*Schink*, 1992; *Stams*, 1994). Acetogens oxidize propionate to acetate, carbon dioxide and hydrogen or to acetate and formate. These oxidations are thermodynamically unfavourable; acetogens can only gain energy from propionate oxidation if the hydrogen partial pressure and the formate concentration are kept low. This is achieved by hydrogen and formate consumers like methanogens or, if sulfate is present, sulfate reducers (*Boone* and *Bryant*, 1980; *McInerney*, 1992).

Until now, *Syntrophobacter wolinii* is the only well described species which degrades propionate syntrophically in coculture with *Desulfovibrio* sp. In a phylogenetic study of *Syntrophobacter wolinii* we have demonstrated that this bacterium is related to the sulfate-reducing bacteria (*Harmsen* et al., 1993). Only very recently this strain was obtained in pure culture (*Wallrabenstein* et al., 1994). *Wu* et al. (1992) described the isolation of a Gram-positive syntrophic propionate-oxidizer, which seems not to be related to *S. wolinii*. No other really defined cultures of syntrophic propionate-oxidizers were described. Isolation of these kind of bacteria remains a difficult task. However, highly purified enrichment cultures of syntrophic propionate-degrading bacteria have been obtained. (*Koch* et al., 1983; *Boone* and *Xun*, 1987; *Mucha* et al., 1988; *Stams* et al., 1992; *Dörner* 1992).

With the availability of molecular techniques (*Olsen* et al., 1986; *Amann* et al., 1992; *Amann* et al., 1994) it is possible to undertake a phylogenetic analysis of enrichment cultures without obtaining pure cultures. The aim of the present study is to analyze the 16S rRNA-genes of the dominant bacteria in two cultures enriched on propionate in a mineral medium. The mesophilic propionate-oxidizing bacterium (MPOB) was described previously (*Stams* et al., 1993; *Plugge* et al., 1993) as being able to use fumarate in the absence of methanogens. The mesophilic propionate-oxidizing bacterium KOPROP1 was described before (*Dörner*, 1992) as being able to grow on propionate and sulfate in the absence of a methanogen.

The 16S rRNA analyses were done by polymerase chain reaction (PCR) amplification of the 16S rRNA-genes from the DNA isolated from the enrichments. The amplification products were cloned in vectors and the right clones were

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selected by sequencing the V6 variable region of a number of clones. Based on abundance and homology one single clone was selected from each enrichment culture and the insert was sequenced. The obtained sequences were confirmed by *in situ* and/or dot blot hybridization with oligonucleotide probes specific for the obtained sequences.

## Materials and methods

*Growth of the cultures.* MPOB was cultured in a basal bicarbonate-buffered medium with 20 mM fumarate as substrate as described before (*Stams* et al., 1993). Strain KOPROP1 was kindly provided by B. Schink (Universität Konstanz, Germany). It was grown on 10 mM propionate plus 10 mM sulfate in the basal bicarbonate-buffered medium described by *Dörner* (1992). Subculturing this culture on propionate and sulfate was difficult. Therefore, KOPROP1 was cultured syntrophically on 20 mM propionate with a *Methanospirillum* species which was still present in the enriched culture. Routinely, bacteria were grown at 37 °C in 120-ml bottles containing 50 ml medium and a gas phase of 80 kPa $N_2/CO_2$ (80:20).

*Nucleic acids isolation, PCR amplification, cloning and sequence analysis.* All enzymes for DNA manipulations were obtained from Life Science Technologies (Gaithersburg, Md., USA). All unlabeled oligonucleotides were obtained from Pharmacia (Uppsala, Sweden). The nucleic acids from all anaerobic cultures were isolated by the following procedure: 10 ml of late logarithmic cultures were centrifuged at 17000 g. The pellet was suspended in 400 µl autoclaved TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and transferred to a 1.5 ml Eppendorf tube. 200 µl Tris buffered phenol pH 8.0 was added together with approximately 300 µl of glass beads with a diameter of 0,11 mm. Cells were disrupted for 5 minutes in a cell homogenizer model MSK (Braun, Meslungen, Germany) under carbon dioxide cooling. After 15 minutes of centrifugation at 15000 g, the aqueous phase of the supernatant was extracted with phenol/chloroform/isoamylalcohol 25:24:1 (v:v:v), followed by chloroform/isoamylalcohol 24:1 (v:v) extraction and precipitation with 1 ml of 96% ethanol and 40 µl of 3M sodium acetate (pH 5.2). After 15 min centrifugation at 15000 g, the nucleic acid pellet was washed with 70% ethanol, dried under vacuum and resuspended in 100 µl of TE buffer. This extract contained DNA and rRNA of good quality, as judged by agarose gel-electrophoresis and ethidium bromide staining. This DNA and rRNA was used for PCR and dot blot hybridization.

The 16S rRNA-genes were amplified by polymerase chain reaction (PCR) as described before (*Harmsen* et al., 1993) using a set of universal 16S rRNA-based primers containing restriction sites to facilitate cloning. The forward primer corresponds to *Escherichia coli* positions 8 to 27 (5' CACGGATCCAGAGTTTGATC(T) (A/C)TGGCTCAG) and the reverse primer corresponds to *E. coli* positions 1493 to 1510 (5'GTGCTGCAGGGTTACCTTGTTACGACT) (*Lane*, 1991). The PCR-amplification products of MPOB were cloned in M13mp18 phages; amplificates of KOPROP1 were cloned in a pUC18 vector by standard procedures (*Sambrook* et al., 1989). DNA sequencing was done using the dideoxy-chain termination method (*Sanger* et al., 1977) using T7 polymerase in the case of M13mp18 or cycle-sequencing with Taq-polymerase according to the manufacturers manual in the case of pUC18.

*Sequence alignment and phylogenetic tree.* The partial nucleotide sequences were aligned with those of other bacterial sequences taking into account sequence similarity and higher order structure. The reference sequences were taken from the ribosomal database project RDP (*Olsen* et al., 1992; *De Rijk* et al., 1992). Evolutionary distance values (Knuc) were calculated according to *Hori* and *Osawa* (1979). The unrooted phylogenetic tree was reconstructed using the neighbor joining method of *Saitou* and *Nei* (1987) as implemented in the program "NEIGHBOR" (Phylip version 3.4) developed by *Felsenstein* (1982) including only sequence positions which are invariant in at least 50% of the entire sequence set. The topology of the tree was evaluated performing parsimony and bootstrapped parsimony analysis using the programs DNAPARS and DNABOOT (*Felsenstein*, 1982).

*Dot blot and in situ hybridizations.* Dot blot hybridization experiments were performed on Hybond N+ filters (Amersham, Little Chalfont, UK). Nucleic acid samples containing approximately 50 ng of rRNA were applied to the membrane with a Hybri.Dot manifold (Life Science Technologies) and immobilized according to *Church* and *Gilbert* (1984). The membranes were pretreated with 10 ml hybridization buffer (0.5 M phosphate buffer, 7% sodium dodecyl sulfate (SDS), 1% bovine serum albumin and 1 mM of EDTA, pH 7.2) for 30 min prior to hybridization with 100 ng of oligonucleotide probes 5' labeled with 10–20 µCi of [$\gamma^{32}$P]ATP (3000 Ci/mmol, Amersham). All membranes were hybridized at 40 °C and washed in 1% SDS, 1 × SSC (0.15 M NaCl, plus 0.015 sodium citrate, pH 7.0) washing buffer at 55 °C for the probes EUB338 and MPOB1 and at 45 °C for the probes KOP1 and MPOB2 (for probe sequences see *Amann* et al. 1992 and Table 1). The membranes were dried and exposed to a phospho storage screen for 90 min and the screen was scanned for radioactive response on a Phosphor Imager (Molecular Dynamics, Sunnyvale, USA). The digital signals were processed by the manufacturers software (ImageQuant). For *in situ* hybridization, cells were fixed, bound to vectabond-coated (Vector Laboratories, Inc., Burlingame, USA) slides and hybridized according to *Amann* et al., 1992. The oligonucleotide probes MPOB1 and KOP1 were synthesized and labeled at the 5' position with rhodamine using an aminolinker, by Eurogentec (Seraing, Belgium). The hybridization conditions were as follows: 45 °C incubation overnight followed by washing for 30 minutes at 48 °C in hybridization buffer (0.9 M NaCl, 0.1% SDS, 20 mM Tris/HCl pH 7.2). The cells were viewed either with a Bio-Rad

| Name oligo | Target | Sequence | Td |
|---|---|---|---|
| MPOB1 | MPOB (222) and KOPROP1 (222) | 5'-ACGCAGGCCCATCCCCGAA* | 63 |
| MPOB2 | MPOB (464) | 5'-CGTCAGCCATGAAGCTTAT | 53 |
| KOP1 | KOPROP1 (460) | 5'-TCAAGTCCCCAGTCTCTTCGA | 61 |

* The underlined A matches with the U of the MPOB 16S rRNA, but mismatches with an A of the KOPROP1 16S rRNA.

Table 1. The sequences of the specific oligonucleotides directed against the 16S rRNA sequences of the propionate oxidizing bacteria MPOB and KOPROP1. The number in brackets in the target column refers to the start of the target-site (*E. coli* numbering). Td is the experimentally determined dissociation temperature in °C

MRC-600 confocal laser scanning microscope equipped with a krypton/argon laser (emission peak at 588 nm) or with a conventional Nikon epifluorescence microscope.

## Results

For the isolation of the nucleic acids we used highly purified enrichment cultures. The MPOB enrichment culture contained lemon-shaped Gram-negative rods as the dominant bacteria. The MPOB culture was free of methanogens, but it contained a small thin rod as contaminant (*Stams* et al., 1993). The KOPROP1 enrichment culture contained lancet-shaped Gram-negative rods as the dominant bacteria and *Methanospirillum*-like methanogens (*Dörner*, 1992). PCR amplification of the 16S rRNA-genes from the two enrichments gave products with the expected size of about 1.5 kb. However, when the products were digested with the restriction enzymes BamHI and PstI to facilitate cloning, half of the amount of the product of KOPROP1 was cut into two fragments of approximately 0.6 and 0.9 kb. The 0.9 kb fragment hybridized with the group-specific probe 804 for *Desulfobacterium, Desulfobacter* and other bacteria designed by *Devereux* et al. 1992 (data not shown). These two fragments were not sequenced. Cloning of the other products resulted in twenty to fifty clones of each culture. Five clones of each culture, containing the correct insert, were selected for sequencing with the universal primer 1115 (*E. coli* 1100–1115). Both MPOB and KOPROP1 clones gave five times the same sequence and both sequence types were homologous to the sequence of *Syntrophobacter wolinii* (*Harmsen* et al., 1993). For each of the enrichments the insert of one clone was sequenced completely. This resulted in two sequences of the same length of 1486 bp with a homology of 95.3%. Both sequences were 92.7% homologous to the *Syntrophobacter wolinii* sequence. The MPOB sequence itself was deposited in the EMBL database under the accession number X82874. The KOPROP1 sequence was deposited under the EMBL accession number X82875.

### *Phylogenetic analysis*

The obtained sequences of the 16S rRNA genes of the enrichment cultures MPOB and KOPROP1 were aligned to other sequences available from the data bases (*Olsen* et al., 1992; *De Rijk* et al. 1992). Comparative sequence analyses revealed that both sequences cluster phylogenetically with the sulfate-reducing bacteria (SRB). A consensus tree based on distance matrix and parsimony analyses for selected members of the delta subclass of proteobacteria is depicted in Fig. 1. *Escherichia coli* and *Bacillus subtilis* were included as an outgroup. Both MPOB and KOPROP1 are highly related. Their relation to *S. wolinii* is less, but still profound. The three sequences form a group which share a common ancestor with *Desulfomonile tiedjei* and *Desulfoarculus baarsi*, two species which are only moderately related to the former group.

### *Design of specific oligonucleotide probes and hybridization with the target organisms*

The 16S rRNA-gene sequences of MPOB and KOPROP1 were used to develop specific oligonucleotide probes. The base composition of the probes is given in Table 1. The dissociation temperatures Td were determined as described by *Devereux* et al. (1992). The probes were developed against regions which had more than 3 mismatch-

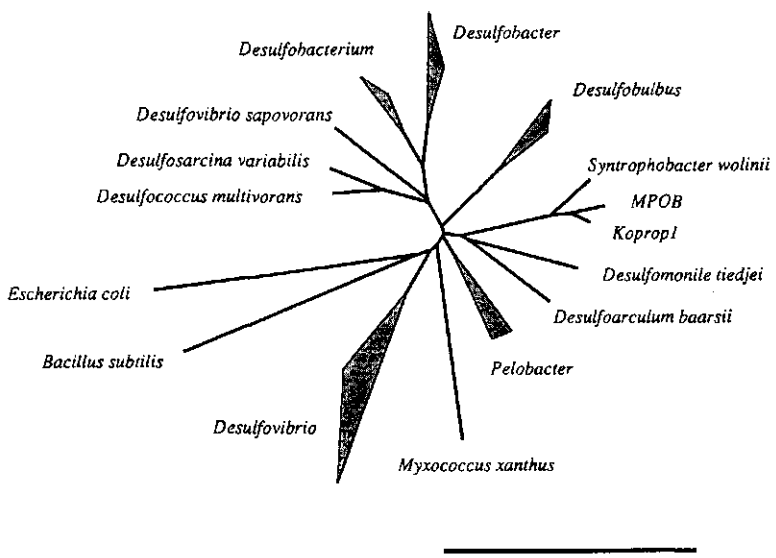



Fig. 1. Unrooted phylogenetic tree from 16S rRNA sequence divergence of MPOB, KOPROP1, *Syntrophobacter wolinii* and the major groups of the delta-subclass of Proteobacteria with *Escherichia coli* and *Bacillus subtilis* as outgroups. Only positions were used that had 50% invariance in all sequences. The bar represents 10% estimated sequence divergence.

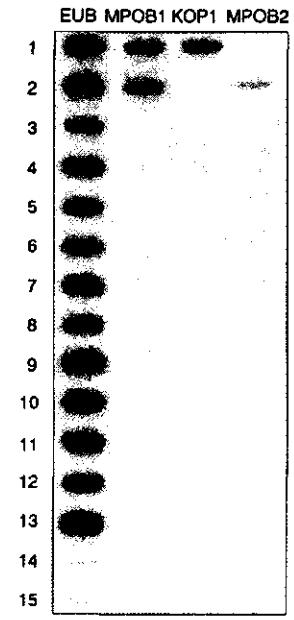


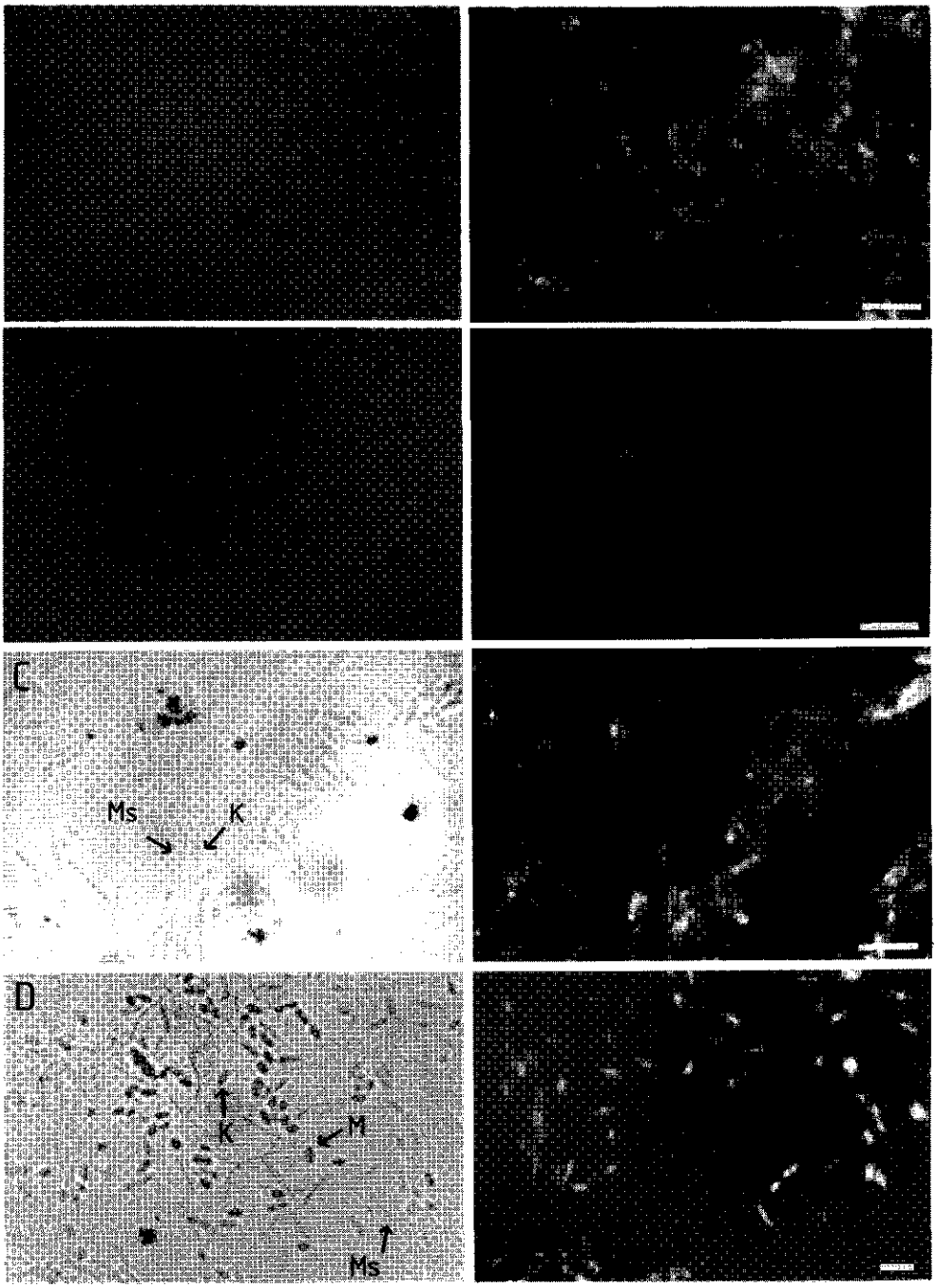

Fig. 2. Dot blot hybridization of four identical filters containing nucleic acids (DNA and rRNA) of fifteen bacteria and methanogens. Each column represents the radioactive signals from one filter. Ten ng of nucleic acids from the following organisms were bound to the filter; 1: KOPROP1 enrichment, 2: MPOB enrichment 3: *Syntrophobacter wolinii* DSM 2805, 4: *Desulfobulbus* sp. strain isolated in this laboratory, 5: *Desulfobulbus propionicus* DSM 2505, 6: *Desulfovibrio* sp. strain G11 isolated from DSM 2805, 7: *Desulfovibrio vulgaris* DSM 8303, 8: *Bacteroides xylanoliticus* X51 DSM 3808, 9: *Clostridium* DP-1 DSM 6566, 10: *Clostridium butyricum* DSM 552, 11: *Clostridium granularum* EE121 isolated in this laboratory, 12: *Syntrophospora bryantii* DSM 3014B, 13: Propionate-oxidizing enrichment isolated in this laboratory, 14: *Methanospirillum hungatei* DSM 864, 15: *Methanotrix soehgenii* DSM 2139.

es with the sequences present in the database. At the time the MPOB1 probe was developed, the sequence of KOPROP1 was unknown and the probe seemed to be specific for MPOB. When it turned out that the MPOB1 probe had only one mismatch with the corresponding regions of the KOPROP1 16S rRNA-gene, a new specific probe, MPOB2, was developed for MPOB. The specificity of the probes MPOB1 and MPOB2 for MPOB and KOP1 for KOPROP1, was tested by dot blot hybridization. Figure 2 shows that the MOPB2 and the KOP1 probes hybridized only with their respective target organisms, indicating that the obtained rRNA sequences originated from the main population in the enrichments. As expected, the MPOB1 probe cross-reacted with KOPROP1 rRNA. To prove that the 16S rRNA sequence of MPOB was the one belonging to the majority of the bacteria in the enrichment, *in situ* hybridization with probe MPOB1 was performed with fixed cells of the enrichment culture. Only the dominant lemon-shaped bacteria showed fluorescence (results not shown). The specificity of the MPOB1 probe was shown in a mixture of different anaerobic bacteria. While all bacteria light up with the bacterial probe EUB338 (*Amann* et al., 1992) (Fig. 3A), only the MPOB cells reacted with the probe MPOB1 (Fig. 3B). Cell smears of the KOPROP1 enrichment culture were hybridized with a rhodamine-labeled MPOB1 probe. The probe reacted with all the lancet-shaped rods and not with the *Methanospirillum* sp. (Fig. 3C). In addition, a cell smear of a mixture of the MPOB and the KOPROP1 enrichment cultures was also hybridized with the rhodamine-labeled KOP1 probe and only the lancet-shaped KOPROP1 rods gave fluorescent signals (Fig. 3D).

## Discussion

MPOB is a propionate-oxidizing bacterium which is able to grow on fumarate in the absence of methanogens (*Stams* et al., 1993). In our laboratory, attempts were made to obtain a pure culture of MPOB from the enrichment culture. When the roll tube technique was applied to a culture containing about $10^8$ cells/ml, colonies were formed till $10^{-4}$ to $10^{-5}$ dilution. At these dilutions, a small rod-shaped contaminant was present in the colonies. The contaminant became dominant when yeast extract was added to the medium (unpublished results). This contaminating bacterium is either parasitic or grows on cell exudates of MPOB cells. When yeast extract was omitted, generally approximately 99% of the cells were MPOB. For classification, a pure culture or a defined coculture is highly desired if not essential. The research described in this article was undertaken to elucidate the phylogeny of this bacterium. The KOPROP1 enrichment was included in this study to get more evidence for a phylogenetic group of syntrophic propionate- oxidizing sulfate-reducers.

Fig. 3. *In situ* hybridization on different mixtures of fixed cells with rhodamine-labeled probes; comparison of phase-contrast (left) and fluorescent (right) micrographs of identical fields. A. A mixture of cells of MPOB, *Desulfobulbus propionicus* DSM 2505, *Desulfovibrio* sp. strain G11 isolated from DSM 2805, *Clostridium granularum* EE121 isolated in this laboratory and *Desulfomonile tiedjei* ATCC 49306 hybridized with probe EUB338. All bacteria present are fluorescently labeled. B. The same mixture of cells as in A but now hybridized with probe MPOB1. Only the MPOB cells are labeled. C. Cells of the KOPROP1 enrichment, containing both KOPROP1 and *Methanospirillum* sp., hybridized with probe MPOB1. Only the KOPROP1 cells are labeled. D. A mixture of MPOB, KOPROP1 and *Methanospirillum* sp. hybridized with probe KOP1, specifically labeling KOPROP1 cells.
MPOB cells are marked with M, KOPROP1 cells are marked with K and *Methanospirillum* sp. are marked with Ms. Micrographs A to C were viewed a with confocal laser scanning microscope, the micrographs D were viewed using a conventional epifluorencence microscope. The bars represent 5 μm.


C
Ms
K
D
K
M
Ms

The tree presented in Fig. 1 clearly shows that the dominant propionate-oxidizing bacteria in the enrichment cultures, obtained at different laboratories, are closely related. The bacteria MPOB and KOPROP1 form one group with *S. wolinii*. All three bacteria have similar physiological properties; apart from their ability to oxidize propionate syntrophically, they can oxidize propionate with sulfate as electron acceptor (*Dörner, 1992*; *Wallrabenstein* et al., 1994; *van Kuyk,* personal communication). However, recently in our laboratory a bacterium was isolated, which is not able to grow syntrophically on propionate, but which is closely related to *S. wolinii* based on 16S rRNA analyses (*Oude Elferink* et al., in prep.). This indicates that this phylogenetic group of sulfate reducers is physiologically heterogeneous. The group of syntrophic propionate oxidizers belong to group 7 according to the grouping of *Devereux* et al. (1989). Group 7 also includes *Desulfomonile tiedjei* and *Desulfoarculum baarsii*. This is a phylogenetically distinct group belonging to the line of sulfate-reducing bacteria which oxidize organic compounds completely to carbon dioxide.

Because the sequences were derived from cloned PCR-products obtained from enrichment cultures, convincing evidence was needed that each sequence indeed came from the dominant propionate-oxidizing bacterium in the culture. Prove is clearly given by dot blot and *in situ* hybridization. Fig. 2 shows that the signals of the specific oligonucleotide probes on the dot blot with the nucleic acids isolated from the enrichment cultures come from the 16S rRNA of the dominant bacterium and not from the contaminant. Even if differences are considered between the specific activity of the bacterial probe and the specific probes, the signal could never come from the contaminant. The specific activity of the MPOB2 probe which reacts only with MPOB rRNA was always five times lower than the specific activity of the other probes. The reason for this is not clear, but it could be due to steric hindrance by secondary structure in combination with a relative low melting temperature. The *in situ* hybridization with the MPOB1 probe on the MPOB culture gave only a signal with the dominant lemon-shaped bacterium, but not with the morphologically distinct contaminant. The negative results of the hybridization with the KOP1 probe with the MPOB cells indicates that the fluorescent signal of the MPOB1 probe was not an artifact. These experiments confirm that the sequence comes from the MPOB bacterium. The *in situ* hybridization signals of the MPOB1 probe and KOP1 probe with cell smears of the KOPROP1 enrichment culture confirmed that the KOPROP1 sequence was the correct one.

The finding that syntrophic propionate-oxidizing bacteria form a group within the sulfate-reducing bacteria justifies the creation of a genus, which includes the species *S. wolinii*, MPOB and KOPROP1. According to the rules for nomenclature, the name should be the genus name of the first isolated bacterium, *Syntrophobacter*. But there are two arguments against keeping this genus name. Firstly, these bacteria are sulfate-reducers and secondly, as mentioned above, there is at least one other related sulfate-reducer, that can not oxidize propionate syntrophically. Therefore, the ability to grow syntrophically on propionate is an unsuitable phylogenetic marker for these bacteria. This raises the question whether these bacteria should be named according to their relation with sulfate-reducers. Before answering this question, more syntrophic propionate-oxidizing bacteria have to be isolated or enriched and their phylogeny and physiology studied.

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# Chapter 4

## Enrichment and phylogenetic analysis of sporeforming syntrophic propionate-oxidizing bacteria.

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# Enrichment and phylogenetic analysis of sporeforming syntrophic propionate-oxidizing bacteria.

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**Abstract**

Sporeforming syntrophic propionate-oxidizing bacteria were enriched from freeze-dried anaerobic granular sludge obtained from an upflow anaerobic sludge bed reactor treating sugarbeet wastewater. The culture oxidized propionate in presence of hydrogen/formate consuming *Methanospirillum hungatei*. The 16S rRNA genes of the dominant bacteria were cloned and characterized. This revealed that at least two different bacterial 16S rRNA sequences were present in the culture. These two sequences had 96.8 % sequence similarity with each other and were related to the Gram-positive sulfate reducing genus *Desulfotomaculum*. Specific 16S rRNA-based oligonucleotide probes were designed and used for slot blot hybridization. This revealed that both sequences were present in the enrichment culture in equal amounts.

