Stellingen

1. De doorgaans gebruikte incubatietijd van maximaal 3 maanden bij Most Probable Number (MPN) experimenten kan bij het tellen van anaërobe micro-organismen leiden tot een onderschatting van het aantal micro-organismen in het monster.

   Dit proefschrift.

2. Glutamaatafbraak naar acetaat via de β-methylaspartaat route is niet voorbehouden aan een homogene groep bacteriën in cluster I en II van de *Clostridia*.

   Dit proefschrift.

   Buckel, W. (1980). Analysis of the fermentation pathways of clostridia using double labelled glutamate. Arch. Microbiol. 127, 167-169.

   Buckel, W. and Barker, H.A. (1974). Two pathways of glutamate fermentation by anaerobic bacteria. J. Bacteriol. 117, 1248-1260.

3. Het toekennen van metabole capaciteiten aan microbiële soorten die slechts geïdentificeerd zijn aan de hand van het 16S rDNA, dient met de grootst mogelijke terughoudendheid te geschieden.

   Achenbach, L.A. and Coates, J.D. (2000). Disparity between bacterial phylogeny and physiology. ASM News 66, 714-715.

4. Aangezien *Trichlorobacter thiogenes* phylogenetisch verwant is aan *Geobacter chapellii* had op zijn minst het vermogen ijzer te reduceren onderzocht dienen te worden.

   De Wever, H., Cole, J.R., Fettig, M.R., Hogan, D.A. and Tiedje, J.M. (2000). Reductive dehalogenation of trichloroacetic acid by *Trichlorobacter thiogenes* gen. nov., sp. nov. Appl. Environ. Microbiol. 66, 2297-2301.

5. De Donald Duck heeft een hogere "impact factor" dan Science of Nature.
6. Als het winnen van medailles op Olympische Spelen en andere internationale sportwedstrijden door de publieke opinie zeer belangrijk wordt geacht, moet men de intensiteit van het gymnastiekonderwijs op scholen drastisch opvoeren.
7. De oorsprong van de Impressionistische schilderkunst zou kunnen liggen in de bijziendheid van een schilder.
8. Mist en andere slechte weersomstandigheden veroorzaken geen verkeersslachtoffers; de auto doet dat.

Stellingen behorende bij het proefschrift:
"Syntrophic degradation of amino acids by thermophilic methanogenic consortia" van Caroline Plugge.
Wageningen, 11 mei 2001.

Syntrophic Degradation of Amino Acids by Thermophilic Methanogenic Consortia

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Syntrophic Degradation of Amino Acids by Thermophilic Methanogenic Consortia

Caroline Plugge

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
prof. dr. ir. L. Speelman,
in het openbaar te verdedigen
op vrijdag 11 mei 2001
des namiddags te half twee in de Aula

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General Introduction

1) Anaerobic mineralisation of organic matter under methanogenic conditions.

In the absence of inorganic external electron acceptors the mineralisation of complex organic compounds occurs via a cascade of reactions ultimately leading to CH_4 and CO_2. In the sequence of redox reactions, CO_2 is the ultimate terminal electron acceptor to form CH_4. Methanogenic environments are widely distributed in nature. Wetlands, fresh water sediments (swamps and rice paddies), digestive tracts of ruminants and insects are environments that give rise to high amounts of methane. Man-made systems, such as anaerobic bioreactors and landfills are other sources of methane production.

Anaerobic bioreactors are commonly applied world-wide to treat high strength (industrial) wastewater, such as wastewater from sugar factories, slaughter houses, beer breweries, paper mills, potato starch factories and other types of waste. In some reactor types the hydraulic retention time is uncoupled from the biomass retention time. In fluidised and fixed bed reactors the biomass is immobilised on solid surfaces, whereas in upflow anaerobic sludge bed (UASB) reactors and expanded granular sludge bed (EGSB) reactors the biomass is formed by self-immobilisation. The mechanism of this microbial self-immobilisation is complex and has been studied by many researchers (Lettinga et al. 1980; Wiegant 1986; Isa et al. 1986; Dolfing 1987; Hulshoff Pol 1989; Grotenhuis 1992). Reactor design, ionic strength and composition of the influent wastewater, hydrophobicity of the microorganisms and the presence of inorganic salts are all factors involved in the granulation process. This self-immobilisation process yields methanogenic granular sludge in which the microorganisms are very densely packed. The sedimentation of the granules prevents the granules from being washed out of the reactors. The UASB reactor is applied world-wide with great success. Over a 1000 full-scale UASB reactors are operative.

Most methanogenic bioreactors are operating at mesophilic temperatures of 30 - 37^0C, but there are also examples of reactors that are operated at moderately thermophilic conditions (50 - 65^0C). The choice for thermophilic conditions can be dependent on several factors. In tropical areas, where ambient temperatures are around 30^0C and sanitary conditions are poor, thermophilic conditions can be chosen to kill pathogenic organisms. Especially when manure and household waste is treated in these reactors the growth of pathogenic organisms is likely to occur under mesophilic conditions. The die-off rate of *E. coli*, an indicator organism, occurs via a first-order process (Catunda et al. 1994): $dN/dt = -K_b \cdot N$, where N is the decimation and K_b the die-

off constant. K_b increases with increasing temperatures. The contact period in the reactor to kill pathogens can be reduced from several weeks under mesophilic conditions to several hours under thermophilic conditions (Bendixen 1994). Furthermore, at elevated temperatures the overall process is faster, enabling a higher loading rate of the reactor. The main application of thermophilic treatment is when industrial wastewater produced at elevated temperatures has to be cleaned. Examples are waste waters produced from pulp and paper industries, alcohol distilleries, palm oil production plants and canneries.

For the complete conversion of organic matter three physiological groups of microorganisms are involved (Figure 1). One metabolic type of organism, in theory, might be capable of complete oxidation of complex carbon to CO_2 and H_2O. However, in anaerobic methanogenic environments such organisms have never been found. The first group in the food chain consists of a wide variety of fermentative bacteria. They excrete hydrolytic enzymes that degrade biopolymers, such as polysaccharides, nucleic

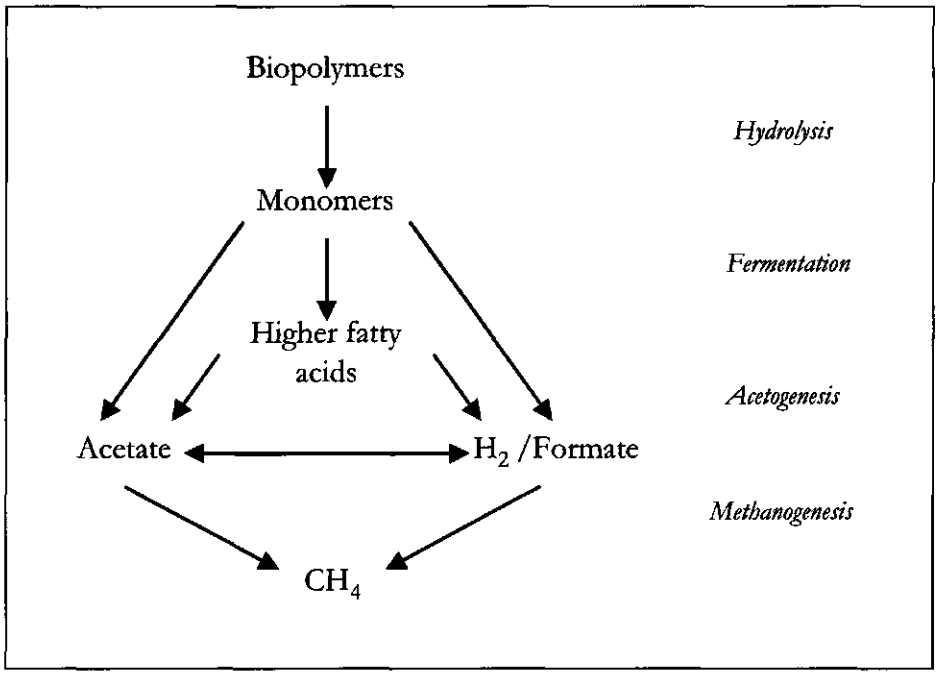


Figure 1. Reaction scheme for the anaerobic degradation of organic matter to methane (adapted after Gujer and Zehnder 1983).

acids, lipids and proteins to their corresponding monomeric substances. Subsequently, these compounds are fermented to reduced organic compounds (short chain fatty acids, alcohols, lactate and alanine), and to compounds that can be used directly by methanogenic archaea (acetate, formate, CO_2 and H_2). The reduced compounds are further oxidised to acetate, formate, CO_2 and H_2 by acetogenic bacteria, the second trophic group. Ultimately, the third group, which consists of methanogenic archaea, metabolises the acetate, formate and CO_2 plus H_2 to form CH_4. These three groups of bacteria have strong metabolic interactions; the H_2 removal by the methanogens enables a faster metabolism of the fermentative and acetogenic bacteria. Especially the acetogenic bacteria are strongly dependent on a low hydrogen partial pressure to catabolise their substrates.

The most important organic substrates in anaerobic wastewater are carbohydrates, proteins, nucleic acids and lipids. The degradation of lipids and proteins in methanogenic environments has been little studied, especially when compared with the degradation of carbohydrates. The role of interspecies of H_2 transfer between bacteria degrading proteins and lipids and methanogenic archaea has received little attention.

2) Syntrophic conversions.

The first example of a syntrophic conversion was described by Bryant et al. (1967). They discovered that the previously isolated pure culture of an ethanol-oxidising methanogen *Methanobacterium omelianskii* (Stadtman and Barker 1949) consisted of two microorganisms: one organism termed the "S-organism", which oxidised ethanol to acetate and H_2 and another organism, *Methanobacterium bryantii*, that converted H_2 and CO_2 to CH_4. The "S-organism" was found to be dependent on the presence of *M. bryantii* for its growth. The dependency can be explained using the changes in Gibbs' free energy ($\Delta G^{0'}$) for the oxidation of ethanol to H_2, CO_2 and acetate. Under standard conditions (gases at 10^5 Pa pressure, 1 M of products/substrates, a pH of 7 and 298K) this value is positive (+9.6 kJ/reaction), indicating that the reaction can not take place, but it becomes negative when the pH_2 (hydrogen partial pressure) is low. This first example of interspecies hydrogen increased our knowledge on the degradation of organic matter under methanogenic conditions. The majority of the acetogens in the anaerobic food web, that oxidise e.g. butyrate, propionate or ethanol, can not grow without the presence of a methanogen, or any other hydrogen scavenger, which keeps

the pH_2 at a low level (10^{-3} to 10^{-5} atm.). These acetogens are obligate syntrophic organisms. Cocultivation of fermentative hydrogen-producing bacteria with methanogens often results in a shift in product formation. Several examples are known to illustrate this phenomenon (Ianotti et al 1973; Weimer and Zeikus 1977). When the fermentative bacteria *Clostridium thermocellum* or *Ruminococcus albus* are cultivated in pure culture on glucose acetate, hydrogen and reduced end products (lactate and ethanol) are formed. At a high hydrogen partial pressure, the bacteria are no longer able to form hydrogen through NADH or ferredoxin oxidation. These components are common redox mediators in these bacteria. The redox couples NAD/NADH and $Fd_{(ox)}/Fd_{(red)}$ are –320 mV and –398 mV, respectively. The redox couple H^+/H_2, however, is lower with –414 mV. This means that the electron transfer from the electron mediators to protons is energetically unfavourable. However, when the hydrogen partial pressure is kept low, the reactions can occur. The Gibbs' free energy change of the redox reaction can illustrate this. Calculation of Gibbs' free energy change can be done with the following equation:

$$\Delta G' = \Delta G^{0'} + RT \ln([\text{products}]/[\text{reactants}]) \qquad (1)$$

$\Delta G'$ is the change in Gibbs' free energy under conditions, where substrate and product concentrations do not equal 1 M or 1 atmosphere. $\Delta G^{0'}$ is the change in Gibbs' free energy under standard conditions (T = 298 K; 1 M solutes; 1 atm. gases; pH = 7) and R is the gas constant (8.3144 kJ * mol^{-1}* K^{-1}). $\Delta G^{0'}$ can be calculated from the equation

$$\Delta G^{0'} = -n * F * \Delta E^{0'} \qquad (2)$$

where n is the number of electron transferred; F is the Faraday constant and $\Delta E^{0'}$ is the difference between the redox couples. In the case of Fd and NADH oxidation coupled to proton reduction the reactions are as follows:

$$2\ Fd\ (red) + 2\ H^+ \rightarrow 2\ Fd\ (ox) + H_2 \qquad \Delta G^{0'} = +3.1\ kJ/\ mole \qquad (3)$$

$$NADH + H^+ \rightarrow NAD^+ + H_2 \qquad \Delta G^{0'} = +18.1\ kJ/mole \qquad (4)$$

From these reactions it becomes clear that only at hydrogen partial pressures, which are lower than 10^{-1} atm. the ferredoxin oxidation and 10^{-3} atm. the NADH oxidation become possible. The reducing equivalents formed by *Ruminococcus albus* growing on glucose are disposed directly upon acetyl-CoA or pyruvate to form ethanol and lactate, respectively. When these bacteria are cultivated in the presence of hydrogen-consuming organisms, less of these reduced products is formed, because ferredoxin and NADH oxidation can proceed. Consequently, more acetate and coupled to this, more ATP is formed.

An important factor in syntrophic interactions is the distance between the hydrogen producing and the hydrogen-consuming organism. Schink and Thauer (1988) describe that the diffusion distances for the transfer of metabolites should be as short as possible. The diffusion of hydrogen from producer to consumer can be described by a simple equation: $Flux_{H_2} = -A*D*(c_2 - c_1)* d^{-1}$ mol*sec^{-1} (A is the surface area of the hydrogen producer ($4\pi r^2$), D is the diffusion constant for hydrogen ($4.9*10^{-5}$ cm^2*sec^{-1} at 298 K, c is the concentration of hydrogen in water and d is the distance between producer and consumer). Aggregation of bacteria from different metabolic groups enables a faster degradation of organic material. In granular sludge containing bioreactors the extreme high cell densities and the short interbacterial distances can explain the high rates of methane formation.

3) Anaerobic metabolism of amino acids.

Much of our knowledge of anaerobic protein and amino acid degradation has been obtained through studies on ruminants, since protein is an important dietary product for ruminants (Allison 1970; Bryant 1977; Hobson and Wallace 1982). Proteins in the rumen are hydrolysed by extracellular proteases and intracellular peptidases (Hazlewood and Nugent 1978) to single amino acids, peptides and ammonia. The input of proteins into anaerobic digesters can be large. The amount of protein of various wastewaters, used as substrates for the digesters, varies from 10 - 90% of the total carbon (McInerney 1988). The degradation of protein in anaerobic digesters is probably similar to that found in the rumen. Many mesophilic anaerobic bacteria are known to hydrolyse proteins (Buchanan and Gibbons 1975). Although this proteolytic activity is a common trait of mesophiles, very few thermophilic anaerobic proteolytic bacteria have been characterised. In the past 15 years four novel genera have been described, namely

Thermobrachium (Engle et al. 1996), *Anaerobranca* (Engle et al. 1995), *Coprothermobacter* (Ollivier et al 1985; Kersters et al. 1994; Etchebehere et al. 1998) and *Caloramator* (Tarlera et al. 1997, see section 4.3) which are capable of hydrolysing different proteins under strict anaerobic conditions.

The degradation of mixtures of amino acids or single amino acids can be performed by many fermentative organisms. Usually the first step in the degradation of amino acids is a deamination (Barker 1981; McInerney 1988; Andreesen et al. 1989). This reaction can be performed in three ways by anaerobic bacteria (Figure 2). Oxidative deamination, which occurs via transaminases or dehydrogenases, results in the production of the corresponding keto acid, ammonia and reducing equivalents. The second mechanism is a reductive deamination. This mechanism is found only in anaerobes. Reducing equivalents are used to convert the amino acid to its corresponding fatty acid, with the concomitant production of ammonia. The third mechanism, a redox-neutral reaction, is an α,β-elimination, resulting in the production of the corresponding keto acid.

The Stickland reaction is an important mechanism of anaerobes to convert a mixture of amino acids. It is a coupled oxidation-reduction reaction, which was first observed by Stickland (1934). Several Clostridia and some other fermentative anaerobes can perform this reaction (Seto 1980; McInerney 1988). An example of the Stickland reaction is the oxidation of valine to isobutyrate, bicarbonate, ammonia and hydrogen coupled to the reduction of glycine to acetate and ammonium:

Oxidation: valine + 3 H_2O → isobutyrate$^-$ + HCO_3^- + NH_4^+ + H^+ + 4 [H]

Reduction: 2 glycine + 4 [H] → 2 acetate$^-$ + 2 NH_4^+ + 2 H^+

Overall:

valine + 2 glycine + 3 H_2O → isobutyrate$^-$ + 2 acetate$^-$ + HCO_3^- + 3 NH_4^+ + 3 H^+

The amino acids alanine, histidine, isoleucine, leucine, serine and valine can be used as electron donor and arginine, glycine, proline and tryptophan can serve as electron acceptors in the Stickland reaction (Seto 1980; Andreesen et al. 1989). The aromatic amino acids and leucine can serve as oxidants as well as reductants. Several proteolytic clostridia can carry out the Stickland reaction with leucine alone. Reduction of the leucine leads to 4-methylvalerate formation and oxidation leads to 3-methylbutyrate

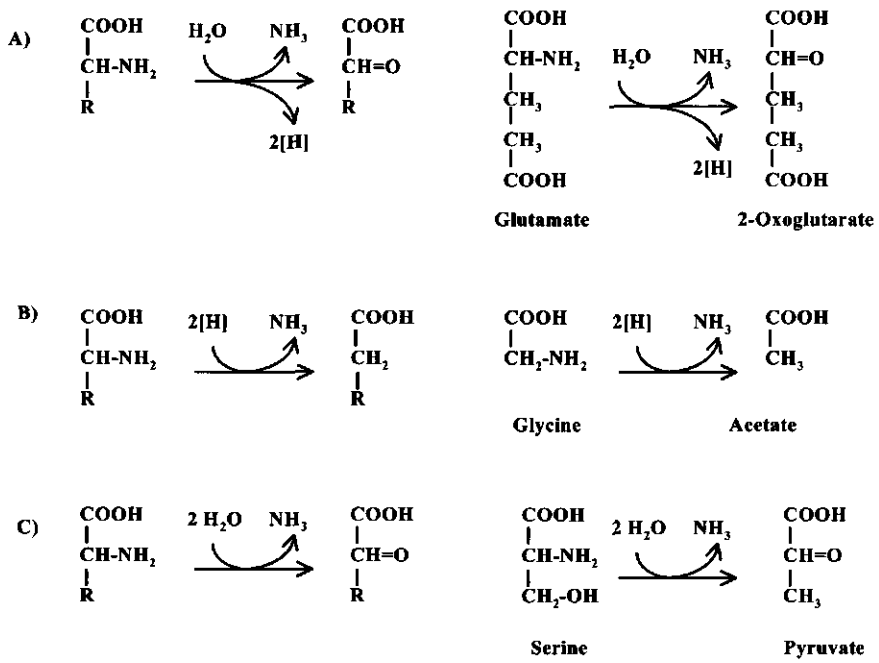


Figure 2. Three examples of amino acid conversion to illustrate the mechanisms of deamination. A) oxidative deamination of glutamate; B) reductive deamination of glycine; C) redox-neutral deamination of serine.

formation. Other mechanisms that can occur in amino acid degradation, such as vitamin B_{12} dependent carbon-carbon rearrangements or decarboxylation prior to deamination will be discussed later in this chapter in connection with glutamate degradation pathways (section 4.4).

4) Anaerobic metabolism of glutamate.

4.1) Glutamate fermentation.

In this thesis the catabolism of moderate thermophilic glutamate-degrading organisms was studied. Emphasis was given on the degradation of glutamate by syntrophic consortia. Below a short overview is given about what has been reported in the literature on this topic. Table 1 gives an overview of glutamate-degrading microorganisms that are discussed in this whole chapter.

Under anaerobic conditions glutamate can be metabolized in different ways. Glutamate is fermented by a variety of *Clostridium*, *Fusobacterium* and *Peptococcus* species into acetate, butyrate, NH_4^+, CO_2, and H_2. Glutamate can also be fermented by a few homoacetogenic *Sporomusa* species yielding only acetate (Dehning et al. 1989). In *Anaeromusa (Selenomonas) acidaminophila*, glutamate is converted to acetate and propionate, where propionate is formed reductively through the succinate pathway (Nanninga et al. 1987). A few sulphate reducing bacteria have been reported to convert glutamate with the production of acetate and sulphide (Baena et al. 1998b; Rees et al. 1998). A special group is formed by those organisms that dispose reducing equivalents exclusively as molecular hydrogen. Interspecies hydrogen transfer affects the metabolism of these organisms. In Table 2 reactions involved in the anaerobic glutamate degradation are listed.

4.2) Syntrophic glutamate degradation.

The importance of syntrophic associations in the degradation of amino acids has received little attention in the past, since isolation and enrichment techniques were used which selected for relatively fast-growing bacteria. Nagase and Matsuo (1982) were the first to show metabolic interactions between amino acid-degrading bacteria and hydrogenotrophic methanogens. Several anaerobic bacteria degrading amino acids have been described in the last 20 years the growth of which is dependent on hydrogen removal (Stams and Hansen 1984; Nanninga and Gottschal 1985; Wildenauer and Winter 1986; Zindel et al. 1988; Cheng et al. 1992; Örlygsson 1994; Baena et al. 1999a, 2000). Bacteria that grow syntrophically grow slower than fermentative bacteria on the same substrate (Nanninga et al. 1986). Moreover, acetogens from syntrophic cultures are usually not able to grow on agar plates or in agar shakes and therefore have to be purified in dilution series using liquid media (Stams et al. 1994; Wallrabenstein et al. 1995). This requires patience and accurate labour. Reported maximum specific growth rates of syntrophic amino acid degrading consortia are lower than those of other amino acid fermenting bacteria. The μ_{max} of *Clostridium* sp. growing on glutamate is 0.3-0.6 h^{-1} (Laanbroek et al. 1979) and that of *Anaeromusa acidaminophila* is 0.13 h^{-1} (Nanninga et al. 1987). These bacteria convert the glutamate to acetate and butyrate and acetate and propionate, respectively. However, the μ_{max} of *Acidaminobacter hydrogenoformans* growing on glutamate in syntrophic association with a hydrogen scavenger is

Table 1. An overview of glutamate-degrading anaerobes discussed in this chapter

Species	*Origin*	*Products from Glutamate*[*)]	*Products from glutamate coculture*	*Temperature optimum (^{0}C)*	*Saccharo-lytic*	*References*
Acidaminococcus fermentans		A, B, (H)		37	Yes	Rogosa 1969; Buckel and Barker 1974
Aminobacterium colombiense	Anaerobic lagoon	No growth	(A), P, H	37	No	Baena et al. 1998a
Aminobacterium mobile	Anaerobic lagoon	No growth	(A), P, H	37	No	Baena et al. 2000
Acidaminobacter hydrogenoformans	Anaerobic marine black mud	A, (P), H	A, P, H	30	No	Stams and Hansen 1984
Aminomonas paucivorans	Anaerobic lagoon	A, (P), H	(A), P, H	37	No	Baena et al. 1999a
Anaeromusa acidaminophila	Anaerobic digestor	A, P		35	No	Nanninga et al. 1987; Baena et al. 1999b
Clostridium tetanomorphum		A, B, (H)		30-37	Yes	Barker 1961; Buckel and Barker 1974
Desulfovibrio aminophilus	Anaerobic lagoon	Pglut[**)], A, HS^-		35	No	Baena et al. 1998b
Desulfobacterium vacuolatum		HS^-		37	No	Rees et al. 1998
Fusobacterium ssp.	Oral cavity; GI Tract	A, B, (H)		37	Yes	Gharbia and Shah 1991
Sporomusa malonica	Anoxic mud	A		28 - 32	Yes	Dehning et al. 1989

Table 1. Continued

Species	*Origin*	*Products from glutamate*	*Products from glutamate coculture*	*Temperature optimum (^{0}C)*	*Saccharo-lytic*	*References*
Tindallia magadii	Soda lake	n.m.***	n.m.	37	Yes	Kevbrin et al. 1997
Caloramator proteoclasticus	Methanogenic sludge	A, Al, H	A, H	55	Yes	Tarlera et al. 1997
Thermanaerovibrio acidaminovorans	Methanogenic sludge	A, (P), H	A, P, H	55	Yes	Cheng et al. 1992; Baena et al. 1999b

*) NH_4^+ and bicarbonate are always formed from glutamate degradation. A, acetate; P, propionate; B, butyrate; Al, alanine; H, hydrogen;, Pglut, pyroglutamate; HS^-, sulfide. Products in parentheses represent minor products.

**) Pyroglutamate formed in the absence and acetate plus HS^- formed in the presence of sulphate

***) n.m. not measured

only 0.10 h^{-1} (Stams and Hansen 1984). The growth rate of *Acidaminobacter hydrogenoformans* in pure culture on glutamate is even lower. The μ_{max} of a *Campylobacter* sp. growing on aspartate is 0.15 h^{-1} (Laanbroek et al. 1978), whilst the μ_{max} of *E. acidaminophilum* in coculture with a methanogen is below 0.1 h^{-1} (Zindel et al. 1988). Nevertheless high numbers of bacteria can be counted which grow syntrophically with methanogens in environments like methanogenic granular sludge. This phenomenon can be explained in different ways. Firstly, the syntrophic consortia might have a higher affinity for the substrate, resulting in higher growth rates of the consortia at low substrate concentration. In densely packed granules the substrate availability, especially in the centre of the granule, can be very low due to diffusion limitation (Grotenhuis et al. 1986). Secondly, the syntrophic consortia could grow on mixtures of substrates rather than on single substrates. In the past, fatty acid-degrading syntrophs were described to be very specialised in their substrate utilisation. However, more recent reports show that syntrophs are much more versatile than thought before (Wallrabenstein et al. 1994; Harmsen et al. 1998). Thirdly, due to inappropriate cultivation conditions, in situ growth rates of the consortia could be higher than the ones measured. The reported growth rates were measured in suspended cultures, whilst in densely packed methanogenic granules the growth rates might be higher because of the short distances between the bacteria (Schink and Thauer 1986). The rate-limiting step might be the transport of the hydrogen from producer to consumer.

The overall conversion of glutamate to CH_4, NH_4^+ and CO_2 according to the equations given in Table 2 is an energy yielding process. It yields under standard conditions 131.8 kJ per mole glutamate, when glutamate is converted via reactions 4, 7 and 8 (Table 2). However, the amount of energy formed in the overall reaction has to feed three different trophic groups: the glutamate-degrading acetogen, the acetoclastic methanogen and the hydrogenotrophic methanogen. The question arises how the glutamate-fermenting bacterium manages its energy metabolism, this especially since the oxidation of glutamate involves several highly endergonic reaction steps.

From Table 2 it also becomes clear that not only the hydrogen partial pressure has an effect on the energy released from glutamate degradation, but also the

Table 2. Some reactions involved in the methanogenic degradation of glutamate and the Gibbs' free energy changes[a)]

Reaction	ΔG^{0}' (25°C)	ΔG' [b)] (55°C)	ΔG' (25°C)	ΔG' (55°C)
$Glutamate^- + 2\ H_2O \rightarrow 2^1/_4\ acetate^- + {}^1/_2\ HCO_3^- + {}^3/_4\ H^+ + NH_4^+$	-59.9	-64.0	-59.9	-64.0
$Glutamate^- + 2\ H_2O \rightarrow acetate^- + HCO_3^- + 0.5\ H^+ + NH_4^+ + 0.5\ butyrate^-$	-57.9	-61.1	-57.9	-61.1
$Glutamate^- + 2\ H_2O \rightarrow 1^2/_3\ acetate^- + {}^1/_3\ propionate^- + {}^2/_3\ HCO_3^- + {}^2/_3\ H^+ + NH_4^+$	-60.5	-69.0	-60.5	-69.0
$Glutamate^- + 3\ H_2O \rightarrow 2\ acetate^- + HCO_3^- + H^+ + NH_4^+ + H_2$	-33.9	-41.6	-62.4	-73.1
$Glutamate^- + 4\ H_2O \rightarrow propionate^- + 2\ HCO_3^- + H^+ + NH_4 + 2\ H_2$	-5.8	-16.0	-62.8	-79.0
$Glutamate^- + 7\ H_2O \rightarrow acetate^- + 3\ HCO_3^- + 2\ H^+ + NH_4^+ + 5\ H_2$	+70.0	+47.9	-72.5	-109.6
$Acetate^- + H_2O \rightarrow CH_4 + HCO_3^-$	-32.0	-35.8	-32.0	-35.8
$4\ H_2 + HCO_3^- \rightarrow CH_4 + H_2O$	-135.6	-125.0	-21.6	+1.0
$Propionate^- + 3\ H_2O \rightarrow acetate^- + HCO_3^- + H^+ + 3\ H_2$	+76.5	+64.7	-9.0	-29.8

a) Energy changes were calculated using the van 't Hoff equation, standard enthalpy values of compounds (Chang 1977; Hanselmann 1984) and Gibbs' free energy changes at 298°K (Thauer et al. 1977)

b) ΔG' calculated with $pH_2 = 10^{-5}$ atm.

temperature at which the conversions occur. In many cases the thermophilic methanogenic archaea, serving as hydrogen scavengers in syntrophic consortia, grow faster than their mesophilic counterparts (Muralidharan et al. 1997) and also exceed the growth rate of the hydrogen-producing organism. *Methanococcus maripaludis*, *Methanobacterium thermoautotrophicum* THF and *Methanococcus jannaschii* are representatives of mesophilic, moderate thermophilic and hyperthermophilic methanogens (Jones et al. 1983a; Jones et al. 1983b; Zinder and Koch 1984). Their respective doubling times are 120, 80 and 25 minutes. Since hydrogen production and consumption are growth rate associated, the dynamics of interspecies hydrogen transfer may be different under thermophilic conditions than at mesophilic temperatures.

4.3) Syntrophic glutamate converting organisms.

Besides the previously mentioned glutamate-degrading organisms a few anaerobic bacteria are known which can grow on glutamate and dispose reducing equivalents as hydrogen. In general these organisms show a shift in their metabolism when they grow on glutamate in the presence of a methanogenic archaeon and/or they grow faster.

Acidaminobacter hydrogenoformans is a non-sporulating asaccharolytic amino acid fermenting organism (Stams and Hansen 1984). Glutamate is converted in pure culture to mainly acetate, bicarbonate, formate, NH_4^+ and traces of propionate. A remarkable shift in the product formation is observed when this organism is grown in the presence of a hydrogen scavenger. While acetate is still the major organic end product, large amounts of propionate are formed, besides the usual other products. Besides glutamate, *Ac. hydrogenoformans* can utilise several other amino acids and organic acids when grown in coculture with a hydrogenotrophic organism. In pure culture only a limited number of these substrates can be fermented.

Aminomonas paucivorans is a mesophilic glutamate-degrading anaerobe, which can use a limited number of substrates (Baena et al. 1999a). Besides glutamate, it is only capable of using arginine, histidine, threonine and glycine. In pure culture these substrates are mainly converted to acetate, NH_4^+ and in some cases formate and hydrogen is formed. When glutamate is converted also some propionate is formed. *Am. paucivorans* shows a shift in product formation when co-cultivated on arginine, histidine and glutamate with the hydrogen and formate scavenger *Methanobacterium formicicum.*

Relatively more propionate is formed. This trait is similar to *Acidaminobacter hydrogenoformans*. Interestingly, the metabolism of the pure culture of *Am. paucivorans* is only slightly inhibited by hydrogen in the atmosphere. Large amounts of formate are formed, when growing on histidine and glutamate under a hydrogen atmosphere. This indicates the presence of an active formate dehydrogenase. Experimental data on the biochemistry of *Am. paucivorans*, however, are lacking to confirm this.

Aminobacterium colombiense is an amino acid-degrading organism unable to grow on glutamate in pure culture (Baena et al. 1998a). However in coculture with *M. formicicum* it is capable of glutamate degradation. Glutamate is converted to acetate and propionate. It is unclear why *Ab. colombiensis* is unable to grow on glutamate in pure culture. It might be that the energetic barrier to convert glutamate to α-ketoglutarate is too high. Under standard conditions it costs +59.9 kJ/mole glutamate to overcome this step.

Aminobacterium mobile, an other mesophilic anaerobic asaccharolytic amino acid-utilising bacterium is phylogenetically related to *Am. colombiense* (Baena et al. 2000). Its metabolism is strongly affected by the presence of a methanogen. Glutamate can only be degraded in the presence of *M. formicicum*, yielding acetate, propionate, NH_4^+ and CH_4. However, after 21 days of incubation at 37^0C only part of the added glutamate (about 40%) was degraded. *Ab. mobile* is specialised in serine conversion. With this substrate, an unusual fermentation pattern was found. In pure culture serine is converted to acetate, alanine NH_4^+ and traces of hydrogen but in the presence of *Methanobacterium formicicum* serine is converted almost exclusively to acetate, NH_4^+ and CH_4. The formation of alanine by *Ab. mobile* is similar to that of *Pyrococcus furiosus* (Kengen and Stams 1995) where alanine is formed as a reduced end product during sugar and pyruvate fermentation.

Tindallia magadii is an anaerobic alkaliphilic bacterium isolated from a soda lake deposit in Kenya (Kevbrin et al. 1998). It is capable of using a limited number of amino acids and some organic acids for growth. It is highly specialised in arginine and ornithine conversion. This might suggest that the organism is strongly adapted to the presence of proteinaceous deposits in the soda lake. Most probably cyanophycine, a storage compound from cyanobacteria with many ornithine moieties, is released in the environment providing the carbon and energy source for *Td. magadii*. In pure culture the organism shows poor growth on glutamate, with the production of acetate, propionate,

NH_4^+ and traces of hydrogen. Based on the formation of hydrogen it can be speculated that *T. magadii* would grow better on glutamate when a hydrogen-consuming organism is present. However, such experiments have not yet been performed.

Only a limited number of thermophilic anaerobic syntrophic glutamate-degrading organisms are described to date. In general these organisms are versatile in their metabolism. No asaccharolytic thermophilic glutamate-degrading organisms are known. *Thermanaerovibrio acidaminovorans* (formerly known as *Selenomonas acidaminovorans*) is a versatile thermophilic proton-reducing anaerobe (Cheng et al., 1992; Baena et al., 1999b). It can ferment a number of amino acids, including glutamate, to acetate, propionate, NH_4^+ and hydrogen. In coculture with *Methanobacterium thermoautotrophicum* ΔH the acetate to propionate ratio shifted in favour of propionate formation. A remarkable feature of *T. acidaminovorans* is its ability to ferment arginine in pure culture, yielding ornithine and citrulline as the only products. This property is also found in various lactic acid bacteria (Poolman et al. 1987). The arginine is metabolised via the arginine deiminase (ADI) pathway. There is one energetically difficult step is this pathway: the phosphorolysis of citrulline yielding ornithine and carbamylphosphate. The $\Delta G^{0'}$ of this reaction, catalysed by ornithine carbamyltransferase, is +28.5 kJ/mole. One ATP is formed from the conversion of carbamylphosphate into carbon dioxide plus ammonium. When *T. acidaminovorans* is cultured in the presence of *M. thermoautotrophicum* arginine is converted to acetate, propionate, ammonium and CH_4. Although there is no influence of the hydrogen partial pressure in the phosphorylysis of citrulline, the conversion of ornithine is influenced by the presence of a methanogen.

