The Effect of Trace Elements on the Metabolism of Methanogenic Consortia

Bo Jiang

Promotor:	**Prof. Dr. ir. A. J. M. Stams** persoonlijk hoogleraar bij het laboratorium voor microbiologie
Promotiecommissie:	**Prof. Dr. H. L. Drake** University of Bayreuth, Germany **Prof. Dr. H. P. van Leeuwen** Wageningen Universiteit; University of Geneva, Switzerland **Prof. Dr. ir. G. Lettinga** Wageningen Universiteit **Dr. ir. J. C. T. Vogelaar** Paques Natural Solutions, Balk

Dit onderzoek is uitgevoerd binnen de onderzoekschool WIMEK.

The Effect of Trace Elements on the Metabolism of Methanogenic Consortia

Bo Jiang

Proefschrift

ter verkrijging van de graad van doctor

op gezag van de rector magnificus

van Wageningen Universiteit

Prof. Dr. M. J. Kropff

in het openbaar te verdedigen

op vrijdag 17 maart 2006

des namiddags te half twee in de Aula

B Jiang

The Effect of Trace Elements on the Metabolisms of Anaerobic Microbial Consortia
Thesis Wageningen University, 2006 – with summary – 122 pp

ISBN: 90-8504-360-3

Contents

Abstract

Jiang B. (2006) **The Effect of Trace Elements on the Metabolisms of Anaerobic Microbial Consortia.** PhD Thesis, Wageningen University, Wageningen, The Netherlands. p122.

Trace metals are essential for the growth and metabolism of anaerobic microorganisms, duo to their roles in key enzymes or cofactors of metabolic pathways. The requirement of trace metals has been recognized. But, proper dosing of these metals in anaerobic treatment system as nutrient still is a great challenge, since dosing of a metal at a high concentration is toxic for growth of microorganisms, and dosing of a specific metal may lead out-compete of one group of microorganisms by the other. In order to obtain knowledge for optimization of metal dosing of anaerobic treatment system, the influence of trace metals, like cobalt, nickel, tungsten and molybdenum on the conversion of methanol and propionate were studied in this research.

By using cobalt-sufficient medium, a methanogenic enrichment culture was enriched from a thermophilic lab-scale UASB reactor fed with methanol as carbon and energy source. From which a novel thermophilic obligate methylotrophic methanogenic archaeon, strain L2FAW^T, was isolated and characterized as *Methanomethylovorans thermophila*. The growth of strain L2FAW^T on methanol is stimulated by the addition of cobalt; the optimal cobalt concentration is 0.5 to 2 μM. therefore, cobalt is important for direct methanol conversion by this methanogen.

On the other hand, a syntrophic coculture of methanol degradation was enriched from the same sludge by using cobalt deficient medium, which consisted of a homoacetogen and a hydrogenotrophic methanogen. This coculture degrades methanol partially to acetate and partially to methane, depending on the presence of cobalt. Acetate is the main product when cobalt is presence at high concentration; otherwise methane is formed as dominant products. Therefore, cobalt plays a role in the regulation of the pathway of methanol conversion. The optimal cobalt concentration of the coculture for complete methanogenesis from methanol is about 0.1 μM. A thermophilic spore-forming bacteria, strain AMP, was isolated from the coculture, and it is most closely related to *Moorella thermoacetica* based on 16S rRNA analysis. Despite its high DNA-DNA homology with *M. thermoacetica*, strain AMP differs from *M. thermoacetica* on its inability to use glucose, formate and H_2/CO_2, and its unique hydrogenogenic growth on CO. Moreover, strain AMP can grow on formate in a coculture with a hydrogenotrophic methanogen. It is described for the first time that a bacterium can grow on the conversion of formate to H_2 and bicarbonate provided that hydrogen is consumed by a methanogen.

The effect of cobalt and nickel on the corrinoid and F430 content and on growth of *Methanosarcina barkeri* on methanol was studied. Cobalt and nickel limitation was achieved and competition between cobalt and nickel uptake was observed. Uptake efficiency of cobalt was high at low cobalt concentration and decreased when the cobalt concentration in the medium was increased. Corrinoid and F430 content correlated positively with the cell content of the corresponding metal, but incorporation in the corrinoid and F430 was significant less at low cell metal contents, ranging from 35% to 80% for corrinoid and 5% to 15% for F430.

The trace elements tungsten and molybdenum play an essential role in the growth of anaerobic microorganisms. Depletion of tungsten and/or molybdenum in the media did not affect axenic growth of *Syntrophobacter fumaroxidans* on propionate+fumarate, indicating under these conditions this organism does not have a high tungsten or molybdenum requirement. However, growth of *Methanospirillum hungatei* on either formate or hydrogen and carbon dioxide required tungsten, and molybdenum can replace tungsten to some extent. Growth of the *Syntrophobacter-Methanospirillum* coculture on propionate is significantly affected by the addition of these two metals. Measurement of enzyme levels in cell extracts of syntrophically grown cells indicated that the levels of hydrogenase and formate dehydrogenase activity were correlated with the methane formation rates by the cocultures, which suggests both hydrogen and formate play important role in syntrophic propionate oxidation.

Chapter 1

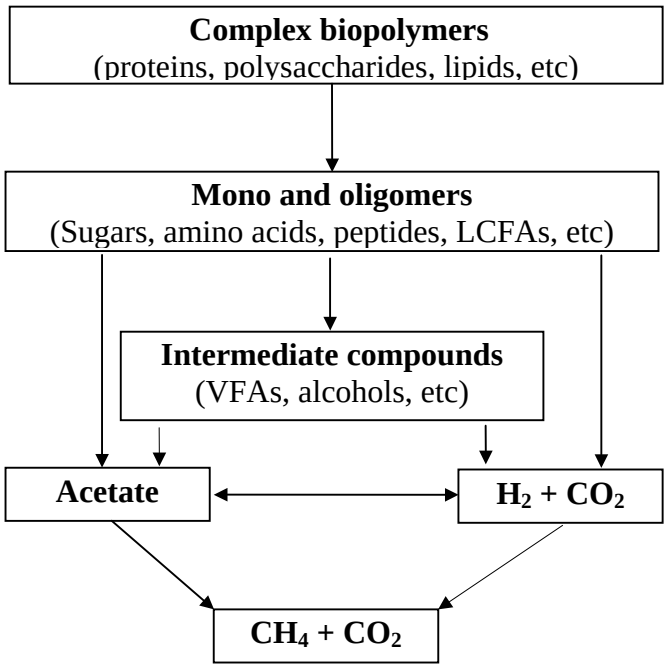
General introduction

Anaerobic environmental technology

Anaerobic wastewater treatment

Anaerobic treatment of wastewater is presently accepted as a proven technology (94, 139). Interest in anaerobic treatment of industrial wastewater is increasing rapidly, primarily due to the positive energy balance of the anaerobic treatment process, the low excess sludge production and the development of inexpensive and high rate treatment systems. Various advanced anaerobic treatment configurations have been developed, such as the packed bed, fixed film, expanded/fluidized bed, membrane and upflow anaerobic sludge blanket reactor (72).

Fig. 1 Flow of carbon in the anaerobic digestion of organic matter, after Gujer & Zehnder (51). LCFAs: long chain fatty acids; VFAs: volatile fatty acids.