## Introduction.

Syntrophic processes play an important role in the mineralization of organic compounds under methanogenic conditions (Schink, 1992). Fatty acids such as butyrate and propionate can only be degraded by acetogenic bacteria when cocultured with hydrogen- and/or formate-consuming methanogens. The methanogens are needed to keep the partial hydrogen pressure and formate concentration sufficiently low. (Stams, 1994).

A large variety of acetogenic bacteria have been isolated that perform such syntrophic conversions in coculture with methanogens. In recent years several of these syntrophic bacteria have been phylogenetically characterized. *Syntrophomonas wolfei* (McInerney et al. 1981) and the sporeforming bacterium *Syntrophospora bryantii* (Stieb and Schink 1985) that both grow syntrophically on long chain fatty acids, are related to Gram-positive bacteria (Zhao et al., 1990). *Syntrophus buswellii* and *Syntrophus gentianae*, two bacteria that degrade benzoate syntrophically, were recently shown to be related to gram-negative sulfate-reducing bacteria, although both were not able to reduce sulfate (Wallrabenstein et al., 1995a). Recently, three syntrophic propionate-oxidizing bacteria have been characterized, that are closely related, and form a single group within the Gram-negative sulfate-reducing bacteria (Harmsen et al., 1993; 1995a). These bacteria, *Syntrophobacter wolinii*, *Syntrophobacter pfennigii* (previously designated KOPROP1) (Wallrabenstein et al., 1995b) and *Syntrophobacter fumaroxidans* (previously indicated as MPOB), can also oxidize propionate in pure culture using sulfate as electron acceptor (Wallrabenstein et al., 1995b; van Kuijk and Stams, 1995). So far only one sporeforming syntrophic bacterium capable of oxidizing propionate, was isolated from granular sludge, but apart from its grouping within the Gram-positive bacteria, no further phylogenetic characterization was reported (Wu et al., 1992). This study describes the enrichment and phylogenetic analysis of a culture containing at least two different sporeforming syntrophic-propionate oxidizing bacteria that are related to Gram-positive bacteria.

## Materials and methods.

*Medium, analytical methods, and enrichment.* A basal bicarbonate-buffered medium containing 20 mM propionate, used for the primary enrichment and subculturing, was prepared according to Stams et al. (1993). Propionate, acetate, methane and hydrogen were measured by gas chromatography as described previously (Stams et al., 1993).

The propionate-oxidizing culture was enriched from freeze-dried granular sludge form an upflow anaerobic sludge bed reactor (CSM, Breda The Netherlands). After freeze-drying, the sludge had been stored aerobically for more than 2 years at room temperature. Bottles (120 ml) with 50 ml medium and a $N_2/CO_2$ (80:20 % v/v) gasphase were inoculated with 0.2 g of freeze-dried granular sludge and incubated at 37 °C. The propionate-oxidizing bacteria were further purified by repeated pasteurization for 30 min at 85 °C and subculturing in medium at 37 °C. To these cultures 10 % (v/v) of a $H_2/CO_2$ (80:20 % v/v) grown culture of *Methanospirillum hungatei* JF1 (DSM 864) was added.

*Nucleic acid isolation, PCR amplification, cloning and sequence analysis.* The nucleic acids were isolated from 10 ml of a late logarithmic enrichment culture as described previously. (Harmsen et al. 1995a). The microbial 16S rRNA genes present in the enrichment culture were amplified by polymerase chain reaction (PCR) as described before (Harmsen et al., 1993) using a set of universal 16S rRNA-based primers containing restriction sites (in italics) to facilitate cloning: the forward primer (5'CAC*GGATCC*GGACGGGTGAGTAACACG) corresponded to *Escherichia coli* positions 106 to 124 and the reverse primer (5'GTG*CTGCAG*GGTTACCTTGTTA-CGACT) to *E. coli* positions 1493 to 1510. Recombinant plasmids from the PCR amplification products were obtained as described previously using pUC18 as cloning vector and *E. coli* TG1 as host (Harmsen et al., 1995b) DNA sequencing was done using the dideoxy-chain termination method (Sanger et al., 1977) adapted for cycle-sequencing with Taq-polymerase as described by the Life Science Technologies manual. All enzymes for DNA manipulations were obtained from Life Science Technologies (Gaithersburg, Md., USA). Unlabelled oligonucleotides were synthesized by Pharmacia (Uppsala, Sweden).

*Sequence alignment and phylogenetic tree.* Partial 16S rRNA nucleotide sequences were aligned with those of other bacterial sequences taking into account sequence similarity and higher order structure using the alignment tool of the ARB

program package (Van de Peer et al., 1994; Strunk et al., in prep.). The reference sequences were taken from the ribosomal database project RDP (Maidak et al., 1994). The tree is based on the results of a distance matrix analysis including only sequence positions which share the same nucleotides in at least 50% of sequences from the relevant members of Gram-positive bacteria (ARB; PHYLIP, Felsenstein, 1982). The tree was corrected according to the results of maximum parsimony and maximum likelihood analyses (fastDNAml, Maidak et al., 1994).

*Slot blot hybridizations.* Slot blot hybridization- experiments were performed on Hybond N+ filters (Du Pont). Nucleic acid samples containing approximately 50 ng of rRNA were applied to the membrane with a Hybridot manifold (Life Science Technologies) and immobilized according to Church and Gilbert (1984). The membranes were pretreated with hybridization buffer (0.5 M phosphate buffer, 7 % sodium dodecyl sulfate, 1 % BSA, 1 mM of EDTA, pH 7.2) for 30 min prior to hybridization with 100 ng of [$\gamma$-$P^{32}$]ATP-labeled probes. All membranes were hybridized at 40 °C and washed in 1 % SDS, 1 x SSC (0.15 M NaCl, plus 0.015 sodium citrate, pH 7.0) wash buffer at 55 °C for the probe EUB338 (Stahl and Amann, 1991) and at 50 °C for probe SPA and SPB (Table 1), the latter one being the experimentally determined optimal washing temperature for these probes. The membranes were exposed to a Kodak X-ray film or exposed to a phospho-imager screen. This screen was scanned for radioactive response on a Phosphor Imager (Molecular Dynamics, Sunnyvale, USA). The digital signals were processed by the manufacturers software (ImageQuant).

## Results

*The enrichment of the sporeforming syntrophic propionate- oxidizing culture.* To enrich for syntrophic propionate-oxidizing bacteria, freeze-dried granular sludge was inoculated in a 125 ml bottle with 50 ml medium with 20 mM of propionate. After 3 months of incubation at 37 °C, the propionate was depleted, and methane gas was produced. Acetate was detected only in low amounts. This culture was further purified by repeated rounds of pasteurization at 85 °C and subculturing of the pasteurized culture in dilution series in the presence of *Methanospirillum hungatei*. The $10^8$ dilution culture was usally used for sulculturing. After 6 transfers, the culture consisted of two morphotypes of microbes. One was *M. hungatei* and the other was a rod shaped

bacterium, 1 $\mu$m by 2-2.5 $\mu$m in size, which formed endospores (Fig. 1). No other bacteria were observed microscopically. However, when the culture was grown on glucose, long rod shaped bacteria were detected. These bacteria remained present, even after additional rounds of pasteurization and subculturing. The doubling time of the culture was approximately 7 days and the culture had a lag phase varying from 1 up to 8 weeks. It converted propionate stoichiometrically into acetate and methane. Attempts to grow this culture on propionate and sulfate failed so far. Scanning electron microscopy micrographs suggests that the rod-shaped dominant bacteria contained flagella, indicating a motility. However, this was never observed with phase-contrast microscopy.

*PCR amplification, cloning and sequence analysis.* An enrichment culture which has been pasteurized and subcultured for at least 15 times, was used for phylogenetic analysis. PCR-amplification of the 16S rRNA genes from the nucleic acids isolated from this culture resulted in a product of the expected size of 1.4 kb. This fragment was digested with *Bam*HI and *Pst*I and ligated in pUC18, linearized with the same enzymes. The ligation products were transformed into *E. coli* TG1 cells. This resulted in 38 recombinant plasmids.

Ten of the recombinant plasmids were characterized by sequence analysis of the insert DNA using the universal 16S rRNA primer 1115 (*E. coli* 1100-1115) (Lane, 1991). Four of these plasmids contained inserts with identical sequences of the V6 region of the 16S rRNA gene, indicated as type A sequences. Five plasmids contained identical sequences indicated as type B. One plasmid contained an unidentified sequence. Two plasmids containing a type A or B 16S rDNA sequence, were selected for further analysis, and their inserts were sequenced completely, resulting in sequences of 1361 bp or 1362 bp, respectively, which were deposited in the EMBL-database under the accession numbers X91169 (type A) and X91170 (type B). The organism from which the type A or B sequences were derived will be referred to as SporeA or SporeB, respectively.

*Phylogenetic analysis.* The 16S rRNA sequences of the SporeA- and SporeB-type were aligned to other sequences available from the data bases (Maidak et al., 1994; Van de Peer et al., 1994). Comparative sequence analyses revealed that both sequences cluster phylogenetically with the genus *Desulfotomaculum*. A consensus tree

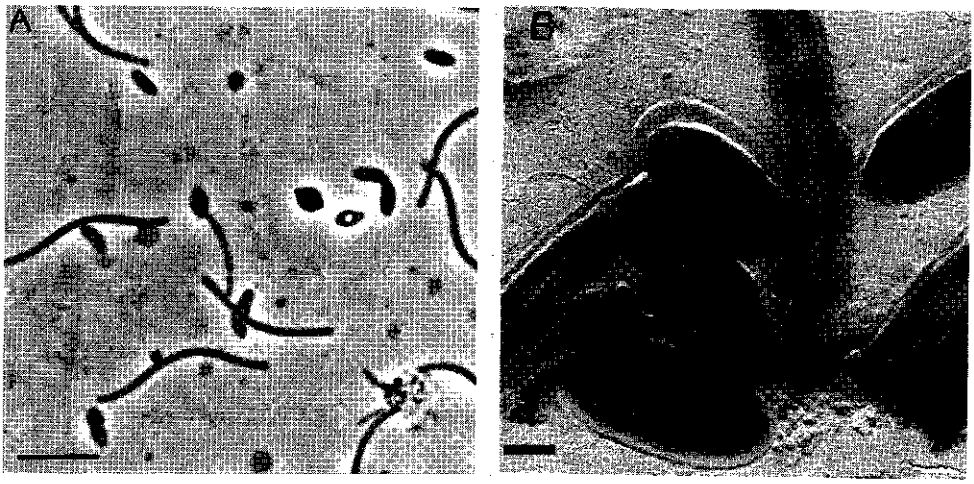


Fig. 1. Microscopic images of the sporeforming syntrophic propionate-oxidizing culture. The micrographs were made with a Leitz phasecontrast microscope on Kodak film (A), or with a Jena scanning electron microscope (B). The bar in A is 10 $\mu$m, the bar B represent 0,5 $\mu$m.

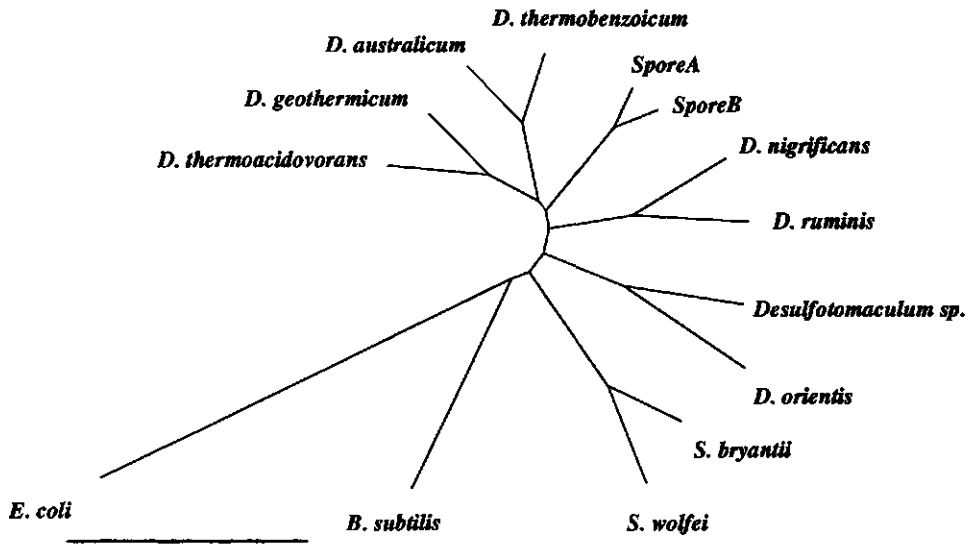


Fig. 2. Unrooted phylogenetic tree from 16S rRNA sequence divergence of SporeA, SporeB, members of the genus *Desulfotomaculum* and other members of the group of Gram-positive bacteria with *Escherichia coli* a an outgroup. Only positions were used that had 50 % invariance in all sequences. The bar represents 10% estimated sequence divergence.

based on distance matrix and parsimony analyses for selected members of the Gram-positive bacteria is depicted in Fig. 2. *E. coli* was included as an outgroup. The phylogenetic tree shows that both SporeA and SporeB are closely related to each other, and belong to the genus *Desulfotomaculum*. The sequence similarity of the SporeA with the SporeB 16S rRNA sequence was 96.8% and the closest relatives of SporeA and SporeB are *D. thermo-acidovorans* and *D. thermobenzoicum*, respectively (Table 1).

*Slot blot hybridization of the recombinant plasmids and nucleic acids isolated from the enrichment culture.* Specific oligonucleotide probes were designed against the V6 region of the 16S rDNA sequences of SporeA and SporeB (SPA and SPB, respectively; Table 2). A slot blot containing approximately 100 ng of each of the 38 plasmids was hybridized with both probes, to determine the ratio in which the two sequence types were represented by the clone library. In addition, a comparable amount of DNA obtained from five different PCR amplifications was added to the blot to determine if selective cloning had appeared (data not shown). Thirteen of the 38 plasmids gave a positive signal with the SporeA specific probe SPA. Seventeen plasmids gave a positive signal with the SporeB specific probe SPB, and eight plasmids remained unidentified. All five PCR-products hybridized with both probes with approximately the same efficiency, indicating that the two 16S rDNA sequences were present in equal amounts after amplification.

When nucleic acids isolated from the enrichment culture were hybridized with probes SPA and SPB, both probes gave similar radioactive signals. Granular sludge from an UASB reactor treating potato-processing wastewater, was enriched for syntrophic propionate-oxidizing bacteria by repeated cultivation on propionate (Harmsen et al., 1995b). However, the hybridization of the nucleic acids isolated from these enrichments with both probes SPA and SPB gave no significant signal (results not shown). Remarkably, the SPB probe did hybridize with nucleic acids isolated from an enrichment using the same sludge, which was obtained by repeated pasteurization and cultivation on propionate in the presence of *M. hungatei* (Fig. 3). The SPA probe gave only a very week signal when hybridized with these nucleic acids.

Table 1. 16S rRNA sequence similarity between the two sporeforming propionate-oxidizing bacteria and the genus *Desulfotomaculum*, as being their closest relatives, other Gram-positive bacteria and *E. coli*.

| Similarity in % | SporeA | SporeB |
|---|---|---|
| SporeB | 96.8 | 100 |
| *D. thermoacidovorans* | 88.9 | 89.2 |
| *D. thermobenzoicum* | 87.8 | 89.3 |
| *D. australicum* | 88.5 | 89.1 |
| *D. geothermicum* | 87.9 | 88.8 |
| *D. nigrificans* | 87.7 | 87.1 |
| *D. ruminis* | 87.3 | 87.6 |
| *D. orientis* | 87.4 | 88.0 |
| *Desulfotomaculum* sp. | 86.0 | 85.3 |
| *S. bryantii* | 83.9 | 85.3 |
| *S. wolfei* | 83.3 | 84.2 |
| *B. subtilis* | 83.9 | 84.2 |
| *E. coli* | 78.1 | 77.6 |

Table 2. Alignment of the 16S rRNA sequences of the target-sites of the probe SPA, underlined in the SporeA sequence and the SPB probe, underlined in the SporeB sequence. The nucleotide sequences of the V6-domain, corresponding to the *E. coli* positions 991-1049, of several species of Gram-positive bacteria are aligned. The data were taken from the Ribosomal Database Project (Maidak, 1994).

| | |
|---|---|
| SporeA | uacaucccugaugacuguggaaacacagaguuuugcc....uucg...gguaaacagggagaca |
| SporeB | uacauccucugacagccuaugaaaguagguuuucuauc....uucg...gauggacagggagaca |
| *D. geothermicum* | uacaucncccgccagccaugaaacauggucuuuccc.....uuac...ggga.acggguagaca |
| *D. thermoacid.* | uacaucccuggccgcuguggaaacacaguuuuccauc....uucg...gauggacagggagaca |
| *D. australicum* | uacaucucucugacguucguggaaacacggauuucacgcgggguaacccgugugacagggagaca |
| *D.thermobenzoicum* | uacaucccugacagccugugaaaagcaggguuuu.....cuau....agaggcagggagaca |
| *D. nigrificans* | uacaucccgcgcaccacucuagagauagaggguucugcc...uuag...gguagacgcgcagaca |
| *D. ruminis* | uacauccgucunaaaaguauagggauauacucuugcc.....uucg...gggnnacagagagaca |
| *Desulfotomac. sp.* | uacauccauagaaucccguggaacaugggagugccc.....uucg.....ggagcuaugagaca |
| *S.bryantii* | uacauccaacgaacccuuaugaaaguagggnnugnc.....uucg.....ggagcguugagaca |
| *S.wolfei* | uacauccuacggauuucuaugaaaguagaaaagugcc.....uucg.....ggagcguagagaca |
| *B.subtilis* | uacauccucugacaauccuagagauaggacgucccc.....uuc.....ggggcagagugaca |
| *E.coli* | uacauccacggaaguuuucagagaugagaaugugcc.....uucg.....ggaaccgugagaca |

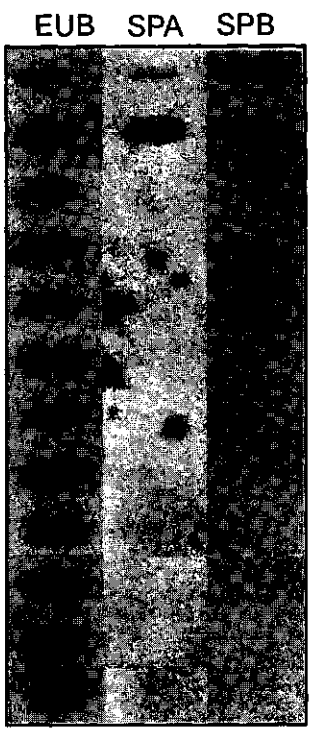


Fig. 3. Slot blot hybridization of three identical filters containing nucleic acids (DNA and rRNA) of two enrichment cultures and ten reference bacteria. The filters were hybridized with the eubacterial probe EUB338 (EUB), a specific probe for SporeA (SPA), and a specific probe for SporeB (SPB). Each column represents the radioactive signals from one filter. Approximately 10 ng of nucleic acids from the following organisms were bound to the filter; 1: Propionate-oxidizing culture enriched by pasteurization from granular sludge from a UASB reactor treating potato-processing wastewater, 2: The sporeforming syntrophic propionate-oxidizing enrichment culture described in this study, 3: *Syntrophobacter fumaroxidans* (DSM 10017), 4: *Syntrophobacter wolinii* (DSM 28005), 5: *Desulfobulbus* sp. strain isolated in this laboratory, 6: *Desulfobulbus propionicus* (DSM 2505), 7: *Desulfovibrio* sp. strain G11 isolated from (DSM 2805), 8: *Desulfovibrio vulgaris* (DSM 8303), 9: *Desulfobacterium autotrophicum* (DSM 3382), 10: *Clostridium butyricum* (DSM 552), 11: *Clostridium granularum* EE121 isolated in this laboratory, 12: *Escherichia coli* MRE 600 (DSM 3901).

## Discussion

We were able to enrich for sporeforming syntrophic propionate oxidizers which were related to Gram-positive bacteria. This indicates that syntrophic growth on propionate is not restricted to Gram-negative bacteria such as *Syntrophobacter wolinii*. There has been only one other report on the isolation of a sporeforming syntrophic propionate-oxidizing bacterium (Wu et al., 1992). This strain PT was isolated from granular sludge in coculture with *Methanobacterium formicicum*. Although somewhat smaller in length, it had the same characteristics as the bacteria in the enrichment described here. It formed endospores in the late logarithmic phase, it had a doubling time of 7 days, and it was also not able to reduce sulfate in the presence of propionate.

The SporeA and SporeB sequences are closely related to each other and clearly belong the Gram-positive sulfate-reducing genus *Desulfotomaculum*. Several *Desulfotomaculum* species are thermophilic. However, it is not known whether the syntrophic sporeformers are capable of growing at temperatures above 45 °C in coculture with a thermophilic methanogen such as *Methanobacterium thermoautotrophicum*. *M. hungatei* used here does not grow above 42 °C. Thermophilic syntrophic propionate-oxidizers have been enriched before in coculture with *M thermoautotrophicum* or *M. thermoformicicum* at 55 °C, but a phylogenetic characterization was not yet performed (Stams 1992). Although related to sulfate-reducing bacteria, experiments to grow the sporeforming syntrophs on propionate and sulfate failed so far. The sporeforming syntrophs were not related to the other syntrophic bacteria belonging to Gram-positive bacteria growing syntrophically on butyrate or longer chained fatty acids, such as *Syntrophospora bryantii*.

There were two closely related 16S rRNA sequences obtained from the culture, which seem to be present in comparable amounts. The most likely explanation for this is that there are two closely related organisms with identical numbers of rRNA operons present in this culture, which had the same growth characteristics and hence reached the same numbers. However, it could be possible that both 16S rRNA sequences were present in a single bacterium. If so, this would resemble the situation reported previously for the archaeon *Haloarcula marismartui* (Mylvaganam and Dennis, 1992). However, this possibility can not be reconciled with the observation that only the SPB probe reacted with nucleic acids isolated from a culture which was enriched by pasteurization from potato-starch-fed granular sludge (Fig. 3). In this case the SporeA sequence was absent, indicating that the two 16S rRNA genes can be separated.

The 16S rRNA sequences of the sporeforming syntrophs were never detected by slot blot hybridization of nucleic acids isolated from granular sludge, using the SPA and SPB probe. Only the SporeB sequences could be detected after enrichment of pasteurized material. Therefore, it is not likely that these bacteria play an important role in the syntrophic propionate degradation in granular sludge, unlike the *Syntrophobacter*-like bacteria that are present in high numbers. However, since the endospores will be more tolerant to high temperatures and aerobic conditions, these bacteria may be more versatile than the obligately anaerobic and mesophilic *Syntrophobacter*-like syntrophs.

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Microbial composition and characterization of prevalent methanogens and acetogens isolated from syntrophic methanogenic granules. Appl. Microbiol. Biotechnol. 38, 282-290 (1992)

# Chapter 5

## Detection and localization of syntrophic propionate-oxidizing bacteria in granular sludge by *in situ* hybridization using 16S rRNA-based oligonucleotide probes.

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Submitted

# DETECTION AND LOCALIZATION OF SYNTROPHIC PROPIONATE-OXIDIZING BACTERIA IN GRANULAR SLUDGE BY *IN SITU* HYBRIDIZATION USING 16S rRNA-BASED OLIGONUCLEOTIDE PROBES.

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## ABSTRACT

The microbial architecture of granular sludge in two labscale upflow anaerobic sludge blanket reactors was investigated by fluorescently labeled oligonucleotide probing. The reactors had been fed for several months with either sucrose or a mixture of volatile fatty acids. Sections of the granules were hybridized with 16S rRNA-targeted universal oligonucleotide probes for bacteria and methanogens, and specific oligonucleotide probes for two syntrophic propionate-oxidizing strains MPOB and KOPROP1. Cells of the syntrophic strain KOPROP1 were not detected in both types of sludge granules. Hybridizations of the sucrose-fed granules showed an outer layer of mainly bacterial microcolonies with different morphology. More inwards of these granules, a layer of different methanogenic microcolonies mixed with large colonies of the syntrophic strain MPOB could be detected. The MPOB colonies were intertwined with hydrogen- or formate-consuming methanogens indicating their syntrophic growth. The granules fed with volatile fatty acids showed an outer layer of mainly bacteria followed by a thick layer of *Methanosaeta*-like methanogens mixed with few bacteria followed by a layer of methanogens mixed with syntrophic MPOB-microcolonies. The centre of both sludge types consisted of large cavities and methanogenic microcolonies. These results indicate a juxtapositioning of syntrophic bacteria and methanogens and provide new evidence for a layered microbial architecture of anaerobic granular sludge.

## INTRODUCTION

Anaerobic degradation of organic matter leads to intermediate formation of alcohols and fatty acids. The oxidation of these products under methanogenic conditions is coupled to proton reduction and can only proceed at low hydrogen partial pressures and low formate concentrations. This is achieved by interspecies transfer of hydrogen or formate between syntrophic consortia of bacteria. Acetogens convert propionate into acetate, hydrogen/formate and carbon dioxide while methanogens use these products in the production of methane (4, 14). A specific spatial orientation of acetogens and methanogens has been suggested because the distance between these microorganisms should be sufficiently small to obtain a low partial hydrogen pressure (23). The architecture of syntrophic consortia has been studied in methanogenic granular sludge, where microorganisms are immobilized in densely packed granules and high conversion rates of alcohols and fatty acids are reached (6, 12, 25). Immunogold labeling techniques with antibodies against methanogens and acetogens, suggested a juxtaposition of propionate degrading micro-colonies throughout the entire granule, although there were no antibodies that were sufficiently specific to detect the acetogens (6, 11, 16). Additionally, a layered structure of the granule was proposed, in which a central core of aceticlastic methanogens is surrounded by a layer of hydrogen/formate-producing acetogens and hydrogen/formate-consuming methanogens (12). In the outer layer of the granule most likely the hydrolyzing and acidifying bacteria are present.

The studies of the spatial distribution of the microorganisms in methanogenic granular sludge are hampered by the lack of identification methods that allow to differentiate between the different groups of microorganisms. Modern molecular biological techniques provide tools to investigate these issues in anaerobic ecosystems *in situ* (2, 21, 17). *In situ* hybridization with fluorescently labeled oligonucleotide probes based on rRNA sequences has proven to be a powerful tool to identify and localize microorganisms in their natural habitat (24).

Recently, we described the phylogenetic characterization of three syntrophic bacteria, *Syntrophobacter wolinii* and the strains MPOB and KOPROP1 (7, 8). Analysis of their 16S rRNA sequences revealed that these syntrophic bacteria are closely related and belong to the delta subclass of proteobacteria. These bacteria were also closely related to *Desulforhabdus amnigenus* a sulfate reducing bacterium isolated from sulfidogenic granular sludge, which is not able to grow syntrophically on propionate (15). Specific oligonucleotide probes were designed to detect these bacteria.