Caloramator proteoclasticus is a thermophilic proteolytic and saccharolytic bacterium (Tarlera et al. 1997; Tarlera and Stams 1999). It grows on various proteinaceous compounds and produces hydrogen and different organic compounds. In coculture with *Methanobacterium thermoautotrophicum* Z245 the proteolytic activities are 3 times higher as compared to growth in the pure culture. In monoculture, glutamate is completely degraded to acetate, formate, hydrogen, NH_4^+ and alanine. This organism also forms alanine as a reduced end product. When *C. proteoclasticus* is cocultivated with a methanogen glutamate is completely converted to acetate, hydrogen and NH_4^+, while no alanine is formed. Besides *C. proteoclasticus* only a few other thermophilic

proteolytic bacteria have been isolated and described (Kersters et al. 1994; Engle et al. 1996; Etchebehere et al. 1998).

A thermophilic propionate-oxidising culture TPO was described by Stams et al. (1992). It is capable of converting glutamate into one acetate and CH_4. It is not yet clear how the glutamate is converted to acetate, neither is it sure whether the conversion is carried out by one organism.

During the past 15 years research on hyperthermophilic microorganisms has led to the description of many new genera and species. In their hot biotopes these hyperthermophiles are reported to be capable of growth on many complex substrates, such as proteins, polysaccharides etc. However, a detailed description of the physiological characteristics with respect to the use of amino acids as single substrates is often lacking. To date, only a few members of the genus *Thermococcus* have been described to be capable of converting a mixture of amino acids (Dirmeier et al. 1998). Growth on single amino acids has never been reported. The growth of *Tc. acidaminovorans* on a mixture of amino acids was independent of the hydrogen pressure. This can be explained by the fact that with mixtures of amino acids oxidative and reductive conversions occur simultaneously (see section 3).

4.4) Glutamate fermentation pathways.

The degradation of glutamate under methanogenic conditions can be performed via different pathways. Buckel and Barker (1974) were the first to completely unravel two pathways, the β-methylaspartate and the hydroxyglutarate used by different bacteria. Hitherto, seven pathways have been described.

The following pathways will be discussed: β-methylaspartate, hydroxyglutarate, citric acid cycle, reversed citric acid cycle, methylmalonyl-CoA pathway, direct oxidation via methylmalonyl-CoA and two versions of the aminobutyrate pathway. The pathways can be distinguished experimentally by clarifying certain characteristics such as the presence of key enzymes, the stoichiometry of the conversion and the position of labelled carbon atoms from glutamate in end products.

The pathways are schematically depicted in Figures 3.1-7 and 4.1-2. In the illustrations only acetate or propionate formation is shown, although butyrate can also be formed directly from crotonyl-CoA or by condensation of two acetate moieties.

Furthermore the enzymes of each route which can be used to discriminate between the different pathways are indicated in the figures.

4.4.1) β-Methylaspartate pathway.

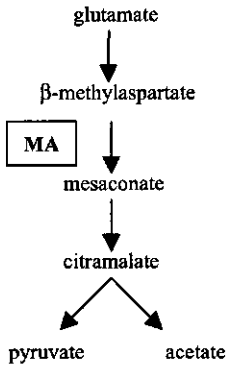
The β-methylaspartate pathway, sometimes indicated as the mesaconate pathway, was the first glutamate pathway described in detail by studies with strain *Clostridium tetanomorphum* (Barker et al. 1959; Barker et al. 1964; Blair and Barker 1966; Wang and Barker 1969; Buckel and Barker 1974). The conversion of glutamate to methylaspartate is mediated by coenzyme B_{12} (adenosylcobalamin) and results in the formation of a branched carbon chain. In the subsequent conversion ammonia is released leading to the formation of mesaconate. Mesaconate is converted to citramalate, which is cleaved to acetate and pyruvate. Acetate is always formed as an end product, but depending on the organisms biochemical capacities, pyruvate can also be converted to form butyrate, propionate or acetate.

4.4.2) Hydroxyglutarate pathway.

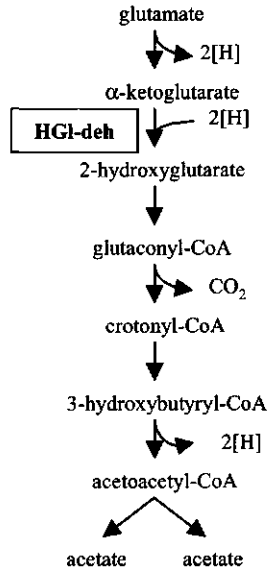
Buckel and Barker (1974) proposed the hydroxyglutarate pathway for the degradation of glutamate by *Peptococcus aerogenes*. Evidence could only be found for the first two steps: from glutamate to α-ketoglutarate and from α-ketoglutarate to hydroxyglutarate. In later research Buckel and coworkers confirmed the course of this proposed pathway with cell free extracts of *Acidaminococcus fermentans* (Buckel 1980; Buckel et al. 1981). The first conversion leading to the formation of α-ketoglutarate is an energetic barrier in this pathway because the Gibbs' free energy for this reaction is highly positive (+55.9 kJ/mol glutamate). In the next step hydroxyglutarate is formed, dehydrated to glutaconyl CoA and decarboxylated to crotonyl-CoA (Sweiger and Buckel 1984). In the last step crotonyl-CoA is cleaved to two acetate or converted to butyrate.

4.4.3) Citric acid cycle.

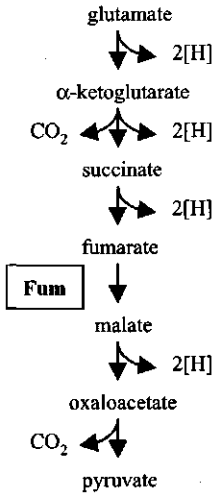
So far, no anaerobic bacteria have been isolated and characterised, which use this pathway for glutamate degradation, although this pathway is energetically feasible under methanogenic conditions. The first step is the conversion from



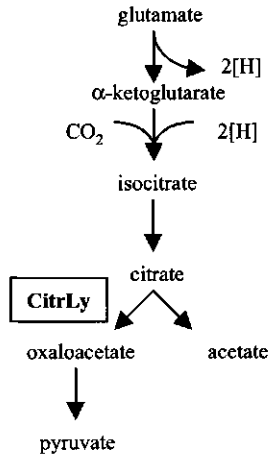
1. ***β-methylaspartate pathway***



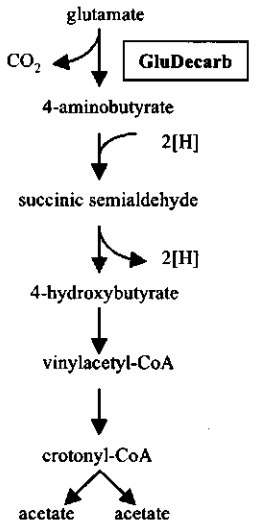
2. ***Hydroxyglutarate pathway***



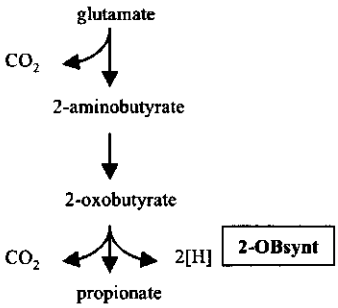
3. ***Citric acid cycle***



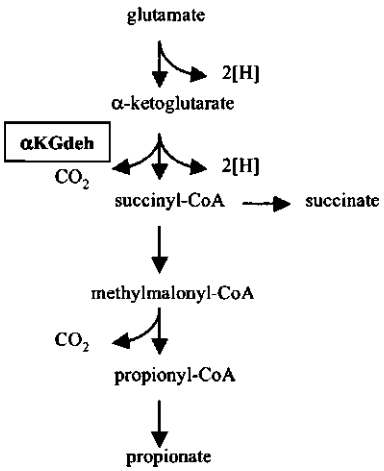
4. ***Reversed citric acid cycle***



5. ***4-Aminobutyrate pathway***

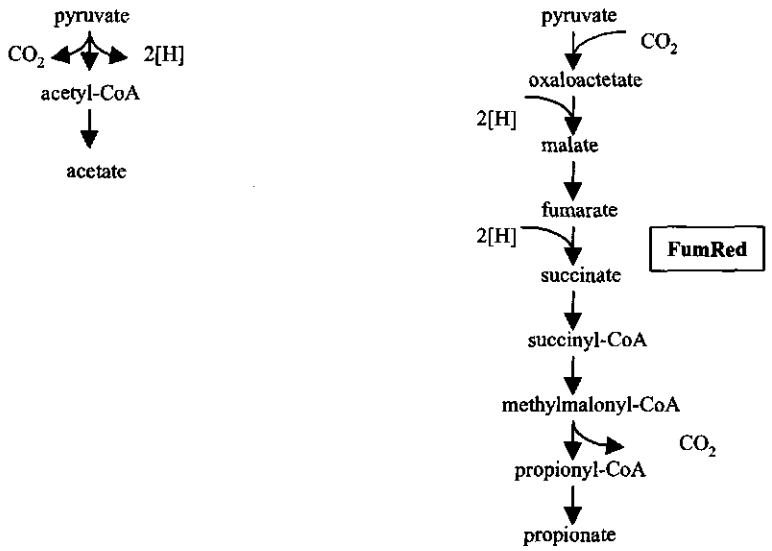


6. ***2-Aminobutyrate pathway***



7. ***Direct oxidation via methylmalonyl-CoA***

Figure 3. An overview of glutamate degradation pathways. Enzymes to demonstrate the respective pathways are indicated in boxes. MA, methylaspartase; HGl-deh, hydroxyglutarate dehydrogenase; Fum,fumarase; CitLy, citrate lyase; αKG deh, HSCoA dependent α-ketoglutarate dehydrogenase; GluDecarb, glutamate decarboxylase; 2-OBsynt, 2-oxobutyrate synthase.



1. Acetate formation from pyruvate

2. Reductive formation of propionate from pyruvate

Figure 4. Acetate and propionate formation from pyruvate. Enzymes to demonstrate the pathways are indicated in boxes. FumRed, fumarate reductase.

glutamate to α-ketoglutarate. α-Ketoglutarate is decarboxylated to succinyl-CoA and subsequently to succinate. From succinate fumarate is formed which is an energetic unfavourable oxidation (van Kuijk 1998). Malate is formed by hydratation of fumarate. Malate is converted to oxaloacetate, which is then decarboxylated to pyruvate. Pyruvate is ultimately converted to acetate. Overall 10 moles of reducing equivalents are formed during glutamate fermentation. These 10 moles could give rise to 5 moles of hydrogen.

4.4.4) Reversed citric acid cycle.

Some bacteria possess the ability to ferment glutamate in more than one way (Stams et al. 1994). *Acidaminobacter hydrogenoformans*, in pure culture, forms acetate via the β-methylaspartate pathway and also forms some propionate. However, when the hydrogen partial pressure is low, in case of growth in coculture with the hydrogen utilising *Methanobrevibacter arboriphilus*, propionate formation via direct

oxidation via succinyl-CoA is favoured and consequently the ratio acetate to propionate decreases. Moreover, acetate formation under methanogenic conditions does not occur via the β-methylaspartate pathway but via the reversed citric acid cycle (Stams et al. 1998). Two key enzymes, isocitrate dehydrogenase and citrate lyase, could be detected in syntrophic cocultures while some enzymes of the methylaspartase pathway were absent. So, several steps of the reversed citric acid cycle play a major role. Glutamate is deaminated to α-ketoglutarate and then carboxylated to isocitrate. Isocitrate is converted to citrate that is then cleaved into acetate and oxaloacetate. The latter compound is converted to pyruvate and eventually to acetate (Stams et al. 1998).

4.4.5) Aminobutyrate pathway.

Gharbia and Shah (1991) elucidated the glutamate degradation pathways used by *Fusobacterium* species. All species possess enzymes for the hydroxyglutarate pathway, some species possessed a key enzyme, β-methylaspartase, for the β-methylaspartate pathway as well. However, two species, *F. varium* and *F. mortiferum*, possess besides the enzymes mentioned, enzymes from the aminobutyrate pathway. In the first step glutamate is decarboxylated to 4-aminobutyrate. The latter compound is deaminated to succinic semialdehyde and then converted further to hydroxybutyrate. Via 4-hydroxybutyryl-CoA, vinylacetyl-CoA and crotonyl-CoA, finally butyrate or acetate is formed.

Another, theoretical, option could be that glutamate is first decarboxylated to 2-aminobutyrate. The 2-aminobutyrate is further converted to 2-oxobutyrate, leading to the formation of propionate.

4.4.6) Oxidative and reductive formation of propionate.

As stated previously, *A. hydrogenoformans* forms more propionate at a low hydrogen partial pressure than at high partial pressure. In this pathway glutamate is first converted to α-ketoglutarate. The latter compound is decarboxylated to succinyl-CoA. The conversion of succinyl-CoA to methylmalonyl-CoA is coenzyme B_{12} dependent. Methylmalonyl-CoA is decarboxylated to propionyl-CoA that is converted further to propionate. In this last step coenzyme A is released and recycled to form succinyl-CoA. The term direct oxidation is meant to distinguish this pathway from the

reduced propionate pathway. This reductive pathway is much more elaborate than the direct oxidation, but also leads to the formation of propionate. The course of the latter pathway is followed by many propionate forming or, in the reverse direction, by propionate-oxidising bacteria (Stams and Hansen 1984, Houwen et al. 1990, Van Kuijk 1998). *Anaeromusa* (*Selenomonas*) *acidaminophila* uses this reductive pathway to form propionate (Nanninga et al. 1987). Glutamate is converted via the β-methylaspartate pathway to acetate and pyruvate. Part of the pyruvate is then converted to oxaloacetate via a transcarboxylase. Malate, fumarate, succinate, succinyl-CoA, methylmalonyl-CoA are intermediates leading to the formation of propionate. The presence of high activities of fumarate reductase in glutamate grown cells of *Anaeromusa acidaminophila* and the excretion of small amounts of succinate indicate the presence of the methylmalonyl-CoA pathway (Nanninga et al. 1987).

5) Outline of this thesis.

The aim of the research presented in this thesis was to study the physiological and biochemical aspects of bacteria involved in the anaerobic metabolism of amino acids by syntrophic consortia. We used glutamate as the model substrate. The importance of interspecies hydrogen transfer in the degradation of glutamate under moderate thermophilic conditions was emphasised.

Chapters 2 and 3 present the relative importance of syntrophic glutamate-degrading consortia as opposed to fermentative glutamate-degrading organisms in methanogenic granular sludge. With the use of a dialysis membrane reactor it became possible to enrich a highly specialised consortium of microorganisms. In Chapters 4 to 7 the most predominant organisms isolated from several glutamate-degrading consortia are described. These organisms have either novel properties, are novel species or belong to a new genus.

Chapter 8 describes the catabolic pathways involved in the degradation of glutamate by three anaerobic bacteria. These three bacteria are all dependent to a various extent on the presence of a hydrogen scavenger for optimal growth on glutamate and all have a different stoichiometry. The metabolism of these bacteria is studied with the use of ^{13}C-NMR spectroscopic techniques and enzymatic measurements.

Chapter 9 describes the arginine metabolism of *Thermanaerovibrio acidaminovorans*. In pure culture this organism can convert arginine to citrulline,

ornithine and ammonia. Although there is no influence of hydrogen in this conversion, the addition of a methanogen has a clear effect on the arginine conversion.

This thesis is concluded by a summary of the obtained results (chapter 10).

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The role of interspecies hydrogen transfer on glutamate degradation in methanogenic granular sludge

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Abstract

To get insight into glutamate degradation by a mesophilic and a thermophilic methanogenic sludge, anaerobic glutamate-degrading bacteria were enumerated using the most probable number technique (n=3). Quantification of glutamate-degrading microorganisms was highly dependent on the cultivation conditions. The numbers of glutamate-degrading bacteria counted at 55°C per ml of thermophilic granular sludge were 1.9×10^8 when extra methanogens were added, 5.6×10^7 without special additions and 7.2×10^6 when an inhibitor of methanogenesis was added. Under these conditions, the numbers per ml. mesophilic sludge were at 37°C were 7.9×10^8, 3.8×10^8 and 1.8×10^7, respectively. The numbers of glutamate-fermenting bacteria counted were 2-10 fold higher after 6 months of incubation compared with 3 months incubation. The most abundant glutamate-degrading bacteria were physiologically different from the well-known fast-growing butyrate-forming glutamate-fermenting bacteria. For optimal growth these bacteria were dependent on the presence of hydrogen-scavenging methanogens. Thus, interspecies hydrogen transfer plays a crucial role in the degradation of glutamate in methanogenic granular sludges.

INTRODUCTION

Glutamate is an abundant amino acid in protein [1]. Under methanogenic conditions glutamate can be metabolised in different ways (Table 1). Glutamate can be fermented by a variety of *Clostridium*, *Fusobacterium* and *Peptococcus* species to acetate, butyrate, NH_4^+, CO_2 and in some cases H_2. Reducing equivalents formed during the conversion of glutamate to acetate are disposed off by formation of butyrate mainly [1-3]. Glutamate can also be fermented by some homoacetogenic *Sporomusa* species yielding only acetate, CO_2 and NH_4^+ as products [4]. In *Selenomonas (Anaeromusa) acidaminophila*, glutamate is converted to acetate and propionate, where propionate is formed reductively through the succinate pathway [5]. Reducing equivalents produced in glutamate degradation can also be released as molecular hydrogen. The metabolism of the hydrogen-producing bacteria is influenced by the presence of hydrogen-scavenging methanogens. Such metabolic interactions between amino acid-degrading bacteria and hydrogenotrophic methanogens were described for the first time by Nagase and Matsuo [6]. Several anaerobic amino acid-degrading bacteria have been described, the growth of which is dependent on hydrogen removal [7-13]. Under standard conditions the oxidative deamination reactions involved in amino acid degradation are endergonic or only slightly exergonic (Table 1). Therefore, these bacteria grow in syntrophic association with methanogens, which are responsible for hydrogen consumption. Syntrophic degradation of amino acids has received little attention in the past. This in particular because most isolation and enumeration techniques select for relatively fast growing bacteria, which are largely independent of hydrogen removal. Bacteria growing syntrophically in general grow slower than other fermenting bacteria growing on the same substrate [14].

To study the fate of glutamate in anaerobic methanogenic sludge granules, we quantified the number of glutamate-degrading bacteria from two methanogenic sludges. To obtain more insight into the impact of syntrophic conversions in granular sludge special attention was given to techniques that enable the enumeration of slow-growing glutamate-degrading bacteria living in syntrophic association with methanogens.

MATERIALS AND METHODS

Source of inoculum and organisms. As inoculation material 2 types of anaerobic granular sludge were used: granular sludge from a UASB reactor treating sugar beet waste water at 35°C (Centrale Suikermaatschappij, Breda, The Netherlands: CSM Sludge) and granular sludge from a 5-l laboratory scale UASB reactor degrading a mixture of fatty acids and sugars at 55°C (Thermo sludge). This sludge was a gift of J.B. van Lier (Wageningen University, The Netherlands). *Methanospirillum hungatei* JF1 (DSM 864) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). *Methanobacterium thermoautotrophicum* ΔH (DSM 1053) was a gift from J.T. Keltjens (University of Nijmegen, The Netherlands).

Media composition. A bicarbonate-buffered medium with the following composition was used (per litre): 0.4 g KH_2PO_4; 0.53 g Na_2HPO_4; 0.3 g NH_4Cl; 0.3 g NaCl; 0.1 g $MgCl_2.6H_2O$; 0.11 g $CaCl_2$; 1 ml alkaline trace element solution; 1 ml acid trace element solution; 1 ml vitamin solution; 0.5 mg resazurin; 4 g $NaHCO_3$; 0.25 g Na_2S. 7-9 H_2O. The trace elements and vitamins were as described previously [16]. All compounds were heat sterilised except for the vitamins, which were filter sterilised.

Table 1. The change in Gibb's free energy for the methanogenic degradation of glutamate by anaerobes. (Values calculated from Thauer [15])

Reaction	$\Delta G^{o'}$ kJ/ reaction*)	$\Delta G'$ kJ/ reaction*)
$Glutamate^- + 2\ H_2O \rightarrow acetate^- + HCO_3^- + 0.5\ H^+ + NH_4^+ + 0.5\ butyrate^-$	-57.9	-57.9
$Glutamate^- + 3\ H_2O \rightarrow 2\ acetate^- + HCO_3^- + H^+ + NH_4^+ + H_2$	-33.9	-62.4
$Glutamate^- + 2\ H_2O \rightarrow 2^1/_4\ acetate^- + {}^1/_2\ HCO_3^- + {}^3/_4\ H^+ + NH_4^+$	-59.9	-59.9
$Glutamate^- + 2\ H_2O \rightarrow 1^2/_3\ acetate^- + {}^1/_3\ propionate^- + {}^2/_3\ HCO_3^- + {}^2/_3\ H^+ + NH_4^+$	-60.5	-60.5
$Glutamate^- + 4\ H_2O \rightarrow propionate^- + 2\ HCO_3^- + NH_4^+ + 2\ H_2$	-5.8	-62.8
$Propionate^- + 3\ H_2O \rightarrow acetate^- + HCO_3^- + H^+ + 3\ H_2$	+76.5	-9.0
$Butyrate^- + 2\ H_2O \rightarrow 2\ acetate^- + 2\ H_2 + H^+$	+48.1	-8.9
$Acetate^- + H_2O \rightarrow CH_4 + HCO_3^-$	-32.0	-32.0
$4\ H_2 + HCO_3^- \rightarrow CH_4 + 2\ H_2O$	-135.6	-21.6

*) $\Delta G^{o'}$: Gibbs' free energy at pH = 7, 25°C, 1 M or 1 atm. for substrates/products; $\Delta G'$: $pH_2 = 10^{-5}$ atm.

Incubations were done in serum bottles sealed with butyl rubber stoppers (Rubber bv, Hilversum, The Netherlands) and a gas phase of 182 kPa (1.8 atm) N_2/CO_2 (80:20, vol/vol). For the cultivation of methanogens a gas phase of 182 kPa (1.8 atm) H_2/CO_2 (80:20, vol./vol.) was used and after growth the gas phase was changed to N_2/CO_2.

Carbon sources were added from anaerobic sterile stock solutions to a concentration of 20 mM (unless otherwise stated). $FeCl_2$ was added from a sterile anaerobic stock solution to a concentration of 0.05 mM to stabilise the methanogens [16]. In some cases enrichments were further purified by application of the roll tube technique [17], using the same medium as described above, but supplemented with 2-3% agar noble (Difco, Detroit, MI, USA).

Most Probable Number (MPN) countings and enrichment procedures. The sludge samples were diluted 1:1 with anaerobic medium without substrate. All manipulations were done under anaerobic conditions. Granules were gently homogenised and disintegrated with a 5-ml syringe by repeatedly taking up and ejecting the suspension. Granules were treated thereafter with a Potter homogeniser (Tamson, Zoetermeer, The Netherlands). This disintegration procedure causes a negligible cell lysis [18]. Glutamate-degrading bacteria and hydrogenotrophic methanogens in the obtained suspensions were quantified using the most probable number (MPN) technique (n=3). Serial dilutions were made with and without 20 mM bromoethane sulphonic acid (BES, a specific inhibitor of methanogenesis) and one dilution row was made in hydrogen pregrown cultures of methanogens.

The MPN counts were done in 120-ml serum vials (Prins, Schipluiden, The Netherlands) containing 50 ml of medium. All incubations were done in the dark over a period of 6 months at 37 and 55°C. After 3 and 6 months of incubations glutamate-degrading bacteria were quantified. After 6 months of incubation gas and product formation and substrate depletion were measured in all bottles with visible growth, using gas chromatography and HPLC methods. The cultures were also examined microscopically using a phase contrast microscope.

The lowest and highest dilutions where growth had occurred were subcultured several times in liquid media to determine the fermentation pattern and the roll tube technique was used to obtain pure cultures or defined cocultures.

Analytical methods. Glutamate and ammonium were determined with glutamate dehydrogenase [19]. Organic acids and formate were determined by HPLC (LKB-Pharmacia) with a Chrompack organic acid column at 60°C and a flow rate of 0.6 $ml.min^{-1}$ using 0.01 N H_2SO_4 as the eluent. Volatile fatty acids were analysed by gas chromatography using a Chrompack gas chromatograph (Model CP 9000) equipped with a Chromosorb 101 column (80/100 mesh, 2m x 2mm) and a flame ionisation detector at 240°C. The column temperature was 150°C and the injection port temperature was 220°C. The carrier gas was N_2 saturated with formic acid. Gases were determined using a Packard-Becker 417 gas chromatograph equipped with a thermal conductivity detector at 100°C and a molecular sieve column. The column temperature was 100°C and the carrier gas was argon at a flow rate of 25 ml/min. The dry weight content was determined after heating a sample overnight at 105°C.

RESULTS

Most probable number counts. After 3 and 6 months of incubation at 37°C and 55°C the numbers of glutamate-degrading bacteria in both sludge samples were highest when extra methanogens were added and lowest when BES was added (Table 2). In the sludge sample originating from the thermophilic reactor higher numbers were counted at 55°C compared to the mesophilic sludge. At 37°C higher numbers were counted in the sample originating from the mesophilic reactor compared to the thermophilic sludge. Slow-growing glutamate-degrading bacteria were most abundant in all methanogenic incubations, since a 2-10 fold higher number of bacteria was counted when the incubation period was prolonged to 6 months.

In all methanogenic incubations, glutamate was completely converted to CH_4, CO_2 and NH_4^+ in the lowest dilutions. In the highest dilutions with growth, after 6 months incubation, glutamate had been converted to CH_4, CO_2, NH_4^+, propionate and acetate. In the non-methanogenic, BES-containing incubations glutamate had been converted into acetate, butyrate, some propionate, some H_2, NH_4^+ and CO_2 in case of the lowest dilution at 55°C and the lowest and highest dilutions at 37°C. The highest dilution with growth at 55°C yielded besides the previously mentioned end products also traces of formate, but no butyrate.

Table 2. Number of glutamate-degrading bacteria an hydrogenotrophic methanogens per ml. sample counted via the MPN method after 3 and 6 months of incubation at 37 and 55^{0}C

Sample *)	Methanogens added		No additions		BES added		H_2/CO_2	
	3 months	6 months	3 months	6 months	3 months	6 months	3 months	6 months
CSM-55#)	1.4×10^{6} +)	6.8×10^{6}	0.22×10^{6}	1.2×10^{6}	0.025×10^{6}	0.025×10^{6}	1.2×10^{6}	1.2×10^{6}
Thermo-55	190×10^{6}	495×10^{6}	3.1×10^{6}	56×10^{6}	7.2×10^{6}	7.2×10^{6}	8.3×10^{9}	8.3×10^{9}
CSM-37	0.69×10^{8}	7.9×10^{8}	0.12×10^{8}	3.8×10^{8}	0.18×10^{8}	0.18×10^{8}	6.0×10^{10}	6.0×10^{10}
Thermo-37	1.3×10^{6}	2.4×10^{8}	2.6×10^{5}	2.8×10^{5}	0.16×10^{5}	0.16×10^{5}	2.8×10^{5}	2.8×10^{5}

*) *Methanobacterium thermoautotrophicum* ΔH was used at 55^{0}C and *Methanospirillum hungatei* JF1 was used at 37^{0}C.

#) sample description see materials and methods section

+) number of microorganisms per ml. original sample was calculated via the dry weight content of the first dilutions.

Enrichments and isolates. The first dilutions and the highest dilutions with growth were analysed further to investigate which types of organisms predominated. The morphology of the bacteria in the highest and lowest dilutions of the methanogenic MPN rows differed in most dilution series, indicating that the most predominant and the fastest growing bacteria were probably different species.

The first dilutions and the highest dilutions with growth from the two sludge samples were enriched further. In all methanogenic enrichments stable cultures developed, with 2, 3 or 4 morphological types, but no pure cultures could be obtained.

From the methanogenic enrichment cultures obtained the stoichiometry of glutamate conversion was determined (Table 3). Additional tests showed that all enrichment cultures mentioned in Table 3 were inhibited in their growth when 20 mM BES was added to stop methanogenesis, but in all cultures some glutamate degradation could be measured. The mesophilic enrichments degraded only 10-19% of the glutamate, the thermophilic enrichments degraded only 25-30% of the glutamate. In all cases H_2 accumulated in the gas phase and acetate, propionate, CO_2 and NH_4^+ were the other end products. In one enrichment (37-SPIR-1) some butyrate was formed besides the other fermentation products.

From the BES enrichments at 37°C two pure cultures were obtained. Both isolates were the fastest growing as well as the most predominant organisms. The strains were both gram positive, rod shaped and spore-forming and produced acetate, butyrate, NH_4^+ and traces of hydrogen from glutamate. Based on these characteristics the strains might belong to the genus *Clostridium*. The fermentation pattern of both isolates was not influenced by the presence of a methanogen. Both strains had a doubling time of approximately 2 hours. The BES enrichments at 55°C were very hard to subculture. Both first and last dilutions lost their ability to grow after 6 months of incubation. No identification of the organisms could be made based on their morphology. The experiments were repeated, but at a shorter incubation time (3 months) to reveal the identity of the bacteria. Two, microscopically pure cultures were obtained from these enrichments. Both strains (55-BES-1 and 55-BES-4) produced acetate, propionate, NH_4^+ and H_2 from glutamate.

The hydrogenotrophic methanogens from the highest diluted MPN row on H_2/CO_2 were isolated and obtained in pure culture. The isolated methanogens from the CSM and Thermo sludge incubated at 55°C were similar to *M. thermoautotrophicum* based on their morphology and ability to grow on H_2/CO_2 only. The isolated

methanogens from the incubations at 37°C were able to grow on both formate and H_2/CO_2, but based on their morphology it was not possible to identify them.

DISCUSSION

The lower numbers of glutamate-degrading bacteria counted in the two sludge samples in the presence of BES compared to the numbers counted in the presence of extra methanogens, clearly indicated that the degradation of glutamate is strongly affected by interspecies hydrogen transfer. Inhibition of methanogenesis (by BES) resulted in the enumeration of only 0.3-1% and 1.4-7% of the glutamate-degrading population in the mesophilic sludge and the thermophilic sludge, respectively.

The fastest growing bacteria (isolated from the lowest dilutions) were not the most predominant bacteria since the morphology in the lowest and highest dilution differed. Besides acetate, propionate was formed as an important end product, in the highest dilutions with growth. The formation of butyrate seemed to be of minor importance in all our methanogenic incubations. Other studies showed that at high glutamate concentration butyrate-forming bacteria became predominant, most likely because such bacteria are very fast growers [20, 21]. Doubling times of less than one hour are not exceptional. In our countings the most predominant bacteria, growing in the highest dilutions, did not form butyrate as a reduced end product but these bacteria disposed their reducing equivalents as molecular hydrogen. From a thermodynamical point of view there is a difference in acetate, butyrate and propionate formation. Under standard conditions (Table 1), conversions which do not yield hydrogen are most favourable. However, if the hydrogen partial pressure (pH_2) is kept sufficiently low ($<10^{-4}$ atm., 10 Pa), propionate formation becomes more favourable over acetate formation. The hydrogenotrophic methanogens present keep the pH_2 sufficiently low for propionate-forming glutamate-degrading bacteria to grow. In granular sludge the in situ hydrogen concentration is kept very low, since the inter-bacterial distances are short and the efficiency of hydrogen diffusion is very high.

The results of the MPN counts also showed that the quantification of the number of glutamate-degrading bacteria is highly dependent on the conditions in the test tube. If one disrupts the spatial orientation within a sludge granule, where a hydrogenogenic bacterium and a hydrogenotrophic bacterium are very close to each other, the number of counted bacteria is lower than in the original sample [22]. Probably, the extra addition of

Table 3a. Glutamate conversion by enrichments obtained from MPN vials at 55^0C

Sample *)	glutamate converted+)	acetate formed	propionate formed	butyrate formed	CH_4 formed	H_2 formed	NH_4^+ formed
ΔH-1	18.3	4.9	14.6	-	7.7	-	18.2
N-1	21.3	13.0	3.1	-	8.2	-	21.0
BES-1	19.9	16.7	0.5	-	-	2.6	20.3
ΔH-7	16.1	7.5	9.5	-	8.3	-	15.5
N-6	20.4	6.3	15.6	-	26.1	-	19.5
BES-4	4.9	2.9	3.1	-	-	2.6	4.4

*) ΔH: enrichments cultivated in vials pregrown with *Methanobacterium thermoautotrophicum* ΔH; N: no extra additions; BES: bromoethane sulfonic acid (20mM) added.

+) Substrate converted and products formed in $mmol.l^{-1}$. -: measured, but not detected

Table 3b. Glutamate conversion by enrichments obtained from MPN vials at 37^0C

Sample *)	glutamate converted+)	acetate formed	propionate formed	butyrate formed	CH_4 formed	H_2 formed	NH_4^+ formed
SPIR-1	23.0	27.6	8.6	-	11.4	-	20.8
N-1	18.3	16.8	11.7	-	8.6	-	18.6
BES-1	19.6	16.8	0.6	4.3	-	17.0	19.2
SPIR-7	20.2	7.6	15.6	-	10.2	-	19.9
N-7	19.5	22.5	10.2	-	9.6	-	19.2
BES-6	22.9	19.7	0.5	5.0	-	0.1	22.7

*) SPIR: enrichments cultivated in vials pregrown with Methanospirillum hungatei; N: no extra additions; BES: bromoethane sulfonic acid (20mM) added.

+) Substrate converted and products formed in $mmol.l^{-1}$

methanogens mimics the conditions in a sludge granule better, resulting in higher numbers of counted bacteria [23].

It was observed that under methanogenic conditions 3 months is not long enough to count all bacteria. This can be ascribed to the slow growth of the glutamate-degrading bacteria. It has been shown that bacteria growing syntrophically grow slower than other fermenting bacteria. Laanbroek et al. [21] reported a μmax of 0.3-0.6 h^{-1} for several glutamate-fermenting *Clostridium* sp., whilst *Acidaminobacter hydrogenoformans* growing syntrophically on glutamate has a μmax of 0.10 h^{-1} only [7]. An other example is growth on aspartate by *Campylobacter* sp. and *Eubacterium hydrogenophilum* [8,10]. The first bacterium grows fermentatively at a maximum growth rate of 0.15 h^{-1} and the latter is growing in coculture with a methanogen at a μmax of 0.07 h^{-1}. Assuming a doubling time of 5 days, it will take at least 140 days for one bacterium to grow upto a number of 10^8 cells and around 100 days before the cultures start showing turbidity (at 10^6 cells/ml). In any case one has to wait longer than 3 months to determine reliable numbers.