Anaerobic digestion is a microbial process in which organic matter is decomposed in the absence of molecular oxygen or other inorganic electron acceptors. The main products of this process are methane, carbon dioxide, ammonium and hydrogen sulfide. Anaerobic digestion can be distinguished in four stages, hydrolysis, fermentation, acetogenesis and methanogenesis (51). Methanogenesis is characterized by a sequence of reactions performed

by different physiological types of anaerobic microorganisms: primary and secondary fermenters acetogenic bacteria and methanogens (51). The last two tropic groups are key players in the anaerobic mineralization of organic matter. Methanogens are specialized in the use of H_2/CO_2, formate and acetate as substrates. Consumption of these products is essential for acetogenic bacteria to oxidize products of the fermentation process, like butyrate and propionate. The conversion of these compounds to the methanogenic substrates is thermodynamically unfavorable (112, 114, 122).

Propionate is an important intermediate in the complete decomposition of complex organic matter under methanogenic conditions. It is formed by fermentative bacteria and accounts for up to 15% of total methanogenesis (51). Conversion of propionate by acetogenic bacteria to acetate, CO_2, H_2 and or formate, can only proceed if the products, hydrogen and formate, are removed efficiently by methanogens (112, 122). Therefore, syntrophic propionate oxidation plays an important role in methanogenic decomposition, and in maintaining bioreactor stability and performance.

Most of the known syntrophic propionate-oxidizing bacteria belong to the *Syntrophobacter* genus. These bacteria degrade propionate via the methyl-malonyl-CoA pathway (103). To dispose of electrons released from this pathway, acetogens reduce protons to H_2 or bicarbonate to formate by means of hydrogenase (H_2ase) and formate dehydrogenase (FDH). These electron carriers are oxidized again by methanogens. Interspecies electron transfer by syntrophic propionate-oxidizing consortia has been studied extensively, but it is still unclear whether electrons are transferred mainly as hydrogen or as formate (112, 122). Unlike syntrophic butyrate and ethanol oxidation bacteria, *Syntrophobacter* species can only oxidize propionate in coculture with methanogens that can utilize both H_2 and formate, such as *Methanospirillum hungatei* and *Methanobacterium formicicum*, but not with *Methanobrevibacter* strains that only utilize H_2 (34). Therefore, besides H_2 transfer also formate transfer was considered as a possible mechanism (14, 27, 127). Biochemical evidence for interspecies formate transfer was obtained for a co-culture of *S. fumaroxidans* and *M. hungatei* (28, 35). Both microorganisms possess both H_2ases and FDHs, and exhibit high activities of these enzymes during axenic growth on formate and during syntrophic growth on propionate (27).

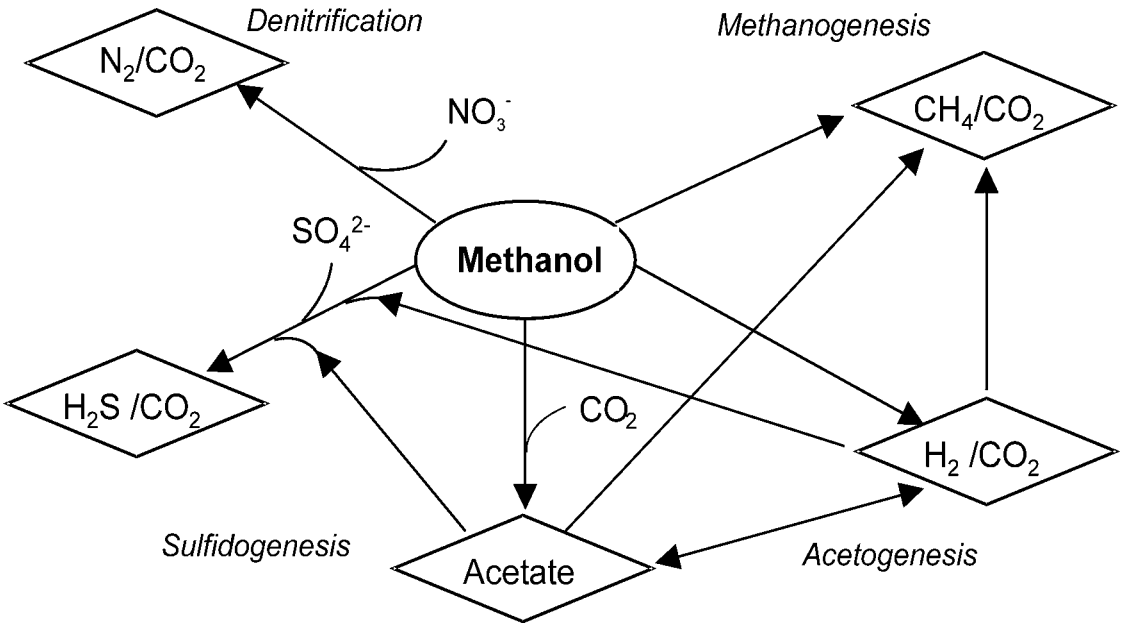
Thermophilic anaerobic wastewater treatment

The possibility of the anaerobic treatment technology could be extended if the process could be applied at high temperature (>50^{o}C). At high temperature the reaction rates proceed much faster than under mesophilic conditions, so that the loading rates of anaerobic bioreactors can be substantially higher (1, 144). Thermophilic treatment could be an attractive alternative of mesophilic treatment, particularly when the wastewater is discharged at high temperatures. Wastewater treatment in agro-industrial processes at high temperature may be useful in closed water circuits, since it avoids the cooling of the process water and thus allows reuse of water without additional heating (71, 133). However, up to now, only a very few thermophilic anaerobic systems are in operation at full-scale. That is because at thermophilic conditions the start-up of a reactor takes much longer and the process is more sensitive to environmental conditions, e.g. temperature and pH changes, shock loading, feed interruptions, etc. (132).

Anaerobic conversion of methanol

Methanol is a one-carbon compound. It is formed during biodegradation of methoxylated aromatics and pectin by fungi and bacteria (96, 113). Methanol is a main

Fig. 2 Four potential degrading processes and end-products of methanol conversion under anaerobic conditions.



pollutant in the evaporator condensate of pulp and paper industries (85) and in some other chemical and food industries, e.g. coal-gasification plants (86), in landfill leachates (134) and in wastewater of potato-starch factories (88). When a cheap and efficient organic electron donor is required, methanol is often selected as a substrate, for example, in sulfate reduction, denitrification and dehalogenation processes (129, 138).

Table 1. Stoichiometries and Gibbs free energy changes of selected biological reactions involved in the anaerobic degradation of methanol at 25°C and 55°C. Values of △G' were calculated after Amend and Shock (2).

	Reactions			**△G' kJ/reaction**	
				25°C	55°C
	Methanogens				
1	$4\ CH_3OH$	$\rightarrow$	$3CH_4 + HCO_3^- + H^+ + H_2O$	-313	-319
2	$CH_3OH + H_2$	$\rightarrow$	$CH_4 + H_2O$	-113	-112
3	$HCO_3^- + 4\ H_2 + H^+$	$\rightarrow$	$CH_4 + 3H_2O$	-136	-125
4	$CH_3COO^- + 2H_2O$	$\rightarrow$	$CH_4 + HCO_3^-$	-31	-35
	Acetogens				
5	$4\ CH_3OH + 2\ HCO_3^-$	$\rightarrow$	$3CH_3COO^- + H^+ + 4\ H_2O$	-219	-214
6	$CH_3OH + 2H_2O$	$\rightarrow$	$3H_2 + HCO_3^- + H^+$	+23	+13
7	$CH_3COO^- + 4H_2O$	$\rightarrow$	$2HCO_3^- + H^+ + 4H_2$	+104	+90
	Sulfate and nitrate reducers				
8	$4\ CH_3OH + 3SO_4^{2-}$	$\rightarrow$	$3HS^- + 4H_2O + 4HCO_3^- + H^+$	-363	-373
9	$5CH_3OH + 6NO_3^-$	$\rightarrow$	$3N_2 + 5HCO_3^- + OH^- + 7H_2O$	-3207	-3207

Possible anaerobic degradation pathways of methanol are shown in Fig. 2. Reaction stoichiometries and Gibbs free energy changes at mesophilic and thermophilic conditions are shown in Table 1. Four distinct groups of anaerobic microorganisms are able to grow on methanol, namely denitrifiers, sulfate-reducing bacteria (SRB), methanogenic archaea (MA), and homoacetogenic bacteria (HAB) (83, 89, 128, 137). All four groups of microorganisms may compete for the available methanol in the mixed communities, depending on the availability of electron acceptors.