The use of these probes to detect and localize MPOB and KOPROP1 in sludge from upflow anaerobic sludge blanket (UASB) reactors is described here. Industrial granular sludge was fed in labscale UASB-reactors with either sucrose or volatile fatty acids. Specific fluorescently labeled oligonucleotide probes based on 16S rRNA sequences were used in combination with fluorescently labeled 16S rRNA-based probes for Bacteria and Archaea. This approach allowed for analyzing the spatial distribution of microorganisms in methanogenic granular sludge, especially of syntrophic propionate-oxidizing bacteria.

## MATERIALS AND METHODS

**The origin of the granular sludge samples.** Sludge samples were taken from two 10-liter UASB-reactors which had been inoculated with sludge from an UASB plant treating industrial sugarbeet wastewater (CSM, Breda, the Netherlands). One reactor was fed with sucrose with a COD load of 5 kg/m$^3$.day and the other reactor was fed with a mixure of volatile fatty acids: butyrate:propionate:actetate 21:16:12 with a COD-load of 5 kg/m$^3$.day. The COD removal was in both cases more the 90 %. The samples were taken six months after inoculation from the lower part of the sludge blankets.

**Fixation, embedding and sectioning of the granules.** Freshly taken granules (3 ml) were gently washed in tapwater (10 ml) and allowed to settle. Subsequently, the granules were fixed in freshly made 4% paraformaldehyde in phosphate buffered saline (PBS) pH 7.2 and left overnight at 4 °C. The fixative was removed by washing the granules twice with PBS. The granules were dehydrated by resuspending in 50% (v/v) ethanol in water for 2 h followed by a treatment with 70 % ethanol overnight at 4 °C. The water/ethanol was replaced with tertiary butyl alcohol (TBA) by consecutively washing the granules for 1 h each at 32 °C using the following solutions: ethanol:water:TBA (v:v:v) 50:30:20, 50:20:30, 50:10:40, 50:0:50, 25:0:75. Subsequently, the granules were transfered to a 1.5-ml tube and incubated overnight in 100% TBA. The TBA was replaced by paraplast at 62 °C by adding 5 pellets of paraplast to the tubes, one pellet every 12 h. The tubes were opened for 2 h to allow for the evaporation of TBA. The granules were then transfered to a 1 cm$^3$ cup with melted paraplast using a small spatula. The cube was allowed to cool down and was then used for sectioning.

The granule cubes were cut with a conventional microtome in sections of 5 to 10 $\mu$m. Sections thinner than 5 $\mu$m showed a loss of structure and the sections broke easily. Sections thicker then 10 $\mu$m resulted in high background especially with conventional epifluorescence microscopy, because of an excess of biomass. The sections (ribbons) were stretched in 50 °C water and transferred to slides coated with Vectabond (Vector Laboratories, Inc., Burlingame, USA). The slides were dried overnight at 42 °C. The sections on the slides were deparaffinated in xylol for 30 min and the xylol was removed by rinsing the slides consecutively for 2 min each in the following solutions; (xylol:TBA:ethanol) (v:v:v) 100:0:0, 75:25:0, 25:75:0, 0:50:50, 0:25:75 and 0:0:100. The sections were stored in a dry box up to 6 months at 4 °C.

***In situ* hybridization and transmission electron microscopy.** The following oligonucleotide probes complementary specific regions of the 16S rRNA were used: (i) EUB338, specific for the domain Bacteria (1). (ii) ARC915 specific for the domain Archaea (20). (iii) MPOB1 specific for MPOB (8, see table 1), (iv) KOP1 specific for the strain KOPROP1 (8), (v) MX825, specific for the genus *Methanosaeta* (18), (vi) MG1200, group-specific for *Methanogenium* and relatives (18), (vii) MB310, group-specific for the order *Methanobacteriales* (18). All the oligonucleotides were synthesized and 5' labeled with FLUOS (a fluorescein derivative) or rhodamine using an aminolinker and subsequently purified using acrylamide gelelectrophoresis by Eurogentec (Seraing, Belgium). The sections of the granules were hybridized in 20 $\mu$l of hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl pH 7.2, 0.01% sodiumdodecyl sulfate) containing 100 ng of labeled oligonucleotide probe. These were incubated in a moist chamber (20) for 3 h at 45 °C and the excess of probe was removed by washing in hybridization buffer at 48 °C for 30 min. The sections were rinsed in water, air dried and subsequently viewed using a Nikon epifluorescence microscope or a Bio-Rad MRC-600 confocal laser scanning microscope equipped with a krypton/argon laser. The transmission electron microscopy was done on a Jeol JEM 100 CX II microscope by a modification of the methods described previously (19).

## RESULTS

**Fluorescent oligonucleotide probing.** The microbial composition and spatial orientation in sections of granular sludge were investigated by fluorescent oligonucleotide probing. Two different sludge samples from labscale UASB-reactors were analyzed; one had been fed with sucrose and another with a mixture of the volatile fatty acids (VFA) acetate, propionate and butyrate. The hybridization with fluorescein-labeled 16S rRNA-based universal probes for Bacteria (EUB338) and for Archaea (ARC915) to detect methanogens, was combined with hybridization with rhodamine-labeled specific probes for the syntrophic propionate-oxidizing strains MPOB and KOPROP1 (MPOB1 and KOP1). The specificity of probe MPOB1 is shown in an alignment of a part of the 16S rRNA sequences of some reference organisms (Table 1). The MPOB1 probe has only one mismatch with the syntrophic propionate-oxidizing strain KOPROP1 or the sulfate-reducing bacterium *Desulforhabdus amnigenus* and hybridizes with the rRNA of these organisms even at high stringency. The probe KOP1 hybridized only to the 16S rRNA of the syntrophic strain KOPROP1, and this probe had at least 3 mismatches with all the other 16S rRNA sequences currently known (8).

**Autofluorescence.** Autofluorescence during epifluorescent microscopy is often a problem when *in situ* hybridization is performed on microbial cells present in complex environments. In the case of the sections of the granule, a considerable amount of autofluorescence was viewed at 520 nm and 580 nm. However, we were able to distinguish the autofluorescence signal from that resulting from fluorescence of the labeled probes, since the autofluorescence gave a bright yellow signal when viewed at 520 nm, while the fluorescence of the fluorescein-labeled probes was green. Usually, fibrous or granular structures showed fluorescense, probably caused by inorganic precipitates. At 580 nm, these structures were as red as the signal from the rhodamine-labeled probes, but viewing the sections at 520 nm afterwards showed that they were not of prokaryotic origin (marked X in Fig. 1 - 4).

**Microbial architecture of sucrose-fed sludge.** Sucrose-fed granules were hybridized with the fluorescein-labeled bacterial probe EUB338 in order to visualize all bacteria (Fig. 1A). Bright microcolonies and individual bacteria at the edge of the granule, showed fluorescence. More inwards of the granule large microcolonies of 2 $\mu$m long thick rods could be visualized. Most bacteria in this area have this morphology. The hybridization of the same sucrose-fed granule with the rhodamine-

Tabel 1. Alignment of the 16S rRNA sequences of the target-site of the MPOB1 probe. The nucleotides corresponding to the *Escherichia coli* positions 222-240 of several species of the delta subclass of Proteobacteria and some reference organisms are aligned. The data were taken from the Ribosomal Database Project (13). The mismathces with the MPOB target-site are marked in bold.

| | | |
|---|---|---|
| MPOB | 5' | UUCGGGGAUGGGCCUGCGU 3' |
| KOPROP1 | | U**A**CGGGGAUGGGCCUGCGU |
| *Desulforhabdus amnigenus* | | UUCGGGGAUG**A**GCCUGCGU |
| *Syntrophobacter wolinii* | | **CA**CGGGGAUG**AGU**CUGCGU |
| *Desulfomonile tiedjei* | | **CCAAA**GGAUGGGC**UC**GCG**G** |
| *Desulfobulbus propionicus* | | **CNUGAAGAGG**GGGUCUGCGU |
| *Desulfosarcina variablis* | | UUU**GAA**GAUGGGCCCGCGU |
| *Desulfovibrio desulfuricans* | | **CGUAA**GGAUG**AGUCC**GCGU |
| *Desulfovibrio gigas* | | **CAAUGA**GAUG**AGUCC**GCGU |
| *Myxococcus xanthus* | | **UAUUCA**GAUG**AGUCC**GCGU |
| *Escherichia coli* | | **CCAUC**GGAUG**U**GCCC**AGAU** |
| *Bacillus subtilis* | | CU**UACA**GAUGGACCCGCG**G** |
| *Methanococcus vannielii* | | **CC**CG**A**GGAU**A**GG**A**CUGCG**C** |
| *Methanosaeta soehngenii* | | **CCUAA**GGAUGGG**U**CUGCG**G** |

labeled probe MPOB1 showed only fluorescence of the large microcolonies more inwards of the granule (Fig. 1B). The morphology of the cells in these microcolonies resembled that of the MPOB cells in the pure culture. The sucrose-fed granules were also hybridized with probe KOP1, specific for strain KOPROP1. This probe did not hybridize to any of the cells (results not shown), indicating that the cells hybridizing with the MPOB1 probe were not KOPROP1 cells but MPOB-like cells.

At a lower magnification, the hybridization of sucrose-fed granules with probe EUB338 (Fig. 2A) and probe MPOB1 (Fig. 2B) showed hardly any individual cells. However, the overall structure of the granule and the localization of the bacteria was more clear. Both probes hybridized with the rRNA of microcolonies located more inwards of the granule, identifying them as MPOB-like bacteria. The microcolonies at the surface of the granule, that were only visualized by the EUB338 probe, showed a brighter fluorescent signal than the MPOB-like microcolonies (Fig. 2A). Additionally, there were individual cells more inwards of the granule that were only visualized by the EUB338 probe.

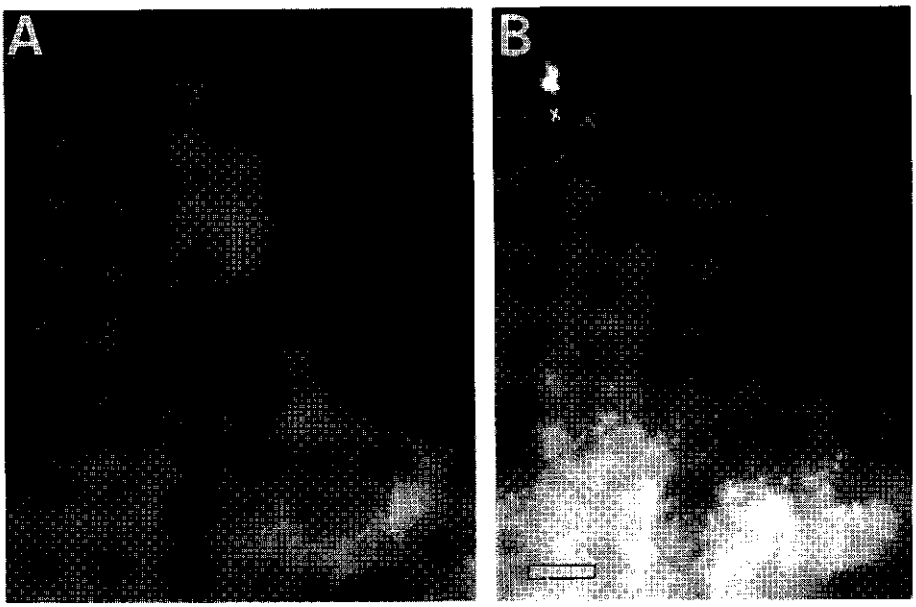


Fig. 1. *In situ* hybridization of sections of sucrose-fed anaerobic granular sludge. The sections were simultaneously hybridized with a fluorescein-labeled oligonucleotide probe universal for bacteria EUB338 and a rhodamine-labeled specific probe MPOB1 and viewed by epifluorescence microscopy with a fluorescein specific filterset (A) and rhodamine specific filterset (B). The photomicrographs were taken at the outer layers of the granule. Various morphotypes of rods and cocci hybridize with the bacterial probe, but only the short rods present in the microcolony in the left corner of the micrographs, are visualized by the MPOB1 probe. Autofluorescence is marked X. Scale bar 10 $\mu$m.

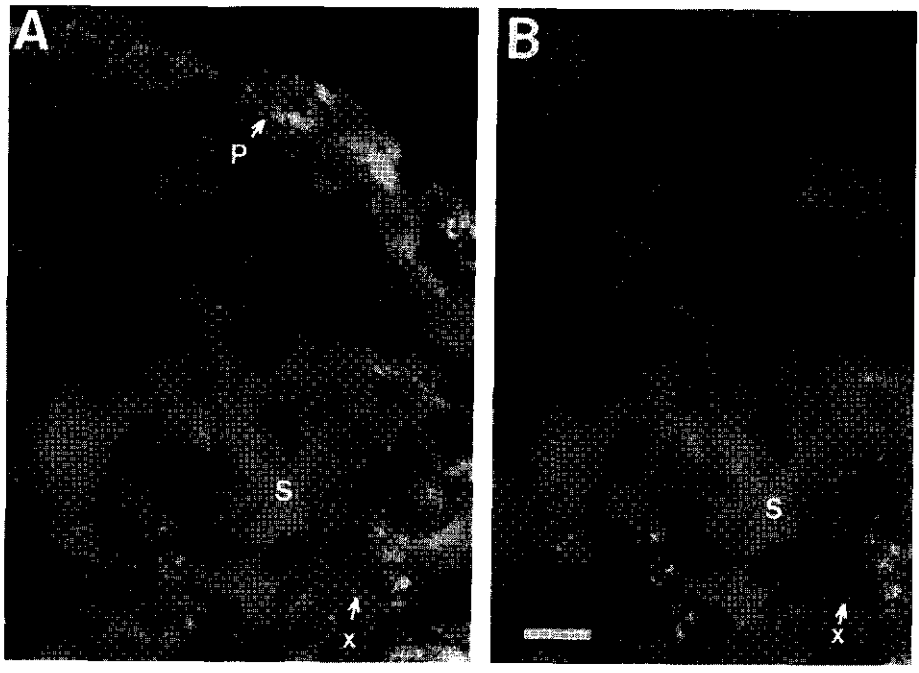


Fig. 2. *In situ* hybridization of a sucrose-fed sludge granule viewed with epifluorescence microscopy at a 200-fold magnification. The fluorescein-labeled bacterial probe EUB338 detects bacterial cells mainly located in the outer layer of the granule (marked P, from peripheric location), and the syntrophic microcolonies more inwards of the granule (marked S, for syntrophic microcolony) (A). The rhodamine-labeled probe MPOB1 detects only the microcolonies inwards of the granule (B). Autofluorescence is marked X. Scale bar 50 $\mu$m.

To visualize all methanogens present in the granule, the fluorescein-labeled archaeal probe ARC915 was used in the hybridization (Fig. 3A). The ARC915 probe hybridized with the rRNA of several microcolonies more inwards of the granule, with different intensity of fluorescent signal. Microcolonies with a bright fluorescent signal are adjacent to those that show a less pronounced signal (Fig. 3A). Hybridization of the same section with the MPOB1 probe visualized large microcolonies on corresponding positions where the methanogenic microcolonies were located that showed a low fluorescent signal with the ARC915 probe (Fig. 3A and B; indicated S from syntrophic microcolony). In all cases this overlap was observed, and, moreover, the MPOB-like microcolonies showed no overlap with the bright methanogenic microcolonies (marked Me in Fig. 3A).

To study the architecture of a possible syntrophic microcolony that hybridized with the ARC915 probe and the MPOB1 probe, magnifications were viewed with a confocal laser scanning microscope (Fig 3C and D). This showed a low fluorescence of methanogens, which were juxta-positioned to the MPOB-like cells. Group-specific probes for *Methanogenium* or *Methanobacter* (18) were used to identify the hydrogen- or formate-utilizing methanogens growing in these syntrophic microcolonies. Both probes hybridized weakly to individual cells and microcolonies outside the syntrophic ones (results not shown). In contrast, only the *Methanobacter* probe showed fluorescence at the positions where the MPOB-like microcolonies were located (results not shown). To confirm the juxta-positioning of the methanogens and the syntrophs, sections of the granules were viewed with transmission electron microscopy, which showed syntrophic microcolonies containing *Methanobacter*-like cells (marked Mb) next to cells hybridizing with the MPOB-probe (indicated Sy from syntrophs). *Methanosaeta*-like cells (marked Me) were found adjacent to cells of the syntrophic microcolony (Fig. 3E).

**Microbial architecture of volatile fatty acids-fed sludge.** Granules fed with VFA were hybridized with probe EUB338 and with probe MPOB1 in order to show the effect of different substrates on the architecture of the granules (Fig. 4). The probe EUB338 hybridized with the rRNA of bacteria present on the surface of the granule, located more inwards, and present around the centre of the granule (Fig. 4A). The structures seemed to be more organized than in the sucrose-grown granules. The MPOB1 probe visualized almost all the bacterial microcolonies located near to the

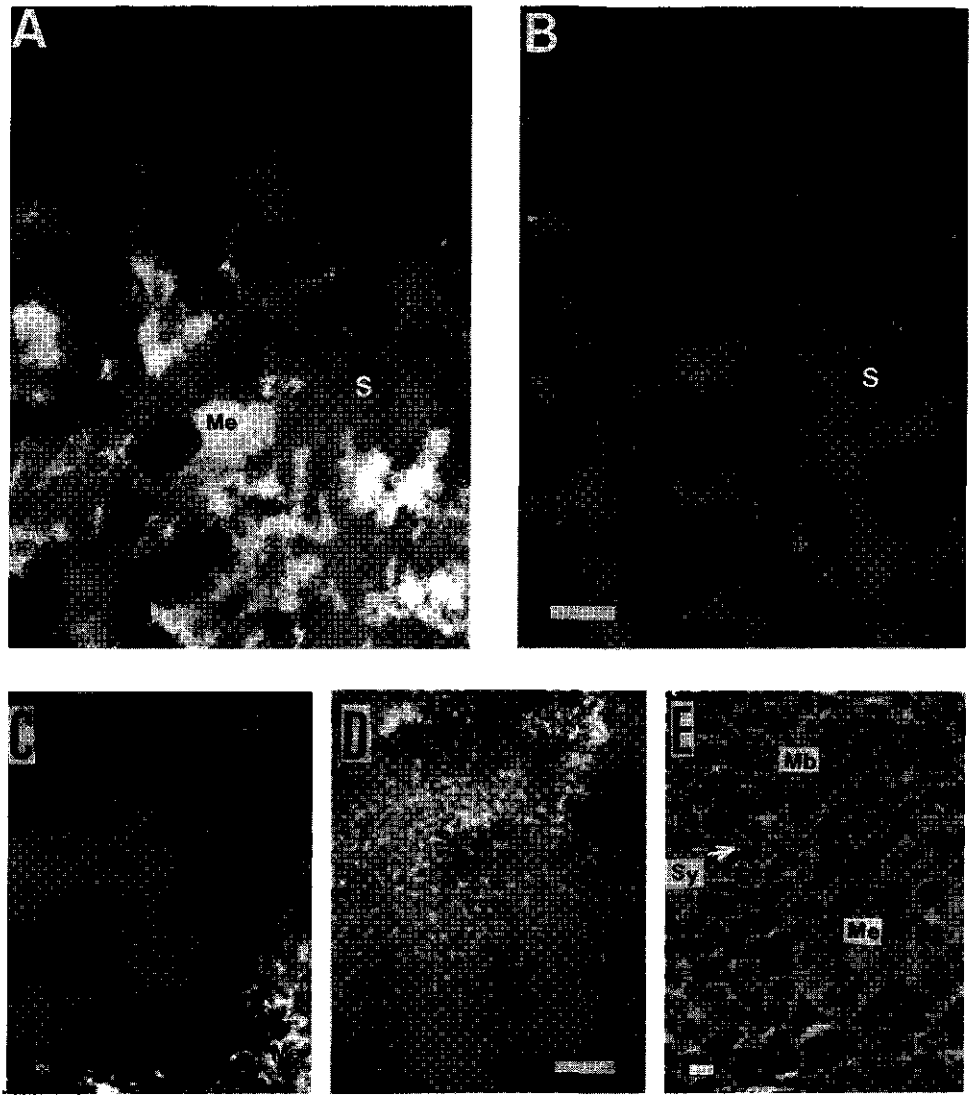


Fig. 3. *In situ* hybridization of a sucrose-fed granule with a fluorescein-labeled archaeal probe ARC915 (A) and the rhodamine-labeled probe MPOB1 (B). Scale bar 50 $\mu$m. A syntrophic microcolony hybridized with the ARC915 probe (C) and the MPOB1 probe (D) is magnified by viewing with a confocal laser scanning microscope. Scale bar 10 $\mu$m. A part of a syntrophic microcolony and the adjacent methanogenic microcolonie is viewed with transmission electron microscopy (E). Scale bar 0.5 $\mu$m. To illustrate the location of the different relevant microbes, the presence of specific microcolonies (A and B) or cells (C to E) is indicated the following way; S for syntrophic microcolonies; Me for *Methanosaeta*-like cells or microcolonies; Mb for *Methanobacter*-like cells; Sy for MPOB-like cells.

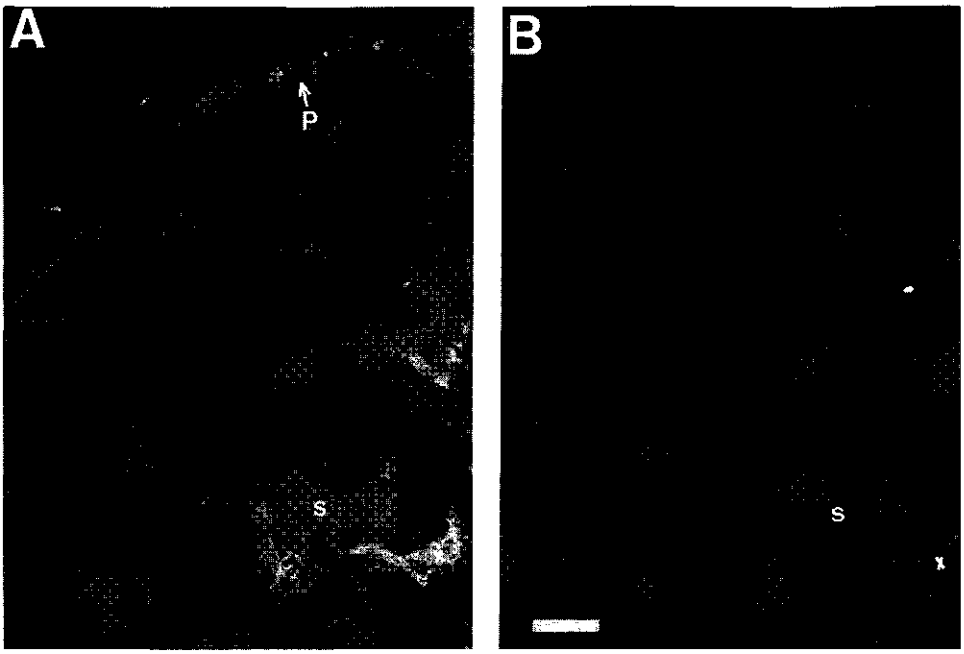


Fig. 4. *In situ* hybridization of a volatile fatty acids-fed sludge granule with the fluorescein-labeled bacterial probe EUB338 (A) and the rhodamine-labeled probe MPOB1 (B). The EUB338 probe visualizes mainly bacterial cells in the outer layer of the granule (marked P from peripheric location) and the microcolonies inwards of the granule (marked S form syntrophic microcolony). The MPOB1 probe visualizes only the inwards microcolonies, marked S. Autofluorescence is marked X. Scale bar 50 $\mu$m.

centre (Fig. 4B). The hybridization of a VFA-fed granule with probe ARC915 and probe MPOB1 is shown in Fig. 5. The methanogens are located entirely throughout the granule (Fig. 5A). The brightest microcolonies are located in the outer layers in a radial way. Most of these microcolonies reacted with a rhodamine-labeled oligonucleotide probe specific for the acetoclastic genus *Methanosaeta* (18) (data not shown). Furthermore, typical microcolonies with a *Methanosarcina*-like morphology were detected in this layer. More inwards, less bright microcolonies are located, next to microcolonies that show a diffuse fluorescence of low intensity. The MPOB microcolonies are located outside the centre of the granule on the corresponding positions where the less fluorescent methanogenic microcolonies are located (Fig. 5B). These are again surrounded by the brighter fluorescing methanogenic microcolonies.

## DISCUSSION

The microbial diversity of granular sludge has previously been studied by using traditional methods such as most probable number counting, immunolabeling techniques and electron microscopy, however, concerning syntrophic interactions with only limited success.(6, 9, 11, 12). *In situ* hybridization of microbes with fluorescent oligonucleotide probes has become a new approach to study the microbial organization and its function in complex ecosystems (2). The application of this technique on sections of granular sludge provides new information on the spatial orientation of acetogens and methanogens in syntrophic consortia.

The hybridizations described have showed that the sucrose-fed sludge consisted of three layers. An exterior layer of mainly bacteria, a second layer where syntrophic microcolonies are mixed with microcolonies of *Methanosaeta* sp. The third layer is the central part with large cavities, anorganic material, and some methanogenic microcolonies. The VFA-fed sludge had one extra layer under the outer layer. This thick layer, which consisted of large quantities of *Methanosaeta* sp., changed gradually in the syntrophic layer. Similar layered structures were proposed previously for anaerobic aggregates from anaerobic digestors (12).

The hybridization with probe EUB338 was used to visualize all bacteria. However, it can not be ruled out that Gram-positive bacteria, like the syntrophic butyrate oxidizing bacterium *Syntrophospora bryantii*, and other bacteria with a rigid

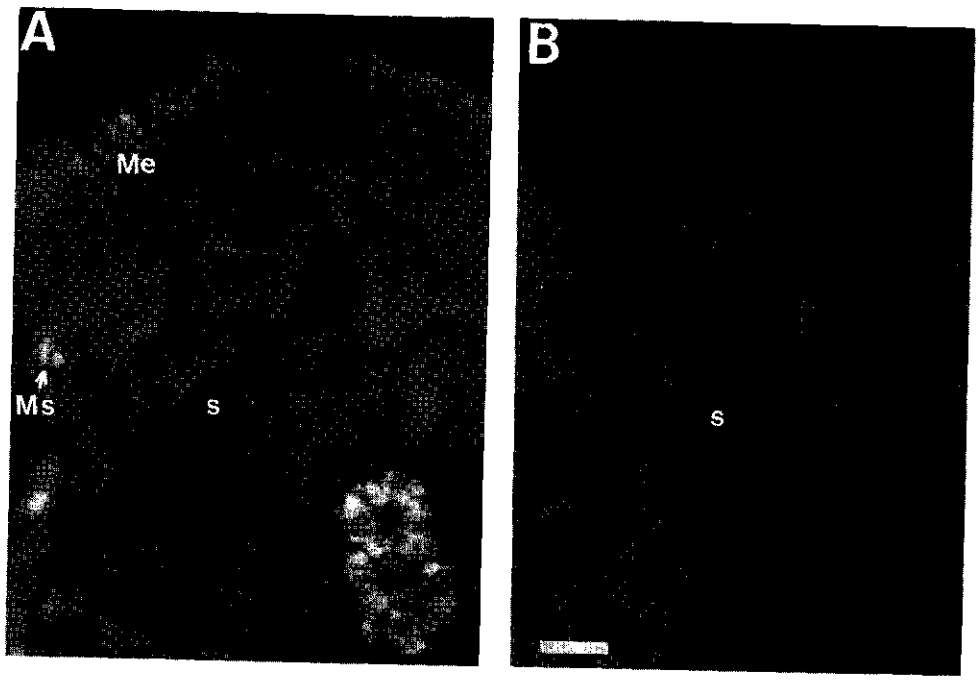


Fig. 5. *In situ* hybridization of a volatile fatty acids-fed sludge granule with the fluorescein-labeled archaeal probe ARC915 (A) and the rhodamine-labeled probe MPOB1 (B), viewed at half the magnification as the previous three figures. The probe ARC915 visualizes a thick layer of methanogens (marked Me), with some microcolonies with a *Methanosarcina*-like morphology (marked Ms). Furthermore it hybridizes with a deeper layer of two types of microcolonies with bright and less fluorescence. The MPOB1 probe detects only the microcolonies on those sites with less fluorescence signals from the ARC915 probe (syntrophic microcolonies are marked S). Scale bar 100 $\mu$m. The small inset is a magnification of a *Methanosarcina*-like microcolony hybridized with the ARC915 probe viewed with a confocal laser scanning microscope. For this micrograph the scale bar is 10 $\mu$m.

cell wall are not detected. The fixation methods used here might not be good enough to permeate all cell wall-types for the probes. However, our methods are sufficienty adequate to detect Proteo-bacteria and methanogens.

