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CHARACTERISTICS OF MICROBIAL COMMUNITIES IN BIOMETHANIZATION PROCESSES

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Abstrakt: Biomethanization of mixed organic substances is the effect of coexistence of numerous groups of microorganisms. Methanogenic degradation of such substances involves at least three different trophic groups of anaerobes, namely fermentative heterotrophs, proton-reducing syntrophs and methanogenic archaea. The development of molecular techniques allowed to detect some new groups of bacteria and archea, which often stay unculturable.
The cultivation of uncultured organisms is of great significance in recognizing the function of these organisms. In the past few years, newly discovered microorganisms have been successfully isolated from anaerobic sludges, and the information regarding their physiology in connection with phylogeny is updated regularly.

Keywords: biomethanization, methanogens, syntrophs

INTRODUCTION

Methanogenesis from complex organic substances is a unique biological reaction catalyzed by mixed populations consisting of diverse arrays of microorganisms (Sekiguchi et al., 2001). Methanogenic degradation of such substances involves at least three different trophic groups of anaerobes, namely fermentative heterotrophs, proton-reducing syntrophs and methanogenic archaea (Schink, 1997).

The complexicity of microflora in anaerobic digestion processes has been known for years, but the development of molecular techniques has made it possible to detect numerous uncultivated microorganisms and to recognize structural and functional relationships between groups of anaerobes.

The cultivation of uncultured organisms is of great significance in recognizing the function of these organisms. In the past few years, newly discovered microorganisms have been successfully isolated from anaerobic sludges, and the information regarding their physiology in connection with phylogeny is updated regularly: examples include carbohydrate degraders (Sekiguchi et al., 2003, Yamada et al., 2006), a protein degrader (Chen and Dong, 2005), fatty acid oxidizers (Imachi et al., 2002; de Bok et al., 2005; Chen et al., 2005; Sekiguchi et al., 2006; Wu et al., 2006 a, b; Zhang et al., 2004,2005), terephthalate oxidizers (Qiu et al., 2006), and methanogens (Jiang et al., 2005; Ma et al., 2005, 2006).

Fermentative heterotrophs

Complex organic molecules are fermented by a variety of fermenting organisms to reduced organic compounds (e.g. lactate, ethanol, propionate and butyrate) and to compounds that can be used by methanogens directly (hydrogen, formate and acetate).

Carbohydrates such as cellulose, starch and pectins are hydrolyzed to monosaccharides mainly as a result of the activity of different *Clostridium* species. Proteins are hydrolyzed to aminoacids by proteases – enzymes excreted by numerous species of anaerobic bacteria such as *Bacteroides*,

Butyrivibrio, *Clostridium*, *Fusobacterium*, *Selenomonas* and *Streptococcus* (Blaszczyk, 2007). Aminoacids are degraded in various pathways to simple fatty acids such as acetic, propionic and butyric acid by different bacteria as *Bacteroides*, *Clostridium*, *Peptococcus*, *Selenomonas* and *Campylobacter*. Various species of *Clostridium* and *Micrococcus* are able to degrade lipids and produce acetylo-CoA.

The significant advance in recognizing of function of abundant microorganisms in anaerobic sludges was the isolation of organisms from subphylum I of the bacterial phylum *Chloroflexi*. The subphylum I was recognized as a clone cluster, members of which were frequently detected in anaerobic environments in abundance (Diaz et al., 2006; Sekiguchi et al., 2001, Yamada et al., 2005).

Recently, four filamentous strains that belong to the *Chloroflexi* subphylum I were successfully isolated and cultured from anaerobic wastewater treatment sludges, and these strains were characterized in detail in order to give them their taxonomic position (Sekiguchi et al., 2003; Yamada et al., 2006). The subphylum I was named as the new class *Anaerolineae* and now contains two thermophilic species of the genus *Anaerolinea*, one mesophilic species of the genus *Levilinea*, and one mesophilic species of the genus *Leptolinea*. These organisms are known to be one of the major populations in mesophilic and thermophilic sludge granules of UASB reactors (Sekiguchi et al., 2001b, Yamada et al., 2005).