Indirect methanogenesis from methanol is occurring when methanol is first converted to either acetate or H_2 by HAB (109, 130), and then acetate or H_2 is used by MA (3) or SRB

(26). Indirect methanol degradation via H_2 as intermediate is unfavorable under standard conditions (Table 1), thus the H_2 level has to be kept low (112, 114, 122). In this respect, the different microorganisms involved depend on each other to degrade methanol, which is called a syntrophic relationship (112, 122). Since utilization of methanol by MA and HAB is independent on electron acceptors, these microorganisms metabolically interact with each other directly or indirectly.

Biotechnology of anaerobic treatment of methanol-containing wastewater

Much research was conducted to investigate the feasibility of anaerobic treatment of methanol containing wastewater. Feasibility of anaerobic treatment of methanolic wastewater was doubted. These doubts concerned the operation of a stable anaerobic treatment process due to unpredictable accumulation of volatile fatty acids (VFAs) in the effluent that may cause the failure of the treatment process (46, 74).

Lettinga and co-workers (46, 73, 74) concluded that environmental factors, such as the cobalt concentration level, the methanol concentration inside the reactor, the presence of exogenous bicarbonate and high concentrations of undissociated VFA, determine the stability of methanogenesis from methanol and the predominance of homoacetogens or methanogens. When bicarbonate and trace metals were applied together, VFAs accumulate in the effluent, consequently, methane production ceases due to a drop of pH (46, 73, 74). When the concentrations of cobalt, bicarbonate and methanol were kept low, methanogens predominated and methanol was converted directly to methane by methylotrophic methanogens and not via the intermediate formation of VFA or H_2. Under other conditions, homoacetogenesis predominated, resulting in the formation of acetate (45). It was found that *Methanosarcina* species dominated under mesophilic conditions in a reactor fed with methanol, indicating direct methanogenesis from methanol (11). If the pH was maintained between the 5.0-6.0, hydrogenotrophic methanogens survived and grew, indicating that methanol was converted syntrophically to methane through H_2 and CO_2 (9, 10). The predominant methanogens in the granular sludge from this reactor resembled the hydrogenotrophic *Methanobacterium* and *Methanobrevibacter*, *Methanosarcina* and acetoclastic *Methanosaeta* (11). From this, the authors concluded that methanol was not converted to methane directly, but was also converted through the intermediate formation of acetate and H_2/CO_2.

Feasibility of thermophilic treatment of methanol-containing wastewater was studied recently (99, 100). Good reactor performance was achieved at organic loading rates (OLR) up to 47.3 g COD/L/day in a bicarbonate buffered medium. About 93% of the methanol was converted to methane, with little acetate accumulation (99). By using specific inhibitors and nuclear magnetic resonance (NMR) spectroscopy, it was found that half of the methanol was directly converted to methane by methylotrophic methanogens and the other half via the intermediates of H_2 and acetate (101). This was confirmed by analysis of the microbial composition of the sludge: the most abundant bacteria and archaea were most closely related to *Moorella glycerini*, *Methanothermobacter thermoautotrophicus* strains and *Methanomethylovorans hollandica* (105). Moreover, it was also found that the cobalt requirement of the thermophilic mixed consortium was lower than that of a mesophilic-methylotrophic consortium (98).

Anaerobic microorganisms involved in methanol conversion

A major part of thesis was focused on thermophilic methanol conversion. Therefore, the microorganisms involved in mainly thermophilic methanol degradation are addressed here.

Thermophilic methanogenic archaea

Methanogenic archaea are microorganisms that use only a few substrates for methane formation, such as H_2/CO_2, acetate and C1-compounds, like formate, methanol and methylated amines (43). Most thermophilic methanogenic archaea can only utilize H_2/CO_2 and (or) formate as substrate, such as species in genera *Methanothermobacter* and a few species of the genus *Methanobacterium*, *Methanocaldococcus* and *Methanosarcina* (135). A few methanogens can utilize acetate for methane formation, like *Methanosaeta thermophila* (93) and *Methanosarcina thermophila* (145). Methanogens capable of methanol utilization almost exclusively belong to the family *Methanosarcinaceae*. Besides the two thermophilic halophilic obligate methylotrophs, *Methanohalobium evestigatum* and *Methanosalsum zhilinae*, *Methanosarcina thermophila* is the only thermophilic freshwater species that can utilize H_2/CO_2, acetate and methanol. *Methanosarcina thermophila* and most *Methanosarina*

species were isolated from anaerobic bioreactors. *Methanothermobacter* species are ubiquitously present in a number of environments such as anaerobic digesters, hot springs and sediments (135).

All methanogenic Archaea examined to date rely on methanogenesis as their sole means of energy conservation (43). Methanogens growing on different methanogenic substrates use a common methanogenic pathway (Fig. 3). This pathway is unique since it has several unique coenzymes, like coenzyme-M (HS-CoM), factor F420 and F430, methanofuran, tetrahydromethanofuran and 7-mercaptoheptanonylthreonine phosphate, which are not commonly present in other groups of microorganisms (108). Activation of methanol in this pathway (reduction route) is carried out by two corrinoid-containing methyltransferases, resulting in a methylated coenzyme-M (23). Methane formation from methyl-CoM is catalyzed by methyl CoM reductase, an enzyme that contains F430. In order to provide reducing equivalents for reduction of methanol to methane, one quarter of methanol has to be oxidized to CO_2. The mechanism of methanol conversion is still not clear yet. An overall pathway is depicted in Figure 3. Several alternatives are possible (63), in which methanol is oxidized to methenyl-H_4MTP by a methanol dehydrogenase and an unknown methyl-carrier. Interestingly, in such a pathway, corrinoid seems not necessary (12). During methanol conversion, a sodium-gradient is generated by methyl transfer from CH_3-H_4MPT to HS-CoM. The sodium-gradient is the driving force for the synthesis of ATP, reverse electron transfer, and solute uptake (7, 13). Recently, an energy conserving hydrogenase was found in *Methanosarcina barkeri*, which could drive a reverse electron transfer process to provide reducing equivalents for the reduction of ferredoxin with H_2 (84).