The isolation and enrichment of different strains of syntrophic propionate-oxidizing bacteria have been described (3, 5, 22, 26). However, there are several reasons to assume that the bacteria hybridizing to the MPOB1 probe are indeed MPOB cells; (i) The KOPROP1-specific probe KOP1 does not hybridize with the MPOB-like cells; (ii) The morphology of the hybridizing cells is similar to that of MPOB grown in pure cultures, and did not resemble the more lancet-shape morphology of Koprop1-cells (8); (iii) *Desulforhabdus amnigenus* can be detected by the MPOB1 probe, but this organism will not be abundantly present in the sludge, since it can only utilize propionate acetate and butyrate coupled to sulfate-reduction (15); (iv) The MPOB culture has been enriched from sludge of the same industrial UASB-reactor (22).
Cells of MPOB do not utilize butyrate or acetate under methanogenic conditions and therefore the main substrate has to be propionate. Under these conditions this bacterium can only oxidize propionate syntrophically. The MPOB-like cells do not grow on the surface of the granule, but more inwards. Moreover, the MPOB-like cells were surrounded by cells of methanogens. This indicates that the MPOB-like cells are indeed growing syntrophically, and depend on the methanogens to remove the reducing equivalents. There are several observations supporting the conclusion that in syntrophic microcolonies MPOB-like cells are juxta-positioned to methanogens that utilize hydrogen and/or formate. (i) MPOB-like colonies were located in areas that showed a less, but significant, fluorescent signal with ARC915 probe (Fig. 3). (ii) MPOB-like cells are never found on other locations. (iii) CLSM-analysis of the data revealed that cells showing a low fluorescence with the ARC915 probe were positioned next to those fluorescencing with the MPOB probe. Additional support for this architecture was obtained from the analysis of sections with transmission electron microscopy that illustrated the position of the individual cells (Fig. 3A).

The bright fluorescent microcolonies surrounding the MPOB-like microcolonies reacted with the group-specific probe for *Methanosaeta* sp., which are obligately aceticlastic. This, strongly suggests that the methanogens next to the MPOB-like cells, that do not hybridize to the *Methanosaeta*-probe, nor show a characteristic *Methanosarcina*-like morphology, are methanogens that do not utilize acetate, and are likely hydrogen and/or formate consuming methanogens. This was confirmed by the

hybridization with fluorescent probe specific for the *Methanobacter*-group that showed fluorescence with the methanogens in the syntrophic microcolonies. Furthermore, the methanogens seen in the syntrophic microcolonies on the transmission electron microscope micrographs resemble the *Methanobrevibacter*-cells, which belong to the *Methanobacter*-group, as seen previously in such syntrophic associations (6).

The bacteria at the surface of the sucrose-fed granule grow either on sucrose, glucose or fructose and produce acetate, butyrate or propionate. Therefore it is likely that they grow independent of other bacteria. Inside the granule they would be hampered by diffusion limitation. They seem to grow as a loosely bound layer on, rather than in the granule. The bacteria at the surface of the VFA-fed granule grow more in the granule and these are next to the MPOB-like microcolonies the most abundant bacteria. Therefore, they could be syntrophic butyrate-oxidizing bacteria. Butyrate oxidation also requires a low partial hydrogen pressure and it has to be degraded syntrophically as well. Acetate will be degraded by the acetoclastic methanogens abundantly present in the two granule types. The methanogens in the thick layer underneath the surface of the VFA-fed granules most likely consumed the acetate diffusing from the outside of the granule. This is supported by the presents of *Methanosarcina*-like microcolonies in this layer, which can utilize acetate. In the sucrose-fed granules this layer is absent because acetate is only produced inside the granule. This is an example of how the architecture of the granule is changed by the substrates utilized by the granule. The stucture of the two types of granules clearly relates to the function that the microorganisms have in these man-made environments. The results shown in this paper indicate that hybridization of granular sludge with fluorescent probes is a powerful tool to study such structure-function relationships *in situ*. This tool can be used to study the population dynamics of such ecosystems during changing nutrient and environmental conditions.

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# Chapter 6

## Enrichment, phylogenetic analysis and detection of syntrophic propionate-oxidizing bacteria from anaerobic granular sludge.

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# Enrichment, phylogenetic analysis and detection of syntrophic propionate-oxidizing bacteria from anaerobic granular sludge.

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**Summary**

Syntrophic propionate-oxidizing bacteria were enriched from anaerobic granular sludge by growing crushed sludge in batch cultures on propionate. Nucleic acids were extracted from samples taken during this enrichment and used in slot blot hybridization with 16S rRNA-based oligonucleotide probes. Since the known syntrophic propionate-oxidizing strains MPOB, KOPROP1 and Syntrophobacter wolinii could not be detected in this sludge, the enrichment culture was further characterized. Following dilution series of this culture, the 16S rRNA gene of the dominant strain, named SYN7, was cloned and characterized. This phylogenetic analysis revealed that SYN7 is a new species with a 16S rDNA sequence related to that of Syntrophus spp. Based on the variable regions of SYN7 16S rDNA specific oligonucleotides probes were designed and tested by slot blot and *in situ* hybridization. *In situ* hybridization with these SYN7 probes visualized a thin rod-shaped bacterium in the purified enrichment culture, localized in flocs together with other bacteria and methanogens. The population dynamics of the enrichment of SYN7 was determined by slot blot hybridizations of nucleic acids extracted from the enrichment cultures, using bacterial, archaeal and SYN7 probes. The total amount of rRNA of the microbial population decreased dramatically during the first transfers. The non-growing microorganisms were diluted and disappeared. After the first transfer SYN7 started to grow and after four transfers the amount of SYN7 rRNA stabilized.

## Introduction

During the anaerobic treatment of industrial wastewater organic matter is broken down to methane and carbon dioxide by microbial aggregates in the form of granular sludge, flocs, or biofilms. In upflow anaerobic sludge blanket (UASB) reactors the biomass immobilizes itself into dense granules (LETTINGA, 1995). These granules consist of different groups of trophic microorganisms that degrade the organic load in a series of bioconversions: hydrolysis, fermentation, acidification and methanogenesis (GUJER and ZEHNDER, 1983). Propionate is an important intermediate in this breakdown, since it accounts for a large proportion of the methane formed. The conversion of propionate into acetate, hydrogen or formate, and carbon dioxide is thermodynamically only possible at a low hydrogen partial pressure and a low formate concentration (STAMS, 1995). In syntrophic propionate-oxidizing consortia acetogens convert the propionate into formate and hydrogen, that are subsequently used by methanogens (MCINERNEY, 1992; SCHINK, 1992). Several syntrophic propionate-oxidizing cocultures have been enriched from different kinds of anaerobic sludges (KOCH et al., 1983; BOONE and XUN, 1987; MUCHA et al.,1988; STAMS et al, 1992; DÖRNER, 1992). For a long time Syntrophobacter wolinii was the only well-described isolated bacterium which could oxidize propionate syntrophically (BOONE and BRYANT, 1980). Recently however, the isolation has been described of a Gram-positive syntrophic propionate-oxidizer which seems not to be related to S. wolinii (WU et al., 1992). Furthermore, two highly enriched cultures of syntrophic propionate oxidizing bacteria have been reported, one containing the strain MPOB and the other containing the strain KOPROP1 (STAMS et al., 1993; DÖRNER, 1992). Phylogenetic analysis based on 16S rRNA genes of S. wolinii and the strains MPOB en KOPROP1, revealed a relationship with sulfate-reducing bacteria (HARMSEN et al., 1993; HARMSEN et al., 1995). All three bacteria are closely related and belong to the delta subclass of Proteobacteria. Furthermore, it was demonstrated that all three bacteria could indeed oxidize propionate in pure culture with sulfate as electron acceptor (DÖRNER, 1992; WALLRABENSTEIN et al., 1994; VAN KUIJK and STAMS, in press). In contrast, pure cultures of Syntrophus buswellii and Syntrophus gentianae, which can oxidize benzoate syntrophically, did not reduce sulfate, although both bacteria were recently shown to be related to sulfate-reducing bacteria (WALLRABENSTEIN et al., 1995).

This report describes the enrichment and characterization of a novel syntrophic propionate-oxidizing strain, named SYN7, from granular sludge, which is not closely related to the Syntrophobacter-like bacteria. By slot blot and *in situ* hybridization using specific oligonucleotide probes based on the variable 16S rRNA sequences of SYN7, its presence in the sludge was demonstrated and its population dynamics during the enrichment was determined.

**Materials and methods.**

*Medium and analytical methods.* A basal bicarbonate-buffered medium containing 20 mM of propionate was used for enrichment, dilution series, and subculturing of the purified enrichment, and was prepared as described previously (STAMS et al., 1993). Propionate, acetate, methane and hydrogen were measured by gas chromatograph as described previously (STAMS et al., 1993).

*Enrichment and sampling.* Methanogenic granular sludge from an upflow anaerobic sludge blanket-reactor (UASB) treating wastewater of a potato-processing factory (Aviko, Steenderen, The Netherlands) was used as starting material for the enrichment of syntrophic propionate-oxidizing bacteria. A sample of 100 ml granular sludge was washed two times with tapwater. The sludge was crushed anaerobically by pressing it through a series of syringes needles with decreasing diameter into serum flasks filled with nitrogen gas. The final diameter of the needles was 0.6 mm.

Five ml of the crushed sludge were used as an inoculum into a 300 ml serum bottle containing 125 ml of basal medium with 20 mM of propionate. The bottle was incubated at 37 °C. The propionate concentration was measured regularly by gas chromatography. Each time the propionate concentration became lower than 1 mM, 10 ml of the culture was transferred into a new bottle with 125 ml of medium and 20 mM of propionate. This procedure was repeated five times. Triplicate samples of 1 ml were taken from the first bottle, at the start of the experiment and just before the transfer. Triplicate 10 ml samples were taken from the other bottles before each of the transfers. The biomass was pelleted by centrifugation and transferred into a sterile 1.5 ml reaction-tube and stored at -20 °C until nucleic acid extraction.

In order to purify the propionate-oxidizing culture, 5 ml of the last transfer were used to prepare a dilution series of ten subsequent ten-fold dilutions. The seventh

dilution was the last dilution where growth was observed. This culture was maintained by regularly transferring 5 ml into 50 ml of fresh medium.

*Isolation of nucleic acids*. Nucleic acids of all samples of the sludge enrichment were isolated simultaneously. The frozen pellets were thawed on ice, and resuspended in 400 $\mu$l TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). The nucleic acids of the purified enrichment culture and all reference strains were extracted by centrifuging 10 ml of a culture at the end of the exponential growth phase at 17,000 g. The pellets were resuspended in 400 $\mu$l of TE buffer and transferred to a 1.5 ml tube. The rest of the procedure was the same for all the extractions and was cooled on ice as much as possible. First, 200 $\mu$l Tris/HCl buffered phenol pH 8.0 was added together with approximately 300 $\mu$l of glass beads with a diameter of 0.11 mm. Subsequently, cells were disrupted for 5 min in a cell homogenizer model MSK (B.Braun, Melsungen, Germany) under carbon dioxide cooling. After 15 min of centrifugation at 15,000 g the aqueous phase of the supernatant was extracted with phenol/chloroform/isoamylalcohol (25:24:1, v:v:v), followed by chloroform/isoamylalcohol (24:1, v:v) extraction and precipitation with 1 ml of 96% ethanol and 40 $\mu$l of 3M NaAc (pH 5.2). After 15 min centrifugation at 15,000 g the nucleic acid pellet was washed with 70 % ethanol, dried under vacuum and resuspended in 100 $\mu$l of TE buffer. The quality of the extracted nucleic acids was analyzed by agarose gel-electrophoresis followed by ethidium bromide staining. These nucleic acids were used for slot blot hybridization, or in the case of the purified enrichment culture, for PCR-amplification.

*PCR amplification, cloning and sequence analysis*. All enzymes for DNA manipulations were obtained from Life Science Technologies (Gaithersburg, Md., USA). Unlabelled oligonucleotides were synthesized by Pharmacia (Uppsala, Sweden). The 16S rRNA-genes of the highly purified culture were amplified by polymerase chain reaction (PCR) as described before (HARMSEN et al., 1993) using a set of universal 16S rRNA-based primers containing restriction sites (in italics) to facilitate cloning: the forward primer (5'CA*CGGATCC*AGAGTTTGAT(C/T)(A/C)TGGCTCAG) corresponded to Escherichia coli positions 8 to 27 and the reverse primer (5'GTG*CTGCAG*GGTTACCTTGTTACGACT) to E. coli positions 1493 to 1510 . The PCR amplification products were digested with the restriction enzymes BamHI and PstI and ligated into a pUC18 vector digested with the same enzymes. The ligation products were transformed into Escherichia coli strain TG1 and the transformants harbouring recombinant plasmids were identified as white colonies on L-broth agar plates

containing X-gal, using established procedures (SAMBROOK et al., 1989). DNA sequencing was done using the dideoxy-chain termination method (SANGER et al., 1977) adapted for cycle-sequencing with Taq-polymerase as described by the Life Science Technologies manual.

*Sequence alignment and phylogenetic tree.* The partial 16S rRNA nucleotide sequence was aligned with those of other bacterial sequences taking into account sequence similarity and higher order structure using the alignment tool of the ARB program package (STRUNK et al., in prep.). The reference sequences were taken from the ribosomal database project RDP (MAIDAK et al., 1994; VAN DE PEER et al., 1994).

The tree is based on the results of a distance matrix analysis including only sequence positions which share the same nucleotides in at least 50% of all available sequences from delta-subclass proteobacteria (ARB; PHYLIP, FELSENSTEIN, 1982). The tree was corrected according to the results of maximum parsimony and maximum likelihood analyses (fastDNAml, MAIDAK et al., 1994).

*Slot blot hybridizations.* Slot blot hybridization- experiments were performed on Hybond N+ filters (Du Pont). Nucleic acid samples containing approximately 50 ng of rRNA were applied to the membrane with a Hybridot manifold (Life Science Technologies) and immobilized according to CHURCH and GILBERT (1984). The membranes were pretreated with hybridization buffer (0.5 M phosphate buffer, 7 % sodium dodecyl sulfate, 1 % BSA, 1 mM of EDTA, pH 7.2) for 30 min prior to hybridization with 100 ng of [$\gamma$-$P^{32}$]ATP-labeled probes. All membranes were hybridized at 40 °C and washed in 1 % SDS, 1 x SSC (0.15 M NaCl, plus 0.015 sodium citrate, pH 7.0) wash buffer at 55 °C for the probes EUB and ARC915 (STAHL and AMANN, 1991) and at 52 °C for probe 177 and S223, the latter one being the experimentally determined optimal washing temperature for these probes. The membranes were exposed to a phospho-imager screen for 90 min and the screen was scanned for radioactive response on a Phosphor Imager (Molecular Dynamics, Sunnyvale, USA). The digital signals were processed by the manufacturers software (ImageQuant).

*Whole cell hybridization.* For whole cell hybridization, cells were fixed, bound to slides coated with Vectabond (Vector Laboratories, Inc., Burlingame, USA) and hybridized according to AMANN et al., 1992. The fluorescent oligonucleotide probes were synthesized and labelled at the 5' position with either FLUOS (a fluorescein

Table 1. The sequences of the specific oligonucleotides directed against the 16S rRNA sequences of the propionate-oxidizing bacteria Syntrophobacter wolinii and strain SYN7. The number in brackets in the target column refers to the start of the target-site (E. coli numbering).

| Name oligo | Target | Sequence |
|---|---|---|
| 177 | SYN7 (175) | 5' AGAAGTCATGCAGTATTATTCGG |
| 177B | SYN7 (1010) | 5' TCCCCGAAGGGCACTTCTCTGT |
| S223 | S. wolinii (223) | 5' ACGCAGACTCATCCCCGTG |

derivative) or rhodamine, using an aminolinker by Eurogentec (Seraing, Belgium). The probe 177 was double-labelled at the 3' and 5' positions by direct coupling of the rhodamine to the oligonucleotide. The hybridization conditions were as follows: 45°C incubation overnight followed by washing for 30 minutes at 48°C in hybridization buffer (0.9 M NaCl, 0.1% SDS, 20 mM Tris/HCl pH 7.2). The cells were viewed with a Nikon epifluorescence microscope and photographed on a black and white Kodak TMY 400-ASA film by exposing for 10 to 30 s.

**Results.**

*Enrichment of syntrophic propionate-oxidizing bacteria.*

Methanogenic granular sludge of an upflow anaerobic sludge blanket (UASB) reactor was crushed and diluted 26-fold into mineral medium with 20 mM of propionate as substrate and incubated at 37 °C to enrich the syntrophic propionate-oxidizing bacteria. After seven days the propionate was degraded, the formation of intermediate acetate was detected, and as end product methane was formed. Subsequently 10 % of this culture was transferred into a new bottle and after depletion of the propionate this was repeated five times. The rate of propionate depletion was lowered following each transfer and after the fifth transfer it took as long as 30 days before the propionate concentration was below detection level. In all cases methane was produced after transfer. However, in the last transfers the acetate was no longer converted into methane.

Syntrophic propionate-oxidizing bacteria like Syntrophobacter wolinii and the strains MPOB and KOPROP1 were not detected in the enrichment cultures (see below). Therefore, a dilution series of the biomass of the last transfer was made in order to purify the dominant strain in the enrichment. The $10^7$ dilution was the highest dilution at which growth was observed. The resulting culture produced acetate and methane from propionate and was used for phylogenetic analysis and whole cell hybridization.

*PCR amplification, cloning and sequence analysis.*

PCR-amplification of the 16S rRNA genes from the nucleic acids isolated from the purified enrichment culture resulted in a product of the expected size of 1.5 kb. This fragment was digested with BamHI and PstI and ligated in pUC18, linearized with the same enzymes. The ligation products were transformed into E. coli TG1 cells. Twelve of the recombinant plasmids were characterized by sequence analysis of the insert DNA using the universal 16S rRNA primer 1115 (E. coli 1100-1115) (LANE, 1991). The majority of these plasmids contained inserts with identical sequences of the V6 region of the 16S rRNA gene. One plasmid, pSYN7 was selected for further analysis and its insert was sequenced completely, resulting in a sequence of 1484 bp, which was deposited in the EMBL-database under accession number X87269. The organism from which this sequence was derived will be referred to as SYN7.

*Phylogenetic analysis.* The sequence of the 16S rRNA gene of SYN7 was aligned to other sequences available from the data bases (MAIDAK et al., 1994; VAN

DE PEER et al., 1994). Comparative sequence analyses revealed that the SYN7 sequence clusters phylogenetically with the sulfate-reducing bacteria. A consensus tree based on distance matrix and parsimony analyses for selected members of the delta subclass of proteobacteria is depicted in Fig. 1. Escherichia coli was included as an outgroup. The phylogenetic tree shows that SYN7 is related to the syntrophic benzoate-oxidizing genus Syntrophus. The sequence similarity of the SYN7 16S rRNA with that of the closest relatives Syntrophus gentianae and Syntrophus buswellii is 95.0 % and 94.4 %, respectively. However, SYN7 is only distantly related to the group of Syntrophobacter-like syntrophs, and its 16S rRNA shares 87.0% sequence similarity with that of Syntrophobacter wolinii.

*Slot blot hybridization of the enrichment.* The 16S rRNA sequence of SYN7 was used to design two specific oligonucleotides probes (Table 1). Probe 177 was directed against the variable V2 region and probe 177B against the V6 region. The specificity of these probes was tested by slot blot hybridization. Both probes only hybridized with the nucleic acids extracted from the purified culture and with pSYN7 DNA (results with probe 177 are shown in Fig. 2).

In order to detect and quantify the syntrophic bacteria with respect to the total microbial population, slot blots with nucleic acids extracted from the enrichment, were hybridized with the bacterial probe EUB338 and archaeal probe ARC915 (STAHL and AMANN, 1991) (Fig 2A and 2B). When the filters were hybridized with specific probes for the syntrophic propionate-oxidizing bacteria Syntrophobacter wolinii, probe S223 (Table 1) (Fig. 2C), MPOB and KOPROP1 (results not shown), no rRNA of these three bacteria was detected. However, SYN7 was detected when the filters were hybridized with probe 177 (Fig. 2D). The radioactive signals coming from the hybridization with the bacterial and the archaeal probes were used to determine the amount of bacterial and methanogenic rRNA in the extracts respectively (Fig. 3). The signals from hybridization with probe 177 were used to determine the amount of rRNA originating from SYN7 relative to the bacterial rRNA. The relative amount of SYN7 rRNA increased from 0.4 % in the original sludge used as primary inoculum to more than 60 % after the fifth transfer (Fig. 3). This corresponds to a significant increase of SYN7 in the population of the cultures due to enrichment on propionate. Attempts to detect the presence of Desulfobulbus with the genus specific probe, D660 (DEVEREUX et al., 1992) showed only a low signal in the first enrichment was detected, indicating that this bacterium was present but not enriched on propionate. The

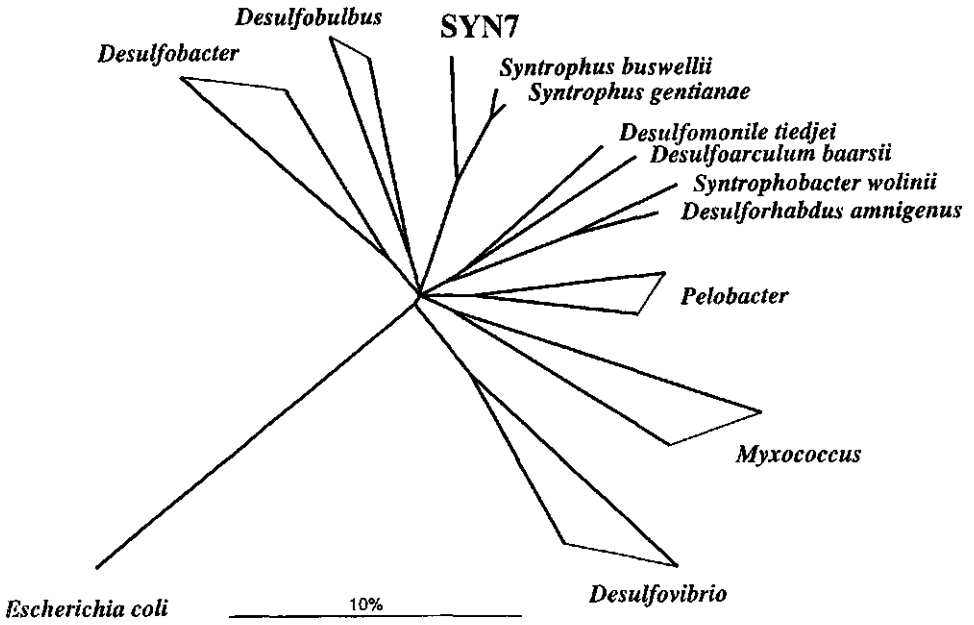


Fig 1. Unrooted phylogenetic tree reflection the relationships of SYN7 and the major groups of the delta-subclass of Proteobacteria with Escherichia coli as outgroup. The triangles indicate phylogenetic groups: Desulfobacter represents Desulfobacter, Desulfobacterium, Desulfobacula, Desulfobotulus, and Desulfococcus. The Pelobacter group also includes Desulfuromonas and Geobacter. Myxococcus represents the myxobacteria, Desulfovibrio also comprises Bilophila, Desulfomonas and Desulfomicrobium. The tree is based on the results of a distance matrix analysis including only sequence positions which share the same nucleotides in at least 50% of all available sequences from delta-subclass proteobacteria. The tree was corrected according to the results of maximum parsimony and maximum likelihood analyses. Multifurcations connect branches for which a relative order could not unambiguously determined or a common pattern was not supported applying different treeing methods. The bar indicates 10% estimated sequence divergence.

influent of the UASB-reactor, from which the sludge originated, contained 5 mM of sulfate, so propionate could have been oxidized by sulfate reduction. Indeed Desulfobulbus sp. were enriched very rapidly when the sludge was fed with 20 mM of propionate and 20 mM of sulfate. But since there was also lactate and butyrate present in the influent of the reactor, the different types of sulfate reducers compete with each other for the available sulfate.

*In situ hybridizations of the purified enrichment culture. In situ* hybridization of the purified enrichment culture with the specific probes was performed to detect the presence of SYN7. Fixed cells of the purified enrichment culture were hybridized with either probe 177B, 5' labeled with rhodamine, or probe 177, 3' and 5' double labeled with rhodamine. Specific hybridization with prokaryotic cells was observed with either of these probes although significant background fluorescence due to crystals in the fixed cells was detected (not shown). These crystals were probably composed of reduced components, such as metal-sulfides precipitates and their interference could be prevented by pretreatment of the slides with the smear of cells using 3 % hydrogen peroxide solution. Hybridization with the fluorescein-labeled bacterial probe EUB338 was done to check if the microbial cells were still intact (Fig. 4A and 4B). The bacterial probe, which did not bind to the anorganic material, hybridized to the same extent to the bacteria before and after the treatment with peroxide. When the pretreated smears were hybridized with either of the two rhodamine-labelled specific probes 177B and 177, the background was reduced significantly and a fluorescent signal was detected of thin rod-shaped cells. Hybridization with the double-labelled probe 177 gave the best fluorescent signal and hence was used in further investigations (Fig. 4D). The hybridization of the same smear of cells with the bacterial probe EUB338, 5' labeled with fluorescein, showed fluorescence of two types of bacteria, indicating that there was at least one more bacterium in the culture present (Fig 4C). When a 5'-FLUOS labelled probe D660, specific for Desulfobulbus, was tested on the pretreated smears the thick rods gave a strong fluorescent signal (Fig. 4E). The hybridization of the pretreated smears with the archaeal probe ARCH915, 5' labeled with fluorescein, visualized one type of methanogen with a Methanospirillum-like morphology (Fig. 4F).