One species (*Anaerolinea thermophila*) is associated with filamentous bulking of methanogenic granular sludge (Sekiguchi et al., 2001b). All strains possess filamentous morphotypes, growing fermentatively with a range of carbohydrates and yeast extract as substrates. These biochemical properties suggest that members of the class might play a key role in the primary degradation of carbohydrates and cellular materials (such as amino acids) in methanogenic digestion processes.

The degradation of reduced organic compounds even long chain fatty acids is often connected with hydrogen production by several syntrophic acetogenic bacteria.

Syntrophs

Acetogenic bacteria, also called obligatory hydrogen-producing acetogens (OHPAs), oxidize the reduced organic products further to hydrogen, formate and acetate, and these are ultimately converted to methane and carbon dioxide by methanogens (de Bok et al., 2004).

In some cold, methanogenic environments the acetate is produced by homoacetogenic bacteria – anaerobic, hydrogen-oxidizing autotrophic microorganisms such as *Clostridium aceticum*, *C. thermoaceticum* or numerous species of *Acetobacterium*. At low temperatures homoacetogens, due to their relatively high growth rate and sufficient psychrotolerance, can successfully compete with methanogens for hydrogen in spite of H_2 thresholds being lower in methanogenic archea (<2Pa H_2) than homoacetogens (<200 Pa H_2) (Kotsyurbenko et al., 2001). Nowadays homoacetogenesis is observed in mesophilic (Nie et al., 2007) and thermophilic digesters (Siriwongrungson et al., 2007) and this process is considered as the alternative pathway for enhance of acetate production.

Oxidation of volatile fatty acids is possible in low concentration of hydrogen and redox potential (<-300mV), therefore many acetogenic bacteria form mutual associations with methanogenic archeons. This symbiotic dependence between *Archaea* and syntrophic bacteria is called "interspecies H_2 transfer". Methanogens receiving hydrogen and CO_2 activate acetogenic syntrophs. The acetoclastic and hydrogenotrophic methanogenic archaea contribute 70% and 30%, respectively, of the methane production in industrial wastewater treatment (Sawayama et al., 2006). On the other hand, the major route of methane production is through a syntrophic relationship between acetate-oxidizing bacteria and hydrogen-utilizing methanogenic archaea (Angenent et al., 2002).

Despite the significant importance of syntrophs, the knowledge of their taxonomic positions, diversity and physiology was insufficient, mainly because of the difficulties in isolating them. Several important proton-reducing syntrophic bacteria affiliated with the group '*Desulfotomaculum*

cluster I' as butyrate-oxidizers (Sekiguchi et al., 2000; Jackson et al., 1999), propionate-oxidizers (Imachi et al., 2000; Liu et al., 1999; Harmsen et al., 1998) and even acetate-oxidizers (Hattori et al., 2000) have been successfully isolated and cultured from methanogenic communities in recent years.

Thermacetogenium phaeum, the thermophilic acetate-oxidizing syntroph isolated and characterized by Hattori et al. (2000) oxidized acetate in co-culture with a thermophilic hydrogenotrophic methanogen. In this connection methanogenic bacteria used hydrogen produced by syntrophic acetate-oxidizing bacteria. Moreover, another study showed that some cultures of acetate-oxidizing, iron-reducing microorganisms could oxidize acetate in co-culture with hydrogen-utilizing sulfate- or nitrate-reducers in the absence of oxidized iron (Cord-Ruwisch et al., 1998).

Propionate-oxidizing *Syntrophobacter*-like bacteria have been identified in micro-colonies in close association with methanogens (Grotenhuis et al., 1991). For kinetic reasons the spatial organization of the microorganisms in aggregated biomass is critical: the efficiency of interspecies electron transfer probably increases with the decrease of the distance between cells. Grotenhuis et al. (1991) observed syntrophic propionate-oxidizing bacteria developing rapidly inside the sludge granule in defined microcolonies with methanogenic archaea. Moreover, the methanogenic partner in the sludge granules, *Methanospirillum*- like cells, was interlaced with all the microcolonies in the granules. This is in contrast with *Methanobrevibacter*-like methanogens that are usually juxtaposed to the hydrogen-producing propionate oxidizers.