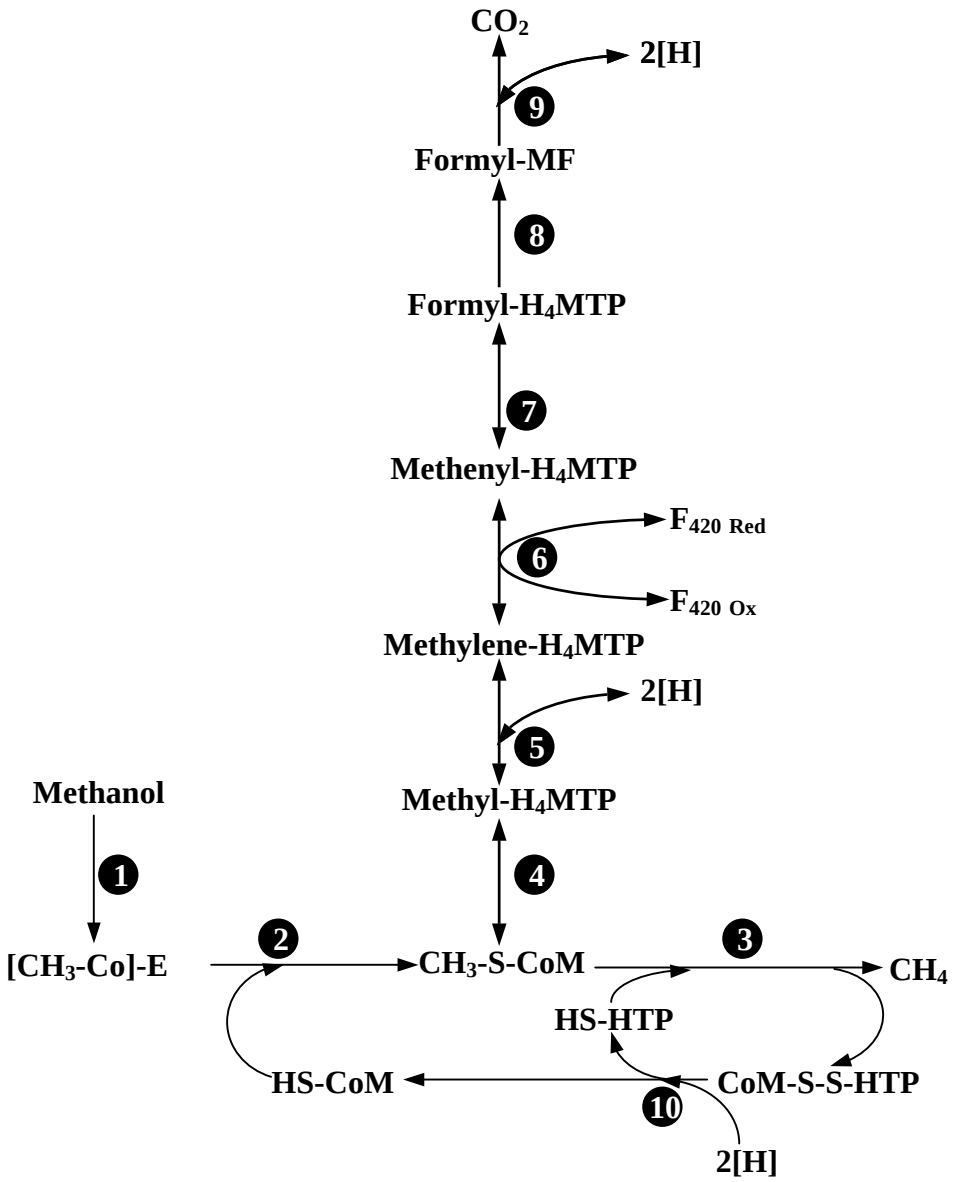


Fig 3. Methanogenic pathway from methanol, after (87) and (24). Abbreviations: MF, methanofuran; H_4MTP, tetrahydromethanopterin; F_{420}, a 8-hydroxy-5-deazaflavin derivative; HS-CoM, coenzyme M; HS-HTP, 7-mercaptoheptanonylthreonine phosphate; [CH_3-Co]-E, corrinoid bound methyl group. Enzymes indicated by 1, methanol:5-hydroxybenzimidazolylcobamide methyltransferase; 2, methylcobamide:HS-CoM methyltransferase; 3, methyl-S-CoM reductase; 4, methyl-H_4MTP:HS-CoM methyltransferase; 5, methylene-H_4MTP reductase; 6, methylene-H4MTP dehydrogenase; 7, methylene-H_4MTP cyclohydrolase; 8, formylmethanofuran:H_4MTP formyltransferase; 9, formylmethanofuran dehydrogenase; 10: $F_{420}H_2$:heterodisulfide oxidoreductase.

Thermophilic homoacetogens

Homoacetogenic bacteria are a group of bacteria that utilize the reductive acetyl-CoA pathway to form acetate (37). They are versatile anaerobes in terms of their substrate profile (36). In the acetyl-CoA pathway CO_2 acts as a sink for reducing equivalents generated in the oxidation of carbohydrates, organic acids, C1 compounds or hydrogen. Around 100 species of homoacetogenic bacteria divided over 21 genera in the *Bacillus-Clostridium* subphylum have been described (22, 37). Well-studied mesophilic methylotrophic utilizing homoacetogens are *Sporomusa ovata*, *Eubacterium limosum*, and *Acetobacterium woodii*. The best-characterized thermophilic methanol-degrading homoacetogens are the heat-resistant spore-producing species *Moorella thermoautotrophica* (140) and *Moorella thermoacetica* (47). Based on which cation is used for energy conservation, homoacetogens are divided into two groups: proton–dependent homoacetogens, like *Moorella* and *Sporomosa* species (49, 62), and sodium-dependent homoacetogens, represented by *Acetobacterium woodii* and *Thermoanaerobacter kivui* (55, 142).

Homoacetogens use the acetyl-CoA pathway (Fig.4) for both catabolism and carbon fixation of C1 compounds, like CO, formate and methanol (37, 76). It is also used by other microorganisms, like sulfate reducing bacteria and methanogens for synthesis of biomass (19, 70, 103). It is also found that the acetoclastic methanogen, *Methanosarcina acetivorans* C2A during growth on carbon monoxide might employ this pathway for energy conservation as a alternative for the methanogenic pathway (107). Therefore, acetyl-CoA pathway is important for all kinds of microorganisms. The key enzymes in the acetyl-CoA pathway are formate dehydrogenase (FDH), methyltransferase and carbon monoxide dehydrogenase/acetyl-CoA synthase complex (CODH/ACS). Formate dehydrogenases contain either tungsten or molybdenum, depending on the species (38), growth substrates (92) or availability of these two metals (8, 18). CODH/ACS complex contains nickel and iron, and has both CO oxidation and acetyl-CoA synthesis activities (76). Tetrahydrofolate is the C1-carrier in the acetyl-CoA pathway (57). It is still unclear how methanol enters this pathway, but likely a corrinoid-containing methyltransferase is involved (130). Alternatively, methanol could be oxidized by a methanol dehydrogenase. In *Moorella thermoautotrophica* oxidation of methanol to formate is done with a methanol dehydrogenase that contains paraquinoline quinone (PQQ) as the prosthetic group (141).