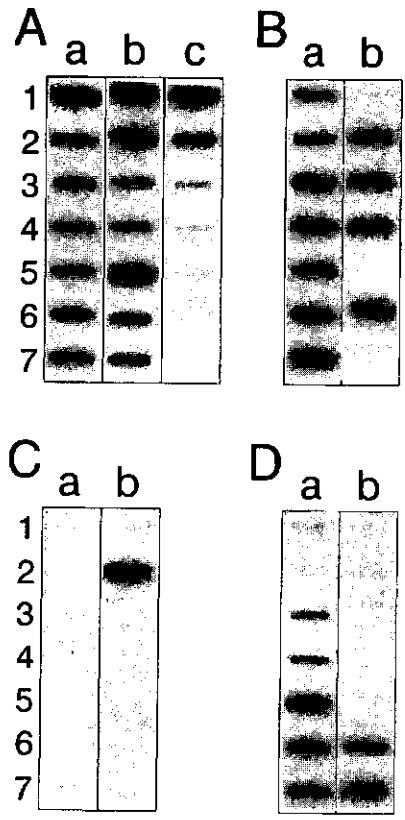


Fig. 2 Slot blot hybridization of four identical filters (A to D) containing nucleic acids (DNA and rRNA) of the enrichment cultures (column a), reference strains (column b), and an Escherichia coli dilution series of rRNA (filter A, column c).

The filters were hybridized with the bacterial probe EUB338 (A), archaeal probe ARC915 (B), the Syntrophobacter wolinii specific probe S223 (C) and the SYN7-specific probe 177 (D). Ten percent of the nucleic acids isolated of the enrichment cultures were bound to the filter as follows: a1: reactor sludge used as inoculum; a2 to a7; the first to the sixth cultures of the enrichment. Representative filters of the triplicate samples are shown. About 30 ng of nucleic acids from the following cultures were also bound to the filters; b1: MPOB enrichment; b2: Syntrophobacter wolinii DSM 2805 in coculture with Methanospirillum hungatei DSM 864; b3: KOPROP1 enrichment; b4: Propionate-oxidizing enrichment isolated in this laboratory; b5: Desulfobulbus propionicus DSM 2505; b6: The purified enrichment culture described in this study; b7: pSYN7. A threefold dilution series starting with 30 ng of E. coli rRNA was bound in column c.

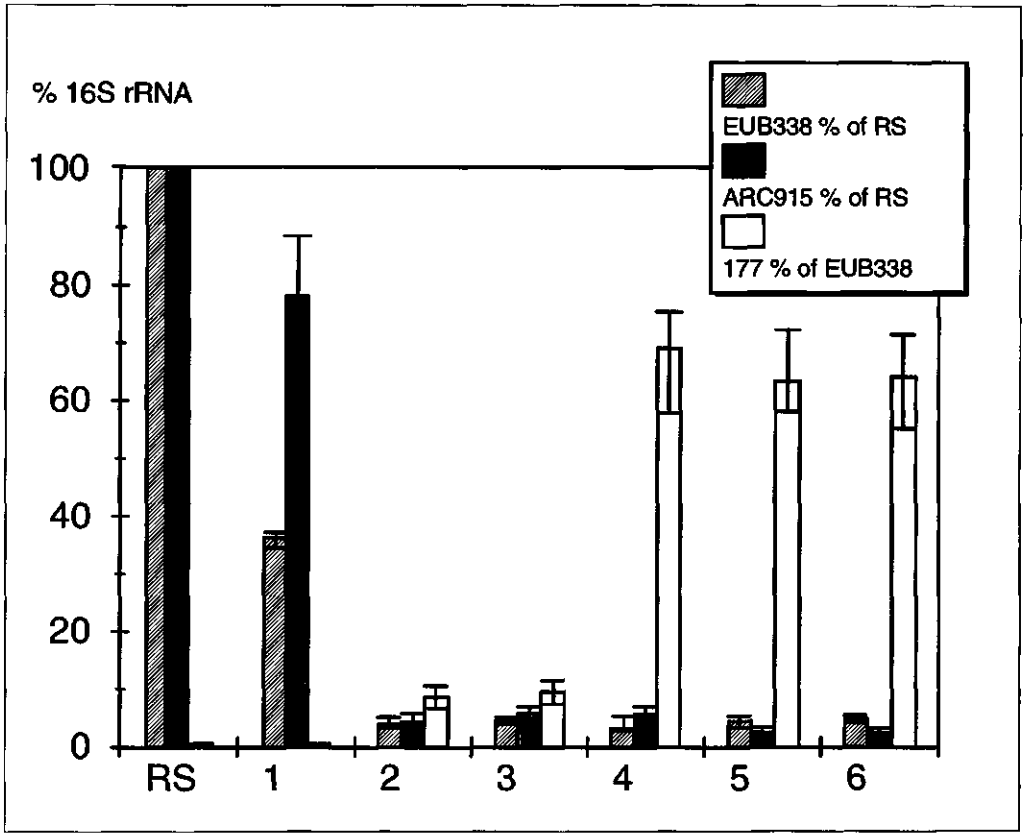


Fig. 3 The population dynamics of bacteria (probe EUB338), methanogens (probe ARC915) and SYN7 (probe 177) in the enrichment cultures as estimated by their rRNA hybridization. The radioactive signals of the hybridizations shown in Fig. 2 were used to quantify the amount of rRNA present in the cultures.

Percentages of bacterial and archaeal 16S rRNA of the cultures are given relative to the samples of the reactor slugde (RS). The percentage of SYN7 16S rRNA is relative to the amount of bacterial rRNA of each culture. The data are the averages of the values of triplicate samples. The error bars represent the maximum and mininum values.

**Discussion**

In studies on syntrophic propionate-oxidation the presence of Syntrophobacter-like organisms is often suggested (BOONE and XUN, 1987; MACLEOD et al., 1990; GROTENHUIS et al., 1991). However, in these cases they have not been identified. We have tried to detect Syntrophobacter wolinii and two Syntrophobacter-like organisms MPOB and KOPROP1, in the granular sludge of an UASB-reactor treating wastewater of potato industry, but these organisms could not be detected. Instead, another bacterium was enriched from this sludge during repeated transfer on propionate. This was a thin slightly-curved bacterium, named SYN7, which had no Syntrophobacter-like morphology. Analysis of its 16S rRNA sequence indicated that SYN7 is related to the genus Syntrophus. However, the two known Syntrophus spp., (i.e. S. buswellii and S. gentianae) do not utilize propionate. Moreover, the 16S rRNA sequences of SYN7 and the Syntrophus spp. share less than 95 % sequence similarity. These arguments favour the conclusion that SYN7 is a new species that does not belong to the genus Syntrophus and could be a member of a new genus, belonging to the group of sulfate-reducing bacteria. SYN7 was detected in low amounts in the original sludge and is likely to be involved in the syntrophic propionate-oxidation in the sludge. However, the presence of other syntrophic propionate-oxidizing bacteria in this sludge can not be ruled out.

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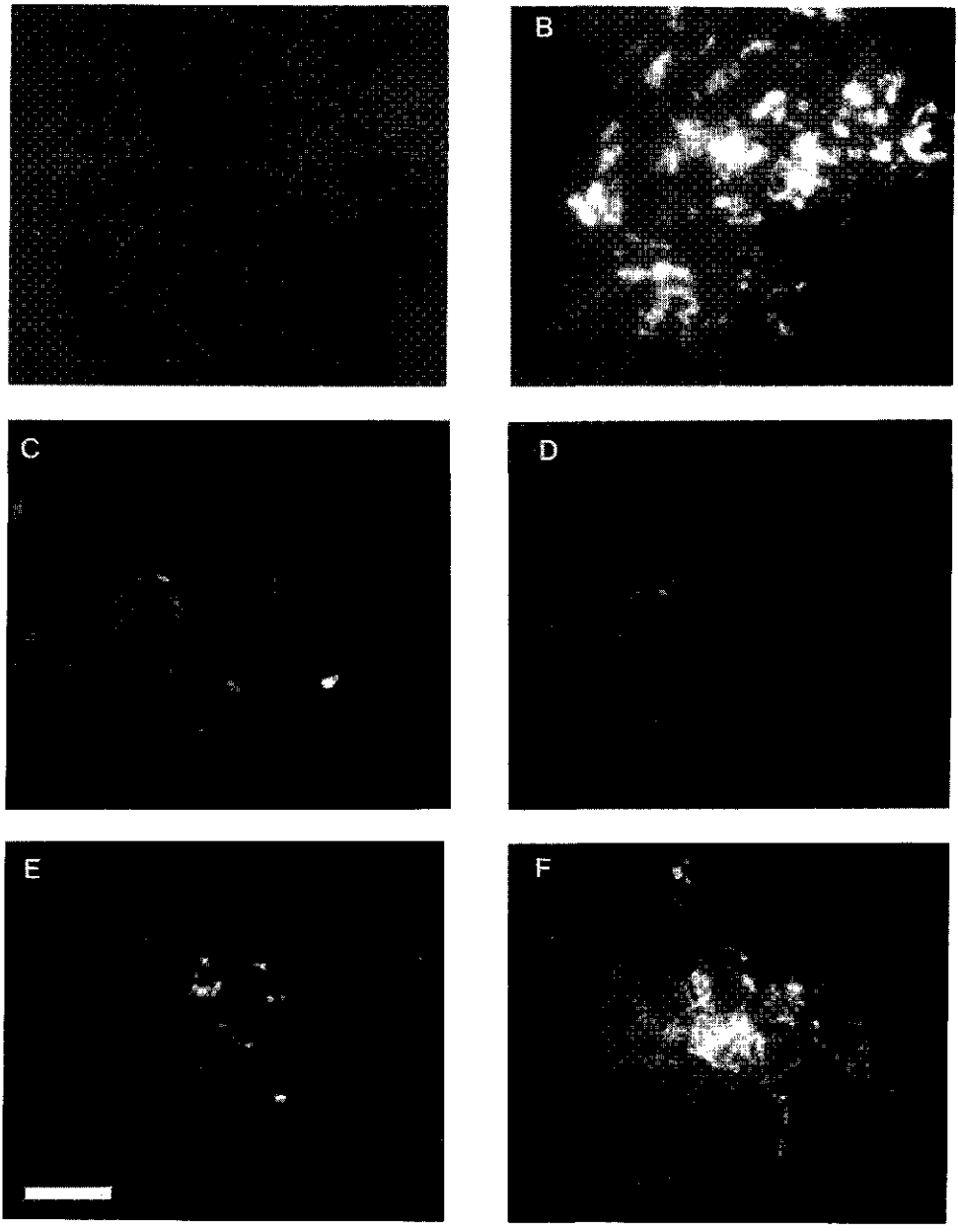
Fig. 4 In situ hybridization on fixed cells of the purified enrichment culture after pretreatment with a peroxide solution, with fluorescently-labeled probes; comparison of phase-contrast (A) and fluorescent (B) micrographs of an identical field hybridized with fluorescein-labeled probe EUB338. The micrographs of an identical field of fixed cells hybridized with probe EUB338 (C), and the rhodamine-labeled probe 177 (D), visualized all bacteria and identified SYN7 cells. The micrographs of different fields of fixed cells hybridized with the fluorescein-labeled probe D660 (E) and the fluorescein-labeled probe ARC915 (F), visualized respectively the Desulfobulbus sp. and the methanogens present in the culture. The bars represent 10 $\mu$m.


B
C
D
E
F

The SYN7 sequence was present in the majority of the analyzed transformants obtained by cloning the PCR-amplified 16S rRNA genes from the enrichment culture growing on propionate. This is in agreement with the finding that the specific probes 177 and 177B hybridized to the nucleic acids from the sludge enrichments. *In situ* hybridization of the purified enrichment culture with these probes indicated that at least half of the bacteria present were visualized. Most of the other bacteria reacted with the specific Desulfobulbus probe. Therefore, it was concluded that the amplified 16S rRNA of the dominant bacterium was obtained. The absence of transformants that contained a Desulfobulbus sequence might be caused by selective PCR-amplification and cloning.

The slot blot hybridizations of the sludge enrichment with the bacterial and archaeal probes showed that the microbial rRNA concentrations decreased in the first enrichment. This suggests that most of the microorganisms became inactive or died, probably due to a lack of substrate. These microorganisms were lost from the culture during the series of transfers. Hybridization of the filters with probe 177, showed that SYN7 was present in the inoculum but lost activity in the first enrichment, since the hybridization signal decreased. It is likely that during the first culture propionate was consumed by either other syntrophic bacteria or respiring bacteria that use alternative electron acceptors present in the inoculum. The relative amount of SYN7 started to increase after the first transfer and remained stable after the fourth transfer, comprising approximately 65 % of the bacterial rRNA. The remaining amount of bacterial rRNA could be derived from other bacteria present, that grew on propionate or its breakdown products. Desulfobulbus spp. were not enriched in the cultures growing on propionate as shown by the slot blot hybridizations. Remarkably, a Desulfobulbus sp. was present in the culture made by dilution of the enrichment, which was used for phylogenetic analysis, as shown by *in situ* hybridization. This culture grew without sulfate, suggesting that this Desulfobulbus sp. was able to grow syntrophically on propionate with about the same doubling time as SYN7. If so, this would indicate that some Desulfobulbus spp. can grow syntrophically on propionate, as was suggested previously (HEPPNER et al., 1992). The enrichments described here are a useful model to study the population dynamics of syntrophic propionate-oxidizing bacteria of granular sludge. We can conclude from these studies that the main syntrophic propionate-oxidizing bacterium in this enrichment was SYN7, a strain belonging to a new species of sulfate-reducing bacteria. SYN7 started growing after the first transfer, when most of the

microorganisms were already lost from the culture.

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# Chapter 7

## Population dynamics of propionate-oxidizing bacteria under methanogenic and sulfidogenic conditions in anaerobic granular sludge

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# POPULATION DYNAMICS OF PROPIONATE-OXIDIZING BACTERIA UNDER METHANOGENIC AND SULFIDOGENIC CONDITIONS IN ANAEROBIC GRANULAR SLUDGE.

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## ABSTRACT

Labscale upflow anaerobic sludge blanket-reactors were inoculated with granular sludge obtained from an industrial wastewater treatment system and fed with either propionate or propionate and sulfate. The population dynamics of the propionate-oxidizing bacteria *Desulfobulbus* sp. and strain SYN7 was studied in the sludge blankets of both sludge-types by dot blot hybridization and *in situ* hybridization with 16S rRNA-based oligonucleotide probes. This revealed a 15-fold increase of the rRNA of *Desulfobulbus* sp. relative to the bacterial rRNA in the reactor fed with propionate and sulfate within six weeks. In the reactor fed with only propionate the amount of rRNA of SYN7 bacteria relative to bacterial rRNA increased tenfold within eight weeks. The bacterial population decreased in both reactors while the methanogenic population remained constant. *In situ* hybridization revealed that the outer layer present in the inoculant sludge, consisting mainly of Bacteria, disappeared during growth on propionate. In the sulfidogenic reactor this layer was replaced by *Desulfobulbus* sp. colonizing the surface of the granules. In the methanogenic reactor the typical bacterial outer layer was lost completely from the granules that contained microcolonies of the propionate-oxidizing syntrophic strain SYN7 intertwined with methanogens belonging to the family of *Methanomicrobiaceae*. Microcolonies belonging to the genus *Methanosaeta* were found in the innerlayers of all types of granules.

## INTRODUCTION

Treatment of industrial wastewaters in upflow anaerobic sludge blanket (UASB)-reactors has increased rapidly in the last two decades. One of the advantages of these type of reactors is the immobilization of the anaerobic microorganisms in dense granules (12). In these granules, complex organic matter is converted into methane and carbon dioxide. Different trophic groups of microorganisms are responsible for this complex bioconversion that includes hydrolysis, fermentation, acetogenesis, and methanogenesis (6). Immobilization stimulates the growth of many of these microorganisms and it is essential for some organisms, because the concentration of intermediates can be kept sufficiently low (21). The composition of the granules is dependent on the type of carbon sources in the wastewater and on the presence of electron acceptors such as sulfate, nitrate and $Fe^{3+}$ (2). In methanogenic bioreactors the only available electron acceptor is carbon dioxide. However, several types of industrial wastewaters contain a significant amount of sulfate, which stimulates the competition between sulfate-reducing bacteria and methanogens (16).

One of the intermediates in the breakdown of organic matter is propionate, of which the degradation is strongly influenced by the absence or presence of sulfate (2, 24). In the absence of sulfate, propionate conversion is thermodynamically only possible at a low partial hydrogen pressure and formate concentration. These conditions are met in syntrophic consortia of acetogens and methanogens, where the acetogens convert propionate into products such as acetate, carbon dioxide and hydrogen and/or formate that subsequently are used by the methanogens. In the presence of sulfate, propionate can be converted without the participation of methanogens. Sulfate-addition and inhibitor studies suggested a role of sulfate-reducing bacteria in the propionate-oxidation (24). Presently, three major groups of bacteria are known to occur in granular sludge that can couple propionate oxidation to sulfate reduction. One group includes sulfate-reducing bacteria, such as *Desulfobulbus* sp., that convert propionate into acetate and hydrogen sulfide. A second group is formed by syntrophic consortia, such as those including *Syntrophobacter wolinii* and sulfate-reducing bacteria like *Desulfovibrio* sp., in which the acetogen converts the propionate while the hydrogen and/or formate is consumed by the sulfate reducer. Recently, a third group of propionate utilizers has been discovered and includes syntrophic propionate-oxidizing bacteria such as *Syntrophobacter wolinii* and the strains MPOB and KOPROP1 that are capable of sulfate reduction and hence can oxidize propionate by using sulfate as

electron acceptor (3, 11, 22). Very little is known about, (i) which of these groups actually converts propionate in granular sludge in the presence of low amounts of sulfate, (ii) whether the numbers and activity of these bacteria can be influenced by varying the sulfate concentration, and (iii) what their spatial distribution is in the sludge.

Recently, we discovered that different syntrophic consortia are present in granular sludge originating from different UASB-reactors (8, 9). In an UASB reactor treating beet-sugar wastewater, the syntrophic propionate-oxidizing strain MPOB was identified as the dominant syntrophic bacterium. *In situ* hybridization with specific 16S rRNA-based fluorescent oligonucleotide probes showed that the syntrophic microcolonies containing MPOB-like bacteria were intertwined with *Methanobacter*-like methanogens (8). In contrast, when granular sludge of an UASB reactor treating potato-processing wastewater was used for enriching syntrophic bacteria on propionate, a new strain, named SYN7, became dominant (9). 16S rDNA sequence analysis of SYN7, showed that it was not related to MPOB or other *Syntrophobacter*-like bacteria, but belonged to a new species, related to the genus *Syntrophus*.

In the present study the population dynamics of the industrial granular sludge used for enrichment of SYN7 was investigated in labscale UASB-reactors fed with propionate in absence or presence of sulfate. Dot blot hybridization with 16S rRNA-based bacterial and archaeal probes and specific probes for SYN7 and *Desulfobulbus* were used to quantify the different groups of microorganisms. *In situ* hybridization with fluorescently-labeled probes was used to localize the different subpopulations in sludge and to investigate which methanogen dominated in the syntrophic consortia that are present in the granular sludge of the UASB-reactors.

## MATERIALS AND METHODS

**Granular sludge types**. Mesophilic methanogenic granular sludge was obtained from a fullscale UASB-reactor treating wastewater of a potato-processing factory (Aviko, Steenderen, The Netherlands). The sludge had been cultivated at 35 °C with wastewater containing starch, lactate, butyrate, propionate, acetate, and approximately 5 mM sulfate. Samples of the sludge were taken and either frozen for nucleic acid isolation, fixed in paraformaldehyde for *in situ* hybridization, or used to inoculate two 500 ml labscale reactors (5). These reactors were fed with a basal mineral medium containing either propionate (20 mM) or propionate (20 mM) and sulfate (15 mM), and operated for 3 months with a liquid retention time of 6 h. The organic load of the two reactors was increased from 4 to 12 kg sodium propionate/$m^3$.day in four weeks. Triplicate samples of 0.25 ml were taken every two weeks and stored at -20 °C prior to the isolation of nucleic acids as described below. After 8 and 12 weeks samples were taken for *in situ* hybridization, which were directly fixed in 4 % paraformaldehyde.

**Oligonucleotide probe synthesis and labeling.** The used oligonucleotide probes and their target organisms listed in (Table 1). The oligonucleotides for dot blot hybridization were synthesized by Pharmacia (Uppsala, Sweden), and 5' end-labeled with [$\gamma^{32}$P]ATP as described previously (7). The fluorescent oligonucleotides were synthesized and 5' end-labelled with FLUOS (a fluorescein derivative) or rhodamine using an aminolinker, and subsequently purified using acrylamide gelelectrophoresis by Eurogentec (Seraing, Belgium). The probe 177 was labelled at the 5' and 3' end by direct coupling of rhodamine to the oligonucleotide.

**Nucleic acids extraction and dot blot hybridization.** The frozen triplicate samples were thawed on ice and the granules were gently crushed. The nucleic acids were extracted using glassbeads and a cell homogenizer in the presence of phenol as previously described (9). The extracts were purified by phenol-chloroform-isoamyl alcohol (25:24:1 [v:v:v]) extraction and nucleic acids were precipitated using 2 volumes ethanol and 1/10 volume of 3M sodium acetate (pH 5.2). The pellets were washed with 70 % ethanol and resuspended in water. The quality of the isolated nucleic acids was judged by agarose gelelectrophoresis followed by ethidium bromide staining and the amount of 16S rRNA was determined by quantifying the appropriate bands using a computer image of the agarose gel, and comparing this with standards containing defined quantities of rRNA using ImageQuant software (Molecular

Table 1. Summary of the oligonucleotide probes used in this study.

| Probe | Target group | Sequence | Ref |
|---|---|---|---|
| EUB338 | Bacteria | 5'GCTGCCTCCCGTAGGAGT | 1 |
| ARC915 | Archaea | 5'GTGCTCCCCCGCCAATTCCT | 20 |
| UNIV-1392 | Virtually all known organisms | 5'ACGGGCGGTGTGT(G/A)C | 15 |
| 177 | SYN7 | 5'AGAAGTCATGCAGTATTATTCGG | 9 |
| S223 | *Syntrophobacter wolinii* | 5'ACGCAGACTCATCCCCGTG | 9 |
| MPOB1 | MPOB | 5'ACGCAGGCCCATCCCCGAA | 7 |
| KOP1 | KOPROP1 | 5'TCAAGTCCCCAGTCTCTTCGA | 7 |
| D687 | *Desulfovibrio* spp. | 5'TACGGATTTCACTCCT | 4 |
| D660 | *Desulfobulbus* spp. | 5'GAATTCCACTTTCCCCTCTG | 4 |
| MX825 | *Methanosaeta* spp. | 5'TCGCACCGTGGCCGACACCTAGC | 18 |
| MG1200 | *Methanomicrobiaceae* and relatives | 5'CGGATAATTCGGGGCATGCTG | 18 |
| MB310 | *Methanobacteriales* | 5'CTTGTCTCAGGTTCCATCTCCG | 18 |

Dynamics, Sunnyvale, USA). Nucleic acids of the reference organisms were isolated as previously described (7). Dot blots were made and hybridized overnight at 40 °C as described previously (9). The blots were washed for 1 h in 1 % SDS, 1 x SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) at different temperatures: 55 °C for the probe EUB338, 50 °C for probe ARC915, 52 °C for probe 177 and 59 °C for probe D660 (for probe sequences see Table 1). The membranes were exposed to phosphor storage screens that were scanned for radioactive response on a PhosphorImager (Molecular Dynamics), followed by processing of the digital signals using ImageQuant.

Total 16S rRNA was quantified by hybridization of the extracted nucleic acids and dilution series of the RNA of the reference organisms, with the universal probe UNI1392 . The resulting standard curves were used to calculate the concentration of the 16S rRNA for the different groups of microorganisms as described previously (17). The following reference organisms were used: *Desulfobulbus propionicus* (DSM 2505), *Syntrophobacter wolinii* (DSM 2805) in coculture with *Methanospirillum hungatei* (DSM 864), the syntrophic propionate-oxidizing strain MPOB (DSM 10017) and KOPROP1, kindy provided by Dr. B. Schink (Universität Konstanz, Germany), *Methanobacterium thermoautotrophicum* (DSM 1053), and pSYN7, a plasmid with a 1.5 kb insert of the 16S rDNA of SYN7 (9).

***In situ* hybridization.** The granular sludge samples were fixed in 4 % paraformaldehyde in phosphate buffered saline (pH 7.2). The granules were embedded in paraplast and sections were made as described previously (8). Slides with thin sections of several granules were hybridized with 100 ng of fluorescent oligonucleotide probes in 20 $\mu$l of hybridization buffer and were hybridized at 45 °C, washed with hybridization buffer at 48 °C, and rinsed with water before drying to the air (8). The slides were mounted in Citifluor (Citifluor, Ltd. London, UK) and viewed with a conventional Nikon epifluorescence microscope or a Bio-Rad MRC-600 confocal laser scanning microscope (CLSM) equipped with a krypton/argon laser.

## RESULTS

**Startup and operation of methanogenic and sulfidogenic labscale reactors.** Industrial granular sludge was used to inoculate two labscale UASB reactors to study the effect of sulfate on the population dynamics of propionate-oxidizing bacteria. The reactors were fed for 12 weeks with either propionate to influence the amount of syntrophic propionate-oxidizing bacteria or propionate and sulfate to stimulate the growth of sulfate-reducing propionate-oxidizing bacteria. Propionate and the formed acetate were found to be utilized for more than 99%. Methane was produced in both reactors while the reactor fed with propionate and sulfate produced hydrogen sulfide as well. The reactor fed with propionate will be referred to as a methanogenic and that fed with propionate and sulfate as a sulfidogenic system. The sludge blankets of both reactors retained their granular structure throughout the experiment and had a constant volume of about one third of the reactor volume.

**Dot blot hybridizations of nucleic acids isolated from industrial and labscale reactors.** To quantify the total amount of rRNA and the relative amount of bacterial and methanogenic rRNA, nucleic acids were isolated from the samples of granular sludge of the industrial, the methanogenic, and the sulfidogenic reactors, and subsequently hybridized with the 16S rRNA-based probes UNI1392, EUB338 and ARC915 (Table 1; 1, 15, 20). The results of the dot blot hybridization showed marked differences in the dynamics of the microbial composition in the two lab-scale reactors (Fig. 1). In the methanogenic reactor the amount of bacterial 16S rRNA relative to the total amount of 16S rRNA decreased from 40 % in the first sample when the reactor was inoculated to 3 % in the last sample taken after 12 weeks. The relative amount of the methanogenic 16S rRNA, detected with the archaeal probe ARC915 increased from 30 % in the first sample to 55 % after 2 weeks and subsequently remained rather constant. In the sulfidogenic reactor the fraction of bacterial 16S rRNA decreased from 40 % to 15 %, while that of the methanogenic 16S rRNA increased from 30% to 50 % in the last sample.