Our results clearly indicate that for a proper enumeration of glutamate-degrading anaerobic bacteria a long incubation time and the extra addition of methanogens is needed. Therefore, cultivation and enumeration methods have to be adapted in such a way that they are suited for syntrophic bacteria. In addition, the most abundant glutamate-degrading anaerobes in mesophilic and thermophilic sludges from bioreactors are not the rapidly growing butyrate-forming organisms. Evidently, bacteria growing in syntrophy with methanogens convert a major part of glutamate.

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Enrichment of Thermophilic Syntrophic Anaerobic Glutamate-Degrading Consortia in a Dialysis Membrane Reactor

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Submitted for publication

Abstract

A dialysis cultivation system was used to enrich slow-growing moderately thermophilic anaerobic bacteria at high cell densities. Bicarbonate buffered mineral salts medium with 5 mM glutamate as the sole carbon and energy source was used and the incubation temperature was 55^0C. The reactor inoculum originated from anaerobic methanogenic granular sludge bed reactors. The microbial population was monitored over a period of 2 years using the most probable number (MPN) technique. In the reactor glutamate was readily degraded to ammonium, methane and carbon dioxide. Cell numbers of glutamate-degrading organisms increased 400-fold over the first year. In medium supplemented with bromoethane sulfonic acid (BES, an inhibitor of methanogenesis) tenfold lower cell numbers were counted, indicating the syntrophic nature of glutamate degradation. After 2 years of reactor operation the most predominant organisms were isolated and characterised. Methanobacterium thermoautotrophicum (strain R43) and a Methanosaeta thermophila strain (strain A) were the most predominant hydrogenotrophic and acetoclastic methanogens, respectively. The numbers at which the organisms were present in the reactor after 24 months of incubation were 8.6×10^9 and 3.8×10^7 ml^{-1} sludge, respectively. The most predominant glutamate-degrading organism (8.6×10^7 ml^{-1} sludge), strain Z, was identified as a new species, Caloramator coolhaasii. It converted glutamate to hydrogen, acetate, some propionate, ammonium and carbon dioxide (Plugge et al. 2000). Growth of this syntrophic organism on glutamate was strongly enhanced by the presence of methanogens.

INTRODUCTION

Most physiological studies on microorganisms have been performed with fast-growing organisms. Little attention has been given to the importance of slow-growing microorganisms in several ecosystems. Slow growing microorganisms are difficult to enrich and isolate. Commonly used culture systems are not selecting for slow-growing organisms. Batch cultures and chemostats are not suitable for studying the behaviour of microorganisms with doubling times longer than 10-12 hours (6). However, the unique and significant role of these slow-growing microorganisms in nature deserves more attention. Anaerobic methanogenic sludge from methanogenic wastewater treatment systems is an environment where slow-growing organisms may prevail.

Amino acid degrading bacteria growing syntrophically, dependent on the removal of hydrogen, grow slower than fermenting bacteria on the same substrate (15,21). A typical example is glutamate. These experiments where done in suspended cultures with high substrate concentrations (>10 mM). In densely packed sludge granules the interbacterial distances are much shorter than in suspended cultures. This may result in higher turn-over rates of the substrate and higher growth rates. The rate-limiting step in the overall process is most probably the transfer of reducing equivalents from producer to consumer. Inside the sludge granules at a certain distance from the granule's outer surface the concentration of amino acids (substrate) will certainly be much lower than the original influent concentration. By maintaining the biomass in a dialysis membrane and pumping the substrate in the reactor and discarding the effluent, no inhibition of growth by end products will occur. In many studies with dialysis cultures high densities of microorganisms could be achieved by effective removal of inhibitory metabolites (19). In the case of glutamate degradation, an increased NH_4^+ concentration could inhibit the deamination of glutamate or inhibit the growth of the whole reactor biomass.

We studied syntrophic degradation of glutamate in anaerobic wastewater systems (5,23,25). The pH_2 (hydrogen partial pressure) strongly affects the degradation of glutamate (16,17). A low hydrogen partial pressure allows complete degradation of glutamate or causes a shift in the metabolism. This can be explained by the Gibbs' free energy of such reactions.

1. $Glutamate^- + 3\ H_2O \rightarrow 2\ acetate^- + HCO_3^- + H^+ + NH_4^+ + H_2$ $\Delta G^{0'} = -33.9$ kJ/mole
2. $Glutamate^- + 4\ H_2O \rightarrow propionate^- + 2\ HCO_3^- + H^+ + NH_4^+ + 2\ H_2$ $\Delta G^{0'} = -5.8$ kJ/mole

When the pH_2 is lowered to 10^{-5} atm. (1 Pa), in the presence from a hydrogen-scavenging methanogen the Gibbs' free energy changes of the above reactions change to –62.4 and –62.8, respectively (data taken from 26).

To study the importance of slow-growing glutamate-degrading syntrophic consortia in anaerobic sludge granules, we developed a reactor system where the substrate concentration was kept low and the biomass retention was high. The reactor was operated during a period of 2 years and the shift in microbial population was monitored. The most predominant physiological groups of microorganisms were isolated and characterised.

MATERIALS AND METHODS

Sources of strains and inocula. Anaerobic sludge granules from a 55^{0}C bench scale UASB Reactor treating a mixture of fatty acids and sugars were a gift from J.B. van Lier (Subdepartment of Environmental Technology, Wageningen, The Netherlands). Anaerobic sludge granules from a UASB treating sugar beet waste water were a gift from the Centrale Suikermaatschappij CSM, Breda, The Netherlands. A 1: 1 mixture of these two granular sludges was used to inoculate the dialysis membrane reactor (DMR). *Methanobacterium thermoautotrophicum* (recently renamed as *Methanothermobacter thermoautotrophicus* (28)) Z245 (DSM 3720) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany. The glutamate-degrading strain Z, the hydrogenotrophic methanogen strain R43 and the acetoclastic methanogen strain A were isolated in this study. Strain Z was recently described as a new species within the *Caloramator* genus (18).

Media composition and cultivation. Anaerobic techniques were used for medium preparation according to Hungate (12). A bicarbonate-buffered medium with the following composition was used (per litre): 0.4 g KH_2PO_4; 0.53 g Na_2HPO_4; 0.3 g NH_4Cl; 0.3 g NaCl; 0.1 g $MgCl_2.6H_2O$; 0.11 g $CaCl_2$; 1 ml alkaline trace element solution; 1 ml acid trace element solution; 1 ml vitamin solution; 0.5 mg resazurin; 4 g $NaHCO_3$; 0.25 g $Na_2S.7\text{-}9\ H_2O$. The trace elements and vitamins were as described

previously (22). All compounds were heat sterilised, except for the vitamins, which were filter sterilised. Medium for the reactor was prepared in 1 litre serum bottles with a liquid volume of 500 ml MPN incubations and enrichments were done in 120-ml serum bottles with 50 ml liquid volume. All bottles were sealed with butyl rubber stoppers (Rubber bv, Hilversum, The Netherlands) and a gas phase of 182 kPa (1.8 atm) N_2/CO_2 (80:20, vol/vol) was applied. For the cultivation of methanogens a gas phase of 182 kPa (1.8 atm) H_2/CO_2 (80:20, vol./vol.) was used and after growth the gas phase was changed to N_2/CO_2.

To mimic the situation in the reactor, growth experiments were performed with strain Z in pure culture, strain Z together with strain A, strain Z together with strain R43 and strain Z together with strain A and strain R43. Strain Z, strain R43 and strain A were pregrown in the bicarbonate-buffered medium as decribed above with glutamate, acetate and H_2/CO_2 as their growth substrates, respectively. Strain Z was pregrown in the presence of 0.02% yeast extract, to enhance the growth rate. The consortia were constructed by adding 10% of each pre-culture to triplicate incubation vials (120-ml serum bottles with 50 ml liquid volume). Finally approximately 10 mM glutamate was added to all incubations.

Reactor set-up. Fig. 1 shows the schematic set up of the DMR. The retention of the biomass in the reactor is very high, since the biomass is kept inside a dialysis bag (pore size 12,000- 14,000 Da; Fisher Scientific, 's-Hertogenbosch, The Netherlands). Fresh medium with 5 mM glutamate is pumped into the reactor at a constant flow rate (50 ml day^{-1}) and effluent with products is discarded at the same rate. An additional effluent stream was built to remove gaseous compounds, since the dialysis membrane was poorly permeable for gaseous methane. The liquid volume of the reactor was 350 ml, the volume of the dialysis bag was 35 ml, and the total reactor volume was 600 ml. The operation temperature was 55^0C. The reactor was operated stable over a period of 2 years. During this period the glutamate conversion was monitored and products were analysed regularly. To monitor the shift in the bacterial population every 6 months a sample was taken from the dialysis bag. The most probable number (MPN) technique (n=3) was used to quantify different metabolic groups of microorganisms.

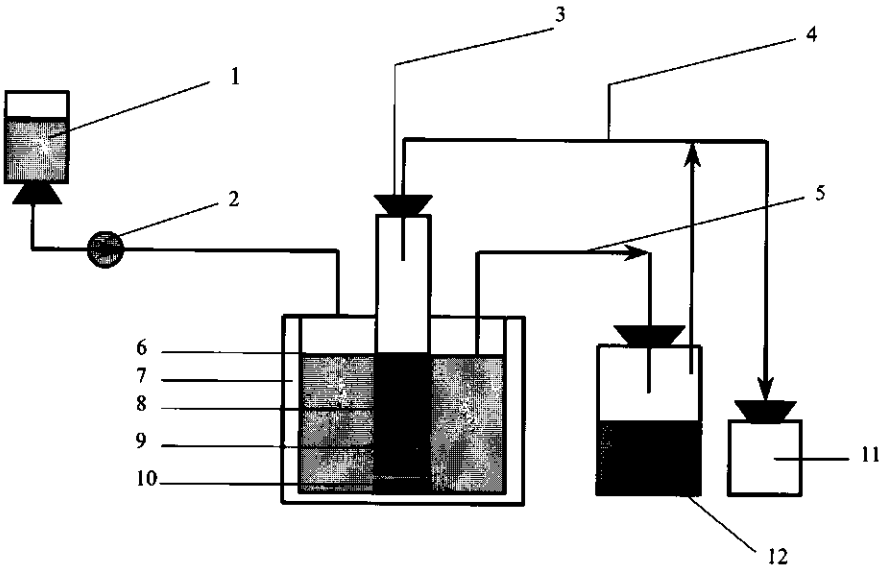

Figure 1. Schematic representation of the Dialysis Membrane Reactor (DMR).
1, Influent; **2,** Peristaltic pump (50 ml. day^{-1}); **3,** Butyl rubber stoppered sampling port; **4,** Gas effluent stream; **5,** Liquid effluent stream; **6,** Liquid/medium surface; **7,** Water mantle (55^0C); **8,** Dialysis tubing; **9,** Biomass; **10,** Teflon coated stirring bar; **11,** Gas collection flask filled with 2 M NaOH to wash away the CO_2 and only collect CH_4; **12,** Liquid effluent collection vial.

Enumeration and isolation of microorganisms from the DMR and the inoculum sludge granules. Samples from the inoculum sludge granules and the DMR were disintegrated by homogenisation in a potter tube and diluted in anaerobic sterile physiologically buffered saline (PBS) to a dilution of 10^{-9}. From these dilution rows triplicate bottles were immediately inoculated for MPN determinations. MPN determinations were done with glutamate, glutamate + 10 mM bromoethane sulfonate (to inhibit methanogenesis), pyruvate, formate, propionate, acetate and H_2/CO_2 (80%/20% v/v) as substrate in the case of the samples from the DMR. An additional triplicate MPN row was inoculated in bottles pregrown with *Methanobacterium thermoautotrophicum* Z245 and glutamate as the substrate. All substrates were added to a concentration of 5 mM, except H_2/CO_2, which was added as a gasphase of 1.8 atm (182 kPa). The MPN bottles were incubated at 55°C in the dark over a period of 6

months. After incubation, the gas phase of the highest dilutions with growth was examined for the presence of methane and/or hydrogen. Furthermore substrate depletion and product formation in the same bottles was measured. The cultures obtained from the glutamate, acetate and H_2/CO_2 enrichments from the MPN counts after 24 months were characterised further.

Activity measurements. Every six months the methanogenic activity of the reactor material was measured. Glutamate, acetate, propionate, H_2/CO_2 and formate were used as test substrates. The tests were performed in anaerobic serum vials (15 ml) with 5 ml bicarbonate buffered mineral salts medium and approximately 10 mg VSS was added to the vials. The reactor sample was pre-incubated overnight with 2 mM of the test compound or in case of H_2/CO_2 an atmosphere of 182 kPa (1.8 atm) was added. After changing the gas phase the substrates were added to a final concentration of 5-10 mM. The vials were incubated at 55^0C in the dark. Gas samples were taken at 3, 6, 9, 12, 22 and 72 h. of incubation.

DGGE analysis. To monitor the population dynamics of glutamate-degrading consortia DGGE analysis was performed with reactor samples taken after 12 and 24 months of incubation, an enrichment culture on glutamate, and pure culture strain Z which was isolated from the reactor. DNA isolation and the amplification of the V6 to V8 regions of the 16S rDNA from these samples were carried out as described previously (28). DGGE analysis of the amplicons was performed on 8% polyacrylamide gels containing a urea plus formamide gradient from 38% to 48% (100% denaturing solution contains 7 M urea and 40% (vol/vol) formamide). Electrophoresis was done in 0.5 x TAE at 85 V at 60°C for 16 hours using the DCode or D GENE System apparatus (BioRad, Hercules, CA). After electrophoresis, gels were silver-stained according to the protocol of Sanguinetti and colleagues (20) with some minor modifications.

Analytical and other techniques. The dry weight and VSS content was determined as described by Grotenhuis et al. (10). Methane, organic and fatty acids were analysed as described previously (18). Glutamate and NH_4^+ were analysed enzymatically according to Bernt and Bergmeyer (4). Morphology of the microorganisms was routineously examined using phase contrast microscopy.

Table 1. Efficiency of glutamate degradation in start-up phase of the DMR.

Day	Feed rate (μmol day^{-1})	% removal of glutamate	CH_4 production (μmol day^{-1})
1	240	33	178
3	"	18	97
5	"	24	13
7	"	92	518
10	275	95	587
11	"	97	600
18	"	102	631
34	262	98	577

RESULTS AND DISCUSSION.

Reactor performance. This investigation was done to evaluate the shift in a microbial population originating from anaerobic methanogenic UASB (Upflow Anaerobic Sludge Bed) Reactors, when it was put under low-strength wastewater conditions, using a Dialysis Membrane Reactor (Fig. 1). Almost directly after the DMR was inoculated with the methanogenic sludge granules the granules disintegrated. However, complete suspended growth was never observed in the DMR. Table 1 shows the efficiency of glutamate degradation during the start-up of the reactor. Within one week a complete conversion of the glutamate to CH_4, CO_2 and NH_4^+ was achieved.

The DMR is a good tool to enrich for slow-growing organisms in high densities. Furthermore, the simplicity of the system made it work without major problems. The glutamate-degrading population, which developed in the reactor, was dependent on the presence of hydrogenotrophic and acetoclastic methanogens for optimal growth. In time the physiological groups present in the reactor shifted to a population converting glutamate almost exclusively via acetate and hydrogen to CH_4, NH_4^+ and CO_2 (Table 2). Propionate, often found as end product from thermophilic

Table 2. Numbers of bacteria. ml^{-1} reactor biomass at different time points on different substrates counted with the MPN method (n=3) after an incubation of 6 months at 55^0C

	0	6	12	24
Glutamate	4×10^5	6.9×10^7	3.3×10^8	8.6×10^7
Glutamate + Z245*)	1.4×10^6	8.4×10^7	5.5×10^8	$3.8\ 10^8$
Glutamate + bres*)	2×10^4	3.8×10^6	2.8×10^7	3.8×10^6
Acetate	9.3×10^7	6.4×10^7	4.6×10^7	3.8×10^7
Propionate	3.5×10^7	3.1×10^6	1×10^5	6.6×10^4
H_2/CO_2	9.6×10^8	3.3×10^9	2.8×10^9	8.6×10^9
Formate	2.4×10^8	4.2×10^9	6.1×10^9	8.6×10^9
Pyruvate	3.5×10^7	6.4×10^7	4.6×10^8	2.4×10^8

*) Numbers of microorganisms counted in the presence of *Methanobacterium thermoautotrophicum* Z245 (+Z245) and in the presence of bromoethanesulfonate (+bres)

glutamate-degrading organisms, played no important role in glutamate conversion in the reactor (24). The numbers of propionate-degrading organisms decreased from 3.5×10^7 to 6.6×10^4 over a period of 2 years.

Glutamate-degrading organisms dependent on the presence of methanogens became predominant. During the two years the numbers of bacteria counted in the presence of the hydrogen consumer *Methanobacterium thermoautotrophicum* Z245 were always highest, whereas numbers of glutamate-degrading organisms grown in the presence of BES were always lowest. The total number of syntrophic glutamate-degraders increased from 1.4×10^6 to 5.5×10^8 in the first year. The total number of glutamate-degraders decreased slightly in the following year.

Methanogens were present in almost constant numbers throughout the whole period. Formate and H_2/CO_2 converting methanogens increased in number by a factor 10, whereas acetoclastic methanogens did not increase in number. In the case of the acetoclastic methanogens, the counted numbers remained more or less constant. Fig. 2 shows a phase contrast picture of reactor biomass. It is clear that *Methanosaeta*-like

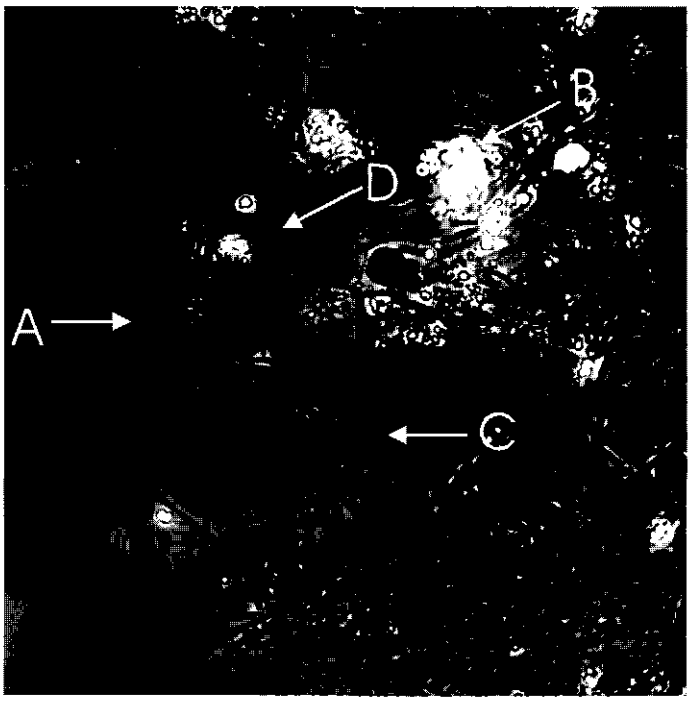

Figure 2. Phase contrast micrograph of reactor biomass with (A) *Methanosaeta*-like organisms, (B) Inorganic precipitates (C) Strain Z and (D) *Methanobacterium*-like organisms.

organisms are very abundant. Due to the filamentous growth of these organisms, MPN counts can only be interpreted as trends, since the filaments can reach a length up to 100 cells, introducing a large error in the MPN counts. Therefore, the exact numbers of the acetoclastic methanogens will be up to a 100 times higher as measured in the MPN counts. Mild sonication before dilution of the samples could not overcome this inaccuracy, since viability of the cells was strongly influenced by this treatment (data not shown). Similar observations were made by Grotenhuis et al. (11), who showed that 20-30% of the biomass of granular sludge in direct counts consisted of *Methanosaeta* sp., but MPN quantifications showed much lower numbers.

Fig. 2 also shows the presence of inorganic precipitates (arrow mark B) in the reactor. Microorganisms adhered to these particles. This resulted in flocculent growth

of the reactor biomass. The presence of cells resembling strain Z and strain R43 is indicated as well in Fig. 2 (arrow marks C and D).

Activity measurements. The initial activity of the reactor biomass (Table 3) on glutamate was low. Acetate, propionate and H_2/CO_2 were converted rapidly, indicating that the organisms capable of converting these compounds were active in the inoculum sludge. After 6 months of incubation on glutamate the activity measurements showed a completely different picture. Methanogenic glutamate conversion activity had increased 500 times, whereas glutamate conversion rates were 20-fold lower, indicating that methanogenic glutamate degradation is the major process in the reactor. Activity on propionate decreased by a factor 10, indicating that glutamate degradation via propionate is of minor importance. Activity on acetate and H_2/CO_2 had remained almost constant. The measurements after 12 and 24 months showed a further decrease in activity on propionate and a slight increase of activity of methanogenic glutamate and fermentative glutamate conversion. Activity on formate was not detectable. This could indicate that in the reactor the reducing equivalents produced during glutamate degradation were released as hydrogen and interspecies formate transfer is not important in the reactor.

Table 3. Potential methanogenic activity on different substrates of reactor biomass at different time points (μmol CH_4. (g VSS)$^{-1}$.h^{-1})

	0	6	12	24
Substrate		time (months)		
Glutamate	0.25	120	150	110
Glutamate + Bes[+)]	<0.1	5.5	7	9
Acetate	90	80	130	120
Propionate	20	10	3	8
H_2/CO_2	180	210	170	200
Formate	n.d.[*)]	<0.1	<0.1	<0.1

+) H_2 formation was used to determine activity in the presence of bromo ethanesulfonate

*) n.d. not determined

Several authors have speculated that formate can serve as an alternative electron carrier during syntrophic degradation (8,16,27). The presence of high numbers of formate-converting methanogens in the MPN counts (Table 2) is most likely due to hydrogenotrophic methanogens also capable of utilising formate after adaptation.

Comparing the activity measurements with the numbers of the bacteria counted in the MPN determinations it is clear that glutamate is degraded via acetate and H_2. The potential methanogenic activities on glutamate, acetate and H_2/CO_2 are considerably higher than the activity in the reactor itself. The daily input of glutamate in the reactor could generate a maximum of ~600 μmol CH_4. The average total VSS content of the reactor biomass during the operation period was 250-300 mg. This could enable a CH_4 formation rate of approximately 80 μmol. g^{-1} VSS. h^{-1}.

Enrichments and isolations. The enrichments obtained from the highest dilutions with growth on glutamate, acetate and H_2/CO_2 from the MPN counts after 24 months were characterised further. The most predominant glutamate-degrading organism, strain Z, was obtained in pure culture (18). Strain Z belongs to the low G + C containing clostridial group of gram-positive bacteria and had relatedness with *Thermobrachium celere* (97.8%), *Caloramator indicus* (97.2%) and *Caloramator proteoclasticus* (96.2%) (7,9,21). A detailed description of strain Z as the species *Caloramator coolhaasii* was given elsewhere (18). A pure culture of strain Z degraded glutamate to acetate, H_2, CO_2, NH_4^+ and traces of propionate. In co-culture with *Methanobacterium thermoautotrophicum* Z 245 glutamate was converted to the same products, but the specific growth rate was 4 times higher. The doubling time of the pure culture of strain Z under optimal conditions on glutamate was 1.6 day. This means that strain Z, under optimal conditions, is not a very slow growing organism. Yet, it was isolated as the most predominant glutamate-degrading organism from the DMR. The organism is versatile in its metabolism and adapted to the conditions in the reactor where it grew much slower than under optimal conditions (18). Strain Z grows on a variety of organic substrates, such as sugars and several organic acids (18). Other thermophilic syntrophic glutamate degraders, such as *Caloramator proteoclasticus* and *Thermanaerovibrio acidaminovorans* posses the same metabolic versatility as strain Z (5,25). With respect to mesophilic glutamate-degrading syntrophs, all of them are asacharolytic and specialised in amino acid degradation.

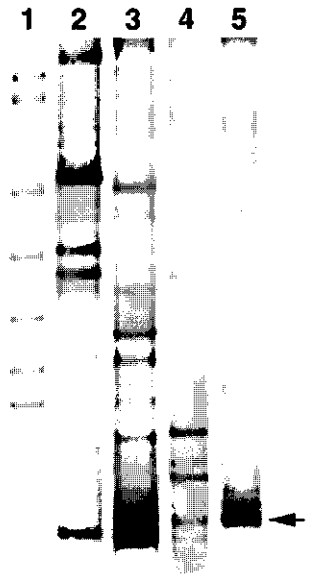

Figure 3. DGGE patterns of PCR products of the 16S rDNA V6 to V8 regions of reactor samples after 12 and 24 months of incubation (lanes 2 and 3), enrichment culture on glutamate (lane 4) and pure culture of strain Z on glutamate (lane 5). Lane 1 represents marker amplicons.

Examples of such organisms are *Aminoobacterium colombiense, Aminobacterium mobile, Acidaminobacter hydrogenoformans* and *Aminomonas paucivorans* (1,2,3,21). A specialised thermophilic glutamate-degrading organism has yet to be isolated.

DGGE analysis of amplified 16S rDNA (V6-V8 region) showed the presence of strain Z in reactor samples taken after 12 and 24 months of incubation (lanes 2 and 3 in Fig. 3) and in the highest dilution with growth from the reactor (Fig. 3, lane 4).

The most predominant acetate-converting organism (strain A) was obtained in a highly enriched culture (>99.99%). The organism was a striking example of a *Methanosaeta* sp. Fig. 2 shows the distinct filaments and the rods with square ends of the organism in the DMR. The enrichment culture stoichiometrically converted acetate to methane with a doubling time of ~ 2.5 day. The growth rate was independent of the

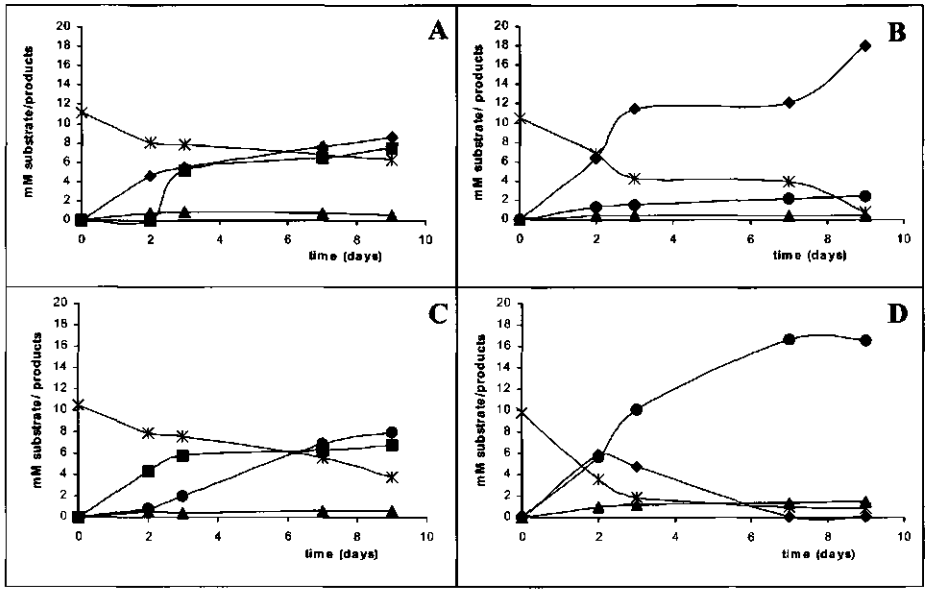

Figure 4. Glutamate degradation (✳) and acetate (◆), hydrogen (■), methane (●) and propionate (▲) formation by (A) strain Z; (B) strain Z + strain R43; (C) strain Z + strain A; (D) strain Z + strain R43 + strain A.

acetate concentration. Growth occurred with acetate concentrations of 5-100 mM. No special requirements for growth were necessary, but addition of 2-mercaptoethane sulphonate (1 mM) and titanium citrate (0.5 mM) decreased the lag phase. The predominance of *Methanosaeta* strain A in the DMR can be explained by the fact that *Methanosaeta* has a high affinity for acetate as opposed to the only other acetoclastic methanogen *Methanosarcina*. Both acetoclastic methanogens have different strategies for growth on acetate. *Methanosarcina* is metabolically more versatile than *Methanosaeta*. The growth rate and growth yield of *Methanosaeta* is low compared to *Methanosarcina* and its K_s is lower (14). *Methanosaeta* like organisms are often present in high numbers in anaerobic methanogenic bioreactors (14).

The most predominant hydrogenotrophic methanogen (strain R43) was obtained in pure culture. The morphology of strain R43 resembled *Methanobacterium thermoautotrophicum*. Growth occurred on H_2/CO_2 as well as on formate, which is in

accordance with the metabolic capacities of *Methanobacterium* ssp.. Acetate, methanol and trimethylamine could not be converted by strain R43.

Reconstitution experiments with strain Z, strain A and strain R43. To mimic the situation in the reactor growth experiments were performed with strain Z in pure culture; strain Z together with strain A, strain Z together with strain R43 and strain Z together with strain A and strain R43. The results are depicted in Fig. 4. Glutamate was converted to acetate H_2, CO_2, NH_4^+ and traces of propionate by strain Z. The acetate and H_2 formed were converted to methane by strain A and strain R43, respectively. When all three organisms were present, glutamate was converted fastest. When the acetoclastic strain A was present, intermediate formation of acetate still could be detected. This was due to the relative long lag phase of the organisms of strain A or an unbalanced ratio between the number of organisms from the different strains. The glutamate degradation by the three different strains isolated as the most predominant organisms nicely reflected the glutamate degradation by the reactor biomass.

ACKNOWLEDGEMENTS

The technical assistance of and stimulating discussions with J.C.M. Scholten are highly appreciated. E.G. Zoetendal is gratefully acknowledged for his assistance with the DGGE experiments.

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Caloramator coolhaasii sp. nov., a glutamate-degrading, moderately thermophilic anaerobe

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Published in:

International Journal of Systematic and Evolutionary Microbiology

50 (2000): 1155-1162.

Abstract

An obligately anaerobic, moderately thermophilic, glutamate-degrading bacterium (strain Z^T) was isolated from an enrichment culture obtained from anaerobic thermophilic granular sludge. The cells are rod shaped to filamentous and showed no motility nor spore formation. The cell wall has a gram-positive structure, which was revealed by electron microscopy. Optimum growth of the strain is observed at neutrophilic conditions and 50 – 55^{0}C. The doubling time of strain Z^T grown in rich medium is approximately 1 hour, at optimal pH and temperature. Strain Z^T is able to grow on a variety of organic compounds. Most carbon sources are converted to acetate, CO_2, H_2 and traces of propionate and lactate. Strain Z^T oxidises glutamate to acetate, CO_2, NH_4^+, traces of propionate and H_2. The doubling time on this substrate is 1.6 days. It can ferment glutamate syntrophically in coculture with Methanobacterium thermoautotrophicum Z245 to the same products, but the coculture had a 4 times higher growth rate. 16S rDNA sequence analysis revealed a relationship with Thermobrachium celere, Caloramator indicus and C. proteoclasticus. The G + C content is 31.7 mol%. Based on its morphological, phylogenetic and physiological characteristics, we propose that strain Z^T should be classified in the genus Caloramator as a new species, Caloramator coolhaasii.

INTRODUCTION

Proteins are important substrates in anaerobic digestion (Barker, 1981; McInerney, 1989). Amino acids, the hydrolysis products of proteins, can serve as carbon and energy sources for many types of anaerobic bacteria. Utilization of amino acids has been used as a classification characteristic for clostridia (Mead, 1971; Elsden, 1979). Several anaerobic bacteria have been described that can grow on glutamate. Most of them are members of the low G + C *Clostridium* subphylum, which convert glutamate to acetate, butyrate, CO_2, NH_4^+ and traces of hydrogen (Buckel & Barker, 1974; Laanbroek *et al.*, 1979). Also fermentative anaerobes are described that utilise glutamate in coculture with hydrogen-scavenging methanogens (Cheng *et al.*, 1992; Tarlera, 1997; Baena *et al.*, 1999a; Nanninga & Gottschal, 1985; Stams & Hansen, 1984). Some of these organisms are mesophilic, but *Selenomonas acidaminovorans*, recently renamed to *Thermanaerovibrio acidaminovorans* (Baena *et al.*, 1999b) and *Caloramator proteoclasticus* are moderately thermophilic. These bacteria grow slowly in pure culture, but a more rapid growth is achieved when grown syntrophically with a partner organism, which removes the hydrogen. These bacteria degrade 1 glutamate to 2 acetate, 1 HCO_3^-, 1 NH_4^+, and 1 H_2 or to 1 propionate, 2 HCO_3^-, 1 NH_4^+ and 2 H_2, where the ratio is dependent on the hydrogen partial pressure.

In this paper we describe the properties of strain Z^T, a novel organism isolated from anaerobic methanogenic granular sludge. This bacterium degrades glutamate in pure culture, but a much faster growth is achieved in coculture with hydrogenotrophic methanogens.

MATERIALS AND METHODS

Organisms, cultivation and isolation procedures. Strain Z^T was isolated from thermophilic methanogenic granular sludge converting glutamate as the sole source of carbon and energy. *Methanobacterium thermoautotrophicum* Z-245 (DSM 3720) and *Thermobrachium celere* (DSM 8682) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *Caloramator proteoclasticus* (DSM 10124) was from our own culture collection.

A bicarbonate buffered anaerobic medium as described previously by Stams *et al.* (1993), supplemented with 0.02% yeast extract, was used for enrichment and cultivation of strain Z^T. Wilkens-Chalgren Broth (Oxoid, Basingstoke, UK; 16 g/l) was used for the isolation of strain Z^T. Direct dilution series in liquid Wilkens-Chalgren Broth (WC Broth) as well as agar plates supplied with WC Broth were used. All incubations were done at 55°C in the dark, unless otherwise stated. For the direct comparison of strain Z^T with *C. proteoclasticus* and *T.celere* all cultures were cultivated on Wilkens Chalgren Broth (16 g/l); bicarbonate buffered anaerobic medium (Stams *et al.*, 1993); bicarbonate buffered anaerobic medium supplemented with 0.02% yeast extract; bicarbonate buffered anaerobic medium supplemented with 0.2% yeast extract and YTG medium. YTG medium contained per liter: Na_2CO_3, 5.3 g; Na_2HPO_4. 2 H_2O, 0.356 g; KCl, 0.075 g; yeast extract 5 g.; tryptone, 10 g.; cysteine-HCl 0.2 g.; Na_2S. 9 H_2O, 0.2 g.; resazurin 1 mg. Before use the pH of the YTG medium was adjusted to 10 by adding sterile 3 N NaOH. Glucose (10mM) was used as the carbon source, except for WC Broth, where no additional carbon source was added.

Determination of growth parameters. The growth rate of strain Z^T on WC broth was determined by measuring the increase in optical density at 600 nm in triplicate in time. The growth rate on glutamate with and without yeast extract was determined by measurement of product formation in time. Growth on different substrates was tested using a bicarbonate buffered anaerobic medium as described previously by Stams *et al.* (1993), supplemented with 0.02% yeast extract. The inoculum size of strain Z^T was 1%. Substrates were added from sterile stock solutions up to a concentration of 10 mM unless stated otherwise. For syntrophic growth tests 1% of strain Z^T was inoculated in hydrogen pregrown cultures of *M. thermoautotrophicum* Z245. In all cases growth was followed by visual examination of culture turbidity and by product formation after 8 weeks of incubation at 55°C. The temperature optimum was determined in WC broth at pH 7 in triplicate bottles at a temperature range of 15 – 80°C. The pH optimum was tested in WC broth adjusted with NaOH or HCl, and triplicate bottles were incubated at 55°C at a pH range of 4 – 10. Both temperature and pH were determined by measuring optical density after 2 weeks of incubation.