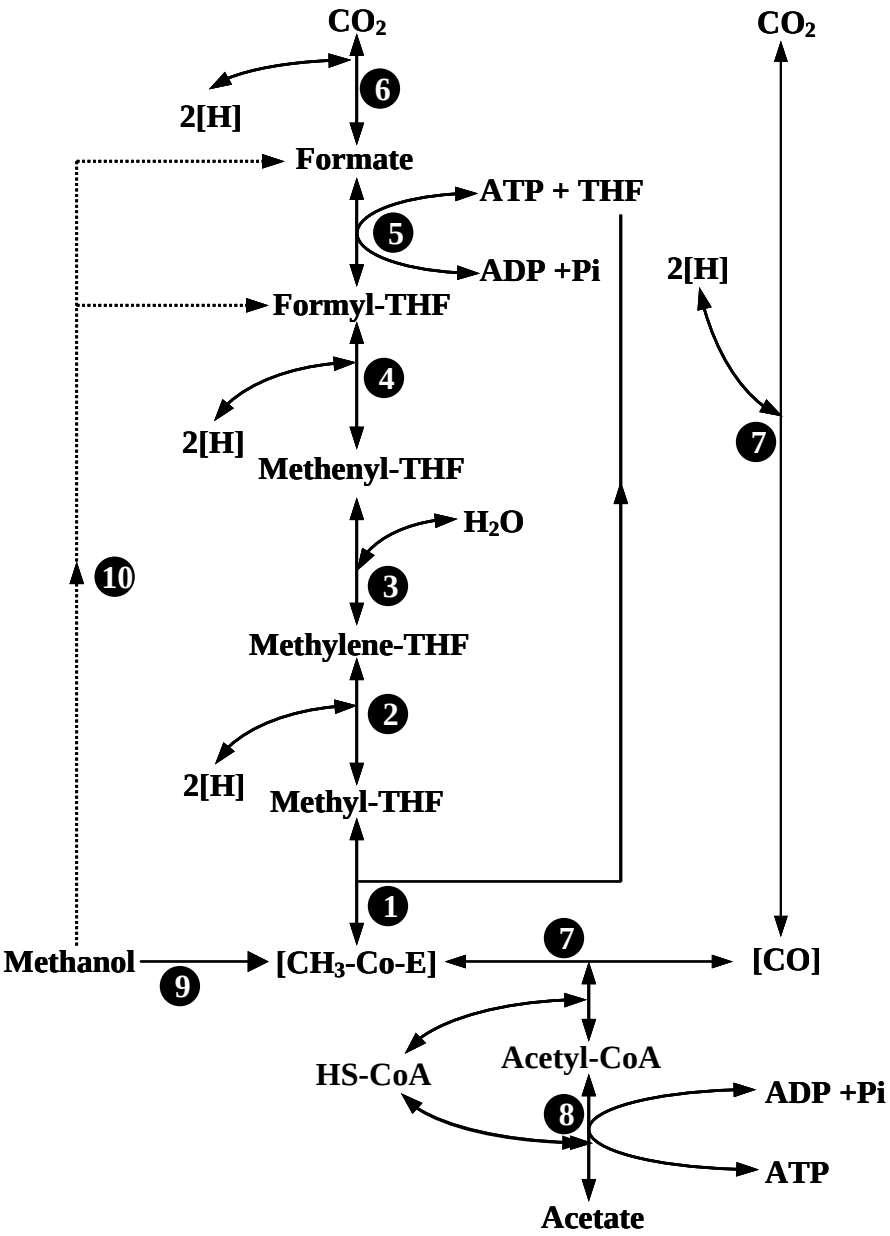


Fig 4. Acetate formation from methanol via the acetyl-CoA pathway in homoacetogens, modified after (54). Abbreviations: THF, tetrahydrofolate; HS-CoA, coenzyme A; CH_3-Co-E, corrinoid bound methyl group. Enzymes indicated by 1, methyltransferase; 2, methylene-THF reductase; 3, methylene-THF dehydrogenase; 4, methenyl-THF cyclohydrolase; 5, formyl-THF synthetase; 6, formate dehydrogenase; 7a, carbon monoxide dehydrogenase; 7b, acetyl-CoA synthase; 8, phosphotransacetylase and acetate kinase; 9, methyltransferase (130); 10, methanol dehydrogenase (141).

Syntrophic degradation of methanol

Conversion of methanol by homoacetogens results in the formation of acetate that is used by aceticlastic methanogens, such as *Methanosaeta* species (93). However, it is also possible that homoacetogens convert methanol to H_2/CO_2, when the H_2 partial pressure is kept low by hydrogenotrophic methanogens or sulfate reducers. As methanol oxidation to hydrogen is thermodynamically unfavorable at standard conditions, consumption of H_2 is essential for syntrophic methanol degradation via H_2 (112, 122). The first syntrophic methanol degrading co-culture was enriched in a attempt to isolate a vitamin B_{12} producer, and consisted of *M. thermoautotrophica* strain Z-99 and *Methanobacterium thermoformicicum* strain Z-245 (97). Such syntrophic methanol conversion was also observed for some other homoacetogens, like *Butyribacterium methylotrophicum*, *Sporomusa ovata*, *Acetobacterium woodii* and *Moorella mulderi* (3, 54), or by a fermentative bacterium *Thermotoga lettingae* (4). Besides methanogens, sulfate reducers (26, 54) and iron reducing bacteria can be syntrophic partners (25).

Trace metals as nutrients in microorganisms

Certain metals play a fundamental role in microbial growth and metabolism, but they may also be toxic to microbial life at higher concentrations (59, 95). A rough estimation of the required amount of trace metals can be obtained from the cell composition of intact microorganisms (45, 111). The requirement of trace metals is dependent on their role in certain enzymes and cofactors in the metabolic pathways of microorganisms (95). Table 2 lists common trace metals required for growth of methanogenic archaea, homoacetogenic bacteria and sulfate-reducing bacteria. Among them, cobalt (24, 130), nickel (33, 76, 104), tungsten (77) and molybdenum (38) are important metals in the methanogenic and homoacetogenic pathways. Most metals exist as a metal center in enzymes or as cofactor, for example, cobalt in corrinoid, nickel in F430, hydrogenase and carbonmonoxide dehydrogenase. Other metals cannot replace these metals. However, tungsten and molybdenum can replace each other in formylmethanofuran dehydrogenase (8, 56).

Table 2. Selected trace elements in enzymes of anaerobic microorganisms and their functions. MA: methanogenic archaea; HAB: homoacetogenic bacteria; SRB sulfate-reducing bacteria

Enzymes or groups	Metals	Exist form	Affected groups	Reactions	Reference
Methyltransferase	Co	Corrinoid	MA, HAB	$MeOH + CoM \rightarrow CH_3\text{-}CoM$	(24, 130)
Methyl-CoM reductase	Ni	F_{430}	MA	$CH_3\text{-}CoM + 2[H] \rightarrow CH_4 + CoM$	(33)
Formylmethanofuran dehydrogenase	W, (Se, Fe) Mo, (Se, Fe)	Tungstopterin Molybdopterin	MA	$CO_2 + MFR \rightarrow CHO\text{-}MFR + H_2O$	(56)
Carbon monoxide dehydrogenase	Ni, Fe	Fe-Ni-S	MA, HAB, SRB.	$CO + H_2O \leftrightarrow CO_2 + 2e^- + 2H^+$	(70, 76, 103)
Hydrogenase	Fe Fe, Ni, Se.	Fe-S Fe-S-Se	MA, HAB, SRB.	$H_2 \leftrightarrow 2e^- + 2H^+$	(53, 104)
Formate dehydrogenase	W, (Se, Fe) Mo, (Se, Fe)	Tungstopterin, Molybdopterin	MA, HAB, SRB	$HCOOH \leftrightarrow CO_2 + 2e^- + 2H^+$	(77, 82, 103)
Carbonic anhydrase	Zn,		MA, HAB	$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$	(121)
Nitrogenase	Mo		MA	$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$	(78, 110)
Superoxide dismutase	Cu, Zn		MA	$H_2O_2 \rightarrow O_2 + 2e^- + 2H^+$	(17, 64)
Cytochrome	Fe	Haem, Fe-S	HAB, SRB	Electron transfer chain	(62)
Ferredoxin	Fe	Fe-S	MA, HAB, SRB	Electron transfer chain	(123)

Cobalt in corrinoid of methyltransferase

Corrinoid sometimes is referred to as cobalamin, or vitamin B_{12}. The catalytic center of the corrinoid is the cobalt ion (124)(Fig. 5). Up to 8 different corrinoid structures have been characterized from anaerobic microorganisms; their occurrence differs from species and the metabolic substrate that is used (124). Synthesis of corrinoid compounds has been studied in most detail in *Propionibacterium shermanii*. Synthesis of corrinoid requires at least 25 enzymes and more than 40 catalytic steps (106). Up to 98% of the total cobalt content may be present in corrinoid (126). The corrinoid content of methanogens and homoacetogens differs dependent on the species, growth substrate and cultivation condition; highest values were obtained when grown on methanol (48, 69, 124). Vitamin B_{12} production by *M. barkeri* was intensively studied (91, 118-120).