Specific probes for the genus *Desulfobulbus* (probe D660) and strain SYN7 (probe 177) were used to quantify the fractions of rRNA derived from the propionate-oxidizing bacteria. Specific probes for *Syntrophobacter wolinii* and related bacteria (probes MPOB1, KOP1 and S223, Table 1) and for the genus *Desulfovibrio* (probe D687, Table 1) (4) were also tested but did not hybridize to the nucleic acids isolated from the sludge. In the methanogenic reactor the amount of 16S rRNA of members of

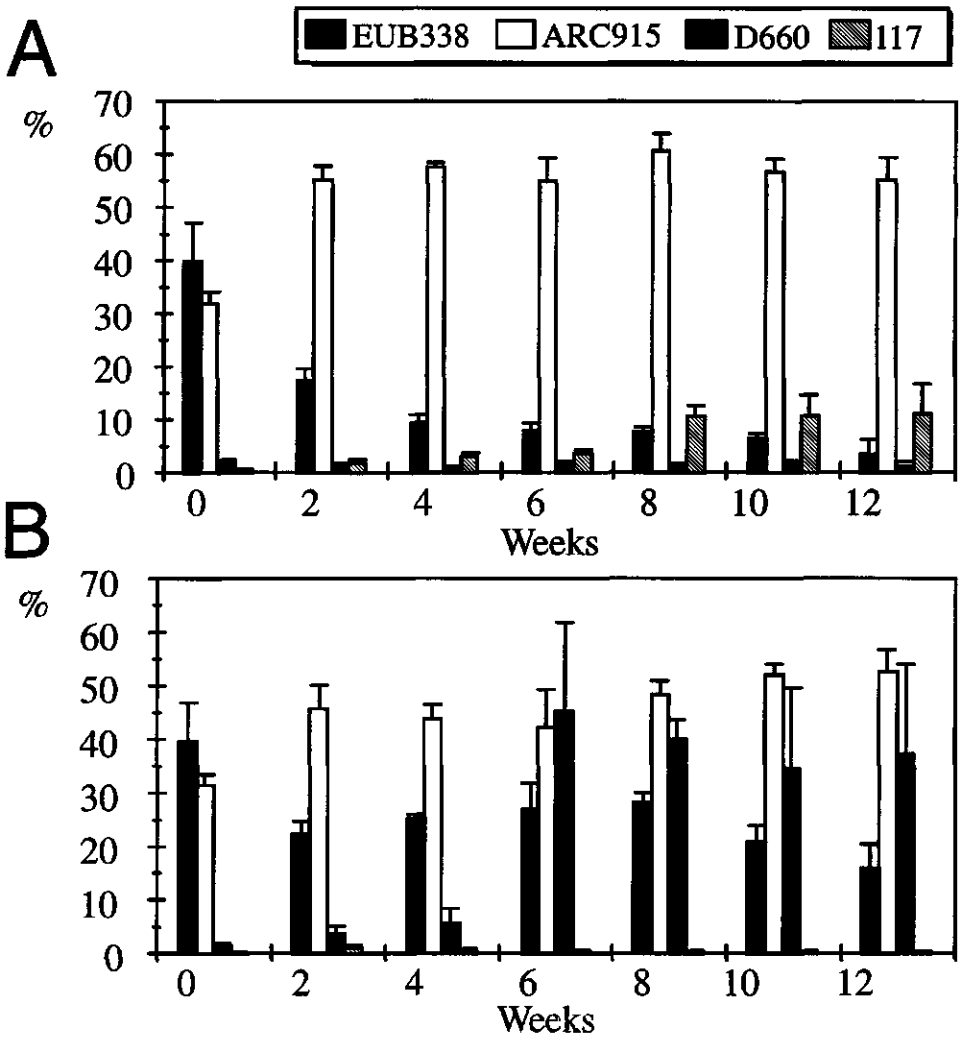



Fig. 1. Population dynamics of granular sludge fed with either propionate or propionate and sulfate. Relative amounts of the 16S rRNA from Bacteria (probe EUB338), Archaea (probe ARC915) and propionate-oxidizing bacteria (probes D660 and 177), isolated from the granular sludge samples of the reactor fed with propionate (A) and the reactor fed with propionate and sulfate (B). The radioactive signals of the probes EUB338 and ARC915 are expressed as a percentage of that obtained with the universal probe UNIV1392. The radioactive signals of the specific probes 177 and D660 are expressed as a percentage of that obtained with the bacterial probe. Error bars indicate the standard deviations.

the genus *Desulfobulbus* as determined with the probe D660 stayed below 2 % relative to the bacterial 16S rRNA, while the relative amount of 16S rRNA of the syntrophic propionate-oxidizing bacterium SYN7 as determined with probe 177 increased from 1 to 10 % of the total bacterial 16S rRNA (Fig. 1A). In contrast, in the sulfidogenic reactor the amount of 16S rRNA of the genus *Desulfobulbus* relative to the bacterial rRNA increased from 2 % to a maximum of 43 % after 6 weeks and subsequently stayed rather constant, while the amount of SYN7 rRNA remained below 2 % of the total bacterial 16S rRNA. (Fig. 1B).

***In situ* hybridization of the industrial reactor sludge.** To study the microbial architecture of the granules present in the industrial UASB reactor, *in situ* hybridization of the inoculum sludge was performed with bacterial and archaeal probes, as well as specific probes for *Desulfobulbus* and SYN7. Hybridization of the industrial reactor-sludge with the fluorescein-labelled probe EUB338 showed a specific layered architecture with microcolonies ordered in concentric circles (Fig. 2A). There was a thick outer layer of bacteria with a large variety of morphologies (Fig. 2B). Hybridization of this sludge with the archaeal probe ARC915 confirmed this layered structure of the granules and showed that the outer layer was almost free of methanogens (Fig. 2C). Below the thick outer layer, other layers were located that contained two types of microcolonies. One type consisted only of methanogens while other microcolonies appeared to consist of bacteria intertwined with chains of methanogens. Hybridization of the sludge with the rhodamine-labelled probe 177 to detect SYN7 bacteria showed no significant signal with any of the microcolonies (results not shown). This confirmed the results obtained with the dot blot hybridizations (Fig. 1). In contrast, hybridization with the fluorescein-labelled probe D660 identified individual cells of *Desulfobulbus* sp., growing in the thick outer layer of bacteria (Fig. 2D).

***In situ* hybridization of sludge from the labscale reactors.** The methanogenic and sulfidogenic labscale reactors were sampled 8 and 12 weeks after inoculation. *In situ* hybridizations of sections of the sludge from both labscale reactors with the different probes showed that the composition of the granules sampled after 8 weeks was comparable to those sampled after 12 weeks. This indicated that after at least 8 weeks a stable situation was reached.

Hybridizations of the sludge from the sulfidogenic reactor with the bacterial EUB338 probe showed a strong signal with an outer layer of bacteria with a

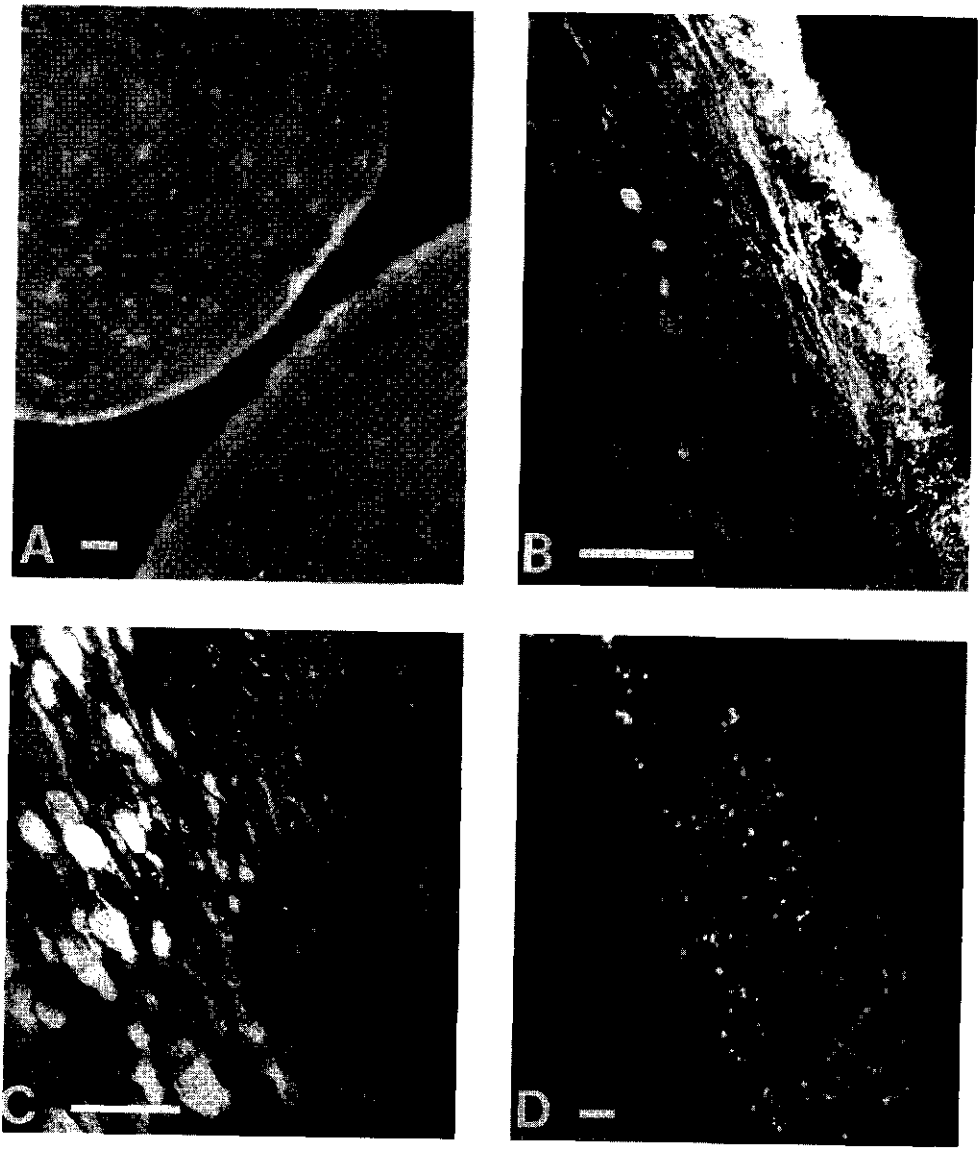



Fig. 2. Photomicrographs of *in situ* hybridization of sections of the industrial anaerobic granular sludge. Sections were hybridized *in situ* with fluorescein-labeled oligonucleotides probes universal for Bacteria (probe EUB338; A and B), Archaea (probe ARC915; C), and a fluorescein-labeled specific probe for the genus *Desulfobulbus* (probe D660; D). Photomicrograph A was viewed by epifluorescence microscopy and shows a section of half the granule (A). The micrographs B to D were viewed with CLSM, and show the outer layers of the granule. Scale bars A to C are 50 $\mu$m, D is 10 $\mu$m

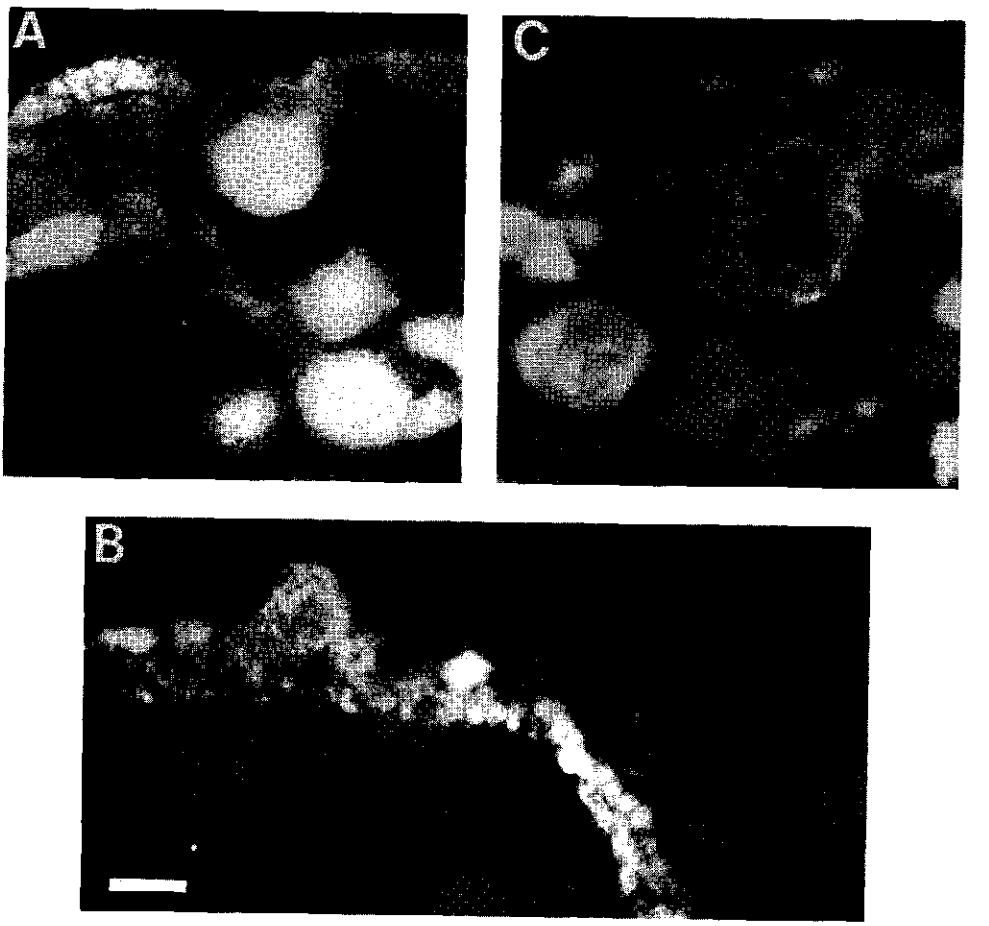
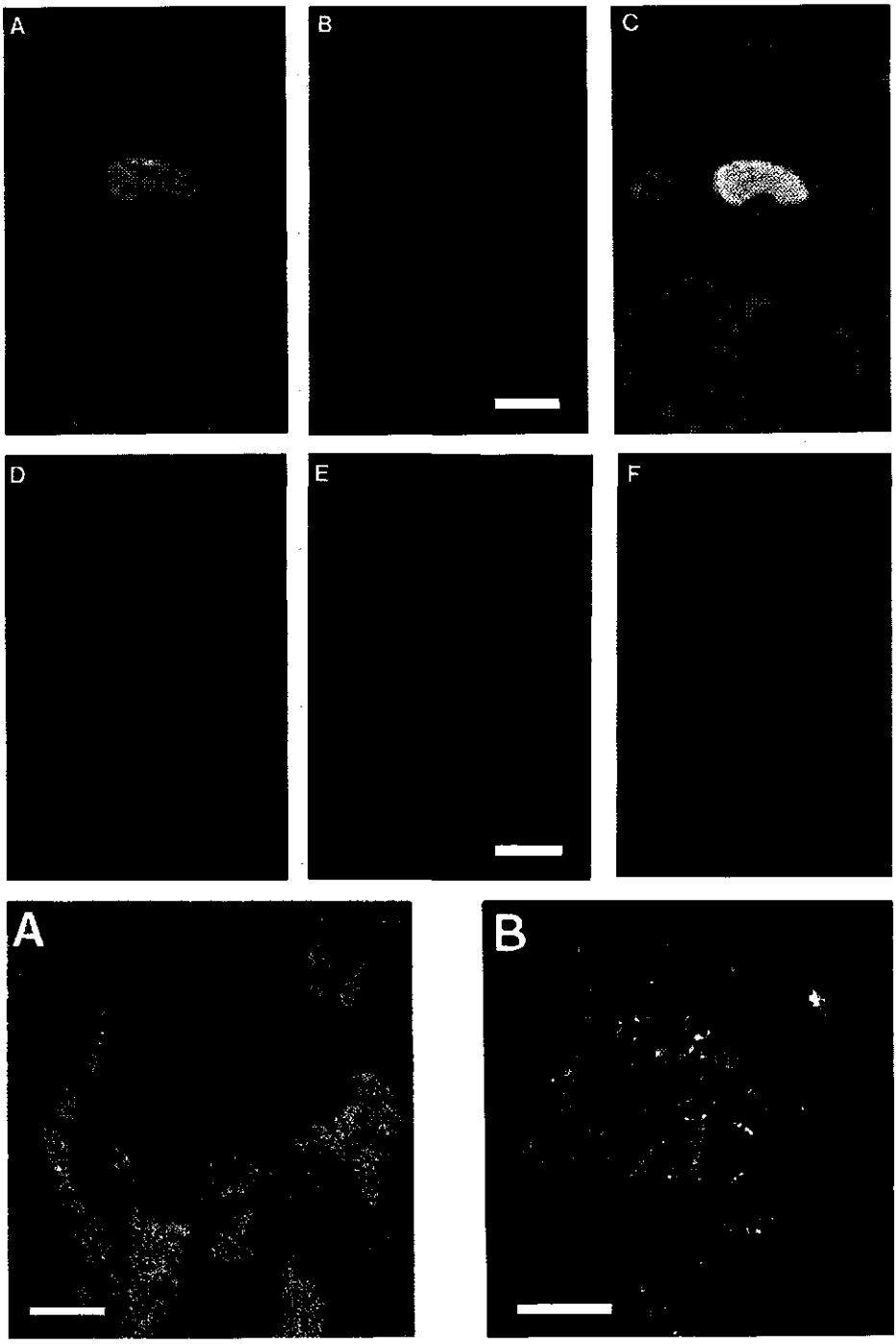



Fig. 3. Photomicrographs of *in situ* hybridizations of granules obtained from the sulfidogenic reactor. Sections showing the outer layers of the granules, were hybridized with fluorescein-labeled probes for Bacteria (probe EUB338; A), specific for *Desulfobulbus* (probe D660; B), and for Archaea (probe ARC915; C). Scale bar 50 $\mu$m.

characteristic morphology (Fig. 3A). This layer was present in roughly half of the granules and covered other fluorescing bacterial microcolonies. Hybridization with probe D660 revealed that the bacteria in this outer layer belonged to the genus *Desulfobulbus* (Fig. 3B). This probe did not hybridize to other microcolonies in the granule. Hybridizations with the archaeal probe ARC915 showed that methanogens were present in large microcolonies below the surface (Fig. 3C). Probe 177, specific for the syntrophic SYN7 bacteria, did not hybridize with any of the microcolonies present in the granules (results not shown). This was also expected from the results obtained with the dot blot hybridization (Fig. 1B).

Hybridization of the methanogenic sludge with the EUB338 probe showed that the granules had changed and lacked the typical bacterial outer layer found in the sulfidogenic reactor (Fig. 4A). The new outer layer consisted of several bacterial microcolonies with a relatively low fluorescence signal. However, few microcolonies showed very bright fluorescence. The number of these microcolonies varied but never exceeded 10 microcolonies per granule. Hybridization of these sections with both the fluorescein-labeled probe EUB338 and the rhodamine-labeled probe 177 revealed that the microcolonies, which showed the bright fluorescence with the EUB338 probe, hybridized to the 177 probe, indicating that these are SYN7 microcolonies (Fig. 4B and 4C). A combined hybridization of these granules with the ARC915 and the 177 probe showed that the SYN7 microcolonies were intertwined with methanogens, illustrating their syntrophic character (Fig. 4D to F).

Confocal laser scanning microscopy (CLSM) was used to investigate in more detail the syntrophic character of microcolonies of SYN7 intertwined with methanogens in the methanogenic sludge (Fig. 5). This technique was used to view a hybridization with three oligonucleotide probes, the rhodamine-labelled EUB338 probe, the fluorescein-labelled ARC915 probe, and the rhodamine-labeled probe MX825 (Table 1), specific for the aceticlastic genus *Methanosaeta* (18), to discriminate acetate-utilizing methanogens from those growing on hydrogen or formate (Fig. 5A). The images obtained included only signals from a single focus plane of 0.7 $\mu$m thick, thus reducing the background. Digital signals obtained with the two specific filtersets for the fluorescent labels were merged into one figure. The syntrophic microcolonies showed a red and green fluorescence due to the presence of the propionate-oxidizing acetogens and the hydrogen or formate consuming methanogens, reacting with the EUB338 (red) and ARC915 (green) probes, respectively. The microcolonies of the *Methanosaeta* sp.


A
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C
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Fig. 4. Photomicrographs of *in situ* hybridization of granules, obtained from the methanogenic reactor. Sections were simultaneously hybridized with the fluorescein-labeled bacterial probe EUB338 (A) and the rhodamine-labeled SYN7-specific probe 177 (B). The double exposures using both the fluorescein- and the rhodamine-specific filtersets shows the hybridization of both probes (C). Sections of the methanogenic sludge were also simultaneously hybridized with the fluorescein-labeled archaeal probe ARC915 (D) and the rhodamine-labeled SYN7-specific probe 177 (E). The double exposures using both filtersets shows the hybridization of both probes (F). Scale bar 50 $\mu$m.

Fig. 5. *In situ* hybridization of a sludge granule obtained from the methanogenic reactor. Sections were simultaneously hybridized with the fluorescein-labeled archaeal probe ARC915 (green), the rhodamine-labeled bacterial probe EUB338 (red), and the rhodamine-labeled for probe *Methanosaeta* sp. MX825 (red). The double (red and green) labeling of the *Methanosaeta* sp. results in a yellow fluorescence (A). Simultaneous hybridization of the sections with the fluorescein-labeled probe MG1200 specific for *Methanomicrobiaceae* (green) and the rhodamine-labeled SYN7 probe 177 (red), identifies the microbes present in the syntrophic microcolonies (B). The CLSM micrographs were made by merging two digital images and show details of sections containing syntrophic microcolonies. Scale bar 20 $\mu$m.

coloured yellow in the merged image as a result from the red and green fluorescence, and were always located outside the syntrophic microcolonies. Therefore, the methanogens that were located in the syntrophic microcolonies did not belong to the genus *Methanosaeta*. To identify the methanogen present in the syntrophic consortia, the granules were hybridized with a combination of rhodamine-labeled SYN7 probe 177 and the fluorescein-labeled probe MG1200 (Table 1). This latter probe is specific for the family *Methanomicrobiaceae* and relatives (18), to which the genera *Methanogenium* and *Methanospirillum* belong, that predominantly utilize hydrogen and/or formate. The results indicate that the large SYN7 microcolonies were intertwined with chains of methanogens hybridizing with the MG1200 probe, that resembled *Methanospirillum* (Fig. 5B). Hybridization of the methanogenic sludge sections with the probe MB310 (Table 1), specific for *Methanobacteriales* (18), showed fluorescence with some individual cells, but not with cells in the syntrophic consortia (results not shown). Hybridization of the methanogenic granules with the *Desulfobulbus* probe D660, showed no significant fluorescence, as expected from the dot blot hybridizations that showed the absence rRNA derived from cells belonging to this genus (Fig. 1).

## DISCUSSION

By enriching industrial granular sludge on propionate with or without sulfate as electron acceptor, stable lab-scale UASB-reactors were obtained that allowed us to study the effect of sulfate on the architecture and dynamics of its microbial population. The inoculum sludge was found to convert propionate via both methanogenesis and sulfate reduction, with no apparent lagphase. Propionate seemed to be always limiting in the reactor since it was completely converted from the first day on. By regularly sampling the sludge blankets, extracting nucleic acids and hybridizing these with domain-, genus- and species-specific oligonucleotide probes, marked differences were observed in the microbial population dynamics in the two labscale reactors. The most important difference is the enrichment of the newly discovered SYN7 bacteria (9) in the methanogenic reactor while in the sulfidogenic reactor *Desulfobulbus* sp. were enriched. This may be explained by the finding that *Desulfobulbus* sp. do not grow syntrophically on propionate (23) and that SYN7, although it may be capable of growing on propionate by sulfate reduction (9), can not outcompete *Desulfobulbus* in the presence of sulfate.

The hybridization of the nucleic acids isolated from the sludge with the universal 16S rRNA probe UNI1392, enabled us to correlate the amount of archaeal rRNA to the amount of bacterial rRNA. The radioactive signals on the dot blots of the bacterial probe and the archaeal probe added up to about 70% of that of the universal probe. Although the presence of rRNA of Eukarya can not be ruled out completely, it is likely that this difference is due to the calculation of the specific activities of the probes, since small differences in the standard curves have a large impact on the calculated relative amounts of rRNA.

In both reactors, the relative amount of archaeal rRNA increased slightly, while the relative amount rRNA of the bacteria decreased rapidly. This is most likely due to the disappearance of the hydrolyzing and fermenting bacteria that grew on starch and other biopolymers present in the potato-processing wastewater treated by the industrial UASB reactor. Most of these bacteria probably did not utilize propionate, stopped growing or died, and hence were washed out of the reactor, as was evidenced from the disappearance of the thick bacterial outer layer from the granules. The increasing relative amount of methanogenic rRNA can be explained by the fact that the amount of bacterial rRNA relative to the total rRNA decreased. The bacterial population in the sulfidogenic reactor remained larger than in the methanogenic reactor. More energy can be gained from the oxidation of propionate via sulfate reduction (-37.7 kJ/mol) than via syntrophic propionate oxidation (-10.9 kJ/mol for the bacterial population at a partial hydrogen pressure of 1 Pa) (21). Therefore, *Desulfobulbus* should gain 4 times more energy from the propionate oxidation than SYN7. This may explain the higher fraction of rRNA from *Desulfobulbus* in the sulfidogenic reactor that exceeded that derived from SYN7 in the methanogenic reactor. The remaining bacterial rRNA in either of the reactors that did not originate from *Desulfobulbus* or SYN7 was probably derived from non-active microorganisms. In contrast to the bacteria located in the outer layers, these organisms are not washed out of the reactor because they are immobilized in the granules and hence may only disappear after a long period of time by decay or breaking of the granules. The results clearly indicated that *Desulfobulbus* does not utilize propionate syntrophically in this sludge, in contrast to what has previously been suggested for other anaerobic sludge types utilizing propionate (10, 24). In the sludge derived from the potato-starch plant studied here, the syntrophic SYN7 was enriched. It can not be excluded that other unknown syntrophic propionate-oxidizing bacteria were active in the methanogenic sludge as well, but we did not detect them with the probes

for presently known syntrophs.

The *in situ* hybridizations very clearly demonstrated the different location and organization of propionate-oxidizing bacteria growing in sludge. SYN7 grows syntrophically with methanogens inside the granule in defined microcolonies, while individual cells hybridizing with the *Desulfobulbus* probe colonize the surface of the granule and may use the granule only as a substratum to prevent them from washing out from the reactor.

In previous studies in which we hybridized granular sludge with probes for syntrophic bacteria, it was difficult to show that the syntrophic microcolonies were bacterial colonies intertwined with methanogens (8). In the methanogenic sludge used here, the methanogens in the syntrophic microcolonies gave a strong signal with the ARC915 and the MG1200 probes. Unlike the juxta-positioned *Methanobacter*-like methanogens found previously in the syntrophic microcolonies (8), the *Methanospirillum*-like cells here formed chains throughout the microcolonies that consisted of syntrophic SYN7 bacteria. Most of the bacterial microcolonies inside the granules were intertwined with methanogens. It is possible that these microcolonies were also involved in the syntrophic oxidation of propionate or other substrates such as butyrate or ethanol.