Cellular characterisation. The gram type was determined using gram staining and electron microscopy. Cells from active cultures were stained for gram type using 2% (w/v) crystal violet and 2% safranin S (w/v) as counter stain. Additionally a 2% (w/v) KOH solution was used to test lysis of bacteria. For transmission electron microscopy cells were fixed for two hours in 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH = 7.2) at 0^0C. After rinsing in the same sodium cacodylate buffer, a post fixation was done in 1% (w/v) OsO_4 and 2.5% (w/v) $K_2Cr_2O_7$ for 1 hour at room temperature. Finally the cells were poststained in 1% (w/v) uranyl acetate. Micrographs were taken with a Philips EM400 transmission electron microscope. Motility was determined using Gray's method for flagella staining (Gerhardt *et al.*, 1981). For negative staining, cells were fixed during 1 hour in 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH = 7.2). A Formvar coated grid (150 mesh) was placed on a drop of a dense cell suspension for 20 minutes. After rinsing with de-ionised water the cells were stained with 1% (w/v) uranyl acetate and the grid was air-dried. Cells were examined in a JEOL transmission electron microscope.

G + C content. The guanine-plus-cytosine (G + C) content of the DNA was determined at the DSMZ (Braunschweig, Germany) by high performance liquid chromatography (HPLC) (Mesbah *et al.*, 1989).

SDS-PAGE analysis for whole cell protein profiles. The strains were grown at 55°C in bicarbonate buffered anaerobic medium supplemented with 0.2% yeast extract and 10 mM glucose. Samples from cells in the logarithmic phase (corresponding to OD_{600} values of approximately 0.25) were centrifuged for 5 min. at 16.000 × g. The pellets were resuspended in 100 mM Tris HCl buffer (pH 7.2). Protein samples were diluted in denaturing loading buffer and boiled for 15 min. Cell debris were removed by centrifugation (5 min. at 16.000 × g) and samples were stored at 4°C until electrophoresis. SDS-polyacrylamide running gels with 10% acrylamide and 4% acrylamide stacking gels were run in a Bio-Rad Mini Protean II system at 20 mA for 2 h.. Approximately 10 µg protein was loaded in each lane. High-molecular-weight markers for SDS gel electrophoresis were from LKB-Pharmacia. The gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R250 in methanol/ deionized water

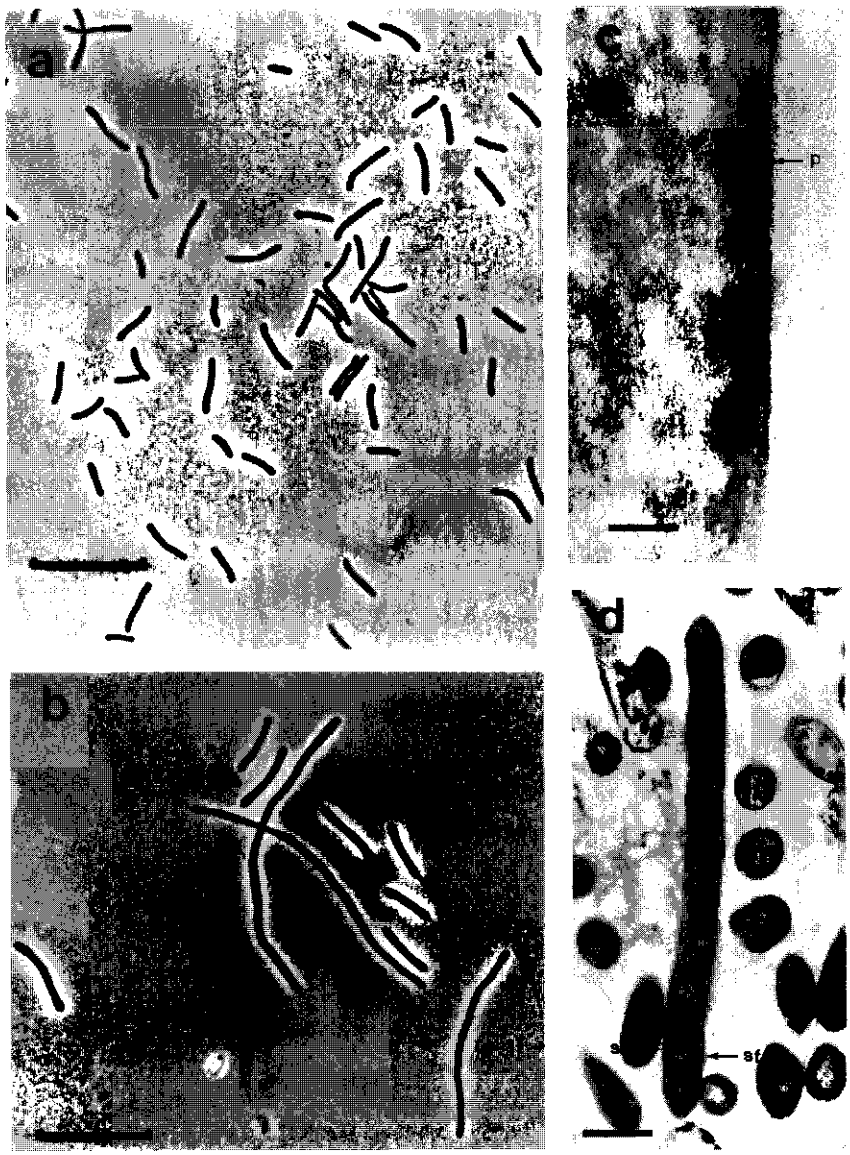

Figure 1. (A, B) Phase-contrast micrographs of cells of strain Z^T during growth on glutamate (A) and Wilkens Chalgren broth (B). Bar, 10 µm. (C, D) Electron micrographs showing the single-layered structure of the cell wall (C; bar, 0.1 µm) CM: cytoplasmic membrane; P: peptidoglycan layer and cell division (D; bar, 1 µm) SF: septum formation. Electron dense structures are probably intracellular protein precipitates (D).

/acetic acid (4:5:1, v/v/v) over night and destained in methanol/deionized water/acetic acid (4:5:1; v/v/v) for 3 h.

16S rDNA sequence analysis. Genomic DNA was isolated from strain Z^T, and the 16S rDNA was amplified using the bacterial primers 8f and 1510r (Lane, 1991) under conditions as previously described (Harmsen *et al.*, 1995). PCR products were purified and concentrated using a QIA Quick Kit (Qiagen GmbH, Hilden, Germany). Approximately 1 μg of purified PCR product was used for sequence analysis using the Sequenase T7 sequencing kit (Amersham, Slough, U.K.) according to the manufacturer's instructions. Infrared Dye 41 (MWG-Biotech, Ebersberg, Germany) labelled primers 515r, 338f (Lane, 1991) and 968f (Nübel *et al.*, 1996) were used as sequencing primers. The sequences were automatically analysed on a LI-COR (Lincoln, NE., USA) DNA sequencer 4000L and corrected manually. The software of the ARB package (Strunk & Ludwig, 1991) was used to check reading errors and for phylogenetic analysis. A neighbor-joining tree was constructed with the closest relatives. FASTA homology searches were done using the GenBank and EMBL databases. The homologies were checked with the ARB programs.

Nucleotide accession number. The 16S rDNA of strain Z^T has been deposited in the Genbank database under accession number: AF104215.
The EMBL database accession numbers of the strains closest related to strain Z^T are *Caloramator proteoclasticus* (DSM 10124) X90488; *Caloramator indicus* (ACM 3982) X75788; *Thermobrachium celere* (DSM 8682), X99238; *Clostridium botulinum* X68187; *Clostridium homopropionicum* X76744; *Clostridium acetobutylicum* X78071 and *Clostridium algidicarnis* X77676.

Other analysis. Substrate and fermentation product concentrations were measured using GC and HPLC methods as described previously (Stams *et al.*, 1993). Amino acids were measured as described by Kengen & Stams (1994), with an extension of the derivatisation time to 30 minutes. Sulfide was analysed as described by Trüper & Schlegel (1964). Anions were analysed as described by Scholten & Stams (1995).

RESULTS

Enrichment and isolation of strain Z^T Strain Z^T was enriched from anaerobic methanogenic granular sludge fed with glutamate as the sole carbon and energy source. Strain Z^T was the predominant glutamate-degrading organism in most probable number determinations of diluted biomass. Methanogens were present as the only microscopically visible impurity. The enrichment obtained from the MPN determinations was serially diluted in the presence of 5 mM bromoethane sulphonate to inhibit the methanogens. In the absence of methane production growth of strain Z^T on the mineral salts medium was poor. Strain Z^T was therefore serially diluted and transferred to anaerobic bottles containing Wilkens Chalgren broth (16 g/l). The growth yield and growth rate of strain Z^T were considerably higher in this medium. To remove traces of methanogens strain Z^T was plated on WC broth agar. This resulted in the development of circular, brownish, shiny colonies. Four colonies were picked from the highest-diluted plate with growth and transferred to a dilution series in WC broth. The highest dilution with growth was used to inoculate WC broth agar plates again, resulting in the development of the type of colonies as described above. This procedure was repeated once more, and resulted in a pure culture of strain Z^T when checked by light and electron microscopy.

Morphology and cell structure Cells of strain Z^T growing in WC broth were rod to filamentous shaped, with a size of 2-40 μm by 0.5 - 0.7 μm as measured by light microscopy (Fig. 1A and 1B). During exponential growth the cells were very long. Rapid lysis was observed as soon as the cells entered the stationary phase. However, in mineral salts medium with glutamate, cells were smaller and did not lyse in the stationary phase. Spores were never observed, and no growth occurred after pasteurisation of the culture (20 min., 90°C). Cells stained gram-negative, but the cell wall ultra structure resembled that of gram-positive anaerobic bacteria (Fig 1C).

Physiological characteristics and metabolism. Strain Z^T was a strictly anaerobic bacterium. No growth occurred when traces of oxygen were present in the medium, as indicated by the pink colour of the medium in which resazurin was present. Its optimal growth temperature was 50-55°C with a lower and upper limit of 37 and 65°C,

Table 1. Growth and glucose fermentation products of strain Z^T, *C. proteoclasticus* and *T. celere* on different media.

Medium	Strain Z^T	*C.proteoclasticus*	*T.celere*
Wilkens Chalgren Broth	+*)	+	+
Bicarbonate buffered glucose medium	acet, lact, H_2#)	-	-
Bicarbonate buffered glucose medium (0.02% yeast extract)	acet, lact, H_2	acet, etoh, form, lact, H_2	-
Bicarbonate buffered glucose medium (0.2% yeast extract)	acet, (etoh), form, lact, H_2	acet, etoh, form, lact, H_2	acet, (etoh), form, H_2
YTG medium, pH 10	-	-	acet, (etoh), form, H_2

*) -: no growth; +: growth

#) acet: acetate; lact: lactate; form: formate; etoh: ethanol. Products between brackets represent minor products

respectively. The pH optimum was 7-7.5. Below a pH of 6 and above a pH of 8.5 no measurable growth was observed. Growth of strain Z^T was possible in a mineral salts medium supplemented with vitamins and glutamate as the sole source of carbon and energy. However, growth was enhanced when 0.02% yeast extract was added. The doubling time of strain Z^T in glutamate media without and supplemented with 0.02% yeast extract was approximately 3.5 and 1.6 days, respectively. The doubling time of strain Z^T in Wilkens Chalgren broth was about 1 h.

Strain Z^T grew with complex substrates like yeast extract, casamino acids, gelatin, casitone, peptone (2%, w/v, of these compounds was added) and Wilkens Chalgren broth.

The following single substrates supported growth of strain Z^T: glutamate, aspartate, alanine, arginine, methionine, glucose, galactose, fructose, mannose, maltose, ribose, starch (0.5%, w/v), xylose, cellobiose (5 mM) and pyruvate. Growth on glutamate, aspartate, alanine, arginine, methionine, mannose, ribose and pyruvate was enhanced when cocultivated with *M. thermoautotrophicum* Z245. It produced acetate (13.9 mM), H_2 (10.1 mmol/l medium) and traces of propionate (1.3 mM) from glutamate (9.8 mM). The carbon and electron recoveries were 97 and 84%, respectively, assuming that glutamate conversion to 2 acetate yields 1 CO_2, while 2 CO_2 are formed when glutamate is converted to propionate. From the glutamate originally added 60-70% was degraded. Glutamate metabolism was influenced by the presence of *Methanobacterium thermoautotrophicum* Z245; glutamate (18.6 mM) was

converted to acetate (30.7 mM), CH_4 (8.6 mmol/l medium; equivalent to 34.4 mmol H_2 /liter medium) and traces of propionate (1 mM). The carbon and electron recoveries were 102 and 109 %, respectively, and glutamate was completely converted. The growth rate was about 4 times higher when *Methanobacterium thermoautotrophicum* Z245 was present. When strain Z^T was grown on glutamate under H_2/CO_2 (80/20) only 7-10% of the glutamate was converted. Under these conditions it was not possible to obtain a closed electron balance. Glucose was fermented to acetate, lactate, CO_2 and H_2. When the amount of yeast extract in the medium was increased to from 0.02% to 0.2% formate and traces of ethanol could be detected as reduced end products besides the usual products. The following components did not support growth of strain Z^T: glycine, leucine, cysteine, arabinose, mannitol, melobiose, rhamnose, casein (0.5% w/v), cellulose (0.5% w/v), citrate, iso-citrate, 2-oxoglutarate, fumarate, malate, benzoate, methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol and acetoin neither in the absence nor in the presence of *Methanobacterium thermoautotrophicum* Z245. A Stickland reaction was not observed with H_2, alanine and leucine as electron donor and glycine, arginine and proline as electron acceptor. The combination of alanine and arginine resulted in a higher production of hydrogen as compared to alanine alone, indicating an oxidative deamination of arginine. Yeast extract stimulated growth on glutamate, but was not

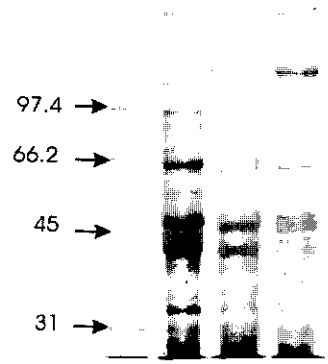

Figure 2. SDS-PAGE of whole-cell proteins of strain Z^T and closely related strains. Lanes show, from left to right: set of marker protein with their molecular masses; *C.proteoclasticus*; Strain Z^T; *Thermobrachium celere.*

required. Sulphide-reduced media were required for growth. Oxygen, nitrate, sulphate, thiosulphate and fumarate could not serve as an electron acceptor for growth on glutamate; no measurable decrease in these compounds could be found after incubation.

Direct comparison of strain Z^T with *C. proteoclasticus* and *T.celere*. Strain Z^T, *C. proteoclasticus* and *T.celere* grew well in Wilkens Chalgren Broth and anaerobic bicarbonate buffered medium supplemented with 0.2% yeast extract and 10 mM glucose. Strain Z^T and *C. proteoclasticus* grew well in a bicarbonate buffered medium supplemented with 0.02% yeast extract and 10 mM glucose and strain Z^T was the only strain capable of growing in bicarbonate buffered medium with glucose without yeast extract. Fermentation products of strain Z^T, *C. proteoclasticus* and *T.celere* grown in glucose yeast extract medium were different (Table 1).

The total cell proteins of strain Z^T, *C. proteoclasticus* and *T.celere* were separated on SDS-PAGE gels (Fig. 2). The pattern of the three strains differed from each other indicating that these organisms are different species.

Phylogeny. The nucleotide sequence (1454 base pairs) of a 16S rRNA gene of strain Z^T was analysed and revealed that this organism belongs to the subphylum of gram-positive clostridia with low G + C content. Sequence analysis showed that strain Z^T is closely related to *Thermobrachium celere, Caloramator indicus and Caloramator proteoclasticus* (Engle *et al.*, 1996; Christostomos *et al.*, 1996; Tarlera *et al.*, 1997) with levels of similarity of 97.8, 97.2 and 96.2%, respectively. A phylogenetic tree showing the relationship of strain Z^T and other related species is depicted in Fig. 3. The G + C content of strain Z^T was 31.7 ± 0.4 mol%.

DISCUSSION

Phylogenetically, strain Z^T belongs to the low G + C containing clostridial group and has close relatedness with *T. celere, C. indicus* and *C. proteoclasticus* (Engle *et al.*, 1996; Christostomos *et al.*, 1996; Tarlera *et al.*, 1997). Within this group all members are closely related, but are described and approved as new species. Based on 16S rDNA analysis *C. indicus* and *T. celere* seem to be almost 100% related.

Table 2. General characteristics of strain Z^T compared to *T.celere*, *C. indicus* and *C.proteoclasticus**).

	Strain Z^T	*T. celere*	*C. indicus*	*C. proteoclasticus*
Gram stain	-	+	-	-
Cell wall type	+	+	+	+
G + C%	31.7	31	25.6	31
Temp. opt.	50-55	62-67	60-65	55
Temp range	37-65	43-75	37-75	30-68
pH opt.	7-7.5	8-8.5	8.1	7-7.5
Motility	-	+	-	+
Spore formation	-	-	-	+
Habitat	Thermophilic granular sludge	Several locations	Thermophilic aquifer	Mesophilic granular sludge
Geographical location	Wageningen, The netherlands	Sevral locations	Gujarat, India	Montevideo, Uruguay

*) data taken from Engle et al. (1996), Christostomos et al. (1996), Tarlera et al.(1997)

Despite the similarity of the 16S rDNA with these mentioned species, there are several differences between strain Z^T and the other members within the genus *Caloramator* (Collins *et al.*, 1994). There are several differences (Table 2), such as the absence of endospores, the immobility, optimum growth temperature and optimum pH for growth. Furthermore, the physiological characteristics of strain Z^T also differ considerably from the other species (Table 3). All four isolates have a broad saccharolytic activity, but strain Z^T is the only strain capable of fermenting xylose. *C. proteoclasticus* is not able to ferment alanine, but forms alanine as a reduced end product from glutamate fermentation (Tarlera,1997). Strain Z^T has no proteolytic activity on casein. Direct comparison of strain Z^T, *C. proteoclasticus* and *T.celere* shows that strain Z^T is the only strain able to grow in a bicarbonate buffered anaerobic medium with glucose as the sole source of carbon and energy. Strain Z^T and *C. proteoclasticus* show no grow on YTG medium at pH 10, whereas *T.celere* grows very well at this pH.

The glucose fermentation products of the three strains also differ; *T. celere* forms only traces of ethanol and no lactate in a bicarbonate buffered medium with 0.2% yeast extract whereas strain Z^T forms traces of ethanol and does produce lactate. *C.proteoclasticus* forms ethanol and lactate as major fermentation products. The whole cell protein profiles of strain Z^T, *C. proteoclasticus* and *T.celere* on SDS-PAGE is different. These differences justify the creation of a new species in the *Caloramator* genus. The choice for the *Caloramator* genus was made because this genus was validly published prior to the genus *Thermobrachium* (Collins *et al.*, 1994; Engle *et al.*, 1996).

The glutamate metabolism of strain Z^T is clearly enhanced by the presence of a hydrogenotrophic methanogen. Complete conversion of glutamate occurred under methanogenic conditions, whereas in pure culture hydrogen formation inhibited glutamate degradation. *Selenomonas (Thermanaerovibrio) acidaminovorans* (Cheng *et al.*, 1992), another glutamate-degrading, hydrogen-producing moderate thermophile that was tested in coculture with a methanogen, showed a complete glutamate conversion, both in pure as well as in coculture. However, *S. acidaminovorans* degraded alanine, isoleucine, leucine and valine only in the presence of a methanogen, indicating that its metabolism is also inhibited by hydrogen formation. Similar observations were made with *Clostridium acetireducens*, another moderate

Table 3. Selected growth substrates that differentiate strain Z^T from *T.celere, C. indicus* and *C.proteoclasticus*[*].

	Strain Z^T	*T. celere*	*C. indicus*	*C. proteoclasticus*
glucose	+	+	+	+
arabinose	-	-		
fructose	+	+	+	+
galactose	+	+		
mannose	+	-	+	+
maltose	+	+		
rhamnose	-	-		
ribose	-	-		
xylose	+	-		-
sucrose	+	+	+	
lactose	-	-	+	-
casamino acids	+	-		+
yeast extract	+	+		+
casein	-			+
casitone	+			
starch	+		+	+
cellulose	-		-	-
cellobiose	+	-	+	+
peptone	+			+
2-oxoglutarate	-			-
glutamate	+			+
W.C. broth	+			
glycine	-			+
leucine	-			+
aspartate	+			+
alanine	+			-
pyruvate	+	-		+
Stickland reaction	-			+

*) data taken from Engle *et al.* (1996), Christostomos *et al.* (1996), Tarlera *et al.*(1997)

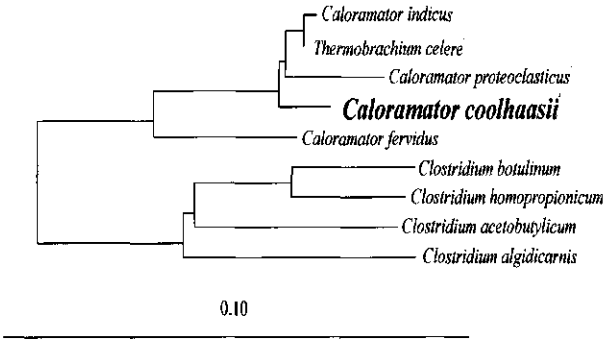

Figure 3. Dendrogram showing the phylogenetic position of strain Z^T among representatives of the genus *Clostridium*. Bar = 0.1 (evolutionary distance).

thermophile, which is unable to grow on glutamate (Örlygsson *et al.*, 1996). Strain Z^T did not show a shift in product formation when it was co-cultivated with a methanogen, but a more rapid and complete degradation of glutamate occurred.

Taxonomy. *Caloramator cool.haas'.i.i.* M.L. gen. sp. nov.. coolhaasii of Coolhaas. In honor of Caspar Coolhaas, a Dutch microbiologist, who was the first to describe thermophilic protein degradation under methanogenic conditions (Coolhaas, 1927).
Rods to filamentous cells that are 2 – 40 µm long by 0.5 – 0.7 µm wide. Gram stain negative, with gram-positive cell wall, non-motile, no spore formation. Strictly anaerobic chemo-organoheterotroph. Utilises glutamate, aspartate, alanine, arginine, methionine, glucose, galactose, fructose, mannose, maltose, ribose, xylose, casamino acids, yeast extract, gelatin, casitone, starch, peptone, cellobiose, pyruvate and Wilkens Chalgren broth for growth. Growth on glutamate, aspartate, alanine, arginine, methionine, mannose, ribose and pyruvate is enhanced in the presence of the methanogen *M. thermoautotrophicum* Z245. Produces acetate, CO_2, H_2 and traces of propionate from glutamate. Produces acetate, lactate, CO_2 and H_2 from glucose. Yeast extract stimulated growth on glutamate, but was not required. No Stickland reaction could be observed. Sulphide-reduced media were required for growth. No reduction of oxygen, nitrate, sulphate, thiosulphate or fumarate.

Moderately thermophilic. Growth range 37 – 65^{0}C, optimum 50 – 55^{0}C. Growth in pH range 6 – 8.5, with an optimum of 7 – 7.5.

The G + C content was 31.7 ± 0.4 mol%.

The type strain is strain Z (= DSM 12679).

Habitat: anaerobic thermophilic methanogenic sludge.

ACKNOWLEDGEMENTS

We want to thank Ineke Keizer-Gunnink and Marten Veenhuis (University of Groningen, The Netherlands) for performing the electron microscopy. Daniel Gisi (ETH, Zürich, Switzerland) is highly acknowledged for his help and useful advice with the molecular characterisation.

Part of this work was performed during the 1997 Microbial Diversity Course at the Marine Biological Laboratory (Woods Hole, Ma, USA). C.P. is deeply grateful to Ed Leadbetter and Abigail Salyers for giving her the opportunity to participate in this course.

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"Methanosaeta thermophila" strain A, a thermophilic, aggregating, acetoclastic methanogen

Caroline M. Plugge and Alfons J.M. Stams

Abstract

A thermophilic strain of Methanosaeta, strain A, was highly purified from a methanogenic glutamate-degrading consortium in a dialysis membrane reactor after enrichment on acetate. The isolate was able to grow and produce methane from acetate. No growth or methane production was observed when H_2, methanol, formate, trimethylamine or pyruvate were provided as substrates in the presence of bicarbonate. The doubling time of the methanogen was approximately 2.5 days. The optimum pH and temperature for growth were 7.5 and 55-60^{0}C, respectively. Boiled cells enhanced growth of the culture. The isolate showed an unusual granule formation in batch cultures and those granules were difficult to disintegrate. The granule formation makes this organism suitable for application in starting up anaerobic granular sludge bed reactors. 16S rDNA analysis revealed similarity with other Methanosaeta strains, with Methanosaeta thermophila Cals 1 as its closest neighbor.

Key words:
Methanogenesis; Acetate; Methanosaeta; Thermophiles; Granule formation

INTRODUCTION

Acetate is a major intermediate in anaerobic methanogenic digestion. Two-third of the methane produced is derived from acetate (Jetten et al. 1992; Zinder 1993). To date only two genera of methanogenic archaea are described which use acetate as sole source of energy: *Methanosarcina* and *Methanosaeta*. *Methanosarcina* and *Methanosaeta* species can be found in both fresh water and marine environments such as anaerobic digesters, rumen, rice paddies and sediments (Jetten et al. 1992; Zinder 1993). Both genera have a very distinct morphology and can be easily recognised in mixed populations by phase contrast microscopy. *Methanosarcina* has a typical coccoid shape and forms large packets. *Methanosaeta* consists of filamentous, straight, sheathed rods, and the filaments can grow to a length of 100 μm. *Methanosaeta* is one of the most predominant organisms in anaerobic digesters where the biomass is immobilised in granules.

Despite its predominance it is very difficult to obtain pure cultures of *Methanosaeta* strains. The existence of acetoclastic methanogens was described already in 1876 by Hoppe-Seyler. It took, however, until 1980 when the first mesophilic *Methanosaeta* was obtained in pure culture (Zehnder et al. 1980; Huser et al. 1982). Several mesophilic strains have been described afterwards, some as pure cultures, others as highly purified cultures (Patel 1984; Touzel et al. 1988; Otsubo et al. 1991). Two axenic thermophilic *Methanosaeta* strains have been described (Zinder 1987; Kamagata and Mikami 1992) and one highly purified strain (Nozhevnikova and Chudina 1984).

After elaborate discussions the previously described thermophilic acetoclastic *Methanothrix* strains were recently classified as *Methanosaeta thermophila* (Boone and Kamagata 1998). Thermophilic and mesophilic *Methanosaeta* species are homologous not only with respect to their physiological and morphological characteristics, but also with respect to their 16S rDNA sequences. A similarity of 94-99%, on the basis of comparison of partial 16S rDNA sequences was found (Kamagata et al. 1992).

In this paper we describe the properties of a highly purified culture (>99.99%) of *Methanosaeta thermophila* (strain A), obtained from a Dialysis Membrane Reactor (see chapter 3). This strain showed unusual pellet formation.

MATERIALS AND METHODS

Enrichment and cultivation. Enrichment of strain A was as described previously (see chapter 3). A bicarbonate-buffered medium with the following composition was used (per litre): 0.4 g KH_2PO_4; 0.53 g Na_2HPO_4; 0.3 g NH_4Cl; 0.3 g NaCl; 0.1 g $MgCl_2.6H_2O$; 0.11 g $CaCl_2$; 1 ml alkaline trace element solution; 1 ml acid trace element solution; 1 ml vitamin solution; 0.5 mg resazurin; 4 g $NaHCO_3$; 0.25 g Na_2S. 7-9 H_2O. The trace elements and vitamins were as described previously by Stams et al. (1992). All compounds were heat sterilised except for the vitamins, which were filter sterilised. Incubations were done in serum bottles sealed with butyl rubber stoppers (Rubber bv, Hilversum, The Netherlands) and a gas phase of 182 kPa (1.8 atm) N_2/CO_2 (80:20, vol/vol) at 55^0C. Substrates were added from sterile, anoxic stock solutions to a final concentration of 20 mM unless stated otherwise. Growth on solid media was tested in roll tubes and on plates. Agar, Bacto-agar, agar noble, agarose (Difco) were used as solidifying agents at a concentration of 0.5-3% (w/v).

The effect of antibiotics was tested with a range of antibiotics, added from freshly prepared anoxic filter sterilised stock solutions (Table 1).

Determination of growth parameters. Growth rates were determined by measuring CH_4 formation and acetate depletion. The temperature optimum was tested in triplicate bottles with bicarbonate-buffered medium with 20 mM acetate at pH 7.2 at a temperature range of 25-80^0C. The pH optimum was tested in medium without bicarbonate, with 50 mM phosphate. Triplicate bottles were tested at 55^0C and the pH was adjusted with the addition of NaOH or HCl at a range of 4-10. Both temperature and pH optimum were determined by measuring the CH_4 formation after 4 weeks incubation.

Cellular characterisation. Cultures were routineously examined by phase-contrast microscopy using a Wild-Heerbrugg phase contrast microscope. Epifluorescence was checked using a Leitz Dialux 20 EB microscope equipped with an I 2/3 filter block. Transmission electron microcroscopy was performed as described previously (Plugge et al. 2000).

Table 1. Effects of antibiotics on the growth of strain A

Antibiotic	Dose ($\mu g.ml^{-1}$)	Growth inhibition of strain A (%)*
Vancomycin (V)	100	0
Vancomycin	200	5
Neomycin (N)	10	20
D-cycloserine (C)	100	30
Kanamycin (K)	100	90
Penicillin-G (P)	100	0
Penicillin-G	200	0
V + N	100 + 10	0
V + N + C	100 + 10 + 100	50
V + N + P	100 + 10 + 100	10
V + K + C	100 + 100 + 100	100
K + C	100 + 100	100
V + P + K	100 + 100 + 100	100
V + P	100 + 100	0

*) Calculated compared to incubations without antibiotics after 4 weeks of incubation, based on CH_4 formation. The contaminant was not inhibited completely in any of the incubations

Analytical methods. Gases were measured on Packard 417 gas chromatograph equipped with a thermal conductivity detector and a molecular sieve column and argon as the carrier gas. Acetate was measured on a Chrompack CP 9001 gas chromatograph with a flame ionisation detector, equipped with a Chromosorb packed column and nitrogen, saturated with formic acid as the carrier gas.

DNA isolation and 16S rDNA sequence analysis. Total DNA was isolated using the method of Zoetendal et al. (1998) with the following modifications. Prior to bead beating the sample was incubated with buffered phenol at 60^0C for 40 min. and the sodium acetate (300 mM) plus ethanol (96%) precipitation step was overnight at -20^0C. The 16S DNA was amplified from the total DNA by using the following primers 1510r (Lane 1991) and a specific archaeal primer Metf (5'-TTC TGG TTG ATC CTG CCA GA-3') in combination with the Taq DNA polymerase kit from Life

Technologies (Breda, The Netherlands). The PCR products were purified with the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Infrared Dye 41 (MWG-Biotech, Ebersberg, Germany) labelled primers 533 f and 519 r (Lane 1991) were used as sequencing primers. The sequences were analysed automatically on a LI-COR (Lincoln, NE., USA) DNA sequencer 4000L and corrected manually. The sequences were checked for reading errors with the alignment programs of the ARB package (Strunk and Ludwig 1991). Homology searches of the EMBL, and GenBank DNA databases for these partial sequences were performed with FASTA and the homologies were checked with the ARB programs.

The 16S rDNA sequence of strain A has been deposited in the Genbank data base under accession number AF334401.

RESULTS AND DISCUSSION

Enrichment of strain A. Strain A was obtained from the highest positive dilution with acetate of a dialysis membrane reactor (see chapter 3). The highest dilution contained an almost pure culture of an organism resembling *Methanosaeta* as described by others (Nozhvenikova and Chudina 1984; Zinder et al. 1987; Kamagata et al. 1991). Granule formation was a remarkable morphological feature of this enrichment (Fig. 1).

For further enrichment the culture was again serially diluted in acetate containing medium containing 100 µg/ml vancomycin and 1 mM 2-mercaptoethanesulphonic acid. This resulted in a decrease of the contaminants, but not in a pure culture. The contaminants were embedded in the granules of strain A. Other antibiotics were used to remove the contaminants present in the enrichment culture (Table 1). The effect of the antibiotics was calculated from methane formation and not from growth. Optical density was not a reliable parameter to use, since the culture did not grow in suspension. The effect of the antibiotics was found to be strongly time dependent. After 4 weeks of incubation most antibiotics lost their inhibitory effect, whereas after two weeks of incubation the antibiotics were still effective.

Figure 1. Granule formation by strain A; (**A**) *Methanosaeta concilii* flock formation (left) and strain A granule formation (right); (**B**) and (**C**) details. (**C**) smallest square represents 1 mm^2

Touzel et al. (1988) already noted this effect for *Methanothrix soehngenii* FE. It seemed that antibiotics were not chemically stable during the 4 weeks of incubation at 55^0C. Kanamycin was found to have a strong inhibitory effect on the growth of strain A. This was also observed by Touzel et al. (1988). However, the contaminant could not be removed from the highly purified culture of strain A with none of the antibiotics, or mixtures of the antibiotics, used.

The highly purified strain A was unable to grow in serial dilutions further than a 100-fold dilution. To retrieve growth in serial dilution boiled cells were added. An old culture was boiled for 1 hour at 105^0C and 1% (v/v) was added to acetate containing media with 100 µg/ml vancomycin and 1 mM 2-mercaptoethane sulphonic acid. In this way growth up to a dilution of 10^{-7} could be obtained. However, still no pure culture could be obtained. Since the methanogen in the culture was growing in large filaments it was difficult to estimate the relative abundance of the contaminant. Based on microscopical analysis of disintegrated granules we estimated that less than 0.01% of the culture consisted of the contaminant.

Strain A was unable to grow on solid media. None of the solidifying agents used in different concentrations yielded colonies after 3-6 months of incubation at 55^0C.

Morphological characteristics of strain A. Cells of strain A were non-motile, straight, sheathed, gram-negative rods (Fig. 2a). The size of single cells was 5 by 4 µm. Cells remained connected to each other forming filaments, with a length of more than 100 µm. Gas vesicles could be observed in the filaments (Fig.2b). Gas vesicles do occur in all described thermophilic *Methanosaeta* strains (Nozhvenikova and Chudina 1984; Zinder et al. 1987; Kamagata and Mikami 1991). The cells grew in granules, which could not easily be dispersed. In upflow anaerobic sludge bed reactors the upward stream of the influent causes a strong selection on the biomass in the reactor. The biomass is immobilised in granules and microorganisms with good adhering properties remain in the reactor. However, when this strong selection pressure is omitted, there is no need for microorganisms to grow in flocks or granules. The *Methanosaeta* strain described here is capable of forming these granules under all conditions tested. Other *Methanosaeta* strains often grow in flocks or aggregate in bundles. However, shaking gently (Kamagata and Mikami 1991) or intensive shaking

(Huser et al. 1980; Patel 1984), usually dispersed the cells. When incubated on a shaker the granules formed by strain A were even more difficult to disrupt than when grown without shaking.