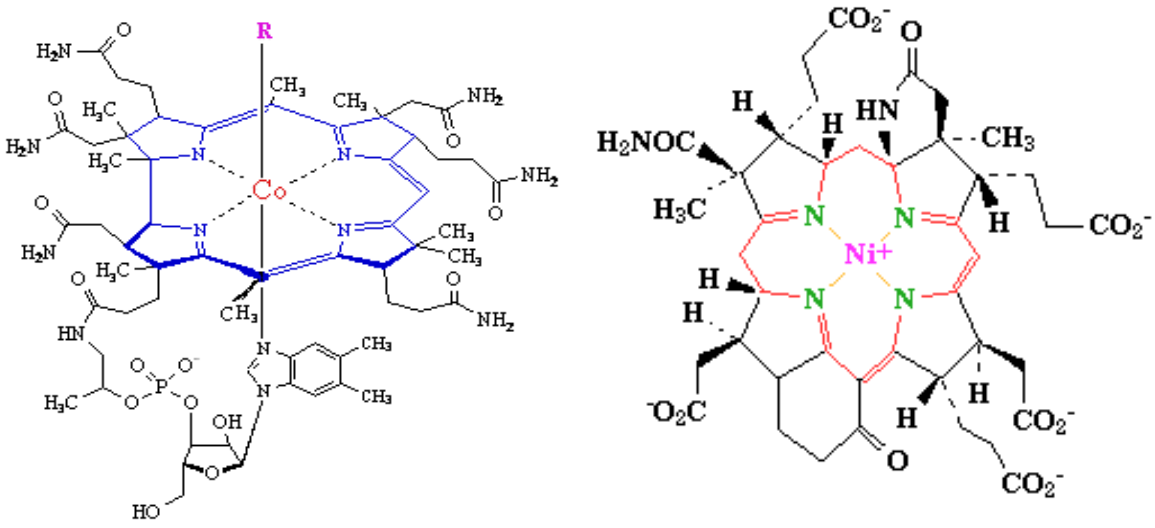


Fig. 5 Structures of corrinoid compounds and cofactor F430 (from (124) and (117), respectively)

Corrinoid-dependent methyl transfer reactions are important in methanogenesis and homoacetogenesis (24, 125, 130). In methanogens, a corrinoid derivate, cofactor III in super reduced Co (I) form accepts the methyl group from methyl-H_4-MTP or methanol, resulting in a methyl-Co(III) intermediate. This intermediate reacts with HS-CoM, resulting in the formation of methyl-CoM and regenerated Co(I) (7). A similar corrinoid-dependent methyl transfer reaction occurs in homoacetogens, except that the methyl donor and methyl acceptor are methyl-THF and carbon monoxide, respectively (76, 130).

Nickel in cofactor F430 of methyl-CoM reductase

Nickel has been found in many enzymes, including urease, hydrogenase, carbon monoxide dehydrogenase of all kinds of microorganisms, and the methyl-CoM reductase of methanogens (53). F430 is a yellow nickel containing tetrapyrrole that does not show fluorescence (116). F430 has a similar structure as corrinoid (Fig. 5). They share almost the same biosynthesis route till the last step before the insertion of the metal (30, 31). Up to 70% of the labeled nickel taken up by *Methanothermobacter thermoautotrophicus* was found to be present in this cofactor (32). Ni(I) in F430 can be methylated to form methyl-Ni(III), which is spontaneously converted to methane and Ni(II)-F430 (50, 61).

Tungsten and molybdenum in formate (formylmethanofuran) dehydrogenase

Molybdenum and tungsten are essential trace elements for growth of a variety of microorganisms, including methanogenic and hyperthermophilc archaea, gram-positive bacteria, sulfate reducers and nitrogen-fixing bacteria (38, 56, 70, 103). Enzymes containing these elements usually catalyze oxidation reactions, such as formate dehydrogenase (FDH), formylmethanofuran dehydrogenase (FMDH). The metal ion is associated with either one or two pterin derivatives to form a cofactor in the enzymes (65). FDH catalyzes the first reaction of CO_2 reduction to formate in the acetyl-CoA pathway (76), and is also important for syntrophic propionate oxidation (103). Formylmethanofuran reductase is an analogue of FDH in methanogens, using methanofuran as formyl group acceptor for CO_2 reduction (56). The stimulation effect of tungsten and molybdenum on homoacetogens and methanogens is mainly related to these two enzymes (65). Isoenzymes with either tungsten or molybdenum can be found in the same species (18, 38). Antagonistic effects were also described (82, 143). Most interestingly, it was found that tungsten could replace molybdenum in the Mo-isoenzymes of FMDH, but replacement of tungsten by molybdenum results in inactive isoenzymes (8, 115). This is the only example that the metal center in enzymes can be replaced by another metal functionally.

Metal speciation and metal uptake

Although essential metals can be added to the growth medium as simple metal salts, the metal ions may be present in the final medium in different forms depending on the chemical properties and concentrations of anions and complexing agents present and the pH.

Under anaerobic conditions, sulfide, carbonate and phosphate may be present at a high concentration. Due to binding with metals to form precipitates, they have a strong effect on metal speciation, metal uptake and growth of microorganisms (20, 21, 58). To keep metals in soluble form, organic ligands, such as EDTA and NTA are often added to the medium (15, 52). As a strategy to facilitate uptake, microorganisms secrete organic products, which have a similar role as the added organic ligands (5, 6, 42). A good example of microbially produced ligands is given by the siderophores secreted by aerobic bacteria to facilitate specifically the uptake of iron (75, 80). Such specific metal ligands are rarely described for anaerobes (16, 44). Due to weak metal binding of most (characterized) compounds, excreted ligands are unlikely effective at low metal concentration (16).

It is generally accepted that only free metal ions can be transported across the cytoplasmic membrane. It is also reported that labile (131), lipophilic and even hydrophilic complexes can be transported (102). Metal ions are taken up by microorganisms via a wide variety of transport systems. Transporters are categorized into two groups: non-specific transporters and specific high affinity transporters (40, 66, 68). Non specific transporter can transport all divalent cations with quite low affinity in a sequence of preference $Mg^{2+}>Co^{2+}>Zn^{2+}>Mn^{2+}>Ni^{2+}>Ca^{2+}$ (60, 90, 136). Therefore, cobalt and nickel uptake is affected by the presence of magnesium (90). When metals are at a growth-limiting concentration, microorganisms express specific high affinity transporters for uptake. Typically ABC transporters and trans-membrane permeases possess an affinity below the μM range (29, 39-41, 67). All these transporters are affected by certain metals at high concentrations, e.g. nickel uptake by the specific transporters, Nik and HoxN, is inhibited by cobalt (29, 41). Uptake of anions, like tungstate and molybdate, is not well studied. They can be transported via a non-specific sulfate transport system and a specific ABC transport system (79, 81).