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# Chapter 8

## Description of *Syntrophobacter fumaroxidans* sp. nov.: A syntrophic propionate-degrading sulfate-reducing bacterium.

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# Description of *Syntrophobacter fumaroxidans* sp. nov.: A syntrophic propionate-degrading sulfate-reducing bacterium.

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**Abstract**

A syntrophic propionate-oxidizing bacterium, strain MPOB, was isolated from a culture enriched from anaerobic granular sludge. It oxidizes propionate syntrophically in coculture with the hydrogen- and formate-utilizing *Methanospirillum hungatei*, and it can oxidize propionate and other organic compounds in pure culture without syntrophic partner, using sulfate as electron acceptor. 16S rRNA sequence analysis revealed a relationship with *Syntrophobacter wolinii* and *S. pfennigii*. DNA-DNA hybridization studies showed less than 26 % hybridization among the different genomes of the *Syntrophobacter* species and MPOB. This justifies the assignment of MPOB to the genus *Syntrophobacter* as a new species, *S. fumaroxidans*.

For a long time *Syntrophobacter wolinii* was the only well-described bacterium which could oxidize propionate syntrophically in coculture with the hydrogen consuming *Desulfovibrio* G11 (Boone and Bryant, 1980). Although, several methanogenic syntrophic cocultures were enriched, the isolation of a defined coculture remained a difficult task. Recently however, *S. wolinii* was obtained in pure culture and found to be able to grow on pyruvate or on propionate and sulfate (Wallrabenstein et al., 1994). Furthermore, a similar bacterium was isolated, *Syntrophobacter pfennigii*, formally indicated as KOPROP1 (Wallrabenstein et al., 1995). This bacterium can grow either syntrophically on propionate in coculture with *Methanospirillum hungatei*, or without syntrophic partner on propionate and sulfate.

A mesophilic propionate-oxidizing bacterium (MPOB) was enriched from granular sludge, which was able to convert fumarate to succinate and carbon dioxide without syntrophic partner (Stams et al., 1993). This strain could oxidize propionate by using fumarate as electron acceptor. Furthermore, it could oxidize propionate in pure culture using sulfate as electron acceptor (van Kuijk and Stams, 1995). Similar to *S. wolinii*, strain MPOB oxidized propionate via the acetyl-CoA cleavage pathway, but in contrast to *S. wolinii*, strain MPOB activates propionate by the use of HS-CoA transferase (Plugge et al., 1993). 16S rRNA sequence analysis of *S. wolinii*, *S. pfennigii*, and strain MPOB revealed that these syntrophic bacteria were closely related and belonged to the delta subclass of proteobacteria (Harmsen et al., 1993; Harmsen et al., 1995). Remarkably, it was observed that another bacterium was related to this group, *Desulforhabdus amnigenus*, a sulfate reducing bacterium which is not able to grow syntrophically on propionate (Oude Elferink et al., 1995).

This report describes the isolation of MPOB from the enrichment culture, its morphological and physiological characterization, and discusses its taxonomic position within the genus *Syntrophobacter*.

**Bacterial strains, cultivation and isolation procedures**. The isolated strain MPOB originated from a culture enriched from granular sludge (CSM, Breda, The Netherlands), which was described previously (Stams et al., 1993). *Syntrophobacter wolinii* (DSM 2805) and *Desulfobulbus propionicus* (DSM 2505) were obtained from the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany). *Syntrophobacter pfennigii* was kindly provided by Dr. B. Schink (Universität Konstanz, Germany). *Desulforhabdus amnigenus* was kindly provided by S. Oude Elferink.

A bicarbonate-buffered medium as described previously was used for isolation and cultivation (Stams et al., 1993). All substrates except amino acids, were measured by gas chromatography as described previously (Stams et al., 1993). Amino acids were measured as described by Kengen and Stams (1994). The previously described enrichment culture (Stams et al., 1993) was purified by application of roll-tube-dilution method (Hungate, 1969), with fumarate as carbon source. Isolation was done by transferring one colony from the roll-tube into a dilution series with medium that contained fumarate as carbon source, supplemented with 0.05 % (w/v) yeast extract (BBL-Becton Dickinson). The culture was checked for purity by growth on Wilkins-Chalgren anaerobe broth (Oxiod, Basingstoke, UK) and by growth in media with 1 % yeast extract and 20 mM glucose. The cultures were examined microscopically.

**Phylogenetic analysis and DNA-DNA hybridization.** Phylogenetic analysis was performed using the ARB program package (Strunk et al., in prep.) as described in Harmsen et al. (submitted). DNA hybridizations were performed by filter hybridization according to Johnson et al. (1981). Modifications of this method on DNA labeling and hybridization conditions were described previously (Klijn et al., 1994). The final stringent washing was done at 65 °C in 0.03 M NaCl and 0.003 M sodium citrate with 1 % SDS. The percentage of binding was determined by measuring the radioactive hybridization signals relative to those found in the homologous hybridizations.

**Isolation and characterization.** The syntrophic propionate-oxidizing bacterium was enriched as a methanogenic coculture from methanogenic granular sludge. The *Methanospirillum* sp. was removed from the coculture by continuous subculturing on malate and fumarate as described previously (Stams, et al. 1993). However, this enrichment culture on fumarate contained contaminating small coccoid bacteria, which could be enriched and isolated on yeast extract. Therefore, the roll-tube technique was applied to the fumarate-oxidizing enrichment culture, to eliminate the contaminating bacteria. A culture, containing about $10^8$ cells/ml was diluted in anaerobic roll-tubes with fumarate as carbon source, and after two months this resulted in the formation of yellow colonies of about 2 mm in diameter in the $10^4$ and $10^5$ dilution. However, when these colonies were cultured in liquid medium with fumarate as substrate and 0.05 % yeast extract, still contaminating bacteria were found. Therefore, dilution series of this liquid enrichment were made, with fumarate as substrate and 0.05 % yeast extract, to purify the culture and check for the growth of the contaminant, simultaneously. In all

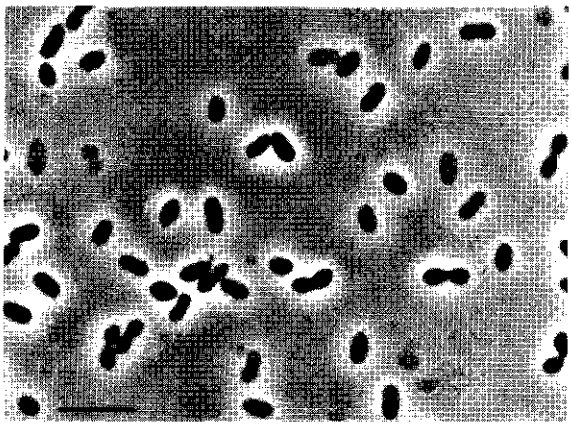

Fig. 1. Phase contrast photomicrograph of cells of strain MPOB, grown on fumarate. Bar is 10 $\mu$M.

Table 1. Organic compounds tested as electrondonors and carbon sources.
The substrate concentrations are given in mM in *parentheses*.

| Utilized: | Products: |
|---|---|
| In coculture with *Methanospirillum hungatei*: | |
| propionate (20). | acetate, methane |
| In pure culture: | |
| fumarate (20),malate (20), | succinate and $CO_2$ |
| pyruvate (20) | acetate |
| In pure culture in the presence of 20 mM sulfate: | |
| propionate (20) | acetate |
| acetate (20), succinate (20), ethanol (20), aspartate (10), glutamate (10). | not determined |

Not utilized:

Tested in the presence of 20 mM sulfate:

Butyrate (10), isobutyrate (10), citrate (10), lactate (20), butanol (10), propanol (20), methanol (10), glucose (20), fructose (10), xylose (10).

the tubes until the $10^8$ dilution growth was observed, but only the tube with the $10^8$ dilution appeared to be free of the contaminating coccoid. This culture consisted only of the gram-negative, non-motile, rod-shaped bacterium described previously and indicated as strain MPOB (Fig. 1).

Strain MPOB grew on fumarate between 20 °C and 40 °C with an optimum at 37 °C. The pH range was from 6.0 to 8.0 with an optimal growth at 7.2. Addition of 0,05 % yeast extract was not necessary, but stimulated growth. Strain MPOB had a growth rate of approximately 0.17 $day^{-1}$ in methanogenic coculture at 37 °C with propionate as substrate. Growth on propionate and sulfate was very slow at a rate of 0.024 $day^{-1}$ (van Kuijk and Stams, 1995). A c-type cytochrome was the only detected cytochrome in cells grown on fumarate. Strain MPOB utilized propionate syntrophically in coculture with *Methanospirillum hungatei*, and in pure culture using sulfate as electron acceptor (Table 1). In both cases it oxidized propionate stoichiometrically to acetate and $CO_2$, to produce either methane or hydrogen sulfide. MPOB fermented fumarate to succinate and $CO_2$, and reduced fumarate to succinate with hydrogen or formate as electron donor (Stams et al., 1993). Preliminary experiments showed that strain MPOB grew chemotrophically with several organic compounds, including amino acids (Table 1). Thiosulfate, could serve as an electron acceptor, but nitrate was not utilized.

**Taxonomy.** Recently, the 16S rRNA sequence of strain MPOB was analyzed, and this showed that it was closely related to *S. wolinii* and *S. pfennigii* (Harmsen et al., 1995). A new phylogenetic tree was constructed, that includes the syntrophic propionate-oxidizing bacteria *S. wolinii, S.pfennigii*, strain MPOB, the sulfate-reducing bacterium *Desulforhabdus amnigenus*, and the members of the delta subclass of proteobacteria, using the 16S rRNA sequence data previously obtained (Harmsen et al., 1993, 1995; Oude Elferink et al., 1995) (Fig. 2). This clearly shows that these syntrophic propionate-oxidizing bacteria form one cluster with *D. amnigenus* and belong, according to the grouping of Devereux et al. (1989) together with *Desulfomonile tiedjei* and *Desulfoarculum baarsii*, to group 7 of the delta subclass of Proteobacteria. This is a phylogenetically distinct group of the line of complete-oxidizing sulfate-reducing bacteria. To investigate this relationship on a species level DNA-DNA hybridizations were performed. Labeled total chromosomal DNA of each of the four strains and *Desulfobulbus propionicus* as an unrelated strain, was hybridized with membrane-fixed total chromosomal DNA of the same strains (Table 2). The

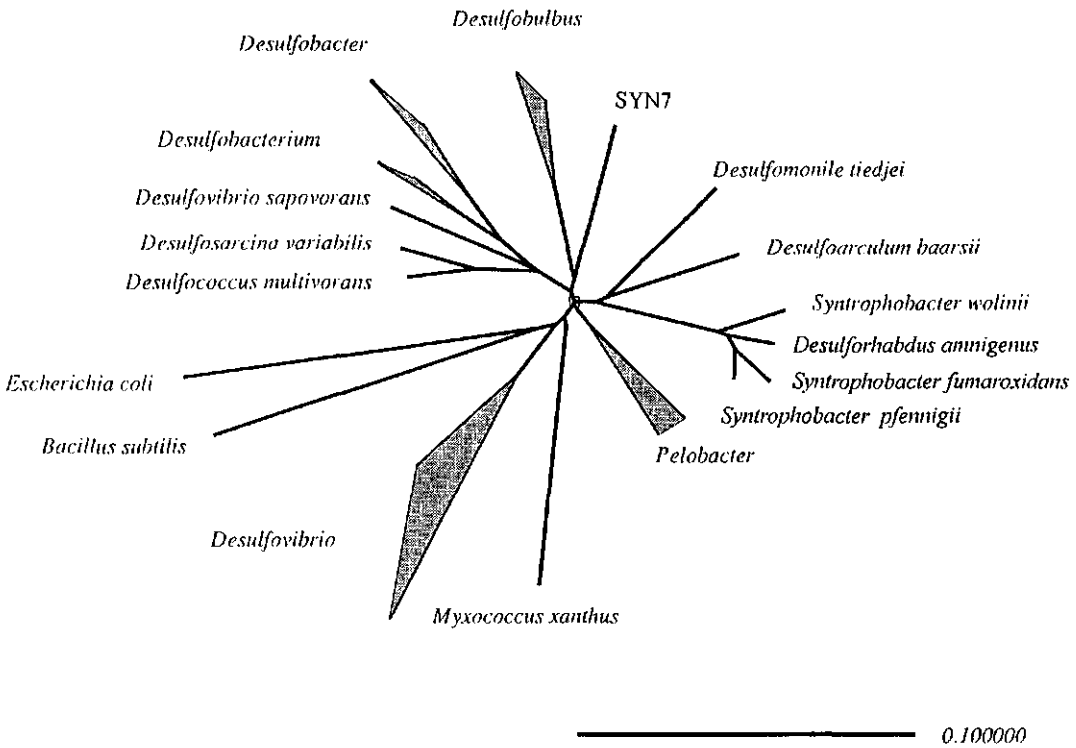


Fig. 2. Unrooted phylogenetic tree from 16S rRNA sequence divergence of MPOB, *Syntrophobacter wolinii*, *Syntrophobacter pfenningii* and the major groups of the delta-subclass of Proteobacteria with *Escherichia coli* and *Bacillus subtilis* as outgroups. The bar represents 10% estimated sequence divergence.

Table 2. Percentage of DNA-DNA hybridization of MPOB, *Syntrophobacter wolinii, Syntrophobacter pfennigii, Desulforhabdus amnigenus* and *Desulfobulbus propionicus*.

| | MPOB | *S.wolinii* | *S.pfennigii* | *D.amnigenus* | *D.propionicus* |
|---|---|---|---|---|---|
| MPOB | 100 | 9.4 | 16.4 | 24.7 | 11.4 |
| *S. wolinii* | 23.2 | 100 | 16.3 | 26.0 | 8.8 |
| *S.pfennigii* | 19.8 | 5.0 | 100 | 13.0 | 6.1 |
| *D.amnigenus* | 14.4 | 2.3 | 8.2 | 100 | 4.2 |
| *D.propionicus* | 22.0 | 6.5 | 15.4 | 8.1 | 100 |

homology observed between the different strains was always below the 26 %, indicating that the strains belong to different species.

Strain MPOB resembles the syntrophic propionate-oxidizing species *Syntrophobacter wolinii* and *Syntrophobacter pfennigii* by its growth on propionate in coculture with *Methanospirillum hungatei* and in pure culture by sulfate-reduction. Furthermore, *S. wolinii* can also utilize fumarate and pyruvate. In addition, in coculture *S. pfennigii* can utilize propanol and lactate. However, the utilization of aspartate, glutamate, ethanol and acetate was not observed with the other two syntrophs. The sulfate-reducing species *Desulforhabdus amnigenus* can not oxidize propionate syntrophically and does not utilize fumarate.

The morphological and physiological differences mentioned above and the results obtained from the DNA-DNA hybridization, showed that the strains do not belong to the same species. Therefore, it appears justified to describe strain MPOB as a new species within the genus *Syntrophobacter* and we propose the name *S. fumaroxidans* sp. nov.

*Syntrophobacter fumaroxidans*. fu.mar.ox'i.dans. The one that oxidizes fumarate, as the ability to oxidize fumarate played an important role in the isolation and physiological characterization of this bacterium. Non-motile, rod- to eye-shaped cells, 1.1 to 1.6 by 1.8 to 2.5 $\mu$m in size, with round ends, single or in pairs. The Gram-negative cells do not form endospores. The strictly anaerobic bacterium grows syntrophically on propionate in the presence of hydrogen and/or formate-utilizing bacteria or methanogens, e.g *Methanospirillum hungatei*. The bacterium ferments fumarate and pyruvate. Propionate, acetate, succinate, ethanol, aspartate and glutamate are utilized via sulfate reduction. Sulfate or thiosulfate serve as electron acceptors, nitrate is not reduced. The growth is optimal in freshwater medium with a pH of 7.0-7.6, and at 37 °C. The habitat is granular sludge from an upflow anaerobic sludge bed (UASB) reactor treating sugar-beat processing wastewater. Type strain: *Syntrophobacter fumaroxidans* strain MPOB (DSM 10017).

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# Summary and concluding remarks.

## Summary and concluding remarks.

The research described this thesis concerns the diversity and phylogeny of syntrophic propionate-oxidizing bacteria and their ecology in granular sludge, from which they were obtained. 16S rRNA was used as a molecular marker to study both the phylogeny and the ecology of these bacteria. Sequence analysis of the 16S rRNA gave information on the phylogeny of the syntrophic bacteria, while specific oligonucleotide probes based on these sequences enabled quantification and detection of these bacteria. *In situ* hybridization techniques were applied to study the architecture of granular sludge. Granular sludge was also enriched for propionate-oxidizing bacteria and studied with dot blot and *in situ* hybridization to combine their presence in the sludge to the physiological processes observed.

In this chapter the results are summarized and discussed in a broader context, starting with the diversity of the syntrophic propionate-oxidizing bacteria. Subsequently, the application of the ribosomal approach for the detection of syntrophs in the sludge is discussed. Next, the ecology and the localization of the microbes in granular sludge fed with different substrates is presented in models of sludge architecture, and finally the main conclusions of the research are presented.

**Diversity of syntrophic propionate-oxidizing bacteria.** For more than one decade *Syntrophobacter wolinii* was the only well-described syntrophic propionate-oxidizing bacterium (Boone and Bryant, 1980). Although other syntrophic propionate-oxidizing cocultures were enriched, no other bacterium could be isolated (Koch et al., 1983; Boone and Xun 1987; Mucha et al., 1988; Stams et al., 1992).

It was initially assumed that *S. wolinii* was an obligately syntrophic bacterium, which only used propionate as carbon source, and only grew in coculture with $H_2$- and/or formate-utilizing microbes such as *Desulfovibrio* G11 or *Methanospirillum hungatei* (Boone and Bryant, 1980). However, at the beginning of the nineties, when the research of this thesis was initiated, this view changed. Stams et al. (1993) enriched a syntrophic propionate oxidizer on malate, which could grow in pure culture on fumarate. This bacterium, strain MPOB, later named *Syntrophobacter fumaroxidans* (Chapter 8), could oxidize propionate using fumarate as alternative electron acceptor. Meanwhile, Schink and coworkers, succeeded to grow *S. wolinii* in the absence of $H_2$- and/or formate-utilizing microbes on propionate and sulfate, and on pyruvate (Dörner,

1992; Wallrabenstein et al., 1994). These findings showed that these syntrophs were not obligately syntrophic, but could be grown in pure culture with other carbon sources as well. The phylogenetic analysis of the 16S rRNA of the syntrophs showed that *S. wolinii*, *S. fumaroxidans* and the later isolated *Syntrophobacter pfennigii*, are not only closely related to each other, but are related to Gram-negative sulfate-reducing bacteria (Chapters 2 and 3). Moreover, they are sulfate-reducing bacteria themselves, as all three *Syntrophobacter* spp. could oxidize propionate by the reduction of sulfate.

The increasing knowledge of this group of syntrophs may lead to the isolation of more related syntrophic propionate-oxidizing species. However, there are presently arguments that syntrophy is not a physiological characteristic of this phylogenetic group. Two sulfate-reducing bacteria have been isolated that were related to the genus *Syntrophobacter*, but which did not use propionate syntrophically under the conditions tested. These include, *Desulforhabdus amnigenus*, isolated by the growth on acetate (Oude Elferink et al., 1995), and *Desulfoacinicum infernum*, a thermophilic bacterium isolated on lactate from a petroleum resevoir (Rees et al., 1995). 16S rRNA sequence analysis revealed a close and a moderate relation, respectively of *D. amnigenus* and *D. infernum* with the genus *Syntrophobacter*, and both most likely share a common ancestor with the latter genus. Whether these bacteria lost the ability to grow syntrophically or that *Syntrophobacter* gained this ability, remains unclear.

16S rRNA analysis of two other cultures enriched from granular sludge showed that syntrophic propionate-oxidation was not restricted to the genus *Syntrophobacter*. The SYN7 bacterium was not related to *Syntrophobacter*, but moderately related to the syntrophic benzoate oxidizing genus *Syntrophus* (Wallrabenstein et al., 1995a) (Chapter 6). Furthermore, the sporeforming syntrophic propionate-oxidizing bacteria SporeA and SporeB (Chapter 4), are related to Gram-positive sulfate-reducing genus *Desulfotomaculum*. Although widely spread throughout the domain of Bacteria, the syntrophic propionate oxidizers that are phylogenetically analyzed so far have one feature in common, they are related to sulfate reducers. It is even possible that they are all sulfate reducers. Such a linkage between syntrophic propionate oxidation and sulfate reduction may originate from common enzymatic pathways, such as the methyl-malonyl CoA pathway, present in the members of the genus *Syntrophobacter* (Houwen et al., 1990; Stams et al., 1993). Also *Desulfobulbus* possess such a pathway (Stams et al. 1984), but so far, has not been shown to grow syntrophically on propionate. It is interesting to find out why *Desulfobulbus* spp. can not oxidize propionate

syntrophically, while *Syntrophobacter* spp. are able to do so. Furthermore, other sulfate-reducing bacteria need to be tested or tested more extensively for the capacity to grow syntrophically on propionate, for instance *Desulforhabdus amnigenus* or members of the genus *Desulfotomaculum*.

The purification of strain MPOB, resulting in the description of *Syntrophobacter fumaroxidans* sp. nov., allowed for the testing of growth on many carbon sources in the presence or absence of sulfate. Preliminary experiments indicated that this *S. fumaroxidans* strain could also grow, although after a long lag-phase, on alcohols and amino acids by the reduction of sulfate. As a consequence it is quite a versatile sulfate reducer and resembles other sulfate reducers in their growth on many different substrates (Widdel and Bak, 1992). However, in methanogenic ecosystems such as granular sludge, propionate appears to be the only substrate available for *S. fumaroxidans*. This bacterium seems to be specialized in the syntrophic oxidation of propionate, and this feature may be of advantage over other sulfate-reducing bacteria present in methanogenic granular sludge.

**16S rRNA sequence analysis of cocultures and enrichment cultures.** Phylogenetic analyses of syntrophic propionate-oxidizing bacteria were performed with mixed cultures. The cultures from which the 16S rRNA genes were obtained, had an increasing complexity. The first culture was a defined coculture consisting of *Syntrophobacter wolinii* and *Desulfovibrio* G11 (Boone and Bryant, 1980). Following the analysis of the 16S rRNA gene of *Desulfovibrio* G11, it was obvious that the other 16S rRNA gene obtained from the culture had to be derived from *S. wolinii* (Chapter 2). This was not the case for the phylogenetic analysis of the highly purified, but not defined cultures MPOB and KOPROP1, later named *S. fumaroxidans* and *S. pfennigii*, respectively (Chapter 8; Wallrabenstein et al., 1995b). Although no other 16S rRNA genes were cloned, it had to be shown that the sequences obtained really came from the dominant syntrophic bacteria. The required evidence was given by *in situ* hybridization with specific oligonucleotide probes (Chapter 3). Because of the higher complexity of the cultures, more elaborate work was needed for the phylogenetic analysis of the dominant syntrophic propionate oxidizers in the less defined enrichments described in the Chapters 4 and 6. In the case of SYN7, several 16S rRNA genes were cloned and characterized, but the SYN7 sequence was the most abundant one. *In situ* hybridization with SYN7-specific probes showed that this sequence was indeed abundantly present in

the culture, next to sequences that hybridized with a *Desulfobulbus*-specific probe. However, these *Desulfobulbus*-like 16S rRNA sequences were not present in the clone library, probably caused by preferential amplification or cloning of the 16S rRNA-genes. This shows that this method of amplification and cloning of 16S rRNA genes may cause biases and should always be followed by feedback methods like *in situ* hybridization. In the case of the sporeforming syntrophs, only one bacterial morphology was observed, while at least two 16S rRNA sequences were derived from the culture, which were derived form bacteria indicated as SporeA and SporeB. *In situ* hybridizations with specific probes for the two sequences were performed to investigate from which bacteria these sequences were derived. Unfortunately, this did not result in unambiguous fluorescent signals, probably because the Gram-positive cell wall could not be made penetrable for the probes with our methods. Therefore, it still can not be ruled out that both sequences were obtained from the same organism. However, dot blot hybridizations showed that only the probe specific for the SporeB sequence hybridized with another enrichment of propionate oxidizers by pasteurization, indicating that the sequences are present in separate organisms.

Despite of these problems, the 16S rRNA sequence-analysis done on these enrichment cultures gave valuable information on the phylogenetic status of the dominant bacteria in the cultures, which may help in the isolation of these and other syntrophic propionate-oxidizing bacteria in the future. Furthermore, specific oligonucleotide probes were designed on the basis of these sequences, which have helped to study their presence and their dynamics in granular sludge as described below.

**Detection of syntrophic propionate-oxidizing bacteria in granular sludge.** Fluorescently-labeled specific 16S rRNA-based oligonucleotide probes were used to detect the syntrophic propionate-oxidizing bacteria in anaerobic granular sludge.
For this, two types of granular sludge were studied, (i) granular sludge that originated from an upflow anaerobic sludge bed (UASB) reactor treating sugarbeet-processing wastewater (Chapter 5) and (ii) sludge from an UASB reactor treating potato-processing wastewater (Chapters 6 and 7). *S. wolinii* and *S. pfennigii* were never detected in these two types of granular sludge, not even after enrichment on propionate. *S. fumaroxidans*-like bacteria were present in large numbers in sludge that originated from the sugarbeet wastewater-treating UASB-reactor. SYN7 was present in

the potato-wastewater sludge, but could only be detected significantly after enrichment on propionate. Both *S. fumaroxidans* and SYN7 were only detected in the sludge from which they were originally enriched. From the two sporeforming syntrophic bacteria, just the SporeB sequence was found in potato-processing wastewater treating sludge, but only after pasteurization and enrichment on propionate .

It seems that each type of sludge possesses its specific syntrophic propionate-oxidizing bacterium, depending on the wastewater to be treated. This could be due to selection caused by the wastewater itself, or due to the different sources of inoculum used to start up the reactors, such as activated sludge or manure. To test this, granular sludge could be transferred from one reactor to another reactor that treats a different type of wastewater, and then follow the population dynamics of the syntrophs. Furthermore, the abundance of *S. wolinii* and *S. pfennigii* could be determined in the sludge from which they were isolated, and more importantly, it could be investigated if also other syntrophs are present in that sludge.

**Architecture of granular sludge.** The architecture of the three investigated sludge types appeared to be dependent on the influent of the reactor. The granules obtained from the UASB reactor treating sugarbeet wastewater, fed with either sucrose or volatile fatty acids (VFA), and the granules from the UASB reactor treating potato wastewater, all had a layered structure in which both syntrophic and methanogenic bacteria were observed. However, there are marked differences in the architecture and number of layers between the three sludge types. Moreover, the abundance of the different trophic groups varied, and the organization of the syntrophic consortia and the microbes present were quite distinct. To illustrate these differences, models are designed that represent active granules of a moderate size. However, the centre of large granules often consisted of large cavities, probably due to substrate transport limitation, which cause lysis of the microbes (Alphenaar et al., 1993).