Salt concentration (0-500 mM) had no effect on granule formation. *Methanosarcina* can grow in clumps, but when it is grown in a medium with high salt

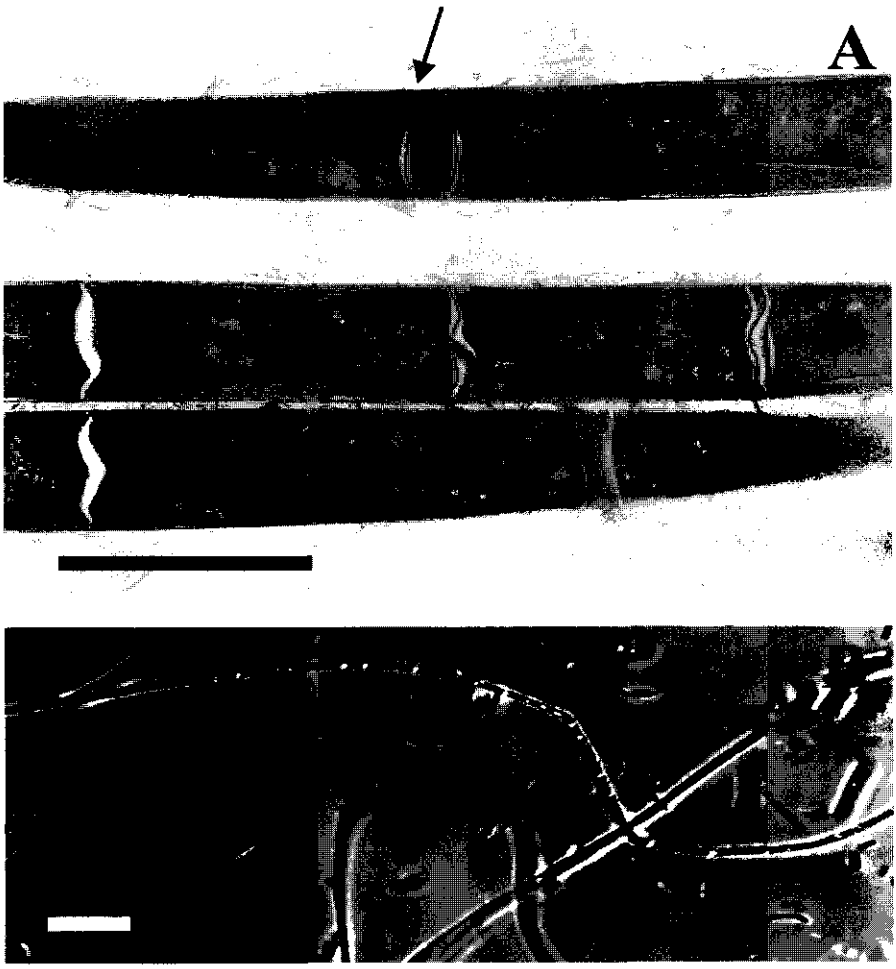

Figure 2. Micrographs of *Methanosaeta thermophila* strain A. (**A**) Thin section showing cell wall structure, the asymetrical aspect of the cell spacers and non-viable cytoplasmic portion (arrow mark). Bar = 0.2 μm (**B**) Gas vesicles in strain A under phase contrast microscopy. Bar = 5 μm.

concentrations the clumps are not formed (Sowers et al. 1993). The specific chelators EDTA (0-5 mM), EGTA (0-5 mM), NTA (0-5 mM) had no effect on granule formation, but only increased the lag phase or inhibited growth. From these observations we conclude that cations do not play an important role in the formationof granules by strain A. Over night incubation of a dense suspension of strain A with 3 mM of the same chelators did not result in any disintegration of the granules. Only a distinct change in colour from blackish to beige of the granules was observed, indicating that the chelators bound the iron and other cations.

Mild ultrasonic treatment (150 Watt, 3 x 30 sec.) partially disintegrated the granules. Microscopic examinations showed that approximately 70% of the biomass consisted of dispersed filaments of strain A. Repeated ultrasonic treatment of the granules (150 Watt, 10 x 60 sec.) led to an almost complete dispersion of the granules. However, a considerable part of the cells was lysed. Inoculation of the suspended biomass in fresh medium leaded to growth in granules, up to a dilution of 10^{-2}. Growth in higher dilutions was only observed when 1% (v/v) boiled cells were added.

Physiological characteristics of strain A. The optimum temperature for growth was 55-60^{0}C, with no methanogenesis detected after 4 weeks of incubation below 40 and above 70^{0}C. The optimum pH was 7.5. No methanogenesis was detected below 5.5 and above 8.5. Acetate was stoichiometrically converted to CH_4 as shown in Fig. 3. The doubling time of strain A was approximately 2.5 days. The growth rate was independent on the acetate concentration (5-100 mM). The doubling time was comparable with that of other thermophilic *Methanosaeta* strains: strain P_t: 1,5 days, strain Cals-1: 1 day and strain Z-517: ~5 days (Nozhvenikova and Chudina 1984; Zinder et al. 1987; Kamagata and Mikami 1991). The molar growth yield of strain A was 1.8 g (mol acetate)$^{-1}$. Acetate was the only compound tested which supported growth and methane formation by strain A. Compounds tested which did not yield methane were methanol, formate, trimethylamine, H_2/CO_2 and pyruvate. However, on pyruvate rapid growth of the impurity present in the culture could be observed. The pyruvate was most probably converted to acetate, which was than further degraded to CH_4 by strain A.

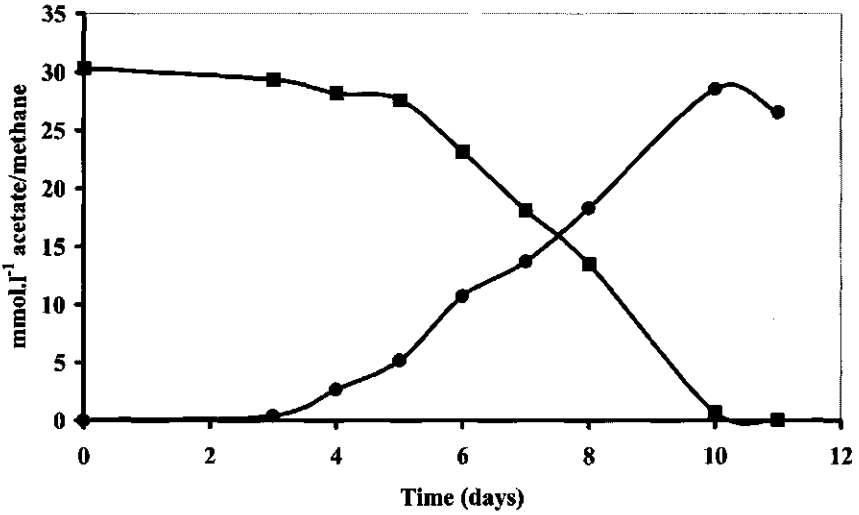

Figure 3. Acetate (■) conversion and CH_4 (●) formation by *Methanosaeta* strain A.

Phylogenetic analysis. The partial nucleotide sequence (550 base pairs) of a 16S rDNA gene of strain A was analysed and revealed that this organism was related with other *Methanosaeta* species. Strain A is 94% related with *Methanosaeta thermophila* strain Cals1 and 93% with *Methanosaeta soehngenii* strain Opfikon as shown in Fig. 4.

Practical applications. The treatment of wastewater in upflow anaerobic sludge bed (UASB) reactors is a very suitable, world-wide applied and appreciated technique. The use of UASB reactors is especially applicable because a very high organic loading rate can be applied. A prerequisite for high-rate reactors is the presence of high concentrations of biomass in the reactor. Usually this biomass is present as granular aggregates. When a new UASB reactor is started up it can be seeded with anaerobic granular sludge from an already active UASB reactor. However, when a reactor has to be started up from active sludge, because granules are not available, it can take months before a good and active granular sludge bed is cultivated. Cells from strain A are capable of forming these granules already in batch cultures and can therefor be used as inoculum for UASB reactors. The aggregates may serve as seeds for new granules.

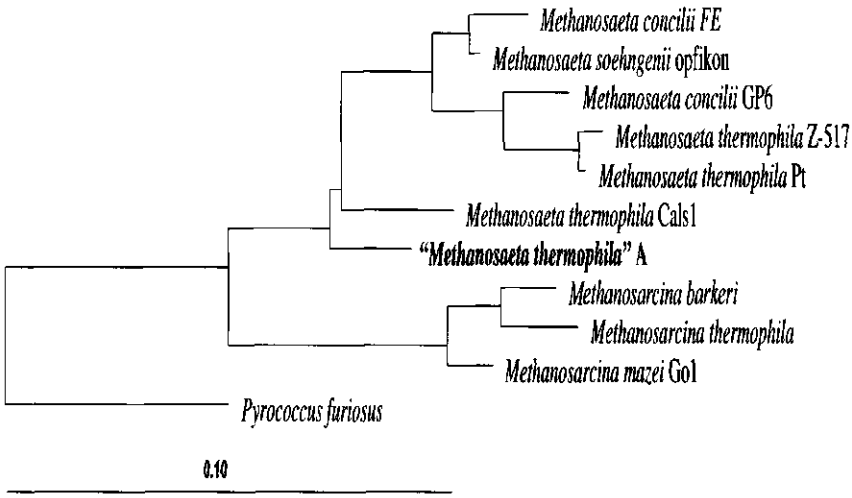

Figure 4. Neighbor joining tree based on 16S rDNA sequences showing the phylogenetic position of strain A among other *Methanosaeta* species. Bar represents evolutionary distance.

The strains that were used for the construction of the tree were: *Methanosaeta thermophila* Cals1, M59141; *Methanosaeta thermophila* Pt, S42728; *Methanosaeta thermophila* Z-517, S42717; *Methanosaeta soehngenii* opfikon, X16932; *Methanosaeta concilii* FE, M59146; *Methanosaeta concilii* GP6, S42679; *Methanosaeta thermophila* strain A, AF 334401

ACKNOWLEDGEMENT

We want to thank Klaas Sjollema (University of Groningen, The Netherlands) for performing the electron microscopy.

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Gelria glutamica, gen. nov., sp. nov., a novel thermophilic obligate syntrophic glutamate-degrading anaerobe.

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Submitted for publication

Abstract

A novel anaerobic, thermophilic, obligate syntrophic, spore-forming, glutamate-degrading bacterium, strain TGO, was isolated from a propionate-oxidising methanogenic enrichment. The cells were rod-shaped and non-motile. The optimal temperature for growth was 50-55^{0}C and growth occurred between 37 and 60^{0}C. The pH range for growth was 5.5 - 8 with an optimum at 7. In pure culture strain TGO could grow on pyruvate, lactate, glycerol and several sugars. In coculture with the hydrogenotrophic methanogen Methanobacterium thermoautotrophicum Z245, the strain could also grow on glutamate, proline and casamino acids. Glutamate was converted to H_2, CO_2, propionate and traces of succinate. Strain TGO was not able to utilise sulphate, sulphite, thiosulphate, nitrate and fumarate as electron acceptor. The G + C% was 33.8. 16S rDNA sequence analysis revealed that strain TGO belongs to the thermophilic, endospore-forming anaerobes, though no close relations were found. Its closest relations were with Moorella glycerini (92%) and Moorella thermoacetica (90%). Strain TGO had an unusual long 16S rDNA gene, with more than 1700 base pairs. The additional base pairs were found as long loops in the V1, V7 and V9 region of the molecule. However, the loops were not found in the 16S rRNA. We propose the name Gelria glutamica for strain TGO as a new species of a new genus.

Key words

Gelria glutamica gen. nov., sp. nov., thermophilic bacteria, interspecies hydrogen transfer, obligate syntrophic glutamate oxidation, proline oxidation, propionate formation

INTRODUCTION

Because proteins are encountered in almost every ecosystem, the biodegradation of amino acids is a very important microbial process. The conversion of amino acids in methanogenic environments has been studied during the last 20 years, especially in mesophilic environments (Barker 1981; Nagase & Matsuo 1982; McInerney, 1989). However, in moderately thermophilic methanogenic environments, the degradation of amino acids has not been studied in detail. Only a few bacterial species are described to degrade amino acids under thermophilic conditions (Cheng *et al.*, 1992; Örlygsson *et al.*, 1994; Tarlera *et al.*, 1997; Plugge *et al.*, 2000).

Glutamate conversion under methanogenic conditions can occur in different ways (see Chapter 1, Table 2). The formation of acetate plus butyrate as the organic end products has been described for many anaerobes that are mainly belonging to the genus *Clostridium*. This type of conversion of glutamate is hydrogen independent. Other examples of hydrogen independent glutamate conversions are the homoacetogenic fermentation (Dehning *et al.* 1989) and the reductive formation of propionate (Nanninga *et al.* 1987). The formation of hydrogen is more likely to occur in methanogenic environments, where hydrogen-scavenging methanogens rapidly convert the hydrogen to methane with the concomitant reduction of CO_2. The presence of these methanogens also makes these hydrogen-generating conversions more favourable, with respect to the Gibbs' free energy (see Chapter 1, Table 2). Under standard conditions, these reactions are yielding small amounts of energy. Especially the formation of propionate from glutamate is very difficult, since the Gibbs' free energy at 55^0C is only –16.0 kJ/mole glutamate (see Chapter 1, Table 2, reaction 5). It is highly unlikely that a single organism can perform this reaction. However, when a consortium is constructed where the hydrogen formed is converted by a methanogenic archaeon via interspecies hydrogen transfer, the energy that becomes available from this reaction is –79.0 kJ/mole glutamate.

Recent results of our work on glutamate degradation indicated the presence of a propionate-forming obligate syntrophic bacterium in a highly enriched thermophilic syntrophic propionate-oxidising consortium as described by Stams *et al.* (1992). From this mixed culture we isolated a bacterium which produced propionate as the major product, in addition to traces of succinate, from glutamate. In this paper we present

detailed information about the organism and we propose to assign the organism to the new genus *Gelria* as a new species, *Gelria glutamica*.

METHODS

Strains and source of organisms. Strain TGO was isolated from a thermophilic syntrophic propionate-oxidising enrichment culture as described by Stams *et al.* (1992). Strain TPO, a syntrophic propionate-oxidising bacterium, was isolated from the same propionate-oxidising consortium. *Moorella thermoacetica* (DSM 521) and *Moorella glycerini* (DSM 11254) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *Methanobacterium thermoautotrophicum* (*Methanothermobacter thermoautotrophicus*) Z245 (DSM 3720) has been used before.

Media and cultivation. A bicarbonate-buffered medium with the following composition was used (per litre): 0.4 g KH_2PO_4; 0.53 g Na_2HPO_4; 0.3 g NH_4Cl; 0.3 g NaCl; 0.1 g $MgCl_2.6H_2O$; 0.11 g $CaCl_2$; 1 ml alkaline trace element solution; 1 ml acid trace element solution; 1 ml vitamin solution; 0.5 mg resazurin; 4 g $NaHCO_3$; 0.25 g Na_2S. 7-9 H_2O; 0.5 g yeast extract. The trace elements and vitamins were as described in Stams *et al.* (1993). All compounds were heat sterilised except for the vitamins and the Na_2S. 7-9 H_2O, which were filter sterilised. Incubations were done in serum bottles sealed with butyl rubber stoppers (Rubber bv, Hilversum, The Netherlands) and a gas phase of 182 kPa (1.8 atm) N_2/CO_2 (80:20, vol/vol). For the cultivation of methanogens a gas phase of 182 kPa (1.8 atm) H_2/CO_2 (80:20, vol./vol.) was used and after growth the gas phase was changed to N_2/CO_2. Carbon sources were added from anaerobic sterile stock solutions to a concentration of 20 mM (unless otherwise stated). To obtain the axenic culture, soft agar (0.7-0.8% agar noble; Difco, Detroit, MI, USA) was added to the same medium as described above, supplemented with 20 mM pyruvate as the carbon source. Light microscopy confirmed purity.

For the reconstitution experiments with axenic cultures strain TGO (0.5%, v/v), strain TPO (2%, v/v) and *Methanobacterium thermoautotrophicum* Z245 (2%, v/v) were inoculated in medium with 20 mM glutamate.

Temperature and pH. The temperature optimum was determined in bicarbonate buffered medium containing 20 mM pyruvate at pH 7, and duplicate bottles were incubated at a temperature range of 30-75°C. The pH optimum was tested in medium by adding 0.15 g/l of KH_2PO_4 instead of sodium bicarbonate. The pH values of the medium containing 20 mM pyruvate were adjusted with NaOH or HCl under the N_2 atmosphere. Duplicate bottles were incubated at 55°C, at a pH range of 4.5-9.5. For temperature and pH optimum determinations OD_{600} as well as acetate production was used to measure growth.

Growth and substrate utilisation. Utilisation of substrates by strain TGO in pure culture and in coculture with *M. thermoautotrophicum* Z245 was determined by monitoring growth and substrate depletion as well as product formation. All incubations were performed at 55°C, pH 7. The effect of electron acceptors on the growth of strain TGO was tested in medium with 20 mM glutamate.

G + C content. Isolation and purification of genomic DNA was carried out according to Marmur (1961). The G + C content of the DNA by thermal denaturation as described by Owen *et al.* (1969).

16S rDNA sequence analysis. Total DNA was extracted from strain TGO as previously described by Zoetendal *et al.* (1998). PCR was performed with the bacterial primers 7f and 1510r (Lane, 1991) by using the Taq DNA polymerase kit from Life Technologies to amplify the bacterial 16S rDNA. The PCR products were purified with the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Infrared Dye 41 (MWG-Biotech, Ebersberg, Germany) labelled primers 538 r, 1100r (Lane, 1991) and 968f (Nübel *et al.*, 1996) were used as sequencing primers. The sequences were automatically analysed on a LI-COR (Lincoln, NE., USA) DNA sequencer 4000L and corrected manually. Phylogenetic analysis and tree construction were performed with the programs of the ARB software package (Strunk & Ludwig, 1991). FASTA homology searches with sequences of the EMBL, and GenBank DNA databases were performed and the results were compared with those obtained with the ARB programs. Because strain TGO has additional loops in several regions of the 16S rDNA, we did the database

comparisons with the complete sequence of 1725 bp and also with the sequence without the additional loops.
The GenBank accesion number for the 16S rDNA sequence of strain TGO is AF 321086.

RNA isolation, RT-PCR and dot blot hybridisations. RNA was extracted from strain TGO as described by Zoetendal *et al.* (1998). Specific probes targetting the V1, V7 and V9 regions of the 16S rDNA of strain TGO were directed to investigate the presence of additional loops in the 16S rRNA. The sequences of these oligonucleotides were: 5'-GCT CTT GGG CCT TTT GAA (V1 region); 5'-GTT AAC CCT CTG GCT TTG (V7 region) and 5'-CTC AAT CCG CAA GTT TAA (V9 region). As the positive control for Eubacteria, primer 538r (Lane, 1991) was used. Dot blot hybridisations were performed with strain TGO, *Moorella thermoacetica, Moorella glycerini* and *E.coli* as described by Oude Elferink *et al.* (1997). All membranes were hybridised overnight at 40^0C.
RT-PCR of the 16S rRNA genes of strain TGO, *Moorella thermoacetica, Moorella glycerini* and *E.coli* was performed with the use of bacterial primers 7f and 1510r using Access RT-PCR System of Promega (Madison, WI, USA). Prior to the RT-PCR amplification the samples were incubated with RNase free DNase (Promega) to remove all traces of DNA. The integrity and size of the nucleic acids was determined visually after electrophoresis on a 1.2% agarose gel containing ethidium bromide, in the presence of markers and compared with the 16S rDNA of strain TGO.

Other methods. Gases and organic acids were analysed by gas chromatography and HPLC as described by Plugge *et al.* (2000). Amino acids were analysed by HPLC as described by Kengen & Stams (1994). Occasionally glutamate was enzymatically determined with glutamate dehydrogenase as described by Bernt & Bergmeyer (1974) Ammonium was analysed using the indophenol blue method by Hanson & Philips (1981). Inorganic compounds tested as electron acceptors were analysed by HPLC as described by Scholten & Stams (1995). Gram and flagella staining were done by standard procedures as described previously (Plugge *et al.*, 2000).

RESULTS

Isolation of the glutamate-oxidising strain TGO. A highly enriched thermophilic syntrophic propionate-oxidising culture as described by Stams *et al.* (1992) was also able to convert glutamate to acetate, NH_4^+, HCO_3^- and CH_4. When the consortium was growing on glutamate, a small rod-shaped bacterium became predominant in the culture. It was not possible to obtain the bacterium in pure culture by adding an inhibitor of methanogenesis (bromoethanesulphonic acid, BES) to the enrichment. No degradation of glutamate was observed when BES was added. The bacterium could be purified on pyruvate by using agar (0.7-0.8%) containing media. The colonies that appeared in the agar and on the surface of the agar were 0.7-1.0 mm in diameter. The colonies were white and round at the surface and lens-shaped in the agar. A single colony picked from the agar grew in pyruvate containing media enriched with 0.05% yeast extract. Repeated transfer from liquid medium to soft agar medium resulted in an axenic culture of a strain designated as strain TGO and this strain was characterised further.

Morphology and cellular characterisation. The isolated strain TGO is a rod shaped, spore-forming organism. The gram stain was positive. The bacterium was 0.5 μm in diameter and 1-1.5 μm in length, when grown on pyruvate. When the bacterium was grown on glucose the size was 0.5 μm in diameter and 3-20 μm in length. Spores were terminally located and were 0.5 x 0.5 μm in size and developed in the late log phase. Motility was never observed, nor were flagella found.

Physiological characterisation. Strain TGO was able to grow on glutamate only in the presence of the methanogenic archaeon *Methanobacterium thermoautotrophicum* Z245. Glutamate was converted to propionate, succinate, NH_4^+, CH_4 and CO_2. The stoichiometry of glutamate degradation was: Glutamate$^-$ (15) → propionate$^-$ (12.9) + succinate (1.0) + NH_4^+ (14.9) + CH_4 (8.9) + HCO_3^- (26.8). The amount of bicarbonate was calculated assuming that 2 moles of bicarbonate were formed from glutamate to propionate and one mole from glutamate to succinate. The carbon recovery was 93% (excluding the biomass formed) and the doubling time of strain TGO in coculture with *M. thermoautotrophicum* Z245 on glutamate was 0.23 day.

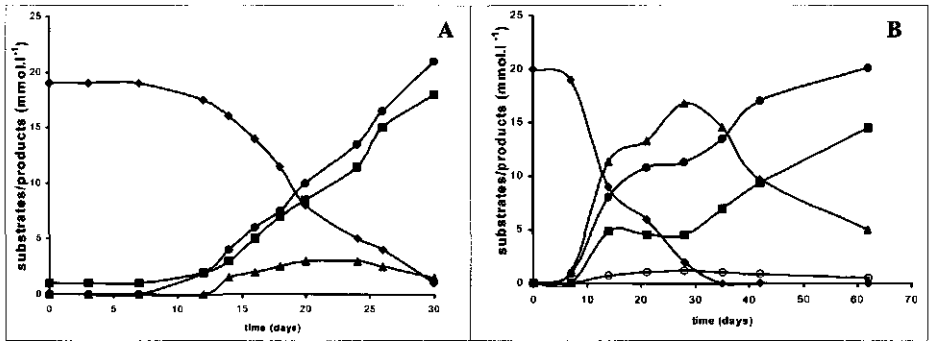

Figure 1. Utilisation of glutamate (◆) and production of propionate (▲), acetate (■), succinate (○) and CH_4 (●) by a consortium of strain TGO, strain TPO and *M. thermoautotrophicum* Z245. **A)** Original consortium as described by Stams *et al.* (1992) **B)** Inoculated with 0.5, 2 and 0.5% (v/v) of pure cultures of strain TGO, strain TPO and *M. thermoautotrophicum*, respectively.

Yeast extract (a minimum of 0.02%) was required for growth. pyruvate was converted by strain TGO to acetate, propionate, succinate (traces), H_2 and CO_2. Glycerol was slowly converted in pure culture, but in coculture glycerol was rapidly converted to acetate, traces of propionate and CH_4.

Other substrates that could be used by a pure culture of TGO are lactate, arabinose, fructose, galactose, glucose, maltose, mannitol, rhamnose and sucrose. Sugars were mainly converted to acetate and propionate with the formation of traces of hydrogen and formate. In coculture with the methanogen *M. thermoautotrophicum* Z245 strain TGO could also grow on casamino acids, α-ketoglutarate and proline. Proline (18.6 mM) was degraded to propionate (17.3 mM), NH_4^+ (18.2 mM) and H_2 (27.2 mM). No growth of strain TGO was observed in pure culture or in coculture with *M. thermoautotrophicum* Z245 on aspartate, alanine, lysine, threonine, leucine, tyrosine, glycine, fumarate, malate, succinate, propionate, acetate, methanol, ethanol, propanol, butanol, acetone, benzoate, starch and H_2/CO_2. The following mixtures of amino acids were tested but not utilised by a pure culture of strain TGO: alanine + glycine, alanine + arginine, alanine + proline, leucine + glycine, leucine + arginine, leucine + proline, H_2 + glycine, H_2 + arginine and H_2 + proline. The strain could not grow in the presence of traces of oxygen, nor could sulphate, sulphite, thiosulphate and nitrate serve as an alternative electron acceptor.

Strain TGO could grow on glucose between 37 and 60^{0}C with an optimum at 50 -55^{0}C. The pH range for growth was 5.5 - 8, with an optimum at 7.

Reconstitution of the original consortium from axenic cultures of strain TGO, strain TPO and *M. thermoautotrophicum* Z245. To investigate the original stoichiometry of glutamate conversion in the propionate-oxidising consortium we performed reconstitution experiments with three axenic cultures: strain TGO, strain TPO (the syntrophic propionate-oxidising organism) and *Methanobacterium thermoautotrophicum* Z245. In the original consortium glutamate was degraded to acetate, H_2, NH_4^+ and CO_2, with the intermediate production of propionate (Fig. 1A). The stoichiometry of glutamate conversion was:

1 glutamate$^-$ ➔ 1 acetate$^-$ + 5 H_2 + 3 HCO_3^- + 1 NH_4^+.

In the reconstitution experiments glutamate was converted according to the stoichiometry as described above (Fig. 1B). The conversion of propionate by strain TPO was the limiting factor in the experiments, since the amount of inoculum was only 2% as opposed to 10-15% which is normally used to grow strain TPO on propionate.

Phylogeny. The nucleotide sequence (1725 base pairs) of a 16S rDNA gene of strain TGO was analysed and revealed that this organism belongs to the subphylum of gram-positive endospore-forming thermophilic anaerobic bacteria. Sequence alignment revealed that strain TGO had additional loops in the V1, V7 and V9 helices of the 16S rDNA. Dot blot hybridisations with specific oligonucleotides against these regions showed no hybridisation reaction with 16S rRNA from strain TGO, *Moorella glycerini, Moorella thermoacetica* and *E.coli*. The positive control with primer 538r reacted with all 16S rRNA's. This indicated that the loops were not transcribed from the 16S DNA to the 16S RNA of strain TGO. Comparison of the size of the 16S rDNA and the 16S RNA showed that the sizes of the molecules were 1700 and 1500 base pairs, respectively. This confirms the absence of the loops in the 16S rRNA, as observed with the dot blot hybridisations. (Fig. 2).

Sequence analysis showed that strain TGO is only distantly related to *Moorella glycerini* and *Moorella thermoacetica* (Slobodkin *et al.* 1997; Collins *et al.*, 1997) with levels of similarity of 92 and 90%, respectively. The similarities were calculated with the use of the 16S rDNA sequence of strain TGO without the additional loops. A phylogenetic tree showing the relationship of strain TGO and other related species is depicted in Fig. 3.

The G + C content of strain TGO was 33.8 mol%.

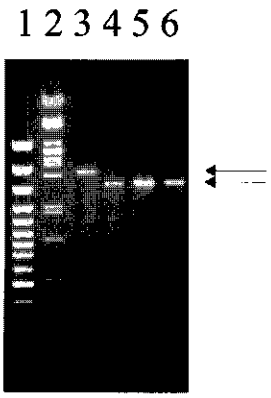

Figure 2. 1.2% agarose gel showing size and integrity of 16S rDNA (lane 3) and rRNA (lane 4) of strain TGO and 16S rRNA of *Moorella glycerini* (lane 5) and *Moorella thermoacetica* (lane 7). Lane 1 and 2 contain markers: a Gene Ruler 100 bp DNA ladder and phage λ digested with Pst.

DISCUSSION

Strain TGO is a thermophilic bacterium that can only degrade glutamate in syntrophic cocultures with hydrogen-utilising methanogens. Several thermophilic bacteria are known to convert glutamate with the concomitant production of H_2, but these organisms also grow in pure culture on glutamate. *Caloramator proteoclasticus, Caloramator coolhaasii,* and *Thermanaerovibrio acidaminovorans* (Cheng *et al.*, 1992; Tarlera *et al.*, 1999; Plugge *et al.*, 2000) are all examples of such thermophilic glutamate-degrading organisms. In pure culture they all form acetate, NH_4^+, CO_2 and

Table 1. Summary of the main characteristics of strain TGO

Cell morphology	Rod shaped
Cell size (μm)	0.5 x 1.5-20 (substrate dependent)
Spore formation	+
Spore size (μm)	0.5 x 0.5
Gram stain	Positive
G + C %	33.8
Temperature optimum (^{0}C)	50-55
pH optimum for growth	7
Substrates used in pure culture	Pyruvate, lactate, glycerol, glucose, rhamnose and galactose.
Substrates used in coculture	Glutamate, proline, several sugars
Stickland reaction	-
Alternative electron acceptors	-
Habitat	Thermophilic methanogenic sludge

H_2. *Thermanaerovibrio acidaminovorans* also forms propionate in pure culture. When *T. acidaminovorans* is grown in coculture with a methanogen the products formed from glutamate conversion shift in favour of propionate formation. However, still considerable amounts of acetate are formed. Examples of mesophilic glutamate-degrading organisms showing the same degradation products as *T. acidaminovorans* are *Anaeromusa (Selenomonas) acidaminophila, Acidaminobacter hydrogenoformans* and *Aminobacterium mobile* (Nanninga *et al.*, 1987; Stams & Hansen, 1984; Baena *et al.*, 2000). Like strain TGO, *A. mobile* only grows on glutamate in the presence of a hydrogen scavenger.

Strain TGO is the first example of an organism that is unable to form acetate from glutamate and forms mainly propionate. As a consequence the organism has to grow in a syntrophic coculture with a methanogen to convert glutamate, since the Gibbs' free energy under standard conditions is only slightly negative (see Chapter 1, Table 2, equation 5). The first step in the conversion from glutamate to propionate is a deamination to α-ketoglutarate, which is strongly dependent on a low hydrogen partial pressure.

$Glutamate^- + H_2O \rightarrow \alpha\text{-ketoglutarate}^{2-} + NH_4^+ + H_2$ $\Delta G' = +59.9$ kJ/reaction.

When the partial pressures of hydrogen this reaction is lowered to 10^{-5} atm. by a methanogen the $\Delta G'$ value is still +31.4 kJ/reaction. The following steps in the metabolism are less affected by the partial pressure of hydrogen. The only hydrogen-generating step is the HSCoA dependent conversion of α-ketoglutarate to succinylCoA. The $\Delta G^{0'}$ of this reaction is –11.1 kJ/reaction.

Why strain TGO does not form acetate from glutamate is unclear, since acetate production could be detected after growth on several sugars, lactate and pyruvate. The formation of traces of succinate during the glutamate conversion suggests that a direct oxidation takes place via α-ketoglutarate and succinyl-CoA. This pathway was also suggested to occur for propionate formation in *T. acidaminovorans* and *A. hydrogenoformans* (Cheng *et al.*, 1992; Stams & Hansen, 1984).

Proline oxidation to solely propionate by strain TGO was remarkable. Proline oxidation has been reported for two other bacteria. *Desulfobacterium vacuolatum* (Rees *et al.* 1998) a versatile amino acid-utilising sulphate reducer and *Geovibrio ferrireducens* (Caccavo *et al.*, 1996), an iron reducing bacterium, are capable of oxidising proline coupled to sulphate reduction and dissimilatory Fe(III)-reduction, respectively. Fermentative proline utilisation by anaerobes has focussed on the use of it as electron acceptor (McInerney, 1989). It might be that strain TGO was able to convert proline to glutamate, with the use of reverse biosynthetic enzymes. Glutamate semialdehyde might play a role as intermediate in this conversion.

16S rDNA analysis showed that TGO has an unusual long ribosomal DNA. There are examples of other organisms with a 16S rDNA gene that exceeds the average length of 1500 base pairs. In thermophiles it is not unusual that the 16S rDNA gene is larger (Rainey *et al.*, 1996). Strain TGO had additional loops in the V1, V7 and V9 regions. Dot blot hybridisation experiments with oligonucleotide probes against specific parts of the V1, V7 and V9 regions showed that the loops were not present in the 16S rRNA. RT-PCR of the 16S rRNA revealed that the larger insertions present in the 16S rDNA were not present. How the transcription of the rRNA is regulated is unclear. Further research is needed at this point.

Taxonomy. Our findings indicate that strain TGO differs physiologically and phylogenetically from previously described species. Strain TGO is phylogenetically most similar to the genus *Moorella* (90-92% similarity), but the phylogenetic relationship is not sufficiently close to classify strain TGO in this genus. Also the ability of strain TGO to form solely propionate from glutamate separates it from members of this genus. Therefore we propose a new genus and a new species, *Gelria glutamica* gen. nov., sp. nov.

Description of *Gelria* gen. nov. *Gelria* (Gel.ri'.a. N.L. n. *Gelria* Gelre or Gelderland, name of one of the 12 provinces in the Netherlands. The city of Wageningen is located in this province.).

Non motile, Gram-positive rods. Spore-forming, with terminal spores. Strictly anaerobic. Moderately thermophilic. Growth by obligate syntrophic catabolism of glutamate. Glutamate and proline oxidised to propionate, H_2, NH_4^+ and CO_2. Saccharolytic growth in pure culture. Products mainly acetate, propionate, CO_2 and H_2. Habitat: methanogenic granular sludge. Type species: *Gelria glutamica.*

Description of *Gelria glutamica* sp. nov. *Gelria glutamica* (glu.ta'.mi.ca M.L. n. *acidum glutamicum* glutamic acid; M. L. adj. *glutamica* referring to glutamic acid, on which the bacterium grows).