Synopsis of the thesis

The objective of the research presented in this thesis was to study microbial aspects of methanogenesis with methanol, and the role of metals on the metabolism of the microorganisms involved. This information is essential to optimize trace metal dosing in anaerobic bioreactors and to steer metabolic pathways in mixed anaerobic microbial communities.

Under moderately thermophilic conditions, direct methanogenesis from methanol by methylotrophic methanogens and indirect methanogenesis from methanol by homoacetogens and hydrogenotrophic methanogens are both important. This indirect methanogenesis from methanol requires less cobalt. Therefore, the first part of the thesis was focused on the isolation and characterization of the microorganisms in sludge of a methanol-fed anaerobic bioreactor operated at 55^{o}C. ***Chapter 2*** described a novel thermophilic methylotrophic methanogen, *Methanomethylovorans thermophila* strain L2FAW. In ***Chapter 3***, a novel carboxydotrophic hydrogenogenic homoacetogen, *Moorella hydrogenoformans* strain AMP was described. Strain AMP grows on formate in a syntrophic association with a methanogen, *Methanothermobacter* strain NJ1 (isolated from the same enrichment culture). This is described in ***Chapter 4***. In the second part of the thesis, the effect of metals on the metabolism of specific microorganisms was studied. In ***Chapter 5***, the effect of cobalt and nickel on the growth of *Methanosarcina barkeri* under conditions of well-defined metal speciation was studied. The relationship between growth rate, cell metal and cofactor content were investigated as well. In ***Chapter 6***, the effect of tungsten and molybdenum on the interspecies electron transfer on syntrophic propionate oxidation was studied. Finally, the results obtained in this thesis are discussed in ***Chapter 7***.

References

1. **Ahring, B. K.** 1995. Methanogenesis in thermophilic biogas reactors. Antonie Van Leeuwenhoek **67:**91-102.
2. **Amend, J. P., and E. L. Shock.** 2001. Energetics of overall metabolic reactions of thermophilic and hyperthermophilic Archaea and Bacteria. FEMS Microbiol Rev **25:**175-243.
3. **Balk, M., J. Weijma, M. W. Friedrich, and A. J.M. Stams.** 2003. Methanol utilization by a novel thermophilic homoacetogenic bacterium, *Moorella mulderi* sp. nov., isolated from a bioreactor. Arch Microbiol **179:**315-20.
4. **Balk, M., J. Weijma, and A. J. M. Stams.** 2002. *Thermotoga lettingae* sp nov., a novel thermophilic, methanol-degrading bacterium isolated from a thermophilic anaerobic reactor. Int J Syst Evol Microbiol **52:**1361-1368.
5. **Barber, W. P., and D. C. Stuckey.** 2000. Metal bioavailability and trivalent chromium removal in ABR. J Environ Eng **126:**649-656.
6. **Barker, D. J., and D. C. Stuckey.** 1999. A review of soluble microbial products (SMP) in wastewater treatment systems. Water Res **33:**3063-3082.
7. **Becher, B., V. Muller, and G. Gottschalk.** 1992. N5-methyl-tetrahydromethanopterin:coenzyme M methyltransferase of *Methanosarcina* strain Go1 is an Na(+)-translocating membrane protein. J Bacteriol **174:**7656-60.
8. **Bertram, P. A., R. A. Schmitz, D. Linder, and R. K. Thauer.** 1994. Tungstate can substitute for molybdate in sustaining growth of *Methanobacterium thermoautotrophicum*. Identification

and characterization of a tungsten isoenzyme of formylmethanofuran dehydrogenase. Arch Microbiol **161:**220-228.

9. **Bhatti, Z. I., K. Furukawa, and M. Fujita.** 1996. Feasibility of methanolic waste treatment in USAB reactors. Water Res **30:**2559-2568.
10. **Bhatti, Z. I., K. Furukawa, and M. Fujita.** 1995. Methanogenic granular sludge formation in an upflow anaerobic sludge blanket reactor treating synthetic methanolic waste. World J Microbiol Biotechnol **11:**631-637.
11. **Bhatti, Z. I., K. Furukawa, and M. Fujita.** 1993. Treatment performance and microbial structure of a granular consortium handling methanolic waste. J Ferm Bioeng **76:**218-223.
12. **Blaut, M., and G. Gottschalk.** 1984. Coupling of ATP synthesis and methane formation from methanol and molecular hydrogen in *Methanosarcina barkeri*. Eur J Biochem **141:**217-22.
13. **Blaut, M., V. Muller, and G. Gottschalk.** 1992. Energetics of methanogenesis studied in vesicular systems. J Bioenerg Biomembr **24:**529-46.
14. **Boone, D. R., R. L. Johnson, and Y. Liu.** 1989. Diffusion of the interspecies electron carriers H_2 and formate in methanogenic ecosystems and Its implications in the measurement of K_m for H_2 or formate uptake. Appl Environ Microbiol **55:**1735-1741.
15. **Bretler, G., and W. Marison Ian.** 1996. A medium optimization strategy for improvement of growth and methane production by *Methanobacterium thermoautotrophicum*. Journal of Biotechnology **50:**201-212.
16. **Bridge, T. A., C. White, and G. M. Gadd.** 1999. Extracellular metal-binding activity of the sulphate-reducing bacterium *Desulfococcus multivorans*. Microbiology **145:**2987-95.
17. **Brioukhanov, A., A. Netrusov, M. Sordel, R. K. Thauer, and S. Shima.** 2000. Protection of *Methanosarcina barkeri* against oxidative stress: identification and characterization of an iron superoxide dismutase. Arch Microbiol **174:**213-6.
18. **Brondino, C. D., M. C. Passeggi, J. Caldeira, M. J. Almendra, M. J. Feio, J. J. Moura, and I. Moura.** 2004. Incorporation of either molybdenum or tungsten into formate dehydrogenase from *Desulfovibrio alaskensis* NCIMB 13491; EPR assignment of the proximal iron-sulfur cluster to the pterin cofactor in formate dehydrogenases from sulfate-reducing bacteria. J Biol Inorg Chem **9:**145-51.
19. **Buchenau, B., and R. K. Thauer.** 2004. Tetrahydrofolate-specific enzymes in *Methanosarcina barkeri* and growth dependence of this methanogenic archaeon on folic acid or p-aminobenzoic acid. Arch Microbiol **182:**313-25.
20. **Callander, I. J., and J. P. Barford.** 1983. Precipitation chelation and the availability of metals as nutrients in anaerobic digestion: II. applications. Biotechnol Bioeng **25:**1959-1972.
21. **Callander, J. J., and J. P. Barford.** 1983. Precipitation chelation and the availability of metals as nutrients in anaerobic digestion: I. methodology. Biotechnol Bioeng **26:**1947-1957.
22. **Collins, M., P. Lawson, A. Willems, J. Cordoba, J. Fernandez-Garayzabal, P. Garcia, J. Cai, H. Hippe, and J. Farrow.** 1994. The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. Int J Syst Bacteriol **44:**812-826.
23. **Daas, P. J. H., W. R. Hagen, J. T. Keltjens, C. van der Drift, and G. D. Vogels.** 1996. Activation mechanism of methanol:5-hydroxybenzimidazolylcobamide methyltransferase from *Methanosarcina barker*i. J Biol Chem **271:**22346-22351.
24. **Daas, P. J. H., R. W. Wassenaar, P. Willemsen, R. J. Theunissen, J. T. Keltjens, C. van der Drift, and G. D. Vogels.** 1996. Purification and properties of an enzyme Involved in the ATP-dependent activation of the methanol:2-mercaptoethanesulfonic acid methyltransferase reaction in *Methanosarcina barker*i. J Biol Chem **271:**22339-22345.
25. **Daniel, S. L., T. Hsu, S. I. Dean, and H. L. Drake.** 1990. Characterization of the H2- and CO-dependent chemolithotrophic potentials of the acetogens *Clostridium thermoaceticum* and *Acetogenium kivui*. J Bacteriol **172:**4464-4471.
26. **Davidova, I. A., and A. J. M. Stams.** 1996. Sulfate reduction with methanol by a thermophilic consortium obtained from a methanigenic reactor. Appl Microbiol Biotechnol **46:**297-302.