The sludge fed with sucrose and VFA that originated from the UASB- reactor treating sugarbeet wastewater, had been operated with a defined influent for several months. This presumably led to a reduction of the diversity of the organisms in the sludge. Based on the results obtained by *in situ* hybridization (Chapter 5), the following schematic architecture of the sucrose-fed sludge is proposed. This sludge probably contains only four trophic groups of microorganisms, that form granules of a very simple architecture (Fig 1A). Fermentative bacteria are present in the loosely bound

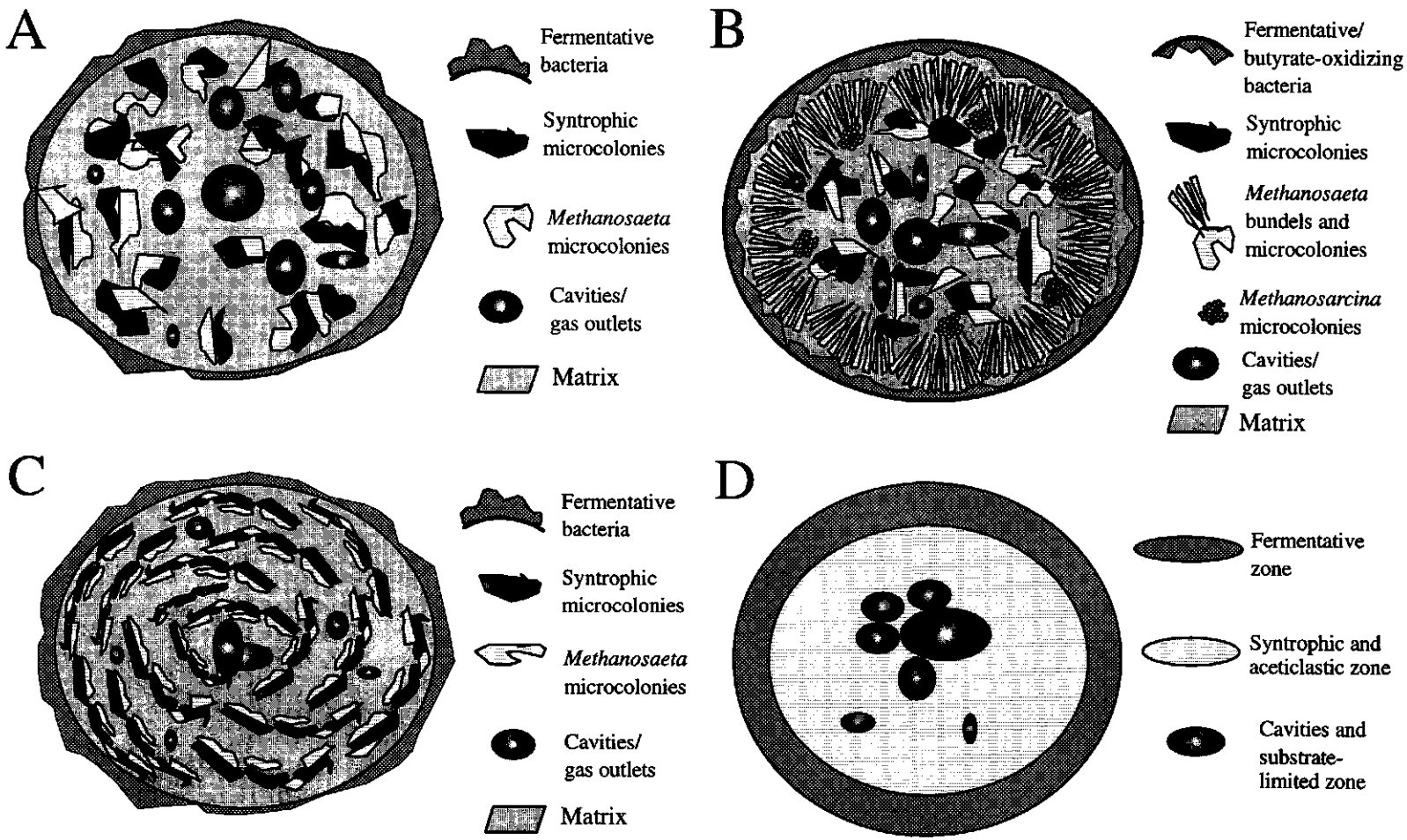


Fig. 1. Schematic architecture of three types of granular sludge (A, B and C) and a general model for granular sludge architecture (D). A. Sucrose-fed sludge ; B. Volatile fatty acids-fed sludge; C. Starch-fed sludge.

outer layer and form propionate and acetate from sucrose. Inside the granule syntrophic microcolonies are present in which the syntrophic acetogens utilize the propionate to form acetate and hydrogen, and carbon dioxide and/or formate. These are then used by the *Methanobacter*-like methanogens also present in the syntrophic microcolonies. The acetate is utilized by *Methanosaeta* sp. microcolonies present adjacent to the syntrophic microcolonies. The results obtained from the VFA-grown sludge (Chapter 5) showed an architecture different from that of the sucrose fed sludge, which leads to the following model (Fig. 1B). The outer layer contains bacteria that are more inside the granule, underneath this layer there is a thick layer of mainly aceticlastic *Methanosaeta* sp., but also *Methanosarcina* microcolonies are seen in this layer. These latter methanogens have a lower affinity for acetate than *Methanosaeta* and will only by present if the concentrations are sufficiently high (Jetten et al., 1990). Underneath this layer there is a layer of syntrophic microcolonies and microcolonies of *Methanosaeta* sp. , degrading propionate and acetate, respectively. The results obtained from the *in situ* hybridizations of granular sludge of the UASB-reactor treating potato-processing wastewater (Chapter 7) was quite different to the granules described above, which leads to the proposal of the following model (Fig 1C). This sludge was fed with industrial potato wastewater containing at least starch, lactate, butyrate, propionate and acetate as substrates. Therefore, the microbial community in the sludge is more diverse. This is clearly shown in the many different morphologies present in the thick outer layer of the sludge (Fig. 2). Underneath the outer layer there is a layer present with small syntrophic microcolonies and *Methanosaeta* microcolonies and maybe other kinds of microcolonies. Remarkably, these microcolonies were all concentrically orientated. *In situ* hybridizations indicated that the microcolonies in the centre were less active than those between the centre and the outer layer, probably due to substrate limitation (Chapter 7). This suggests a development of the granule from the centre towards the outer layer.

The diversity of the potato-starch grown granules was also shown by the enrichment on propionate and on propionate with sulfate, which selected for syntrophic and sulfidogenic propionate-oxidizing bacteria, respectively (Chapter 7). This research showed that both trophic groups of bacteria were present in the inoculum sludge. The sulfidogenic propionate oxidizing *Desulfobulbus* sp. colonized the outer layers of the granules, while the syntrophic SYN7 bacteria formed active microcolonies inside the granule. The morphological diversity in both types of granules decreased dramatically,

due to cultivation on one substrate. This was seen by the disappearance of the diverse outer layer, and by the decrease of bacterial rRNA in the dot blot hybridizations.

The most interesting difference between the sludge that originated from the sugarbeet-wastewater UASB-reactor and the potato-wastewater treating reactor was the organisation and the types of microbes in the syntrophic microcolonies. The sludge grown on sucrose and VFA contained syntrophic microcolonies consisting of thick MPOB-like rods juxta-positioned with rods that hybridized with a *Methanobacteriales*-specific probe, morphologically resembling *Methanobrevibacter* cells. A similar morphological organization of syntrophic microcolonies was previously observed (MacLeod et al, 1990; Grotenhuis et al., 1991; Prensier et al., 1988; Wu et al., 1992). *Methanobrevibacter* only uses hydrogen as substrate, which has a low solubility in the water, and because every MPOB-like cell is surrounded by methanogens, interspecies hydrogen transfer might the mechanism by which the reducing equivalents are transferred. However, MacLeod et al. (1990) measured a high formate utilizing activity in sludge with syntrophic microcolonies containing *Methanobrevibacter*-like cells, although they did not measure hydrogen utilizing activity of the sludge. In contrast to the granules mentioned above, the granules treating potato-wastewater contained syntrophic microcolonies with densely packed small SYN7 rods that were intertwined with chains of cells morphologically resembling *Methanospirillum hungatei*. These cells hybridized with a probe specific for the genus *Methanogenium* and related organisms, including *M. hungatei*. This type of microcolonies have not been reported before. In the SYN7 microcolonies not every syntrophic acetogen is surrounded by methanogens. Since *Methanospirillum* can also utilize formate, it is possible that formate transfer might occur in these microcolonies.

A layered structure of granules was proposed and shown in models by others (MacLeod et al., 1990; Macario et al., 1991). Macleod et al. (1990) suggested for sucrose-fed granules also the presence of four trophic groups of microbes organized as follows; a central core of *Methanosaeta (Methanothrix)* surrounded by a layer of hydrogen-producing acetogens and hydrogen-consuming methanogens, and an outer layer of fermentative bacteria. In the model described here the two inner layers are always mixed, and in the case of VFA grown granules *Methanosaeta* even form a layer around the syntrophic microcolonies. The earlier observation that only *Methanosaeta* cells were present in the central core (MacLeod et al, 1990) could be due to the size of the granules, so that only acetate is able to reach the central core. A general model for

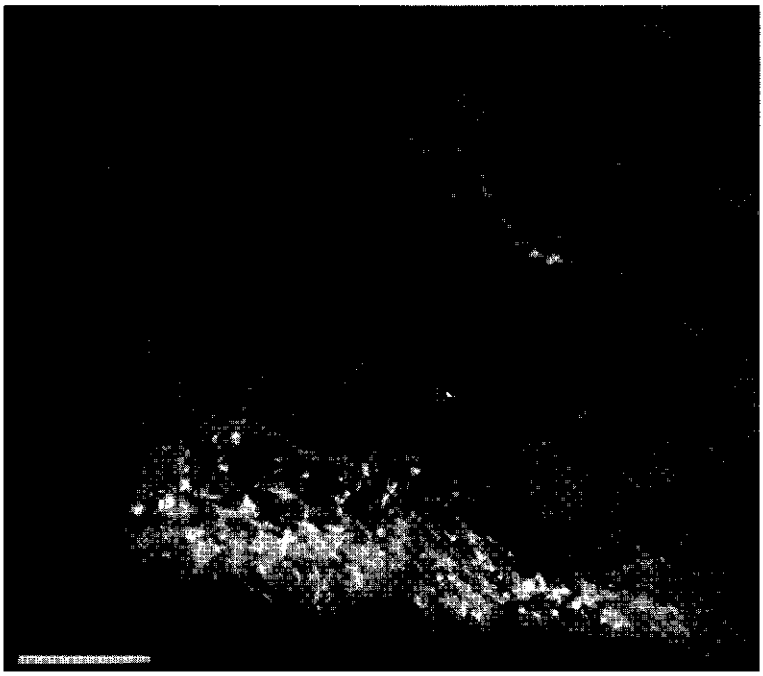

Fig. 2. In situ hybridization of granular sludge from an UASB reactor treating potato-processing wastewater, with the fluorescein-labeled bacterial probe EUB338 (Chapter 7). Bar 50 $\mu$M.

the architecture of granules converting organic matter based on previous models and the observations presented here suggests the presence of two active layers and a substrate-transport limited central core (Fig. 1D). In the outer layers mainly microorganisms are present which degrade the substrates in the surrounding medium or influent, independently from other microorganisms. The inner layers consist of microbes that degrade substrates which are produced by the primary layer, e.g fatty acids, acetate and hydrogen and/or formate, and which are dependent on each other for growth, such as the syntrophic consortia. The presence and size of the central core depends on the size of the granule. In large granules there will be a central core consisting of large cavities occupied by *Methanosaeta* sp. which degrade acetate, produced by the second layer as the terminal intermediate in the breakdown of organic matter. The methane which is produced throughout the granule will disappear from the granule through the numerous cavities and channels in the granule.

**Conclusions.** The main conclusions of the research presented in this thesis are:

i There are at least three phylogenetically different groups of syntrophic propionate-oxidizing bacteria which are all related to sulfate-reducing bacteria. The first group is the genus *Syntrophobacter*, consisting of the species *S. wolinii*, *S. pfennigii* and *S. fumaroxidans*, which are closely related to each other and belong to the delta subclass of Proteo-bacteria. These bacteria were shown to be sulfate reducers themselves. The second group is related to the genus *Syntrophus* also belonging to the delta subclass. The third group are sporeforming syntrophic bacteria related to the Gram-positive genus *Desulfotomaculum*.

ii Granular sludge originating from different sources treating various wastewaters contained specific syntrophic propionate-oxidizing subpopulation. Sludge originating from the UASB reactor treating sugarbeet-processing wastewater, mainly contained *S. fumaroxidans* as propionate-oxidizer, which are juxta-positioned with *Methanobrevibacter*-like cells in syntrophic microcolonies. Sludge from the UASB reactor treating potato-processing wastewater, contains SYN7-like bacteria growing in microcolonies which are intertwined with chains of *Methanospirillum*-like methanogens.

iii The architecture of granular sludge and the presence of specific microbial subpopulations in this sludge is dependent on the substrates and electron acceptors available in the influent. Fermentative bacteria and sulfate-reducing acetogens are located in the outer layers of the granule, while syntrophic consortia and aceticlastic methanogens are located in the inner layers.

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# Samenvatting

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In het hier beschreven onderzoek wordt een overzicht gegeven van de microbiologie van anaëroob korrelslib. Het doel van dit onderzoek was de microbiële diversiteit en de evolutionele verwantschap (fylogenie) van propionaat oxiderende bacteriën in korrelslib te bestuderen en de architectuur van de slibkorrels te beschrijven in relatie tot de metabolische activiteit van deze slibbacteriën. Gezien de beperkingen van de conventionele microbiële detectie methoden is gekozen voor de molekulaire benadering, waarmee micro-organismen op grond van verschillen in het DNA kunnen worden geïdentificeerd. Door gebruik te maken van ribosomaal RNA (rRNA) als marker is het mogelijk geworden de bacteriën fylogenetisch in te delen en om ze specifiek te detecteren in verschillende soorten korrelslib.

**Anäeroob korrelslib in UASB reactoren.** Korrelslib ontstaat in *upflow anaerobic sludge bed* (UASB) reactoren, welke gebruikt worden voor anaërobe zuivering van voornamelijk industriëel afvalwater. In het slib wordt organisch materiaal afgebroken tot methaan en kooldioxide door fermentatieve, acetogene en methanogene micro-organismen. Propionaat is een belangrijk tussenprodukt in deze afbraak route. De energetische ongunstige omzetting van propionaat naar acetaat, kooldioxide, waterstof en/of formiaat, kan alleen dan plaats vinden wanneer de partiële waterstofdruk en/of formiaat concentratie laag gehouden wordt. Dit gebeurt door syntrofe (samenvoedende) consortia van acetogene en methanogene micro-organismen, waarbij de acetogeen de propionaat omzet en de methanogeen de waterstof en/of formiaat omzet. Door het syntrofe karakter van deze propionaat-oxiderende bacteriën zijn ze moeilijk te kweken en te bestuderen en is er van hun fylogenie en hun voorkomen in korrelslib weinig bekend. Daarom is er voor het onderzoek naar deze bacteriën gekozen voor een molekulaire benadering, die niet afhankelijk van kweekmethoden was.

**Het 16S rRNA als marker voor fylogenie en detectie van micro-organismen.** De huidige fylogenie van de micro-organismen is voornamelijk gebaseerd op het 16S rRNA. Dit molekuul maakt deel uit van het ribosoom dat betrokken is bij de aanmaak van eiwitten. Dit 16S rRNA is in elk micro-organisme aanwezig en heeft in al deze

organismen een gelijke basisstructuur. Toch is de volgorde van de nucleotiden waaruit dit molekuul is opgebouwd, voor elk van de organismen specifiek en kan er op basis hiervan een goede fylogenetische indeling gemaakt worden. Daar iedere levende cel over enkele duizenden ribosomen beschikt, kan het 16S rRNA tevens dienen als herkenningsmolekuul voor de identificatie en detectie van micro-organismen in complexe ecosystemen.

De identificatie van bacteriën in een microscopisch preparaat van bijvoorbeeld zuiveringsslib, op basis van morfologie is een onmogelijke zaak zonder het gebruik van specifieke kleuringsmethoden. Daarom is er een techniek ontwikkeld die bacteriën specifiek zichtbaar maakt met behulp van een stukje DNA dat met een fluorochroom gemarkeerd is. Deze zogenaamde oligonucleotide probe herkent een specifiek deel van het 16S rRNA van een micro-organisme of groepen van micro-organismen. Door de cellen eerst chemisch te fixeren en de probe de cel in te laten diffunderen en te laten binden met het 16S rRNA (hybridisatie), kunnen de micro-organismen met behulp van een fluorescentie microscoop specifiek zichtbaar gemaakt worden in hun omgeving (*in situ*). Met behulp van deze oligonucleotide probes kunnen de micro-organismen ook gekwantificeerd worden. Dit gebeurt door eerst het 16S rRNA te isoleren, vervolgens op een filter over te brengen (dot blot) en daarna te hybridiseren met een radioactief gemerkte probe. De radioactiviteit op het filter, die na hybridisatie gemeten wordt, is dan een maat voor de hoeveelheid micro-organismen.

Deze technieken zijn gebruikt voor de bestudering van syntrofe propionaat-oxiderende bacteriën in anaëroob korrelslib. De nucleotidesequentie van het 16S rRNA en de fylogenie van deze bacteriën is bepaald (Hoofdstukken 2, 3, 4 en 6). Specifieke probes op basis van dit 16S rRNA zijn gebruikt om deze bacteriën te detecteren in korrelslib en om de populatie dynamica te beschrijven (Hoofdstukken 5, 6 en 7).

**Fylogenie van syntrofe propionaat oxiderende bacteriën.** Meer dan tien jaar lang was *Syntrophobacter wolinii* de enige beschreven bacterie welke in cocultuur met *Desulfovirbrio* G11 in staat was om propionaat syntroof te omzetten. Aanvankelijk werd aangenomen dat *S. wolinii* alleen syntroof propionaat kon omzetten en geen andere substraten gebruikte. Maar in het begin van deze studie bleek dat *S. wolinii* ook alleen kon groeien, en wel op propionaat en sulfaat. Daarnaast werd er een mesofiele propionaat oxiderende bacterie (MPOB) opgehoopt, later *Syntrophobacter fumaroxidans* genoemd (Hoofdstuk 8), die op fumaraat kon groeien in afwezigheid van methanogene

micro-organismen. Vervolgens werd duidelijk dat deze bacterie onder andere op propionaat, ethanol en aminozuren kon groeien, door middel van sulfaat reductie (Hoofdstuk 8).

16S rRNA sequentie analyse van deze syntrofe bacteriën en de later geïsoleerde *Syntrophobacter pfennigii* onthulde dat deze bacteriën nauw verwant aan elkaar zijn, en behoren tot de groep der Gram-negatieve sulfaat-reducerende bacteriën (Hoofdstukken 2 en 3). Dat de eigenschap van syntrofe propionaat-oxidatie niet tot deze groep van bacteriën beperkt blijft, bleek uit de 16S rRNA sequentie-analyse van twee andere syntrofe propionaat-oxiderende culturen, opgehoopt uit korrelslib. De ene cultuur bevatte sporevormende syntrofe bacteriën welke verwant bleken met het Gram-positieve sulfaat-reducerende geslacht *Desulfotomaculum* (Hoofdstuk 4). De SYN7 bacterie uit de andere cultuur bleek verwant aan het syntrofe benzoaat-oxiderende geslacht *Syntrophus* (Hoofdstuk 6).

**Detectie en populatie dynamica van syntrofe propionaat-oxideerders.** Fluorochroom-gemarkeerde oligonucleotide probes, gebaseerd op het 16S rRNA, zijn gebruikt voor de detectie van de syntrofe propionaat-oxiderende bacteriën in korrelslib. *S. fumaroxidans* bleek in grote getale aanwezig te zijn in korrelslib afkomstig van een UASB-reactor dat afvalwater van een suikerfabriek zuivert (Hoofdstuk 5). In het korrelslib uit de UASB-reactor dat afvalwater zuivert van een fabriek die aardappels verwerkt, bleek stam SYN7 aanwezig te zijn, maar deze kon alleen goed gedetecteerd worden na ophoping van het slib op propionaat (Hoofdstuk 7). *S. wolinii* en *S. pfennigii* zijn in geen van de twee slibsoorten aangetroffen (Hoofdstukken 5 en 7). Sporevormende syntrofe propionaat oxideerders bleken wel aanwezig te zijn in beide slibsoorten, maar speelden waarschijnlijk geen rol van betekenis (Hoofdstuk 4). De populatiedynamica van propionaat-oxiderende bacteriën bij ophoping op propionaat van gemalen korrelslib in fles-culturen is bestudeerd met behulp van dot blot hybridisatie. Hieruit bleek dat SYN7 bacteriën zich pas begonnen op te hopen nadat de meeste bacteriën uit de cultuur verdwenen waren (Hoofdstuk 6).

De invloed van sulfaat op de ophoping van propionaat-oxiderende bacteriën in de korrel is bestudeerd in kleine proef UASB-reactoren waarbij de populaties zijn gevolgd met behulp van dot blot en *in situ* hybridizatie. Uit deze studies bleek dat stam SYN7 zich alleen bij afwezigheid van sulfaat ophoopte in de korrel in micro-kolonies samen met methanogenen. Bij aanwezigheid van sulfaat, hoopte *Desulfobulbus* zich snel

op aan de buitenkant van de korrel.

**Architectuur van verschillende slibsoorten.** De architectuur van korrelslib en de lokalisatie van de verschillende groepen micro-organismen is bekeken met behulp van *in situ* hybridizatie met fluorochroom-gemarkeerde probes. Hieruit bleek dat korrelslib veelal is opgebouwd uit twee of drie lagen, met in het midden open ruimten en dode en/of inactieve bacteriën. Er waren duidelijke verschillen tussen de onderzochte slibsoorten, met name in de architectuur, het voorkomen van verschillende groepen microben en in de opbouw van de syntrofe micro-kolonies. Het slib afkomstig van de reactor die suiker-afvalwater zuiverde, was voor het onderzoek gevoed ofwel met sucrose, ofwel met de vetzuren acetaat, propionaat en butyraat. Om de korrels van het slib dat met sucrose gevoed was bevond zich een dikke buitenlaag van fermentatieve bacteriën, die waarschijnlijk het sucrose omzetten. In de binnenlaag van deze korrel bevonden zich micro-kolonies van syntrofen welke propionaat omzetten en micro-kolonies van acetaat-gebruikende methanogenen, voornamelijk behorende tot de geslacht *Methanosaeta*. Het slib dat met vetzuren was gevoed, had een dunnere bacteriële buitenlaag, meer in, dan rondom de korrel. Daaronder bevond zich een dikke laag van methanogenen, voornamelijk van het geslacht *Methanosaeta,* maar ook micro-kolonies van het geslacht *Methanosarcina*. Onder de tweede laag bevond zich een laag van micro-kolonies van syntrofen en acetaat-gebruikende methanogenen. Het slib uit de reactor die aardappelverwerkings-afvalwater zuivert, bevatte een buitenlaag met een zeer gevariëerd aantal fermentatieve bacteriën en een binnenlaag met kleine micro-kolonies van syntrofen en *Methanosaeta* micro-kolonies. Een opmerkelijk verschil tussen de slib-soorten afkomstig uit de verschillende reactoren was de manier waarop de syntrofe micro-kolonies opgebouwd waren. In het slib uit de UASB-reactor van de suikerfabriek bestonden deze micro-kolonies uit *Syntrophobacter fumaroxidans*-achtige cellen omringd door *Methanobrevibacter*-achtige methanogenen (Hoofdstuk 5). In het slib dat afvalwater van de aardappelverwerkings fabriek, bestonden de micro-kolonies uit SYN7-achtige bacteriën die dicht op elkaar gepakt waren, waar doorheen ketens van *Methanospirillum*-achtige methanogenen groeiden (Hoofdstuk 7).

**Conclusies.**

i Er zijn tenminste drie verschillende groepen syntrofe propionaat-oxiderende bacteriën, welke allen verwant zijn aan sulfaat-reducerende bacteriën. De eerste groep is het geslacht *Syntrophobacter*, dat bestaat uit de soorten *S. wolinii*, *S. pfennigii* en *S. fumaroxidans*. Deze soorten zijn nauw aan elkaar verwant en behoren tot de delta subklasse der Proteo-bacteriën. Deze bacteriën zijn zelf ook sulfaat-reduceerders. De tweede groep is verwant aan het geslacht *Syntrophus* dat ook behoort tot de delta subklasse der Proteo-bacteriën. De derde groep bestaat uit sporevormende, Gram-positieve, syntrofe bacteriën, welke verwant zijn aan *Desulfotomaculum*.

ii Korrelslib afkomstig van verschillende reactoren die elk een ander afvalwater behandelen, bevatten specifieke syntrofe propionaat-oxiderende subpopulaties. Slib afkomstig uit een UASB-reactor waarin suikerfabriek afvalwater wordt gezuiverd, bevatte voornamelijk *S. fumaroxidans* als propionaat-oxideerder; de cellen van deze soort werden omringd door *Methanobrevibacter*-achtige methanogenen. Het slib uit de UASB reactor waarin afvalwater van aardappelverwerkingsfabriek wordt gezuiverd, bevatte SYN7 bacteriën die micro-kolonies vormde waar ketens van *Methanospirillum*-achtige methanogenen doorheen groeiden.

iii De architectuur van korrelslib en de aanwezigheid van bepaalde microbiële subpopulaties hierin, was afhankelijk van de substraten en elektronen-acceptoren die beschikbaar waren in het influent. Fermenterende bacteriën en sulfaat-reducerende acetogenen waren gelokaliseerd in de buitenste laag van de korrel, terwijl de syntrofe consortia en acetaat-verbruikende methanogenen gelokaliseerd waren in de binnenlagen van de korrel.

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...en de lange moeizame weg had een wonderschoon einde...

Hermie.

Curriculum vitae

Hermanus Jozef Martinus (Hermie) Harmsen werd geboren op 5 maart 1965 in Zevenaar. Na de lagere school in Oud-Zevenaar heeft hij het VWO gevolgd op het Liemers College in Zevenaar (1977-1984). Na in 1984 het VWO diploma behaald te hebben, is hij een studie Moleculaire Wetenschappen begonnen aan de Landbouwuniversiteit Wageningen. Zijn afstudeervakken waren moleculaire biologie en bacteriële genetica. Zijn stage heeft hij gevolgd aan het Instituut voor Biochemische Technologie en Microbiologie, Technische Universiteit Wenen, Oostenrijk. In Juni 1990 sloot hij zijn studie af als ingenieur in de Landbouw- en Milieuwetenschappen. In maart 1991 werd hij aangesteld als Assistent-In-Opleiding aan de vakgroep Microbiologie van de Landbouwuniversiteit Wageningen. Tijdens de promotiestudie werd de fylogenie en ecologie van korrelslibbacteriën bestudeerd, wat leidde tot dit proefschrift. Sinds september 1995 is hij werkzaam als Post-Doctoraal medewerker met een beurs van het Centre National de la Recherche Scientifique aan het Station Biologique te Roscoff, Frankrijk. Daar werkt hij aan de microbiële ecologie van maritieme hyperthermale bronnen.