Gram-positive non-motile rods, obligately anaerobic. Single cell dimensions are 0.5 by 0.5-6 μm, dependent on the growth substrate. Spores are located terminally. In pure culture the cells can grow on pyruvate, lactate, glycerol, glucose, rhamnose and galactose. In syntrophic association with a hydrogenotrophic methanogen, the organism can utilise glutamate, α-ketoglutarate, proline, casamino acids and a variey of sugars. Growth occurs between 37 – 60^{0}C with an optimum at 50 – 55^{0}C and pH 5.5 – 8 (optimum 7). The DNA base composition is 33.8%. The type strain is TGO^T (= DSM 14054). A summary of the main characteristics is given in Table 1.

ACKNOWLEDGEMENT

The determination of the G+C % by dr. A. Lysenko at the Institute of Microbiology of the Russian Academy of Sciences in Moscow is highly appreciated.

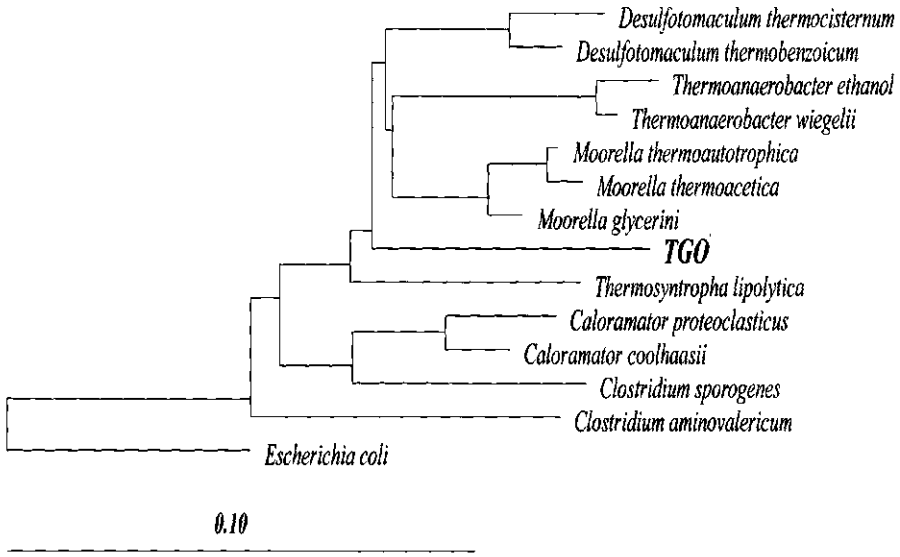

Figure 3. Neigbor-joining tree showing the phylogenetic position of strain TGO among representatives of thermophilic, anaerobic spore-forming genera. Bar = 0.1 (evolutionary distance).

The EMBL and Genbank accesion numbers of the strains used for the construction of the tree were: *Moorella glycerini* (DSM 11254^T), U82327; *Moorella thermoacetica* strain ET-5a, AJ242494; *Moorella thermoautotrophica* (DSM 1974^T), L09168; *Desulfotomaculum thermocisternum*^{T}, U33455; *Desulfotomaculum thermobenzoicum*^{T}, AJ294430; *Thermoanaerobacter wiegelii*^{T}, X92513; *T. ethanolicus*^{T}, L09164; *Thermosyntropha lipolytica*^{T}, X99980; *Caloramator proteoclasticus*^{T}, X90488; *C. coolhaasii*^{T}, AF 104215; *Clostridium sporogenes*, X68189; *Cl. aminovalericum*, M23929.

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Desulfotomaculum thermosyntrophicum sp. nov., a thermophilic syntrophic propionate-oxidising spore-forming bacterium.

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Submitted for publication

Abstract

A novel anaerobic, moderately thermophilic, syntrophic, spore-forming bacterium, strain TPO, was isolated from granular sludge of a lab scale upflow anaerobic sludge bed (UASB) reactor operated at 55^{0}C with a mixture of volatile fatty acids as feed. The cells were straight rods with rounded ends and became eye-shaped when sporulation started. The cells were slightly motile. The optimum growth temperature was 55^{o}C and growth was possible between 45 and 62^{0}C. The pH range for growth of strain TPO was 6 - 8 with an optimum at pH 7 - 7.5. Propionate was converted to acetate, CO_2 and CH_4 by a co-culture of strain TPO with Methanobacterium thermoautotrophicum Z245. An axenic culture was obtained by using pyruvate as sole source of carbon and energy. In pure culture strain TPO could grow on benzoate, fumarate, H_2/CO_2, pyruvate and lactate. Sulphate could serve as inorganic electron acceptor. The G + C content was 53.7 mol%. Comparison of 16S rDNA genes revealed that TPO is related to Desulfotomaculum thermobenzoicum (98%) and D. thermoacetoxidans (98%). DNA-DNA hybridisations revealed 88.2% similarity between TPO and D. thermobenzoicum and 83.8% between TPO and D. thermoacetoxidans. Based on the fact that these organisms differ physiologically from TPO and are not capable of syntrophic propionate oxidation we propose that strain TPO should be classified as new species within the genus Desulfotomaculum as Desulfotomaculum thermosyntrophicum.

Keywords: Desulfotomaculum thermosyntrophicum sp nov., propionate oxidation, syntrophy, interspecies hydrogen transfer

INTRODUCTION

The anaerobic degradation of complex organic compounds to methane and CO_2 is an exergonic process (Zehnder, 1978; Zeikus, 1983). However, there is no single microorganism able to perform this reaction all on its own. Instead the degradation is brought about by a complex food chain which ultimately ends up as CH_4, CO_2 and NH_4^+. Propionate is an important intermediate in this conversion. To completely convert this compound to methane and CO_2 different physiological groups of microorganisms are needed (Stams, 1994; Schink, 1997). Propionate is oxidised by acetogenic bacteria to acetate and CO_2 and reducing equivalents are released as H_2 and/or formate. The energetics of this reaction is highly unfavourable and the reaction is only possible when an external sink is present for the removal of H_2 and/or formate. Methanogenic archaea usually serve as hydrogen and formate scavengers in syntrophic co-cultures with propionate oxidisers.

Several mesophilic, syntrophic propionate-oxidising cultures have been described in the past. *Syntrophobacter wolinii* (Boone & Bryant, 1980) was the first strain described. It degrades 1 mole of propionate to 1 mole acetate, 1 mole CO_2 and 3 moles of H_2. Other examples of syntrophic cultures yielding the same products from propionate conversion are *S. pfennigii* and *S. fumaroxidans* (Wallrabenstein *et al.*, 1995; Harmsen *et al.*, 1998). These *Syntrophobacter* species are phylogenetically related and belong to a physiological heterogeneous group of sulphate reducers. In the presence of sulphate *Syntrophobacter* strains all couple propionate oxidation to sulphate reduction. Recently, Liu *et al.* (1999) described a syntrophic propionate-degrading organism, *Smithella propionica,* which forms besides acetate also butyrate as organic end product. This organism does not group in the cluster with the *Syntrophobacter* strains, but is phylogenetically more related to syntrophic benzoate degraders, such as *Syntrophus buswelli.*

To date, very little is known about thermophilic syntrophic propionate degradation. The sulphate reducer *Desulfotomaculum thermocisternum* was described to be capable of syntrophic growth on propionate in coculture with a methanogen (Nilsen *et al.*, 1996). However, no details are given in this report concerning the products formed from the degradation of propionate. Imachi *et al.* (2000) described the isolation of a moderate thermophilic bacterium, strain SI, which can grow syntrophically on propionate. In this paper only the phylogenetic position of the

bacterium is discussed. Limited information is given on its physiological characteristics.

A thermophilic consortium, enriched by us on propionate (Stams *et al.*, 1992) was able to degrade pyruvate and lactate without the presence of methanogens, indicating that the propionate-oxidising bacterium is able to grow by fermentation. In this paper we describe the isolation of a novel thermophilic spore-forming syntrophic propionate-oxidising bacterium from that culture, strain TPO, with pyruvate as substrate. Its morphological, physiological and phylogenetic characteristics are presented and its taxonomic position is discussed.

METHODS

Organisms, cultivation and isolation procedures. Strain TPO was isolated from a highly purified consortium originating from thermophilic anaerobic granular sludge (Stams *et al.*, 1992). *Desulfotomaculum thermocisternum* (DSM 10259) and *Desulfotomaculum thermobenzoicum* (DSM 6193) were kindly provided by H. Goorissen, University of Groningen, The Netherlands. *Methanobacterium thermoautotrophicum* (*Methanothermobacter thermoautotrophicus*) Z245 (DSM 3720) and *Desulfotomaculum thermoacetoxidans* (DSM 5813) were obtained from the DSMZ (Braunschweig, Germany).

A bicarbonate buffered anaerobic medium as described previously by Stams *et al.* (1992) was used for the cultivation of all strains. Substrates were added from sterile anaerobic stock solutions up to a concentration of 20 mM (unless otherwise stated). Occasionally yeast extract (0.02%, w/v) was added to stimulate growth. For isolation of strain TPO a direct dilution method was used in liquid medium supplemented with 20 mM pyruvate as the sole source of carbon and energy followed by dilution in soft agar media, containing 0.75% agar. Purity was checked by light microscopy and growth in soft agar media.

For syntrophic growth tests 1% of strain TPO, *D. acetoxidans, D. thermobenzoicum* or *D. thermocisternum* were inoculated in hydrogen pregrown cultures of *M. thermoautotrophicum* Z245. Prior to inoculation the gas phase of these bottles was changed to 180 kPa 80% N_2/ 20% CO_2 and 20 mM propionate was added. Unless otherwise stated, all incubations were at 55°C.

Determination of growth parameters. The temperature optimum was determined in a bicarbonate buffered medium containing 20 mM of pyruvate, and a temperature range of 30-75°C. The pH optimum was tested in basal medium by adding 0.15 g/l of KH_2PO_4 instead of sodium bicarbonate. The pH values of the medium containing 20 mM pyruvate were adjusted with NaOH or HCl. In this case, a N_2 atmosphere was applied. Duplicate bottles were incubated at 55°C at a pH range of 4.5-9.5, and acetate production was measured in time.

Utilisation of substrates by strain TPO in pure culture and in coculture with *M. thermoautotrophicum* Z245 was determined by monitoring growth, substrate depletion and product formation. All these incubations were performed at 55°C and pH 7. The effect of electron acceptors on the growth of strain TPO was tested in basal medium with 20 mM propionate.

Cellular characterisation. All methods for cellular characterisation of strain TPO were as described previously (Plugge *et al.*, 2000). Transmission electron microscopy was performed as described before (Plugge *et al.* 2000).

G + C content. Isolation and purification of genomic DNA was carried out according to Marmur (1961). The G + C content of the DNA was determined by thermal denaturation as described by Owen *et al.* (1969).

16S rDNA sequence analysis. DNA was extracted from strain TPO as previously described by Zoetendal *et al.* (1998). PCR was performed with the bacterial primers 7f and 1510r by using the Taq DNA polymerase kit from Life Technologies (Breda, The Netherlands) to amplify the bacterial 16S rDNA. The PCR products were purified with the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Infrared Dye 41 (MWG-Biotech, Ebersberg, Germany) labelled primers 515r, 1100r, (Lane, 1991) and 968f (Nübel *et al.*, 1996) were used as sequencing primers. The sequences were automatically analysed on a LI-COR (Lincoln, NE, USA) DNA sequencer 4000L and corrected manually. The sequences were checked for reading errors with the alignment programs of the ARB package (Strunk & Ludwig, 1991). Homology searches of the ARB, EMBL, and GenBank DNA databases for these partial sequences were performed with FASTA and the homologies were checked with the ARB programs.

DNA-DNA hybridisation experiments. DNA-DNA hybridisations were performed with strain TPO, *Desulfotomaculum thermobenzoicum* DSM 6193^T, *D. thermoacetoxidans* DSM 5813^T and *D.thermocisternum* DSM 10259^T at the DSMZ (Braunschweig, Germany). DNA was isolated by chromatography on hydroxyapatite according to the procedure of Cashion *et al.* (1977). DNA-DNA hybridisation was carried out as described by DeLey *et al.* (1970), with modifications as described by Huss *et al.* (1983) and Escara & Hutton (1980) using a Gilford model 2600 spectrophotometer equipped with a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were computed with the TRANSFER.BAS program by Jahnke (1992).

Nucleotide accession number. The 16S rDNA of strain TPO has been deposited in the Genbank database under accession number: AY 007190. The EMBL database accession numbers of the strains closest related to strain TPO are: *Desulfotomaculum thermobenzoicum* (DSM 6193^T), Y 11574; *D. thermoacetoxidans* (DSM 5813^T), Y11573; *D. kutznetsovii* (DSM 6115^T), Y 11569; *D. thermocisternum* (DSM 10259^T), U 33455; Spore forming syntrophic propionate oxidising enrichments Spore A and Spore B, X 91169 and X 91170 (Harmsen 1996); Strain SI, AB 035723 (Imachi *et al.*, 2000).

Preparation of cell free extracts. Cells of the strain TPO in coculture with *M. thermoautotrophicum* Z245 grown on propionate and in pure culture grown on H_2/CO_2 plus sulphate were centrifuged (23000 g, 10 min, 4°C). Finally, the cell pellet was suspended in 0.1 M Tris.HCl buffer (pH 8.0) containing 0.4 mg Na_2EDTA per ml and 50 µg of lysozyme per ml as described by Wofford *et al.* (1986). Cells were disrupted by ultrasonic disintegration of the cells. Cell debris were removed by centrifugation (15.000 rpm, 15 min, 4^{0}C) and the supernatant was stored anaerobically at 4^{0}C.

Protein concentrations were determined by the method of Bradford (1976) and bovine serum albumin was used as the standard.

Enzyme assays. Enzyme assays were carried out anaerobically at 55°C by using a Hitachi U-2010 UV-VIS spectrophotometer. Cuvettes (1 ml) were closed with rubber

stoppers and flushed with N_2. Hydrogenase (E.C.1.12.1.2) and pyruvate dehydrogenase (E.C.1.2.4.1) were assayed with methyl viologen as electron acceptor according to Odom & Peck (1981). Fumarase (E.C.4.2.1.2), malate dehydrogenase (NAD-dependent, E.C.1.1.1.37 and NADH-dependent, E.C.1.1.1.82), methylmalonyl-CoA:pyruvate transcarboxylase (E.C.2.1.3.1), phosphotransacetylase (E.C.2.3.1.8) and succinate dehydrogenase (E.C.1.3.99.1) activities were assayed according to Stams *et al.* (1984). Propionate kinase and acetate kinase (E.C.2.7.2.1) activities were determined as described by Aceti & Ferry (1988). Carbon monoxide dehydrogenase (E.C.1.2.99.2) and 2-oxoglutarate: methylviologen oxidoreductase (E.C. 1.2.7.3) activities were measured according to Schauder *et al.* (1986).

Analytical methods. Substrate and fermentation product concentrations were measured using HPLC and GC methods as described by Stams *et al.* (1993). Sulphide was measured using the spectrophotometric DMPD method as described by Trüper & Schlegel (1964). Anions were analysed as described by Scholten & Stams (1995).

RESULTS

Enrichment and isolation of strain TPO. The enrichment of the thermophilic syntrophic propionate-oxidising strain TPO from methanogenic granular sludge was described previously by Stams *et al.* (1992). *Methanobacterium thermoautotrophicum* Z 245 was removed from the consortium by pasteurising the culture for 30 minutes at 90^0C and subsequent transfers using pyruvate as carbon source. This resulted in a pure culture of strain TPO when checked by light microscopy.

Strain TPO could not grow on solid media. However, active cultures inoculated from the basal bicarbonate-buffered medium could grow in soft agar media (0.75% agar) containing pyruvate. Colonies, embedded in the soft agar, were white to brownish lens shaped, and reached a size of 0.1-0.2 mm.

Morphological characteristics. Cells of strain TPO were rod shaped, with rounded ends, with a size of 1 µm by 3-11 µm. They occurred as single cells but sometimes pairs were formed. When sporulation started, cells became eye-shaped and ultimately only oval spores remained. Spores were centrally localised and one spore per cell

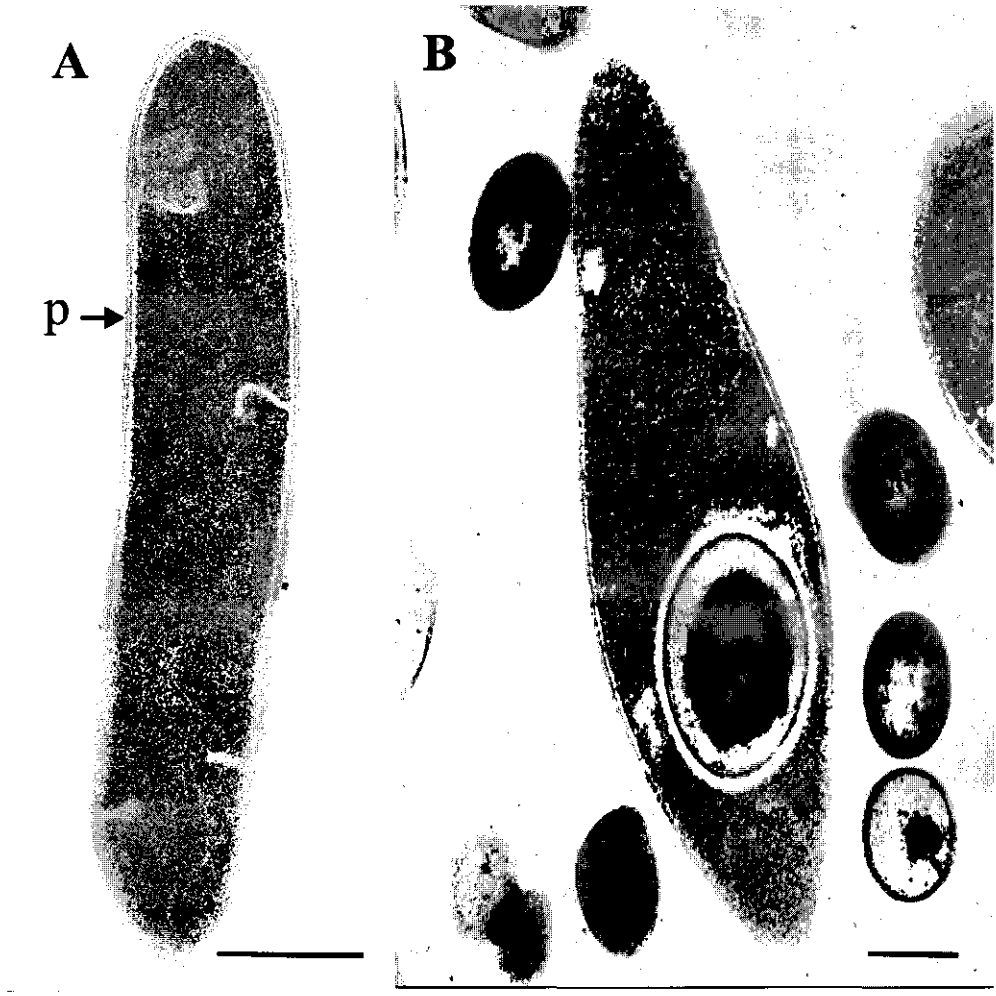

Figure 1. Electron micrographs showing (A) the general morphology of strain TPO and the single-layered structure of the cell wall (cm, cytoplasmic membrane; p, peptidoglycan layer) (B) spore formation. Bar represents 0.5 μm.

(size 1.5 x 1.5 μm) was observed. Cells stained gram negative but the cell wall ultrastructure resembled that of Gram-positive bacteria, as shown in Fig. 1. Weak motility was observed only in actively growing cultures converting pyruvate.

Phylogeny. A total of 1551 base pairs of a 16S rDNA gene from strain TPO were sequenced. To determine the phylogenetic position of strain TPO, we compared the

data with known bacterial 16S rDNA genes (Fig. 3). Sequence analysis showed that strain TPO fell in the gram-positive spore-forming sulphate reducing bacteria group, with the closest neighbours *Desulfotomaculum thermoacetoxidans* (Min & Zinder, 1990) and *Desulfotomaculum thermobenzoicum* (Tasaki *et al.*, 1991) (both 98% similarity). DNA-DNA hybridisations of strain TPO with *D. thermoacetoxidans, D. thermobenzoicum* and *D. thermocisternum* revealed 83.8%, 88.2% and 43.4% similarity, respectively.

The G + C content of strain TPO was 53.7 mol%.

Physiological characterisation and metabolism of strain TPO in pure culture and in coculture with *M. thermoautotrophicum* Z 245. Strain TPO was strictly anaerobic, since no growth occurred in the presence of traces of oxygen. The strain grew in pure culture on pyruvate between 45 and 62^{0}C with an optimum at 55^{0}C. The pH optimum for growth on pyruvate was 7 – 7.5. Below a pH of 6 and above 8 no measurable growth occurred. Under optimum conditions the doubling time of strain TPO in pure culture on pyruvate was 0.33 day. Yeast extract (0.02%) stimulated growth but was not required. Besides pyruvate also lactate, fumarate, H_2/CO_2, and benzoate supported growth of strain TPO in pure culture (Table 1). 10 mM benzoate was converted to 13 mM acetate, 0.5 mM propionate and 1.5 mM succinate. No hydrogen was produced during benzoate conversion. The carbon and redox balance of this conversion was not complete, indicating that other end products should have been formed. Until now we were not able to show the identity of the other components. In addition the bacterium was able to slowly ferment glycine. In coculture with *M.thermoautotrophicum* Z245 strain TPO was able to convert the following compounds: propionate, pyruvate, lactate, fumarate and benzoate. Fig. 2 shows the stoichiometry of propionate conversion by strain TPO in the presence of the methanogen *M.thermoautotrophicum* Z245. In coculture also malate, alanine and glycine were slowly converted. The following single substrates were tested but not utilised for growth by the pure culture nor the coculture of strain TPO: glucose, fructose, ribose, sucrose, xylose, xylitol, acetate (10 mM), citrate (10 mM), succinate (10 mM), butyrate, tartrate, malonate, glutamate, aspartate, methanol, ethanol,

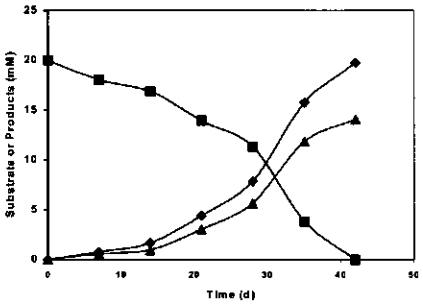

Figure 2. Propionate degradation and acetate plus methane formation by strain TPO in co-culture with *M. thermoautotrophicum* Z245 measured in time.

(■: propionate; ◆: acetate;▲: methane)

propanol (10 mM), butanol, 2,3-butanediol (10 mM), acetoin (10 mM), casamino acids (0.2%, w/v), pepton (0.2%, w/v).

The following mixtures of substrates did not support growth: propionate + glycine, alanine + arginine, alanine + proline, leucine + glycine, leucine + proline, H_2 + glycine, H_2 + arginine, H_2 + proline. In the case of glycine-containing incubations not more acetate was formed as with glycine alone, indicating that glycine could not serve as an electron acceptor. Therefore it seemed that strain TPO was not capable of performing Stickland reactions.

Sulphate could serve as alternative electron acceptor for growth on propionate (Table 1). Besides propionate, also lactate, pyruvate and H_2/CO_2 could be converted coupled to sulphate reduction, forming acetate as the end product. Acetate could not be used as by strain TPO in the presence of sulphate. Thiosulphate, sulphite, nitrate and fumarate could not be utilised as electron acceptor.

Specific enzyme activities measured in cell-free extracts varied in different cell preparations. The lysozyme treatment followed by ultrasonic treatment resulted in extracts with the highest activity. Results of the measurements with cells grown in co-culture with *M. thermoautotrophicum* Z245 on propionate and in pure culture on H_2/CO_2 + sulphate are shown in Table 2. Enzymes from the methylmalonyl CoA pathway of propionate conversion were present in high to moderately high activities in cell extracts of propionate-grown cells. Extracts from H_2 + SO_4^{2-} grown cells showed moderately high activities of carbon monoxide dehydrogenase, whereas no

activity of α-ketoglutarate dehydrogenase could be detected, indicating the presence of the acetyl-CoA pathway during autotrophic growth.

Syntrophic growth on propionate by other strains related to strain TPO. *Desulfotomaculum thermobenzoicum, Desulfotomaculum thermoacetoxidans* and *Desufotomaculum thermocisternum* were all pregrown on propionate + sulphate. These cells were used as the inocula to construct syntrophic consortia with *M. thermoautotrophicum* Z245 to degrade propionate. After 60 days of incubation neither significant methane production, nor propionate disappearance could be detected. The experiments were repeated, this time in the presence of 2 mM sulphate, to stimulate the organisms. Furthermore, the organisms were incubated at their optimum growth temperature (Table 3). In all cases propionate was depleted at a level just enough to reduce all the sulphate present. Thereafter, no further propionate degradation could be measured. A third trial was done, and in this case, besides 2 mM sulphate, also 0.2 mM $FeCl_2$ was added to the cultures. The addition of $FeCl_2$ leads to the formation of

Table 1. Doubling times and OD_{600} measurements of strain TPO grown under different conditions

Substrate#)	Products	Doubling time (days)	OD_{600}
Pyruvate (15.0)	Acetate (16.0) and H_2 (0.3)	0.33	0.067
Fumarate (18.9)	Succinate (13.9), acetate (4.0), Propionate (1.4) and H_2 (0.065)	0.247	0.114
Lactate (12.4)	Acetate (11.3) and H_2 (0.15)	0.138	0.039
Propionate (7.8) + SO_4^{2-} (20)	Acetate (6.8) , HS^- (4.1)	0.099	0.060
Benzoate (10)	Acetate (13), propionate (0.5) and succinate (1.5)	n.d.*)	n.d.
H_2/CO_2 (10^5 Pa)	Acetate (9.7)	n.d.	n.d.
Propionate (18.0) + *M. thermoautotrophicum* Z245	Acetate (18.9), CH_4 (14.1)	0.115	0.144

#) Substrate and product concentrations are given in $mmol.l^{-1}$ in parentheses, except for H_2/CO_2, which is expressed in Pa.

*) n.d.: not determined

FeS precipitates to which many bacteria can adhere (Stams *et al.*, 1992) and brings the acetogenic and methanogenic organisms in closer contact with each other. An additional advantage is that the methanogens remain viable over a much longer period of starvation. These conditions did not result in a measurable propionate degradation after 60 days of incubation either.

DISCUSSION

Strain TPO is a moderate thermophilic syntrophic propionate-oxidising bacterium and it was identified as a spore-forming, gram positive organism. Based on phylogenetic analysis of its 16S-rDNA, strain TPO shows close relationship with members of the *Desulfotomaculum* group. It is most closely related to *Desulfotomaculum thermoacetoxidans* and *Desulfotomaculum thermobenzoicum* (both 98% sequence similarity) and more distantly related to *Desulfotomaculum thermocisternum* (93%). These organisms are all moderate thermophilic sulphate reducers. Strain TPO is also capable of reducing sulphate to sulphide.

Thusfar, there is no detailed description of other thermophilic propionate-oxidising syntrophs from anaerobic environments. Only *Desulfotomaculum thermocisternum* (Nilsen *et al.*, 1996) was described to grow syntrophically on propionate in the presence of *Methanococcus thermolithotrophicus* (DSM 8766). However, no details were given concerning the stoichiometry and propionate conversion rates. We could not grow *D. thermocisternum* on propionate in coculture with *Methanobacterium thermoautotrophicum* Z245. Imachi *et al.* (2000) were also unsuccessful in growing *D. thermocisternum* in a syntrophic coculture on propionate. They used *Methanobacterium thermoautotrophicum* ΔH and a *Methanobacterium thermoformicicum* strain as syntrophic partner. A recent paper by Imachi *et al.* (2000) describes the isolation and phylogenetic position of strain SI, a moderately thermophilic syntrophic propionate oxidiser. A detailed description on its physiological properties is still lacking. Strain SI is phylogenetically closest related to the mesophilic spore-forming propionate-oxidising bacteria "Spore A" and "Spore B" (Fig. 3). It also clusters in the *Desulfotomaculum* group, but seems unable to use sulphate as electron acceptor for growth.

The physiological differences between the mentioned *Desulfotomaculum* species and strain TPO are considerable. Substrate conversion coupled to sulphate reduction of *D. thermoacetoxidans* and *D. thermobenzoicum* is complete, where strain TPO cannot oxidise acetate. Like *D. thermoacetoxidans* and *D. thermobenzoicum*, strain TPO is capable of autotrophic growth on H_2/CO_2, forming acetate. This is confirmed by the presence of carbon monooxide dehydrogenase activity (CODH) in cell free extracts of H_2/CO_2 + sulphate grown cells of strain TPO (Table 2). Strain TPO can only use sulphate as electron acceptor, where *D. thermoacetoxidans* and *D. thermobenzoicum* can also use thiosulphate. *D. thermobenzoicum* can also use sulphite and nitrate. Both *D. thermoacetoxidans* and *D. thermobenzoicum* are unable to transfer their electrons to a syntrophic partner as described by the authors and confirmed by us, whereas strain TPO is a true syntroph.

The metabolic capabilities of strain TPO are somewhat limited; it can only grow, besides on propionate, on lactate, pyruvate, fumarate, malate, benzoate, H_2/CO_2 and glycine.

A remarkable feature of strain TPO is its ability to grow fermentatively on benzoate. In methanogenic environments benzoate generally is oxidised to acetate and H_2. This reaction is highly unfavourable under standard conditions:

$C_6H_5COO^- + 6\ H_2O \rightarrow 3\ CH_3COO^- + 2\ H^+ + CO_2 + 3\ H_2$ $\Delta G^{0'} = +49.5$ kJ/mole benzoate.

($\Delta G^{0'}$ values were taken from Thauer *et al.*,1977).

However, strain TPO does not produce 3 moles of hydrogen from benzoate. Only traces of hydrogen could be measured. Since the fermentation balance on benzoate is far from complete, an unknown reduced end product must have been formed during its conversion. We speculate that the strain is able to reduce the aromatic ring in a similar way as was described to occur in anaerobic sludge by Kleerebezem (1999). Research is in progress to reveal the identity of the unknown compound.

Strain TPO slowly fermented glycine, but was not able to reduce glycine to acetate with glycine reductase. Most likely, glycine is first converted via a glycine decarboxylase complex. The "methylene" group formed may enter the homoacetogenic pathway resulting in the formation of acetate.

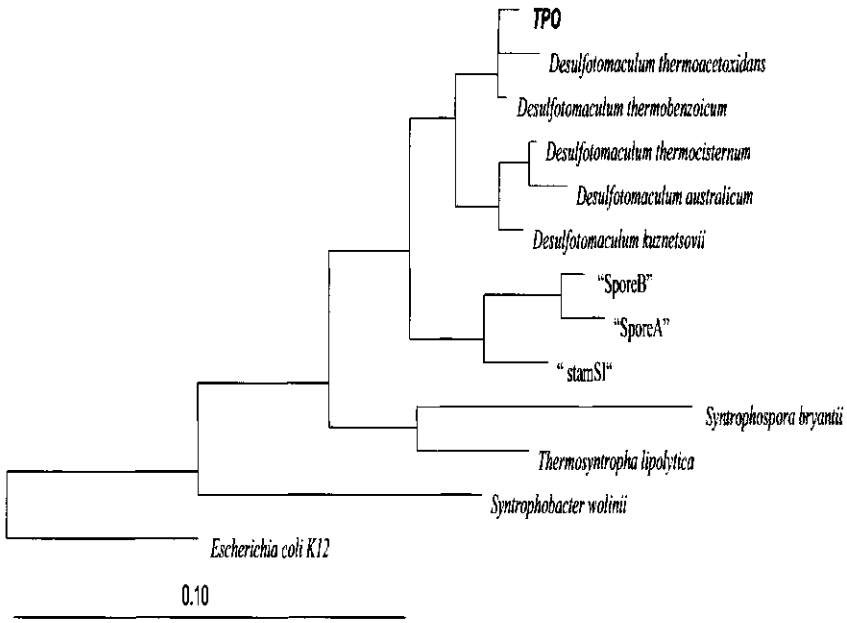

Figure 3. Neighbor joining tree based on 16S rDNA sequences showing the phylogenetic position of strain TPO among representatives of the genus *Desulfotomaculum* and other spore-forming syntrophic microorganisms. Bar (0.1) represents evolutionary distance.

Comparison of strain TPO with other syntrophic organisms. The phylogenetic tree in Fig. 3 shows the relationship of strain TPO with two other spore-forming syntrophic microorganisms: *Syntrophospora bryantii* (Zhao *et al.*, 1990) and *Thermosyntropha lipolytica* (Svetlitshnyi *et al.*, 1996). These organisms branch in the bacterial line of the low G + C gram positive bacteria and are only distantly related to strain TPO. Both organisms are incapable of using propionate in syntrophic coculture with methanogens, but are specialised in the degradation of fatty acids with chain lengths of 4 carbon atoms up to 10 (*S. bryantii*) or even 20 (*T. lipolytica)* carbon atoms. *Syntrophomonas* species are phylogenetically and physiologically related to these two organisms, but do not form spores. Strain TPO is distantly related to the

Table 2. Enzyme activities in cell-free extracts of TPO grown on propionate in coculture with *Methanobacterium thermoautotrophicum* Z245 and on H_2/CO_2 plus sulphate in pure culture. Activities are expressed in μmol. $min^{-1}.mg^{-1}$ protein.

Enzyme	Propionate*)	H_2/CO_2 + sulphate
Propionate kinase	0.93	
Methylmalonyl CoA: pyruvate transcarboxylase	0.56	
Succinate dehydrogenase	<0.001	
Fumarase (dissappearance)	4.8	
Fumarase (formation)	0.16	
Malate dehydrogenase (NAD-dependent)	0.58	
Malate dehydrogenase (NADH-dependent)	0.33	
Pyruvate dehydrogenase (MV^{++})	1.55	0.8
Phosphotransacetylase	1.50	
Acetate kinase	0.66	
Hydrogenase (MV^{++})	0.96	
2-Oxoglutarate dehydrogenase	Not determined	Not detected
Carbon monoxide dehydrogenase (MV^{+})	Not determined	0.6 – 0.7

*) activities were calculated after correction for the protein content of the methanogen

mesophilic spore-forming propionate-oxidising bacteria "Spore A" and "Spore B", as described by Harmsen (1996) and strain SI (Imachi *et al.* 2000). These organisms are unable to use sulphate as terminal electron acceptor, although they group within the *Desulfotomaculum* genus. Further comparison with these cultures is not possible due to their limited physiological description.