The first syntrophic propionate-oxidizing bacterium, *Syntrophobacter wolinii*, was enriched from an anaerobic sewage digester (Boone and Bryant, 1980). The organism was obtained in co-culture with a *Desulfovibrio sp.*, which reduced the sulfate in this culture with the reducing equivalents released from the oxidation of propionate. Other syntrophic propionate-oxidizing bacteria have been described since then, but none of those organisms was obtained in pure or defined co-culture until 1993, when phylogenetic analysis revealed a relationship of *S. wolinii* to sulfate-reducing bacteria (Harmsen et al., 1993). Soon thereafter, *Syntrophobacter wolinii* was obtained in pure culture on propionate plus sulfate, while pyruvate (Wallrabenstein et al., 1994) and fumarate (de Bok et al., 2004) were utilized as well.

Several other syntrophic propionate-oxidizing bacteria have been isolated, including two other *Syntrophobacter* species. The *Syntrophobacter* strains form a cluster within the d-subclass of the proteobacteria (de Bok et al., 2004). All of these strains oxidize propionate to acetate, CO_2 and H_2 or formate, but they differ from each other with respect to morphology and additional substrates used. *Syntrophus aciditrophicus*, isolated by Jackson *et al.* (1999), is a universal syntroph oxidizing fatty acids and benzoate. *S. fumaroxidans* was obtained in pure culture on fumarate, which is an intermediate of propionate-degrading pathway (de Bok et al., 2004).

A markedly different organism is *Smithella propionica*, which was isolated by Liu et al. (1999). This organism produces much less acetate from propionate than the *Syntrophobacter* strains, and besides acetate it produces small amounts of butyrate.

While all of the organisms described above are mesophilic, thermophilic propionate-oxidizing bacteria have also been described, and two of these have been obtained in pure culture so far: *Pelotomaculum thermopropionicum* strain SI, and *Desulfotomaculum thermobenzoicum, subsp. thermosyntrophicum* (Imachi et al., 2000; Plugge et al., 2002). Sekiguchi et al. (2000) isolated a thermophilic butyrate-oxidizer capable of oxidizing saturated fatty acids with four to ten carbon atoms.

Pelotomaculum thermopropionicum was first isolated by Imachi et al. (2000, 2002) from a laboratory-scale UASB reactor operated under thermophilic conditions. This microorganism was affiliated with the sulfate-reducing *Desulfotomaculum* group based on 16S rDNA sequences, although the isolate was not able to reduce sulfate. *In situ* hybridization using a 16S rDNA-based oligonucleotide probe revealed that the propionate-oxidizing syntroph was one of the predominating organisms in the studied thermophilic digester sludge and formed microcolonies associated with hydrogenotrophic methanogens.

In co-culture of *P. thermopropionicum* with *Methanothermobacter thermautotrophicus* Ishii et al. (2005) observed flagellum-like filaments of *P. thermopropionicum* coaggregating cells might have a role in keeping the two organisms closely juxtaposed with each other for efficient interspecies hydrogen transfer.

Later, the obligatory syntrophic, propionate-oxidizing bacterium *Pelotomaculum schinkii* (de Bok et al., 2005), a mesophilic, syntrophic propionate-oxidizing strain MGP [Imachi et al., 2006], two mesophilic, syntrophic phthalate-degrading species *Pelotomaculum terephthalicum* and *Pelotomaculum isophthalicum* (Qiu et al., 2004, 2006), and a mesophilic, syntrophic benzoate-oxidizing species *Sporotomaculum syntrophicum* (Qiu et al., 2003) were also isolated in anaerobic sludges. All these syntrophs lack the ability to dissimilatorily reduce of sulfate, although other members of the '*Desulfotomaculum* cluster I' are known to be sulfate reducers.

Methanogens

Numerous methanogens have been isolated and described (Lyimo et al., 2000; Mori et al., 2000; Joulian et al., 2000; Garcia et al., 2000; Lomans et al., 1999). However, the studies mainly based on 16S rDNA cloning analyses suggest that the number of genera of predominant methanogens in biogas reactors is limited to *Methanobacterium*, *Methanothermobacter* (formerly *Methanobacterium* (Wasserfallen et al., 2000), *Methanobrevibacter*, *Methanosarcina*, and *Methanosaeta* (formerly *Methanothrix*) (Fernandez et al., 1999; Godon et al., 1997; Sekiguchi et al., 1998).