Physiologically, strain TPO has similarities with almost all other mesophilic non-sporulating syntrophic propionate oxidisers. First of all their capability to grow syntrophically on propionate with a syntrophic partner. The use of sulphate as a terminal electron acceptor is an other similarity that strain TPO has with other mesophilic non-sporulating syntrophic propionate oxidisers (Wallrabenstein *et al.* 1995; Van Kuijk & Stams, 1995). Based on the enzyme measurements as shown in Table 2, it seems that strain TPO used the methylmalonyl-CoA pathway for syntrophic propionate oxidation. This pathway is used by most mesophilic syntrophic

Table 3. Characteristics that differentiate strain TPO from other phylogenetically and physiologically related organisms

	TPO	*Desulfotomaculum thermobenzoicum*	*D. thermocisternum*	*D. thermoacetoxidans*
Gram stain	-	+	n.d.	-
Cell wall structure	+	n.d.#)	+	n.d.
Cell morphology	Rod shaped with pointed ends to eye shaped	Rod shaped	Rod shaped	Rod shaped with pointed ends
Colony formation	On soft agar	On agar	On gelrite	Prepurified agar
Motility	+	weak	+	weak
Spore formation	+	+	+	+
Temp. optimum (^{0}C)	55	62	62	55-60
Temperature range (^{0}C)	45 – 62	40 - 70	41 - 75	45-65
pH optimum	7.0	7.2	6.7	6.5
G + C %	53.7	52.8	56-57	49.7
Fermentative growth	Pyruvate, lactate, fumarate, glycine, benzoate	Pyruvate, lactate	Pyruvate	Pyruvate
Substrate degradation coupled to sulphate reduction	Incomplete	Incomplete and complete	Incomplete	Complete
Other electron acceptors				
SO_3^{2-}	-	+	+	-
$S_2O_3^{2-}$	-	+	+	+
S^0	n.d.	-	-	-
NO_3^-	-	+		-
Syntrophic growth on propionate	+	-	-*)	-

*) Nilsen *et al.* (1996) reported syntrophic growth on propionate by *D. thermocisternum*. However, we could not reproduce these results, nor could Imachi *et al.* (2000).

#) n.d. : not determined

propionate oxidisers (Houwen *et al.*, 1990; Plugge *et al.*, 1993; Wallrabenstein *et al.*, 1995). *Smithella propionica* (Liu *et al.*, 1999) has a different stoichiometry of propionate conversion as compared to strain TPO. It forms acetate as well as butyrate as organic end products from propionate conversion. *Smithella propionica* can grow on crotonate in pure culture, but can not use sulphate as terminal electron acceptor.

Taxonomy. Our findings indicate that strain TPO is phylogenetically different from previously described propionate-degrading bacteria. Strain TPO is phylogenetically most closely related to *Desulfotomaculum* species, but the exclusive ability to grow syntrophically on propionate separates it from members of this genus. Based on these differences we propose to place strain TPO as a new species in the genus *Desulfotomaculum*, *Desulfotomaculum thermosyntrophicum*.

Description of *Desulfotomaculum thermosyntrophicum* sp. nov. *thermosyntrophicum* (ther.mo.syn.tro'.phi.cum. Gr. Adj. *thermos*, hot; Gr. prefix. *syn*, together; *trophein*, to eat; *syntrophos*, foster brother or sister; M.L. M. n. *thermosyntrophicum* referring to the capacity of the organism to grow at elevated temperatures on propionate in the presence of a hydrogen-utilising methanogen). Spore-forming, gram positive rods with pointed ends, 3-11 µm long. Weakly motile, strictly anaerobic, moderate thermophilic, neutrophilic. Grows axenically in the presence of sulphate on propionate, lactate, pyruvate and H_2/CO_2. Ferments pyruvate, lactate, fumarate, glycine and benzoate. H_2 plus CO_2 is converted to acetate. Syntrophic growth on propionate with *Methanobacterium thermoautotrophicum* Z245. Habitat: thermophilic granular methanogenic sludge. Strain TPO is deposited at the DSMZ with accession number: DSM 14055.

ACKNOWLEDGEMENTS

The authors would like to thank Erwin G. Zoetendal for his help with the construction of the phylogenetic tree. The G + C% analysis by Dr. A. Lysenko at the Institute of Microbiology of the Russian Academy of Sciences in Moscow is highly appreciated.

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Elucidation of the pathways of catabolic glutamate conversion in three thermophilic anaerobic bacteria

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Submitted for publication

Abstract

The glutamate catabolism of three thermophilic syntrophic anaerobes was compared based on the combined use of [^{13}C] glutamate NMR measurements and enzyme activity determinations. In some cases the uptake of intermediates from different pathways was studied. The three organisms, Caloramator coolhaasii, Thermanaerovibrio acidaminovorans and strain TGO, had a different stoichiometry of glutamate conversion and were dependent on the presence of a hydrogen scavenger (Methanobacterium thermoautotrophicum Z245) to a different degree for their growth. C. coolhaasii formed acetate, CO_2, NH_4^+ and H_2 from glutamate. Acetate was found to be formed through the β-methylaspartate pathway, in pure culture as well as in coculture. T. acidaminovorans converted glutamate to acetate, propionate, CO_2, NH_4^+ and H_2. Most likely, this organism uses the β-methylaspartate pathway for acetate formation. Propionate formation occured through a direct oxidation of glutamate via succinyl-CoA and methylmalonyl CoA. The metabolism of T. acidaminovorans shifted in favour of propionate formation when grown in coculture with the methanogen, but this did not lead to the use of a different glutamate degradation pathway. Strain TGO, an obligate syntrophic glutamate-degrading organism, formed propionate, traces of succinate, CO_2, NH_4^+ and H_2. Glutamate was converted to propionate oxidatively via the intermediates succinyl-CoA and methylmalonyl-CoA. A minor part of the succinyl-CoA is converted to succinate and excreted.

INTRODUCTION

In the past two decades, various anaerobic glutamate-fermenting bacteria have been described that use protons as a sink for reducing equivalents. Nagase and Matsuo (1982) were the first to show that amino acid fermentation could be coupled to proton reduction. *Acidaminobacter hydrogenoformans* (Stams and Hansen 1984), *Aminomonas colombiense* (Baena et al. 1998), *Aminomonas paucivorans* (Baena et al. 1999a) and *Aminobacterium mobile* (Baena et al. 2000) are examples of mesophilic organisms that form hydrogen from amino acid degradation. A few thermophilic anaerobic organisms have been described which ferment glutamate coupled to proton reduction. *Thermanaerovibrio (Selenomonas) acidaminovorans* (Cheng et al. 1992, Baena et al 1999b), *Caloramator proteoclasticus* (Tarlera et al. 1997, Tarlera and Stams 1999) and *Caloramator coolhaasii* (Plugge et al. 2000) are capable of hydrogen formation during glutamate degradation.

All these hydrogen-forming organisms are influenced in their metabolism by cocultivation with a hydrogen scavenger. This interspecies hydrogen transfer can affect the metabolism of the hydrogen producer in two ways. It enables a faster conversion of the substrate and it can influence the product formation. *Acidaminobacter hydrogenoformans* forms H_2, acetate and some propionate from glutamate in pure culture (Stams and Hansen 1984). However, when it is growing in the presence of *Methanobrevibacter arboriphilus*, a hydrogen-utilising methanogen, the acetate to propionate ratio decreases. *Caloramator proteoclasticus*, in pure culture, has a branched metabolism when growing on glutamate. It forms alanine from pyruvate as a reduced end product to discharge reducing equivalents (Tarlera et al. 1997). In coculture with *Methanobacterium thermoautotrophicum* Z245 glutamate is completely converted to acetate.

Different pathways are involved in the anaerobic catabolism of glutamate. The β-methylaspartate pathway and the hydroxyglutarate pathway, both leading to the formation of acetate and/or butyrate, have been studied in detail (Barker 1981, Buckel and Barker 1974). Gharbia and Shah (1991) described a pathway for glutamate degradation via decarboxylation of glutamate, ultimately leading to the formation of acetate and/or butyrate. *Anaeromusa (Selenomonas) acidaminophila* (Nanninga et al. 1987, Baena et al. 1999b) converts glutamate to acetate and propionate, where

propionate is formed reductively. In this pathway oxaloacetate and fumarate are reduced to malate and succinate, respectively. Propionate formation in *Acidaminobacter hydrogenoformans* occurs oxidatively, via α-ketoglutarate and succinyl-CoA (Stams and Hansen 1984). In pure culture this organism forms acetate via the β-methylaspartase pathway. However, when the hydrogen partial pressure is kept low by a hydrogen-scavenging methanogen, *A. hydrogenoformans* uses the reversed citric acid cycle to form acetate (Stams et al. 1994).

In our laboratory we study moderate thermophilic anaerobic glutamate-degrading bacteria from methanogenic ecosystems. Three strains were isolated, which have a different stoichiometry of glutamate conversion and the metabolism of these organisms is strongly affected by the partial pressure of hydrogen. *Caloramator coolhaasii* Z converts glutamate to acetate, traces of propionate, CO_2, NH_4^+ and H_2. When the hydrogen is removed by *Methanobacterium thermoautotrophicum* Z245, glutamate conversion rates increase considerably, but the fermentation pattern remains the same (Plugge et al. 2000). *Thermanaerovibrio acidaminovorans* Su883 metabolises glutamate to acetate, propionate, ammonium, bicarbonate and hydrogen. In the presence of a hydrogen scavenger the metabolism shifts in favour of propionate formation (Cheng et al. 1992). Strain TGO is a thermophilic, syntrophic glutamate-oxidising anaerobe. It was isolated from a syntrophic propionate-oxidising consortium, which was described previously by Stams et al. (1992). Strain TGO catabolises glutamate to propionate, traces of succinate, NH_4^+ CO_2 and H_2 only in coculture with a methanogen. Strain TGO is unable to grow on glutamate in pure culture. The energetics of propionate formation from glutamate in pure culture is unfavourable. Under standard conditions at 55^0C the Gibbs' free energy ($\Delta G'$) of this reaction is only –16.0 kJ/mol of glutamate. The energy released from the reaction changes when the hydrogen partial pressure is decreased by the methanogen to 10^{-5} atm to –79.0 kJ/ mol, allowing growth of strain TGO on glutamate ($\Delta G'$ values calculated from Thauer et al. 1977 and Chang 1977). This research was performed to obtain insight into and compare the glutamate degradation pathways of the three mentioned organisms. Enzyme measurements as well as ^{13}C-NMR spectroscopy were used to study the pathways. Furthermore, the uptake of intermediates from several pathways was tested.

MATERIALS AND METHODS

Growth of the organisms. The three strains, *Caloramator coolhaasii* (DSM12679), *Thermanaerovibrio acidaminovorans* (DSM 6589) and TGO (DSM 14054) were grown in a bicarbonate buffered anaerobic medium as described previously by Stams et al. (1992). The medium was supplemented with 0.05% yeast extract and 20 mM glutamate as the energy source. The cocultures were grown in the presence of *Methanobacterium thermoautotrophicum* Z245 (DSM 3720). This methanogen was recently renamed as *Methanothermobacter thermoautotrophicus* Z245 (Wasserfallen et al. 2000). *M. thermoautotrophicum* Z245 was pregrown with H_2/CO_2 (80%/20%). After growth, the gas phase was changed to N_2/CO_2 (80%/20%) and a 5% (v/v) inoculum of the glutamate-degrading organisms was added.

Preparation of cell extracts. All manipulations were performed at room temperature under anoxic conditions. The cultures were harvested in the late exponential phase by centrifugation at 20,000 g for 30 min., washed twice in 50 mM Tris-HCl, pH 7.5, 1 mM dithioerythreitol (DTE) and resuspended in the same buffer. Cell extracts were prepared by ultrasonic treatment of the cells. The cell debris was removed by centrifugation at 8,000 g for 20 min. Supernatants were stored at 4^0C. The protein content was estimated according to Bradford (1976).

Enzyme activity determinations. All enzymes were assayed using anoxic conditions at 50-55^{0}C using a Hitachi U-2010 spectrophotometer. Enzyme assays were as described previously by others (see Table 1).

NMR measurements. For NMR analysis the three organisms were cultivated (under the conditions as mentioned before) in the presence approximately 10 mM [3-^{13}C] glutamate or 10 mM [1-^{13}C] glutamate and 0.1% yeast extract. Samples were taken from growing cultures and centrifuged (14,000 g, 10 min.). Supernatants were analysed on a Bruker AMX-500 spectrometer located at the Wageningen NMR-Centre. In the case of [1-^{13}C] glutamate 43,000 scans were analysed and with [3-^{13}C] glutamate 7500 scans were analysed. Spectra were obtained as described by van Casteren et al. (1999). Chemical shifts are expressed in parts per million relative to internal dioxane (δ = 67.37 ppm) or ethanol (δ = 58.4 ppm for [1-^{13}C] ethanol and δ =

17.7 ppm for [2-^{13}C] ethanol). Product formation was analysed using HPLC and GC as described previously (Plugge et al. 2000).

Experiments with cell suspensions. Serum bottles with a total volume of 25 ml were filled with 6 ml medium supplemented with 0.05% yeast extract. Intermediates of the β-methylaspartate pathway and hydroxyglutarate pathway were added in concentrations of 10-20 mM. Cells from actively growing cultures of *C. coolhaasii* and *T. acidaminovorans* were concentrated to a volume of 20 ml and divided over small bottles. Samples were taken and products were analysed using HPLC technique and the gas phase was analysed for H_2 and/or CH_4 production.

RESULTS

Enzyme measurements. In general the specific enzyme activities measured in cell-free extracts varied strongly in different preparations of strain *Caloramator coolhaasii* as well as of strain *Thermanaerovibrio acidaminovorans* Su883. In Table 1 the results of the measurements of key enzymes of several glutamate degradation pathways are shown. The results were the same when *C. coolhaasii* and *T. acidaminovorans* were grown in pure culture or in coculture with *M. thermoautotrophicum* Z245, but the measured activities of the enzymes were somewhat higher in the coculture experiments. Only the results of the coculture experiments are shown in Table 1. For strain TGO, only enzyme levels of cocultures could be measured.

Caloramator coolhaasii strain Z

Enzymes from the β-methylaspartase pathway were present in low to moderately high activities (Table 1). Hydroxyglutarate dehydrogenase and α-ketoglutarate reductase (NADH) activities were measured with low activities. Glutamate dehydrogenase activities (NAD- and NADH-dependent) were very high. These high activities could also be measured when *C. coolhaasii* was grown on glucose (Table 2). No activities of key enzymes of other glutamate degradation pathways could be measured.

Thermanaerovibrio acidaminovorans strain Su883

Enzymes catalysing glutamate conversion via direct oxidation to propionate were present in cell free extracts of *T. acidaminovorans* in low to moderately high activities (Table 1). No fumarate reductase activity could be detected. Low activities of β-methylaspartase and citramalate lyase could be measured. No activities of key enzymes of other glutamate degradation pathways could be measured. Similarly to *C. coolhaasii*, a relatively high glutamate dehydrogenase activity was found. In glucose-grown cells the activity was much lower, but still present (Table 2).

Strain TGO

Table 1 shows that the enzymes catalysing the conversions in the direct oxidation via methylmalonyl-CoA were measured in high to moderately high activities. Glutamate dehydrogenase, involved the first step of this pathway, was present in high activities. The key enzymes responsible for subsequent steps, α-ketoglutarate decarboxylase and propionate kinase were present in moderately high activities. Propionyl-CoA carboxylase could not be detected. In the degradation of glutamate by TGO, traces of succinate were formed. However, neither succinate kinase nor succinate thiokinase activities could be demonstrated. Fumarate reductase, characteristic for the methylmalonyl-CoA pathway, was not detected.

Several key enzymes of other glutamate degradation pathways were present as well. β-Methylaspartase, α-ketoglutarate reductase and hydroxyglutarate dehydrogenase were detected in low to moderately high activities. Activity of these enzymes can partly be ascribed to the medium applied to cultivate strain TGO. In these experiments 0.05% yeast extract was used. Traces of acetate were formed from yeast extract by strain TGO (data not shown). Activities of enzymes from the citric acid cycle, the reductive citric acid cycle or the 4-aminobutyrate pathway could not be demonstrated.

NMR experiments.

Caloramator coolhaasii strain Z

When *C.coolhaasii* was grown on 11 mM [1-^{13}C] glutamate, after 16 days of incubation only a minor part of the glutamate (1.2 mM) was converted to acetate

Table 1. Specific activities of key enzymes from different pathways of glutamate degradation in *Caloramator coolhaasii* (Z), *Thermanaerovibrio acidaminovorans* (Su) and strain TGO.

Coculture experiments activities expressed in $\mu mol.min^{-1}(mg\ protein)^{-1}$. Protein contents were corrected for the presence of *M. thermoautotrophicum* Z245

Enzymes involved in acetate formation	Strain			Ref. Assay*)
	Z	Su	TGO	
β-Methylaspartate pathway				
β- Methylaspartase (E.C. 4.3.1.2)	0.1-0.8	0.5-0.8	0.05-0.6	6,9
Mesaconase	1.0-1.8			2
Citramalate lyase (E.C. 4.1.3.22)	0.1-0.2	0.3-0.4		5
Hydroxyglutarate pathway				
Glutamate dehydrogenase (NADH) (E.C. 1.4.1.2)	10-43	7.7-15	38-41	12
Glutamate dehydrogenase (NAD) (E.C. 1.4.1.4)	12-22	8.3-21	3.4	12
α-ketoglutarate reductase (NADH)	0.3-0.6	n.d.#)		6
Hydroxyglutarate dehydrogenase (NAD) (E.C. 1.1.99.2)	0.02-0.06	n.d.	0.17-0.18	10
Citric acid cycle				
Fumarase (E.C. 4.2.1.2) fumarate disappearance	n.d.	n.d.	n.d.	13
Succinate dehydrogenase (E.C. 1.3.99.1)	n.d.	n.d.	n.d.	13
Reductive citric acid cycle				
Isocitrate dehydrogenase (NAD(P)H) (E.C. 1.1.1.42)	n.d.	n.d.	n.d.	4
Citrate lyase (E.C. 4.1.3.6)	n.d.	0.01	n.d.	4
4-Aminobutyrate pathway				
4-hydroxybutyrate dehydrogenase (E.C.1.1.1.61)	n.d.	n.d.	n.d.	6
Miscellaneous				
Pyruvate Fd oxidoreductase (E.C. 1.2.7.1)	0.2	0.3	0.3	11
Acetate kinase (E.C. 2.7.2.1)	6.5	5.8	1.8	1

Table 1. Continued

Enzymes involved in propionate formation	Strain			Ref.
	Z	Su	TGO	Assay*)
Direct oxidation via methylmalonyl-CoA				
α-ketoglutarate dedehydrogenase (MV^{++}) (E.C. 1.2.7.3)	0.05	1.1-2.3	1.4-1.8	11
Propionyl-CoA carboxylase (E.C. 4.1.1.41)			n.d.	8
Succinate thiokinase (E.C. 6.2.1.5)	n.d.	n.d.	n.d.	1
Succinate:propionyl-CoA HSCoA transferase		n.d.	n.d.	7
Reductive formation of propionate				
Fumarate reductase (E.C. 1.3.1.6)	n.d.	n.d.	n.d.	3
Miscellaneous				
Pyruvate Fd oxidoreductase (E.C. 1.2.7.1)	0.2	0.3	0.3	11
Propionate kinase	5.0	7.2	2.2	1

*) References enzyme assays:

1) Aceti and Ferry 1988; 2) Blair and Barker 1966; 3) Boonstra et al. 1973; 4) Brandis-Heep et al. 1983; 5) Buckel and Bobi 1976; 6) Gharbia and Shah 1991; 7) Hilpert et al. 1984; 8) Houwen et al. 1990; 9) Hsiang and Bright 1969; 10) Lemd and Whiteley 1971; 11) Odom and Peck 1981; 12) Stams and Hansen 1984; 13) Stams et al. 1984

#) n.d.: measured, but not detected.

(1.9 mM). When grown on [3-^{13}C] glutamate 3.7mM acetate was formed. It is unclear why growth on labelled glutamate was inhibited. Parallel incubations with unlabelled glutamate showed complete conversion of the glutamate within 16 days.

The label from [1-^{13}C] glutamate is recovered as [1-^{13}C] acetate and from [3-^{13}C] glutamate [2-^{13}C] acetate is formed. The degradation of labelled glutamate by a coculture of *C.coolhaasii* and *Methanobacterium thermoautotrophicum* Z245 resulted in the same labelling pattern of the acetate: [1-^{13}C] acetate was formed from [1-^{13}C] glutamate and [2-^{13}C] acetate was formed from [3-^{13}C] glutamate. In some cases traces of [3-^{13}C] mesaconate were formed from [3-^{13}C] glutamate. Fig. 1A represents the results obtained after growth on [^{13}C] glutamate by *C. coolhaasii*.

Table 2. Specific activities of glutamate dehydrogenases in glucose-grown cells of *Caloramator coolhaasii* and *Thermanaerovibrio acidaminovorans*

(activities expressed in μmol. min^{-1}(mg protein)$^{-1}$)

Enzyme	*C. coolhaasii*	*T. acidaminovorans*
Glutamate dehydrogenase (NAD)	7.5	2.5-3.0
Glutamate dehydrogenase (NADH)	16	1.5-2.0

Thermanaerovibrio acidaminovorans strain Su883

Growth of *T. acidaminovorans* on [1-^{13}C] glutamate led to the formation of [^{13}C] HCO_3^-, but no labelled acetate could be detected. When grown on [3-^{13}C] glutamate, [3-^{13}C] propionate was formed and again no labelled acetate could be detected. HPLC analysis showed that 13 mM glutamate was only partially degraded to 0.8 mM acetate and 4.5 mM propionate. The degradation of labelled glutamate by a coculture of *T. acidaminovorans* and *M. thermoautotrophicum* Z245 resulted in the same labelling pattern from both [1-^{13}C] and [3-^{13}C] glutamate. Fig. 1A and B give an illustration of the results obtained when *T. acidaminovorans* was grown on [^{13}C] glutamate.

Strain TGO

Growth of strain TGO on [1-^{13}C] glutamate led to the formation of [^{13}C] HCO_3^-, as is shown in Fig. 1B, whereas growth on [3-^{13}C] glutamate led to the formation of [3-^{13}C] propionate. Strain TGO also formed some [2,3-^{13}C] succinate from [3-^{13}C] glutamate. HPLC analysis of the products showed that 13 mM [3-^{13}C] glutamate was converted to 10.4 mM propionate, 0.8 mM succinate and 0.5 mM acetate and [1-^{13}C] glutamate was converted to 12.1 mM propionate, 0.7 mM succinate and 0.7 mM acetate. The formation of acetate by strain TGO was caused by the increased amount of yeast extract (0.1%).

Uptake of intermediates from the β-methylaspartate and hydroxyglutarate pathway by concentrated cell suspensions. Intermediates from the methylaspartate pathway, β-methylaspartate, mesaconate and citramalate, were taken up by *C. coolhaasii* in pure culture as well as in coculture with *M. thermoautotrophicum* Z245

(Table 3). All three intermediates were converted to acetate. In the case of citramalate also some mesaconate could be detected, indicating that mesaconase was also active in the reverse reaction. Intermediates from the hydroxyglutarate pathway, α-ketoglutarate and hydroxyglutarate, were not converted by dense cell suspensions. α-Ketoglutarate, however, was taken up by the cell suspensions, but no metabolites could be detected.

The same experiments were conducted with concentrated cell suspensions of *T. acidaminovorans*. However, in concentrated cell suspensions of *T. acidaminovorans* no acetate and propionate were formed from glutamate, neither could any intermediate be detected by HPLC. Instead, the addition of [3-^{13}C] glutamate to concentrated cell suspensions led the formation of metabolites from anabolic reactions such as proline, oxo-proline and ornithine (data not shown).

DISCUSSION

A good method to differentiate between the glutamate degradation pathways is to study the labelling pattern of the end products derived from [^{13}C] glutamate (Stams et al. 1998). According to Table 4 the C-1 of glutamate will end up in the C-1 of acetate when the β-methylaspartate or the hydroxyglutarate pathway is used. To distinguish between these two pathways the acetate derived from [3-^{13}C] glutamate can be measured. When the methylaspartate pathway is operational the label ends up in the C-2 of acetate and in the C-1 of acetate when the hydroxyglutarate pathway is used.

Table 3. The uptake of intermediates from the β-methylaspartate and hydroxyglutarate pathway by concentrated cell suspensions of *C. coolhaasii.*

Intermediate	Uptake	Metabolites
β-Methylaspartate	+	Mesaconate, Citramalate, Acetate
Mesaconate	+	Citramalate, Acetate
Citramalate	+	Mesaconate, Acetate
α-Ketoglutarate	+	n.d.*)
Hydroxyglutarate	-	n.d.

*) n.d.: not detected with the analytical methods used.

From the NMR experiments summarised in Table 5 it becomes clear that the β-methylaspartate pathway is used by *C. coolhaasii* for glutamate degradation: [1-^{13}C] acetate is formed from [1-^{13}C] glutamate and [2-^{13}C] acetate is formed from [3-^{13}C] glutamate. The presence of [3-^{13}C] mesaconate, an intermediate unique for this pathway, confirms this. When the activity of key enzymes from known glutamate-degradation pathways in cell-free extracts were measured, key enzymes of the β-methylaspartate pathway indeed could be measured (Table 1). However, activities of hydroxyglutarate dehydrogenase and α-ketoglutarate reductase, key enzymes of the hydroxyglutarate pathway, could be measured as well. Since the labelling pattern did not show any formation of C-2 acetate from [1-^{13}C] glutamate, we conclude that acetate formation from glutamate in *C. coolhaasii* is through the β-methylaspartate pathway. The experiments with intermediates from the β-methylaspartate pathway and the hydroxyglutarate pathway by concentrated cell suspensions of *C. coolhaasii* confirmed that the β-methylaspartate pathway was present (Table 3). Hydroxyglutarate and α-ketoglutarate could not be metabolised by the suspensions, whereas methylaspartate, citramalate and mesaconate could be metabolised.

Table 4. Label position in acetate, propionate and HCO_3^- during glutamate oxidation via different pathways. Adapted after Stams et al. (1998). Incorporation of label from HCO_3^- is indicated by *.

	Acetate	Propionate	HCO_3^-
Methylaspartate pathway	2/3 1/4		5
	CH_3-COO^-		HCO_3^-
Hydroxyglutarate pathway	2/4 1/3		5
	CH_3-COO^-		HCO_3^-
Citric acid cycle	4/3 3/4		1,2,5
	CH_3-COO^-		HCO_3^-
Direct oxidation		3 4 2	1,5
		CH_3-CH_2-COO^-	HCO_3^-
Methylmalonyl-CoA pathway		4/3 3/4 */5	5
		CH_3-CH_2-COO^-	HCO_3^-

The presence of a relatively high glutamate dehydrogenase activity in *C. coolhaasii* is remarkable, since it is not involved in the β-methylaspartate pathway. The first step in the β-methylaspartase pathway is a vitamin-B_{12}-dependent glutamate mutase and not a glutamate dehydrogenase (Buckel and Barker, 1974). High activities of glutamate dehydrogenase often can be measured, independent of catabolic activities, since glutamate plays a role in both the carbon and nitrogen metabolism. Furthermore, glutamate is also used for the biosynthesis of protein. The measured activities of glutamate dehydrogenase in glucose-grown cells of *C. coolhaasii* confirmed an anabolic function of glutamate dehydrogenase (Table 2).

Propionate formation from labelled glutamate by *T. acidaminovorans* and strain TGO occurred via direct oxidation through methylmalonyl-CoA as shown in Fig. 1B and summarised in Table 5. This was confirmed by the presence of α-ketoglutarate dehydrogenase activity in cell extracts of both organisms. Another key enzyme of this pathway, propionyl-CoA carboxylase could not be detected. This may have been due to the instability of the coupling enzymes (CoA-esters) at 50-55^{0}C. Growth of both bacteria on [1-^{13}C] glutamate led to the formation of [^{13}C] HCO_3^-, whereas growth on [3-^{13}C] glutamate led to the formation of [3-^{13}C] propionate. The occurrence of the citric acid cycle can be excluded since no [2-^{13}C] plus [3-^{13}C] propionate could be detected. The formation of propionate labelled at the 2 and 3 position occurs when fumarate is an intermediate in the metabolism. Due to its symmetrical structure, C-2 and C-3 are identical and in this way the label is randomised over the C-2 as well as the C-3 and ends up in the C-2 and the C-3 of propionate. Furthermore, the formation of labelled succinate, also a symmetrical compound, did not lead to formation of double-labelled propionate. The formation of [2,3-^{13}C] succinate indicated that glutamate is directly oxidised to succinyl-CoA where part of the succinyl-CoA is converted to succinate and excreted, and the majority is further converted to propionate.

It remained unclear how acetate formation in *T. acidaminovorans* occurs. The HPLC analysis of the samples indicated that acetate was formed from glutamate in pure culture as well as in coculture. However, NMR spectra did not show any labelled acetate. Experiments with concentrated cell suspensions of *T. acidaminovorans* unfortunately could not give a decisive answer either. Only the presence of key enzymes from the β-methylaspartase pathway might be indicative for acetate

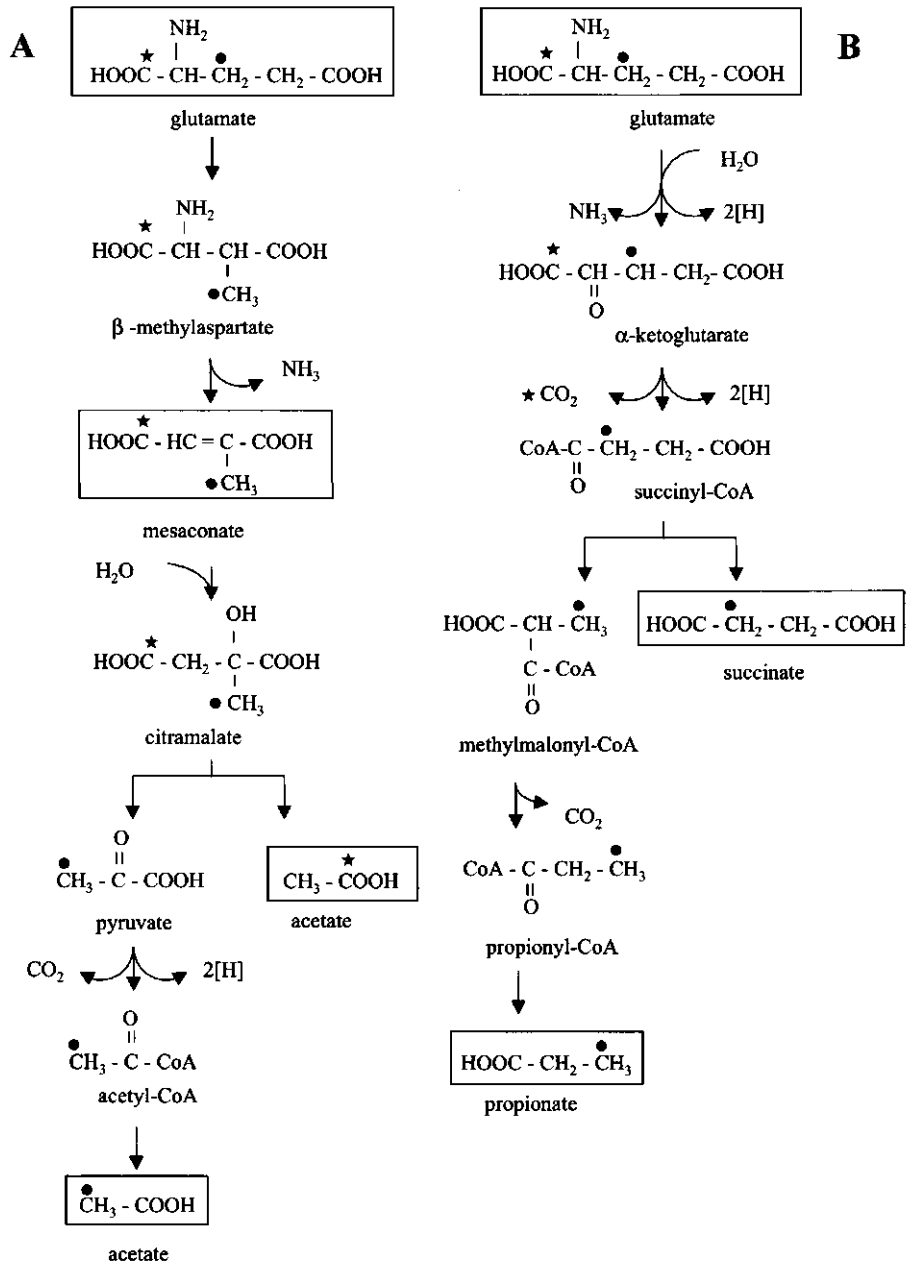

Figure 1. (A) Acetate formation from [1-^{13}C] (★) and [3-^{13}C] (●) glutamate by *Caloramator coolhaasii* and *Thermanaerovibrio acidaminovorans* and **(B)** propionate formation from [1-^{13}C] (★) and [3-^{13}C] (●) glutamate by *Thermanaerovibrio acidaminovorans* and strain TGO. Compounds in boxes are detected in culture supernatants with high-resolution ^{1}H-decoupled ^{13}C-NMR spectra.

formation through this pathway. As in *C. coolhaasii*, *T. acidaminovorans* also had a highly active glutamate dehydrogenase, both the NAD- and the NADH-dependent direction, even when grown on glucose (Table 2).

The effect of interspecies hydrogen transfer on the metabolism of all three organisms did not lead to a regulation on enzyme level. No significant higher or lower activities of key enzymes could be measured. Furthermore, there was no shift in the metabolism as was observed in *A. hydrogenoformans* (Stams et al. 1994), where the organism uses the β-methylaspartate pathway at high hydrogen concentrations and the reversed citric acid cycle at low hydrogen concentrations.

In conclusion, the results of this study revealed that propionate formation from glutamate occurred via direct oxidation in both propionate-forming organisms, rather than via the methylmalonyl-CoA pathway. The reductive formation of propionate as occurring in *Anaeromusa acidaminophila* (Nanninga et al. 1987) clearly can be excluded. The acetate-forming pathways from glutamate by the organisms were rather difficult to clarify, especially for *T. acidaminovorans*. Activities of key enzymes from more than one pathway were present in *C. coolhaasii*. However, the combination of techniques used enabled us to clarify the involvement of the β-methylaspartase pathway in acetate formation by *C. coolhaasii* and *T. acidaminovorans*. The occurrence of enzymes of more than one pathway for glutamate catabolism, however, is not unusual. This has been reported for *Fusobacterium* sp. (Gharbia and Shah 1991) and *Clostridium sticklandii* (Buckel and Barker, 1974). Also *Acidaminobacter hydrogenoformans* shifts from the methylaspartate route to the reversed citric acid cycle dependent on the culture conditions.

Table 5. Label position in products formed after growth on ^{13}C labelled glutamate.

Label added	*C.coolhaasii*	*T. acidaminovorans*	Strain TGO
[1-^{13}C] glutamate	[1-^{13}C] acetate	[^{13}C] HCO_3^-	[^{13}C] HCO_3^-
[3-^{13}C] glutamate	[2-^{13}C] acetate	[3-^{13}C] propionate	[3-^{13}C] propionate
	[3-^{13}C] mesaconate		[2,3-^{13}C] succinate

ACKNOWLEDGEMENT

The assistance of C. Dijkema with the NMR measurements is highly appreciated.

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Arginine catabolism by *Thermanaerovibrio acidaminovorans.*

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Published in:
FEMS Microbiology Letters 195 (2001): 259-262.

Abstract

The arginine catabolism of Thermanaerovibrio acidaminovorans was investigated. T. acidaminovorans was able to produce approximately 0.4-0.5 mole citrulline and 0.5-0.6 mole ornithine from 1 mole of arginine. However, in a methanogenic coculture with Methanobacterium thermoautotrophicum Z245 1 mole arginine was converted to approximately 1 mole of propionate, 0.5 mole acetate, 4 moles ammonia and 4 moles hydrogen; citrulline and ornithine were not formed. Enzyme measurements indicated the presence of the arginine deiminase pathway (ADI) in cells of T. acidaminovorans growing on arginine.

Keywords: Arginine catabolism; ADI (arginine deiminase) pathway; interspecies hydrogen transfer

INTRODUCTION

Arginine can be used by a variety of anaerobic microorganisms as an energy source for growth [1]. Energy rich ureido (R-NH-CO-NH_2) compounds can be formed as intermediates in the energy metabolism of bacteria that can grow on arginine by substrate level phosphorylation. The ureido compounds are degraded by phosphorylytic cleavage to carbamyl phosphate. The carbamyl phosphate is then further metabolised to form ATP via carbamate kinase. The intermediate fermentation product from arginine is citrulline. Furthermore, ornithine, bicarbonate and two moles of ammonia are formed during arginine degradation.

L-Arginine + H_2O + H^+ → citrulline + NH_4^+ $\Delta G^{0'}$ = -37.7 kJ/mol

L-Citrulline + P_i →carbamyl phosphate + L-ornithine $\Delta G^{0'}$ = +28.5 kJ/mol

Carbamyl phosphate + ADP → ATP + carbamate $\Delta G^{0'}$ = -7.5 kJ/mol

Carbamate + H_2O + H^+ → HCO_3^- + NH_4^+ $\Delta G^{0'}$ = -3.3 kJ/mol

Overall:

L-Arginine + 2 H_2O + H^+ + ADP + P_i → L-ornithine + 2 NH_4^+ + HCO_3^- + ATP

$\Delta G^{0'}$ = -20.0 kJ/mol

(Gibbs' free energy changes were obtained from [2].)

The generation of carbamyl phosphate from citrulline is thermodynamically unfavorable. However, the overall process is exergonic and allows the formation of 1 ATP. Degradation of arginine as shown above occurs via the Arginine Deiminase (ADI) pathway, involving the combined activity of arginine deiminase (ADI), ornithine transcarbamylase (OTC) and carbamate kinase (CK). The ADI pathway has been demonstrated in many bacteria, but there are wine lactic acid bacteria that convert arginine to ornithine and ammonia via another pathway. This is the arginase pathway, involving arginase and urease, catalysing the following reactions [3]:

L-arginine + H_2O → L-ornithine + urea $\Delta G^{0'}$= -24.2 kJ/ mole

Urea + H_2O + H^+ → CO_2 + 2 NH_4^+ $\Delta G^{0'}$= -27.6 kJ/ mole

(Gibbs' free energy changes were obtained from [2].)

Both pathways yield the same products: ornithine, ammonia and CO_2. However, only the ADI pathway may yield citrulline, either as an intermediate or as end product.

The function of the ADI pathway in microorganisms is diverse. The ADI pathway can serve as a protectant against acid damage. The ammonia production from arginine by *Carnobacterium* spp. and oral Streptococci protects the organisms against the acid produced from sugar fermentation [4,5]. Carbamylphosphate may serve as a source of phosphate for glycogen synthesis in oral Streptococci [6]. Furthermore, the ADI pathway provides a source of ATP in the conversion of carbamyl phosphate to carbamate in many Lactobacilli.

Thermanaerovibrio acidaminovorans is a versatile thermophilic anaerobe able to grow on a variety of amino acids [7]. The organism is able to ferment arginine, yielding ornithine as a major fermentation product. Up to 60% of the arginine ends up as ornithine. In this study we investigated the pathway used for arginine conversion by *T. acidaminovorans* and the possibility of ATP generation from carbamyl phosphate.

MATERIALS AND METHODS

Cultivation of the organisms. A bicarbonate buffered mineral salts medium with 0.05% yeast extract as described previously [8] was used in all experiments. Cultures of *Thermanaerovibrio acidaminovorans* (DSM 6589) and *Methanobacterium thermoautotrophicum* Z245 (DSM 3720) were incubated at 55^0C in the dark.

Preparation of cell free extracts. Cells were harvested anaerobically by centrifugation and washed twice with 50 mM Tris-HCl (pH 7.2), containing 0.1 mM dithioerytritol (DTE). Cell extracts were prepared anaerobically by ultrasonic disintegration. Cell debris was removed by centrifugation at 13.000 rpm for 20 min. Supernatants were stored anaerobically in the dark at 4^0C.

Enzyme assays. Arginine deiminase (ADI), ornithine transcarbamylase (OTC) and carbamate kinase (CK) were measured according to [9]; Arginase according to [10] and ornithine transaminase according to [11]. Pyruvate: Ferredoxin oxidoreductase was measured as described by [12]. All assays were conducted under anoxic

Table 1. Conversion of arginine by *Thermanaerovibrio acidaminovorans* in the absence and presence of *Methanobacterium thermoautotrophicum* Z245.

	Arginine degraded ($mmol.l^{-1}$)	Products formed ($mmol.l^{-1}$)					
		Acetate	Propionate	H_2	NH_4^+	Ornithine	Citrulline
Pure culture	8.6	<0.5	<0.5	1.1	18.1	3.6	2.9
Coculture	8.2	2.4	8.0	31.8*)	33.2	0	0

*) CH_4 formation was expressed as hydrogen (4 moles H_2 are equivalent to 1 mole CH_4). H_2 was never detected in cocultures.

conditions in the dark at 50-55^{0}C. To calculate the specific activities of *T. acidaminovorans* in cell free extracts of cocultures, a correction was made for the biomass produced by the methanogen, using the molar growth yield (2.2 g dry weight $\times$ (mol CH_4)$^{-1}$) of *M. thermoautotrophicum* on H_2/CO_2.

Analytical methods. Growth yields of the organisms were quantified by measuring the dry weight content. For yield studies triplicate bottles were used for each arginine concentration tested. Growth yields were corrected for the biomass formed from yeast extract. Arginine, citrulline, ornithine and glutamate were analysed using the HPLC technique as described by [13] and NH_4^+ according to [14]. Acetate, propionate, H_2 and CH_4 were analysed as described previously [8]. Protein content was estimated using the method of [15].

RESULTS AND DISCUSSION

Thermanaerovibrio acidaminovorans was able to convert arginine to citrulline, ornithine and NH_4^+ (Table 1). The ratio between citrulline and ornithine varied in different incubations from 1: 1.2 to 1: 2. In coculture with the hydrogen scavenger *Methanobacterium thermoautotrophicum* Z245 arginine was converted to acetate, propionate, NH_4^+ and H_2, the latter being used to reduce CO_2 to CH_4. No intermediate citrulline or ornithine could be detected in these cocultures.

Enzyme measurements showed activity of ADI, OTC and CK in low to moderately high activities (Table 2). In the coculture activities of ADI were somewhat lower and OTC activities somewhat higher. This suggested the presence of the ADI pathway for arginine degradation by *T. acidaminovorans*. No activity of arginase, a key enzyme of the arginase pathway, could be detected. Remarkable is that cells grown on glutamate showed no measurable activity of the enzymes from the ADI pathway, but low activity of arginase could be detected. It has been found in experiments with concentrated cell suspensions of *T. acidaminovorans* that glutamate could be metabolised to proline and ornithine (data not shown). These reactions are probably used for anabolic purposes and might explain the presence of arginase activity in glutamate grown cells.

The absence of ornithine and citrulline after growth of *T. acidaminovorans* in coculture with *M. thermoautotrophicum* is remarkable. The effect of the methanogen is that the hydrogen formed in the degradation of arginine is converted rapidly to reduce CO_2 to CH_4. However, in the conversion of arginine to citrulline and ornithine no proton-reducing reactions occur. So, the effect of interspecies hydrogen transfer on the degradation arginine is caused by the degradation of citrulline and ornithine to H_2,

Table 2. Specific activities of enzymes from ADI and arginase pathway in cell extracts of *Thermanaerovibrio acidaminovorans*, expressed in $\mu mol.min^{-1}mg^{-1}$ protein

Enzyme	*T. acidaminovorans* (arginine)	Coculture**) (arginine)	*T. acidaminovorans* (glutamate)
ADI	0.02 – 0.04	0.005 – 0.02	<0.002
OTC	1.0 – 1.5	2.0 – 2.5	n.d.*
CK	0.1 – 0.22	0.1 – 0.12	n.d.
Arginase	n.d.	n.d.	0.005
Ornithine transaminase	n.d.	n.d.	n.d.
Pyruvate: Ferredoxin oxidoreductase	0.1 – 0.15	0.5 – 1.2	0.5 – 0.7

*) n.d.: measured, but not detected

**) activity was corrected for the presence of the methanogen

acetate and propionate. In *Clostridium sporogenes* similar observations have been made [16]. *C. sporogenes*, in pure culture, converts arginine to 5-aminovalerate, acetate and ornithine as the major products. When it was cocultivated with a methanogen, no ornithine was formed. It was proposed that the presence of the methanogen enabled *C. sporogenes* to further degrade the ornithine to acetate. Based on the stoichiometry of arginine conversion in coculture, *T. acidaminovorans* most probably converted ornithine further to glutamate via glutamate semialdehyde and then to acetate and propionate. As *T. acidaminovorans* is able to convert glutamate in pure culture the bottleneck likely is the conversion of ornithine to glutamate:

$$\text{L-ornithine} + 2\ H_2O \rightarrow \text{L-glutamate} + NH_4^+ + 2\ H_2$$

We were unable to calculate the Gibbs' free energy change for this reaction from available data. However, since *T. acidaminovorans* converts arginine only in coculture completely to acetate, propionate, H_2, HCO_3^- and NH_4^+, we speculate that this reaction might be highly endergonic. The excretion of ornithine and citrulline from arginine may indicate that Arginine/Ornithine or Arginine/Citrulline exchange transport mechanisms as found in Streptococci are present in *T. acidaminovorans* as well [17].

Table 3. Yield of *Thermanaerovibrio acidaminovorans* grown on different arginine concentrations

Arginine converted (µmol)	mg dry weight	Yield (g dw.mole^{-1} arginine)	µmol* dry weight	Citrulline formed (µmol)	Ornithine formed (µmol)	Citrulline Ornithine Ratio
140	3	21.4	12	55	90	0.61
590	11	18.6	44	185	320	0.57
1030	12	11.6	48	260	530	0.49
1260**)	25	19.8	99	0	0	-

*) M_w organic fraction =114; 50% dry weight = organic fraction; 90% organic fraction = protein

**) *T. acidaminovorans* grown in the presence of *M. thermoautotrophicum*

Yield studies with increasing concentrations of arginine resulted in decreasing molar growth yields (Table 3). This was not correlated with a decreasing citrulline/ornithine ratio. More ornithine was formed at higher arginine concentrations. Apparently, there is no correlation between ornithine formation and ATP generation, although all enzymes necessary for ATP generation from ornithine and carbamyl-phosphate were present in cells from *T. acidaminovorans*. The conversion of citrulline to ornithine could be the rate-limiting step. This could be explained by the fact that this conversion is energetically unfavorable ($\Delta G^{0'}$= +28.5 kJ/mole). Another possibility could be that energy was used to take up arginine, which had an effect on the ATP yield.

In conclusion, *T. acidaminovorans* uses the ADI pathway and not the arginase pathway for growth on arginine. It seems that the formation of H_2 is a strict barrier in the arginine metabolism of *T. acidaminovorans*, which can only be overcome by the addition of a methanogen. Only then arginine is completely converted to propionate, acetate, NH_4^+, CO_2 and H_2.

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Summary and concluding remarks

Waste water usually contains large amounts of different organic compounds. A variety of microbial processes is involved in the anaerobic methanogenic treatment of waste water, such as hydrolysis of lipids, polysaccharides and proteins, fermentation of sugars and amino acids, acetogenic conversion of fatty acids and methanogenesis from acetate and H_2/CO_2. The ultimate end product from these microbial processes is biogas, which mainly consists of methane and carbon dioxide. To optimise anaerobic treatment detailed knowledge of the different organisms and their metabolic interactions must be obtained.

This thesis mainly deals with the fate of glutamate in moderate thermophilic methanogenic systems. Glutamate is a major constituent of protein and can be degraded via different pathways.

The introduction (**Chapter 1**) gives a short summary of the anaerobic degradation of amino acids in general and glutamate in more detail. Furthermore, syntrophic degradation of glutamate is discussed. Syntrophism is a special form of symbiosis where two microorganisms cooperate in such a way that one converts the products formed by the other, which enables conversion of the compound. Without the presence of the syntrophic partner no or hardly any degradation can occur, since the thermodynamic conditions are unfavourable. Syntrophic glutamate-degrading organisms known to date are discussed as well as the possible degradation pathways that are used.

To get more insight into glutamate degradation in mesophilic and moderate thermophilic methanogenic granular sludge, anaerobic glutamate-degrading microorganisms were quantified using the "most probable number" (MPN) technique (**Chapter 2**). Quantification of the glutamate-degrading population was dependent on the culture conditions used. When glutamate-degrading organisms were enumerated in the presence of methanogens the highest numbers could be counted. This indicates that hydrogen transfer plays an important role in the degradation of glutamate in methanogenic sludge. Furthermore, the most abundant glutamate-degrading organisms were physiologically different from the well-described butyrate-forming bacteria. When the incubation period of the experiments was prolonged to 6 months more glutamate degraders could be counted as opposed to 3 months of incubation. This indicates the presence of slow-growing glutamate-degrading organisms in the sludge.

Slow-growing glutamate-degrading thermophilic microorganisms were enriched in a special designed reactor system (**Chapter 3**). This dialysis membrane reactor was operated for 2 years and several organisms were isolated from it. **Chapters 4 and 5** describe the isolation and characterisation of two organisms. *Caloramator coolhaasii* is an anaerobic glutamate-converting bacterium, which is capable of complete glutamate conversion to acetate, H_2, CO_2, NH_4^+ and traces of propionate. In the presence of a hydrogen scavenging methanogen, glutamate is converted to the same products, but the growth rate was 4-fold higher. Further research showed that several amino acids and sugars could be converted. However, the degradation was always faster in the presence of a methanogen. *Methanosaeta* strain A (**Chapter 4**) is a methanogenic acetoclastic archaeon. The presence of this organism in the dialysis membrane reactor was expected as *Methanosaeta* has a higher affinity for acetate than *Methanosarcina*. The kinetic properties of *Methanosaeta* enables the bacterium to grow well at low acetate concentrations and low growth rates. The most remarkable property of this organisms is its filamentous growth and especially for strain A its appearance. This organism forms granules, which are difficult to disintegrate, under all growth conditions tested. The property to form granules gives this organism an extra advantage in upflow anaerobic sludge bed reactors. By immobilising in granules wash out from the reactor is prevented.

A glutamate- and propionate-degrading consortium (Stams et al. 1992) was found to consist of two organisms: a glutamate-degrading and a propionate-oxidising bacterium. The isolation and characterisation of both organisms is described in **chapters 6 and 7.** *Gelria glutamica* (strain TGO) can convert a limited number of substrates. In pure culture growth was found on glucose, pyruvate, lactate and glycerol. Besides glutamate, proline was the only other amino acid that could be converted by TGO. Both amino acids were only degraded in the presence of a methanogen. Glutamate and proline were converted to propionate, H_2, CO_2 and ammonia. Traces of succinate were found in the degradation of glutamate. 16S rDNA sequence analysis showed that the organism has an unusually large 16S rDNA gene. The normal size of a 16S rDNA gene is approximately 1500 base pairs, but the 16S rDNA of this bacterium consisted of more than 1700 base pairs. Detailed research showed that large loops were present in the V7 and V9 region of the 16S rDNA.

Desulfotomaculum thermosyntrophicum (strain TPO) is a moderate thermophilic spore forming syntrophic propionate-oxidising bacterium, which is only

capable of growth on propionate in the presence of a methanogen. Propionate is degraded to acetate, H_2 and CO_2. In pure culture, the organism can convert pyruvate, lactate, fumarate, glycine and H_2/CO_2. Remarkable is that TPO is capable of benzoate fermentation to acetate, propionate and an unknown compound, without the production of hydrogen. TPO is also capable of sulphate reduction and it is phylogenetically related to other spore forming thermophilic sulphate reducers.

The glutamate metabolism of the two previously mentioned glutamate degraders and *Thermanaerovibrio acidaminovorans* (Cheng et al. 1992) was studied in detail (**Chapter 8**). *Caloramator coolhaasii* converts glutamate to acetate through the methylaspartate pathway. Enzyme measurements showed, however, only low to moderately high activities of enzymes from the pathway. This in contrast to other organisms known to use this pathway for glutamate conversion. *Th. acidaminovorans* presumable also uses the methylaspartate pathway for the formation of acetate from glutamate, however only preliminary evidence was found for the presence of this pathway. Propionate formation occurs through direct oxidation of glutamate via the intermediates succinyl CoA and methylmalonyl CoA. This pathway also occurs in TGO for propionate formation from glutamate.

The arginine metabolism of *Th. acidaminovorans* was studied in detail and is described in **chapter 9**. In pure culture arginine is converted by *Th. acidaminovorans* to citrulline and ornithine, whereas in coculture with a methanogen acetate, propionate, H_2, CO_2 and ammonia was formed. The arginine deiminase pathway is used for arginine degradation. Although there is an ATP generating conversion in this pathway, yield studies did not confirm this. At higher arginine concentrations the molar growth yield decreased.

Many fast-growing organisms are described to degrade glutamate to acetate and butyrate. The organisms that are isolated and characterised in this research do not form butyrate. Since the organisms originate from methanogenic granular sludge, butyrate formation does not seem to be important in these environments. Studies performed by Baena and coworkers on mesophilic glutamate degraders from methanogenic environments also show that the organisms are specialized in the formation of acetate and propionate from glutamate, rather than in the formation of

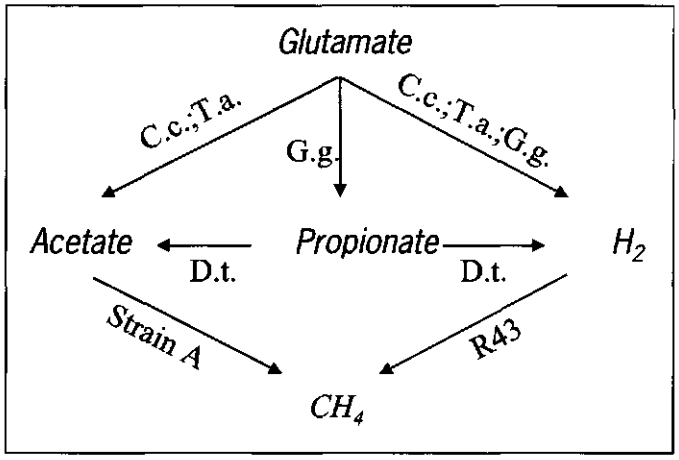

Figure 1. The anaerobic degradation of glutamate in thermophilic methanogenic environments. C.c., *Caloramator coolhaasii*; T.a., *Thermanaerovibrio acidaminovorans*; G.g., *Gelria glutamica*; D.t., *Desulfotomaculum thermosyntrophicum*; Strain A, *Methanosaeta thermophila* cult A; R43, *Methanobacterium* sp. R43

butyrate (Baena et al. 1998; Baena et al. 1999; Baena et al. 2000). Weng and Jeris (1971) investigated glutamate degradation under methanogenic conditions at mesophilic temperatures and showed that butyrate formation was of minor importance.

The ability to grow on glutamate with the concomitant production of hydrogen can be found in anaerobes from different phylogenetic clusters, although most organisms group in the low G+C branch of the Clostridia. In contrast to mesophilic organisms there are no species among the thermophilic syntrophic glutamate degraders that are highly specialised in the degradation of amino acids. All species described thus far are capable of saccharolytic growth.

The degradation pathways of glutamate as performed by *Caloramator coolhaasii*, *Thermanaerovibrio acidaminovorans* and *Gelria glutamica* lead to acetate, bicarbonate, ammonium and hydrogen and to propionate, bicarbonate, ammonium and hydrogen. When *Thermanaerovibrio acidaminovorans* is cocultivated with hydrogen-utilizing methanogens, propionate formation becomes more important

than acetate formation. Acetate formation at low hydrogen partial pressures has ΔG values of –41.6 and –73.1 kJ/reaction at 25 and 55^{0}C, respectively, where propionate formation yields 16.0 and 79.0 kJ/reaction under the same conditions (Chapter 1, Table 2). This shows that propionate formation becomes more favorable at higher temperatures. The presence of organisms in mesophilic methanogenic environments forming only propionate from glutamate, such as *Gelria glutamica* at high temperature, has yet to be demonstrated.

The anaerobic degradation of glutamate to methane under thermophilic conditions as performed by organisms studied in this thesis can be put a reaction scheme as depicted in Fig.1.

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Samenvatting

Afvalwater bevat een grote hoeveelheid sterk uiteenlopende organische verbindingen. Bij anaërobe methanogene zuivering van afvalwater is een groot aantal microbiële processen betrokken, zoals hydrolyse van vetten, polysacchariden en eiwitten, fermentatie van suikers en aminozuren, acetogene omzetting van vetzuren en methaanvorming uit acetaat en koolstofdioxide. Het uiteindelijke product van deze microbiële processen is biogas, bestaande uit methaan en koolstofdioxide. Om anaërobe zuivering zo efficient mogelijk te laten verlopen is een goede kennis van deze microbiële processen onontbeerlijk.

Dit proefschrift beschrijft hoofdzakelijk het lot van glutamaat in methanogene systemen. Glutamaat is een belangrijk bestanddeel van eiwit en kan onder anaërobe omstandigheden op verschillende manieren worden afgebroken.

In de inleiding (**Hoofdstuk 1**) wordt een kort overzicht gegeven van de anaërobe afbraakmogelijkheden van aminozuren in het algemeen en glutamaat in het bijzonder. Tevens wordt aandacht besteed aan zogenaamde syntrofe afbraak van glutamaat. Bij deze afbraak is er sprake van een samenwerking tussen twee micro-organismen, waarbij de een de ander helpt om gevormde producten, zoals waterstof, weg te nemen, zodat afbraak mogelijk wordt. Deze afbraak zou zonder de aanwezigheid van de syntrofe partner niet of nauwelijks mogelijk zijn, omdat de thermodynamische condities ongunstig zijn. De tot nu toe in de literatuur beschreven micro-organismsen, die glutamaat syntroof kunnen omzetten, worden gepresenteerd, alsmede de afbraakroutes die daarbij gebruikt zouden kunnen worden.

Om meer inzicht te krijgen in glutamaatafbraak in mesofiel en gematigd thermofiel methaanvormend korrelslib werden anaërobe glutamaatomzettende micro-organismen gekwantificeerd met behulp van de 'most probable number" (MPN) techniek (**Hoofdstuk 2**). Het bleek dat de kwantificering van deze glutamaatomzettende micro-organismen zeer sterk afhankelijk van de kweekcondities. Wanneer glutamaatomzettende micro-organismen werden gekweekt in aanwezigheid van een overmaat aan methaanvormers konden de hoogste aantallen worden geteld. Dit duidt erop dat in de onderzochte slibsoorten waterstofoverdracht tussen micro-organismen een cruciale rol speelt in de afbraak van glutamaat. Verder bleek dat de meest dominante glutamaatomzetters tot een andere fysiologische groep behoorden dan de tot nu toe goed gedocumenteerde snelgroeiende boterzuurvormende bacteriën. Wanneer de incubatietijd van de experimenten verlengd werd tot 6 maanden konden

meer glutamaatomzetters geteld worden dan na 3 maanden. Dit wijst op prominente aanwezigheid van langzaam groeiende glutamaatomzetters in het korrelslib.

Langzaam groeiende thermofiele glutamaatomzettende bacteriën werden opgehoopt via een speciaal ontworpen reactorsysteem (**Hoofdstuk 3**). Deze dialysemembraan reactor is gedurende 2 jaar in bedrijf geweest en hieruit zijn diverse micro-organismen geisoleerd. **Hoofdstuk 4 en 5** beschrijven isolatie en karakterisatie van een tweetal isolaten. *Caloramator coolhaaasii* is een anaërobe glutamaatvergistende bacterie, die in reincultuur glutamaat volledig omzet naar acetaat, H_2, CO_2, NH_4^+ en spoortjes propionaat. In aanwezigheid van een methaanvormer wordt glutamaat omgezet naar dezelfde producten als in reincultuur, maar de groeisnelheid is 4 maal hoger. Uit verder onderzoek bleek dat diverse aminozuren en suikers konden worden afgebroken. Echter, in aanwezigheid van een methaanvormer verliep de afbraak altijd sneller. *Methanosaeta* stam A (**Hoofdstuk 4**) is een methaanvormende acetaat splitsende archaebacterie. De aanwezigheid van dit organisme in de dialysemembraan reactor was te verwachten, aangezien *Methanosaeta* langzamer groeit bij lage acetaatconcentraties dan *Methanosarcina*. De kinetische eigenschappen van *Methanosaeta* zijn zodanig dat bij lage acetaat concentraties en groeisnelheid dit organisme zeer goed groeit. Het meest aansprekende kenmerk van dit organisme is de filamenteuze uiterlijk en speciaal voor *Methanosaeta* stam A de groeiwijze. Onder alle geteste groeicondities vormt dit micro-organisme stevige korrels die met moeite gedesintegreerd kunnen worden. De eigenschap om korrels te kunnen vormen geeft dit organisme een extra voordeel in zogenaamde opstroom anaërobe slib-bed reactoren. Door te immobiliseren in korrels wordt voorkomen dat de organismen uitspoelen uit de reactor.

Een eerder beschreven glutamaat omzettend methaanvormend consortium (Stams et al., Appl. Environ. Microbiol. 58: 346-352) bleek te bestaan uit een tweetal organismen: een glutamaat omzettende en een propionaat oxiderende bacterie. In **hoofdstuk 6 en 7** worden de isolatie en karakterisatie van beide organismen beschreven. *Gelria glutamica* (TGO) is in staat om slechts op een beperkt aantal verbindingen te groeien. In reinculture kon slechts groei aangetoond worden op glucose, pyruvaat, lactaat en glycerol. Van de geteste aminozuren kon naast glutamaat alleen het aminozuur proline omgezet worden, echter alleen in aanwezigheid van een methaanvormer. Zowel glutamaat als proline worden omgezet naar propionaat, H_2, CO_2 en NH_4^+. Bij de afbraak van glutamaat werd ook in geringe mate succinaat

gevormd. 16S rDNA sequentieanalyse van deze syntrofe bacterie liet een ongewoon groot molecule zien. Waar normaalgesproken het 16S rDNA gen bestaat uit ongeveer 1500 baseparen, bleek het 16S rDNA van deze bacterie te bestaan uit meer dan 1700 baseparen. Nader onderzoek liet zien dat in de zogenaamde V7 en V9 regio van het 16S rDNA extra grote loops aanwezig waren.

Desulfotomaculum thermosyntrophicum (TPO) is een gematigd thermofiele sporevormende syntrofe propionaat-oxiderende bacterie, die slechts in aanwezigheid van een methaanvormer propionaat omzet naar acetaat, H_2 en CO_2. In reincultuur is het micro-organisme in staat pyruvaat, lactaat en H_2/CO_2 om te zetten. Opmerkelijk is dat TPO in staat is om benzoaat te fermenteren tot acetaat, propionaat en een tot nu onbekend eindproduct, zonder dat er H_2 gevormd wordt. TPO is in staat om sulfaat te reduceren en is phylogenetisch verwant aan andere sporenvormende thermofiele sulfaatreduceerders.

Van de twee eerder genoemde glutamaatomzetters en *Thermanaerovibrio acidaminovorans* (Cheng et al., Arch. Microbiol. 157: 169-175) werd het glutamaat metabolisme verder onderzocht (**Hoofdstuk 8**). *Caloramator coolhaasii* bleek glutamaat om te zetten naar acetaat via de methylaspartaat route. Enzym metingen lieten echter zien dat de activiteiten van enzymen uit deze route slechts laag tot matig hoog waren. Dit in tegenstelling tot andere organismen die deze route gebruiken voor glutamaat afbraak. *Thermanaerovibrio acidaminovorans* gebruikt voor de vorming van acetaat hoogstwaarschijnlijk ook de methylaspartaat route, hoewel hiervoor slechts voorzichtig bewijs is gevonden. Propionaatvorming uit glutamaat verloopt door directe oxidatie van het glutamaat via de intermediairen succinyl-CoA en methylmalonyl CoA. Dezelfde route wordt gevolgd door *Gelria glutamica* (TGO) om glutamaat om te zetten naar propionaat.

Het arginine metabolisme van *Th. acidaminovorans* is nader onderzocht en beschreven in **hoofdstuk 9**. In reincultuur zet *Th. acidaminovorans* arginine om naar citrulline, ornithine en ammonia, terwijl in co-culture met een methaanvormer acetaat, propionaat, H_2, CO_2 en ammonia. Arginine bleek omgezet te worden via de arginine deiminase (ADI) omzettingsroute. Hoewel er in deze route een ATP-genererende omzetting voorkomt, kon dit in groeiopbrengst studies niet eenduidig worden aangetoond. Bij een steeds hogere arginine concentratie werd de molaire groeiopbrengst steeds geringer.

Caroline Mariëtte Plugge werd geboren op 10 juli 1961 te Amsterdam als dochter van Robert Henri Plugge en Maryse Henriëtte Lensing. Zij groeide op in Amstelveen, waar zij achtereenvolgens kleuterschool "Kindervreugd", lagere school "Johannes Post" en scholengemeenschap "Hermann Wesselink College" bezocht. Nadat zij in 1979 het eindexamen gymnasium β met goed gevolg had afgelegd, besloot zij te gaan studeren aan de toenmalige Landbouwhogeschool te Wageningen. Daar het theoretische karakter van de studie aldaar haar niet beviel, begon zij in 1981 aan de opleiding tot analist aan de toenmalige Hogere Laboratorium School "Stova" te Wageningen. Hoewel zij de Botanische richting gekozen had, betrof zowel haar stage als afstudeerspecialisatie een microbiologisch onderwerp. Stage werd gelopen bij de Gemeente Waterleidingen te Amsterdam, waar zij onderzoek deed naar de diversiteit van ingewandsbacteriën in de Waterleidingplas te Loosdrecht alsmede naar de algen groeipotentie in dezelfde plas. Haar afstudeeropdracht voerde zij uit bij het Laboratorium voor Microbiologie van de Landbouwhogeschool Wageningen (thans Wageningen Universiteit). Onder de inspirerende begeleiding van mw. dr. M.H. Deinema deed zij onderzoek naar polyfosfaataccumulatie in diverse *Acinetobacter* soorten. In maart 1985 werd de opleiding met goed gevolg afgesloten. In april 1985 werd zij aangesteld als analist bij het Laboratorium voor Microbiologie van de Landbouw Hogeschool in het door de Stichting Technische Wetenschappen gefinancierde project: "Structuur en stabiliteit van methanogeen korrelslib". Samen met Tim Grotenhuis werd door haar voornamelijk gewerkt aan het microbiologisch karakteriseren van onder laboratorium omstandigheden gekweekt anaëroob methanogeen korrelslib. In april 1989 werd zij aangesteld als tijdelijk onderzoeksanalist bij het Laboratorium voor Microbiologie van de Landbouw Universiteit in de "Anaërobe Groep". Deze aanstelling werd na 1 jaar omgezet in een vast dienstverband. In de periode van 1989 tot 1993 werd fysiologisch/biochemisch onderzoek gedaan aan syntrofe propionaat oxiderende bacteriën. Van 1993 tot 1995 werd fysiologisch onderzoek verricht aan diverse anaërobe micro-organismen. In de zomers van 1995, 1996, 1997 en 1999 heeft zij de Zomerschool "Microbial Diversity" aan het Marine Biological Laboratory in Woods Hole, MA, USA bezocht. Eerst als student (1995) en daarna als staflid. Officieus in 1995 en officieel in januari 1996 is zij gestart met de bestudering van syntrofe thermofiele glutamaat oxiderende bacteriën, waarbij daarnaast de werkzaamheden als analist van de werkgroep Microbiële Fysiologie bij het Laboratorium voor Microbiologie gewoon doorgingen. Het resultaat van genoemd onderzoek staat beschreven in dit proefschrift.

Het is niet zo dat voor mijn bijgedragen steentjes aan de wetenschap meer bijzondere, zwaardere wetten gelden dan voor de alledaagse. Dit boekje is de weergave van een aantal jaren werk, niets meer, maar ook niets minder. Ik heb deze periode beleefd als zeer waardevol. Er is voor mij niets mooier dan zelf proeven doen in het laboratorium, kennis overdragen aan anderen en het draaiende houden van een onderzoekslab. De combinatie van de taken die behoorden bij mijn functie als werkgroepanalist en het uitvoeren van "mijn eigen onderzoek" leidden er soms toe dat ik door de bomen absoluut het bos niet meer kon zien. Het heeft me geleerd beter "nee" te zeggen en nog efficiënter met mijn tijd om te gaan.

De gewoonte om in het dankwoord behorend bij een proefschrift familie, vrienden en collega's met naam en toenaam alle lof toe te zwaaien voor bewezen diensten is begrijpelijk, maar tegelijkertijd vaak onvolledig. Daarom wil ik volstaan met een ieder die op welke manier dan ook een bijdrage heeft geleverd aan het tot stand komen van dit proefschrift een oprecht en welgemeend "DANK" toe te roepen.

Als uitzondering, om de regel te bevestigen, wil ik toch één periode uit mijn onderzoek speciaal belichten. In de zomers van 1995, 1996, 1997 en 1999 heb ik de Zomerschool "Microbial Diversity" aan het Marine Biological Laboratory in Woods Hole, MA, USA bezocht. Eerst als student (1995) en daarna als staflid. Ik vond daar, naast een enorme vergroting van mijn algemene microbiologische kennis, waardering voor en erkenning van mijn vakmanschap. Dit heeft voor mij zo veel betekend, dat ik dit niet onvermeld kan laten.

The habit to thank relatives, friends and colleagues in the acknowledgements of a thesis is understandable, but at the same time also often incomplete. I want to limit myself by expressing a sincere "THANK YOU" to everybody who contributed in any way to the accomplishment of this thesis.

As an exception, to confirm the rule, I want to mention specifically one period from the past years. During the summers of 1995, 1996, 1997 and 1999 I visited the Summer School "Microbial Diversity" at the Marine Biological Laboratory in Woods Hole MA, USA. First as a student (1995) an later as a staff member. What I have found there, besides an enormous broadening of my general microbiological knowledge, was great appreciation and acknowledgement of my professional skills. This has meant so much to me that I can not leave it unmentioned.

"Het is gelukt"

Author: Plugge, C.M.
Title: Syntrophic degradation of amino acids by thermophilic methanogenic consortia
ISBN: 90-5808-431-0
Publication year: 2001
Subject headings: anaerobic; syntrophic; interspecies hydrogen transfer; thermophilic; methanogenic; amino acid; glutamate
Thesis Wageningen University, Wageningen, The Netherlands -With references- With summary in Dutch – 171 p.