Approximately 70% of the methane formed during the UASB process is produced by members of the acetoclastic *Methanosarcina* and *Methanosaeta* species. *Methanosaeta spp.* dominated in large-scale mesophilic and thermophilic digesters treating wastewater and sewage sludge (Raskin et al., 1994; McHugh et al., 2003; Sawayama et al., 2004).

The individual fingerprints suggest that the composition of the different types of wastewaters probably had an influence on the methanogens detected in the granules of sludge. Keyser et al. (2006) reported that *Methanosaeta concilii* was detected in the fingerprints of the winery and brewery granules, while *Methanosaeta thermophila* was found in the fingerprint of the brewery granules. *Methanosaeta*, formerly known as *Methanothrix* (Huser et al., 1982), is one of the main species responsible for the conversion of acetate to methane.

The wide tolerance of *Methanosaeta spp.* for environmental factors (nutrients, temperature) provides for common dominance of this genus in anaerobic digesters. Even low temperatures and low acetate concentrations seem to favor representatives of *Methanosaetaceae* versus *Methanosarcinaceae* (Chin et al., 1999; Kotsyurbenko, 2005). It is very important for the digestion process because of the crucial role of this filamentous organism in the formation and maintenance of stable anaerobic granules (McHugh et al., 2003).

Methanosarcina mazeii was only detected in the fingerprint of the winery granules, while *Methanobacterium formicicum* was detected only in the fingerprint of the brewery granules by Keyser et al. (2006). Lack of hydrogen may limit the growth of *Methanobacterium* strains. It occurs in the presence of the fastergrowing non-methanogens such as homoacetogenic bacteria that also utilize hydrogen as substrate. If the *Methanobacterium* cannot survive in the reactor due to a lack of hydrogen to utilize; their absence can have a negative influence on granulation. *Methanobacterium* plays a role in granulation that is responsible for the production of extracellular polymers necessary in binding other bacteria together to form granules (Hulshoff Pol et al., 2004).

Immobilization of microbial biomass is one of the directions in the intensification of the anaerobic digestion, mainly due to the increase of biomass concentration. After the immobilization of anaerobes on polyurethane foam in a thermophilic, fixed-bed, anaerobic digester supplied with acetate, the results of real-time PCR analysis indicated that the major immobilized methanogenic archaea were *Methanosarcina spp.*, and that the major free-living methanogenic archaea were *Methanosarcina* and *Methanobacterium spp.* (Sawayama et al., 2006). Densities of *Methanosarcina spp.* and *Methanobacterium spp.* immobilized on the polyurethane foam, measured by 16S rRNA gene analysis, were 7.6×10^9 and 2.6×10^8 copies/cm^3, respectively. Immobilized methanogenic

archaea could be concentrated 1000 times relative to those in the original anaerobically digested sludge from a completely mixed thermophilic digester supplied with cattle waste. The concentration of immobilized bacteria could increase only 10 times. This disproportion could restrict the hydrogen transport between syntrophic acetate-oxidizing bacteria and hydrogen-utilizing methanogenic archaea in immobilized biomass. Sawayama et al. (2006) reported that the ratio of *Methanobacterium spp.* and *Methanosarcina spp.* among the free-living methanogenic archaea was higher than that among the immobilized methanogenic archaea.

High concentration of free ammonia nitrogen (FAN) influences the structure of the methanogen community. Acetoclastic species of methanogens seem to be more sensitive to free ammonia than hydrogen utilizing ones. Calli et al. (2005) reported free ammonia inhibition thresholds in acclimated sludge of 700 and 1200 mg N/l for acetoclastic and hydrogenotrophic methanogens, respectively. Since about two-thirds of the methane produced in an anaerobic reactor is derived from acetate, a decrease in the activity of acetotrophic methanogens severely affects the anaerobic degradation process.

During adaptation of sludge, Calli et al. (2005) found first a noticeable loss of the activity of *Methanosaeta*-related species, and afterwards the presence of mainly *Methanosarcina*-like acetoclastic methanogens in all of the reactors studied. Following the increase in FAN level above 150 mg/l, single coccus shaped *Methanosarcina* cells tended to form large multicellular structures. The disintegration of large *Methanosarcina* clusters started at 600 mg/l of FAN. The presence of inorganic particles originating from granular sludge seemed to protect *Methanosarcina* clusters.

Uncultured organisms

The complexicity of microflora in anaerobic digestion processes has been known for years, but the development of molecular techniques has made it possible to detect numerous uncultivated microorganisms and to recognize structural and functional relationships between groups of anaerobes. Molecular analyses primarily based on 16S rDNA cloning and gene sequencing analyses displayed constituents of more than 20 bacterial phyla in anaerobic (mostly methanogenic) waste and wastewater sludges (Narihiro and Sekiguchi, 2007; Sekiguchi et al., 2001). For example, 16S rRNA gene clones that were frequently and commonly retrieved from these sludges were distributed in various prokaryotic taxa such as the phyla *Proteobacteria* (mainly in the class *d-proteobacteria*), *Chloroflexi*, *Firmicutes*, *Spirochaetes* and *Bacteroidetes* in the domain *Bacteria*. Clones in the classes *Methanomicrobia*, *Methanobacteria*, and *Thermoplasmata* are typical phylotypes found in such sludges in the domain *Archaea* (Narihiro and Sekiguchi, 2007). Besides these relatively well known taxa, phylotypes belonging to a variety of uncultured candidate phyla (or classes), known as 'clone clusters', were often detected in anaerobic sludges (Collins et al., 2005; Chouari et al., 2005a, b; Leclerc et al. 2004). Within the domain *Bacteria*, diverse uncultivated taxonomic groups, such as OP10, BA024, OP8, TM6, EM3, OP3, and OS-K (named according to the review by Hugenholtz (2002)), were detected in this medium (Narihiro and Sekiguchi, 2007).

Chouari et al. (2005b) found that the predominant population of clone clusters belonged to candidate bacterial phylum WWE1 (named according to the review by Hugenholtz (2002)), and described that 81% of all the bacterial 16S rRNA gene clones retrieved from an anaerobically digested sludge in mesophilic conditions were assigned with the WWE1 group. These WWE1-type cells were found as rod-like and filamentous morphotypes in fluorescence *in situ* hybridization (FISH) analysis, and the rRNA from WWE1 cells accounted for 12% of the total bacterial rRNA. Their high abundance in the sludge suggests they might play a certain role in the digestion process, although the ecophysiological function of WWE1-type bacteria remains unknown.

Another predominant populations detected in anaerobic sludges belong to clone clusters of the bacterial phylum *Deferribacteres*. This phylum (formerly recognized as the phylum '*Synergistes*') contained phenotypically diverse genera such as *Deferribacter*, *Denitrovorans*, *Geovibrio*, *Flexistipes* and '*Synergistes*' (Narihiro and Sekiguchi, 2007). The 16S rRNA gene clones gained

from methanogenic digester sludges, distantly related to known cultivated species, forming distinct clone clusters at the subphylum (class) levels were assigned to four different subphyla of *Deferribacteres*, none of which contains cultured representatives. Diaz et al. (2006) showed that 34% of all the bacterial clones detected from a full-scale methanogenic UASB process treating brewery wastewater were affiliated with uncultured clades of the phylum *Deferribacteres*. Their high occurrence in such methanogenic ecosystems suggests they might play a role in organic matter degradation for the methanization; however, their ecophysiology remains unknown.

With respect to the uncultured archaeal lineages, Chouari et al. (2005a) obtained several archaeal 16S rRNA gene clones affiliated with the candidate taxon WSA2 from a mesophilic methanogenic digester stabilizing sewage sludge. This group is a clone cluster at the subphylum (or class) level within the archaeal phylum *Euryarchaeota* (Narihiro and Sekiguchi, 2007).

Another unique uncultured archaeal taxon also often found in methanogenic sludges is subphylum C2 of the archaeal phylum *Crenarchaeota*. Chouari et al. (2005b) retrieved 16% of the archaeal rRNA gene clones from a mesophilic methanogenic digester belonged to members of *Crenarchaeota*, particularly the subphylum C2. Such C2-type rRNA gene clones were also detected in a full-scale methanogenic (partially sulfidogenic) UASB process treating paper mill wastewater by Roest et al. (2005) and in anaerobic sludges from a variety of methanogenic wastewater treatment systems by Collins et al. (2005). The abundance of C2-type reached 14–78% of the total archaeal clones analyzed.

Finding dominant populations that belong to such uncultured lineages at various taxonomic levels (from species to phylum levels) is one of the major advances in the microbiology of anaerobic digestion processes in the past few years.

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