27. **de Bok, F. A. M., M. L. G. C. Luijten, and A. J. M. Stams.** 2002. Biochemical evidence for formate transfer in syntrophic propionate-oxidizing cocultures of *Syntrophobacter fumaroxidans* and *Methanospirillum hungatei*. Appl Environ Microbiol **68:**4247-4252.
28. **de Bok, F. A. M., E. H. A. Roze, and A. J. M. Stams.** 2002. Hydrogenases and formate dehydrogenases of *Syntrophobacter fumaroxidans*. Antonie Van Leeuwenhoek **81:**283-291.
29. **de Pina, K., C. Navarro, L. McWalter, D. H. Boxer, N. C. Price, S. M. Kelly, M. A. Mandrand-Berthelot, and L. F. Wu.** 1995. Purification and characterization of the periplasmic nickel-binding protein NikA of *Escherichia coli* K12. Eur J Biochem **227:**857-65.
30. **Diekert, G., H. H. Gilles, R. Jaenchen, and R. K. Thauer.** 1980. Incorporation of 8 succinate per mol nickel into factors F430 by *Methanobacterium thermoautotrophicum*. Arch Microbiol **128:**256-62.
31. **Diekert, G., R. Jaenchen, and R. K. Thauer.** 1980. Biosynthetic evidence for a nickel tetrapyrrole structure of factor F430 from *Methanobacterium thermoautotrophicum*. FEBS Lett **119:**118-20.
32. **Diekert, G., B. Klee, and R. K. Thauer.** 1980. Nickel, a component of factor F430 from *Methanobacterium thermoautotrophicum*. Arch Microbiol **124:**103-6.
33. **Diekert, G., U. Konheiser, K. Piechulla, and R. K. Thauer.** 1981. Nickel requirement and factor F430 content of methanogenic bacteria. J Bacteriol **148:**459-464.
34. **Dong, X., C. M. Plugge, and A. J. M. Stams.** 1994. Anaerobic degradation of propionate by a mesophilic acetogenic bacterium in coculture and triculture with different methanogens. Appl Environ Microbiol **60:**2834-2838.
35. **Dong, X., and A. J. M. Stams.** 1995. Evidence for H_2 and formate formation during syntrophic butyrate and propionate degradation. Anaerobe **1:**35-39.
36. **Drake, H. L., and S. L. Daniel.** 2004. Physiology of the thermophilic acetogen *Moorella thermoacetica*. Res Microbiol **155:**869-83.
37. **Drake, H. L., K. Küsel, and C. Matthies.** 2002. Ecological consequences of the phylogenetic and physiological diversities of acetogens. Antonie van Leeuwenhoek **81:**203-213.
38. **Durfor, C. N., P. J. Wetherbee, J. C. Deaton, and E. I. Solomon.** 1983. Characterization and spectroscopic properties of reduced Mo and W formate dehydrogenase from *Clostridium thermoaceticum*. Biochem Biophys Res Commun **115:**61-7.
39. **Eitinger, T., O. Degen, U. Bohnke, and M. Muller.** 2000. Nic1p, a relative of bacterial transition metal permeases in *Schizosaccharomyces pombe*, provides nickel ion for urease biosynthesis. J Biol Chem **275:**18029-33.
40. **Eitinger, T., and M. A. Mandrand-Berthelot.** 2000. Nickel transport systems in microorganisms. Arch Microbiol **173:**1-9.
41. **Eitinger, T., L. Wolfram, O. Degen, and C. Anthon.** 1997. A Ni^{2+} binding motif is the basis of high affinity transport of the *Alcaligenes eutrophus* nickel permease. J Biol Chem **272:**17139-44.
42. **Fang, H. H. P., and J. X. Shan.** 1998. Soluble microbial products (SMP) of acetotrophic methanogenesis. Bioresr Technol **66:**235-239.
43. **Ferry, J. G.** 1999. Enzymology of one-carbon metabolism in methanogenic pathways. FEMS Microbiol Rev **23:**13-38.
44. **Finney, L. A., and T. V. O'Halloran.** 2003. Transition metal speciation in the cell: insights from the chemistry of metal ion receptors. Science **300:**931-6.
45. **Florencio, L., J. A. Field, and G. Lettinga.** 1994. Importance of cobalt for individual trophic groups in an anaerobic methanol-degrading consortium. Appl Environ Microbiol **60:**227-34.
46. **Florencio, L., P. Jenicek, J. A. Field, and G. Lettinga.** 1993. Effect of cobalt on the anaerobic degradation of methanol. J Ferm Bioeng **75:**368-374.
47. **Fontaine, F. E., W.H. Peterson, E. McCoy, and M.J. Johnson.** 1942. A new type of glucose fermentation by *Clostridium thermoaceticum* nov. sp. J Bacteriol **43:**701-715.
48. **Gorris, L. G. M., and C. Van Der Drift.** 1994. Cofactor contents of methanogenic bacteria reviewed. Biodegradation **4:**139-145.

49. **Gottwald, M., J. R. Andreesen, J. LeGall, and L. G. Ljungdahl.** 1975. Presence of cytochrome and menaquinone in *Clostridium formicoaceticum* and *Clostridium thermoaceticum*. J Bacteriol **122:**325-8.
50. **Goubeaud, M., G. Schreiner, and R. K. Thauer.** 1997. Purified methyl-coenzyme-M reductase is activated when the enzyme-bound coenzyme F430 is reduced to the nickel(I) oxidation state by titanium(III) citrate. Eur J Biochem **243:**110-114.
51. **Gujer, W., and A. J. B. Zehnder.** 1983. Conversion Processes in Anaerobic-Digestion. Water Sci Technol **15:**(8-9)127-167.
52. **Hartung, H. A.** 1992. Stimulation of anaerobic digestion with peat humic substance. Sci Total Environ **113:**17-33.
53. **Hausinger, R. P.** 1987. Nickel Utilization by Microorganisms. Microb Rev **51:**22-42.
54. **Heijthuijsen, J., and T. A. Hansen.** 1986. Interspecies hydrogen transfer in cocultures of methanol-utilizing acidogens and sulfate-reducing or methanogenic bacteria. FEMS Microbiol Ecol **38:**57-64.
55. **Heise, R., V. Muller, and G. Gottschalk.** 1992. Presence of a sodium-translocating ATPase in membrane vesicles of the homoacetogenic bacterium *Acetobacterium woodii*. Eur J Biochem **206:**553-7.
56. **Hochheimer, A., R. Hedderich, and R. K. Thauer.** 1998. The formylmethanofuran dehydrogenase isoenzymes in *Methanobacterium wolfei* and *Methanobacterium thermoautotrophicum*: induction of the molybdenum isoenzyme by molybdate and constitutive synthesis of the tungsten isoenzyme. Arc Microbiol **170:**389-93.
57. **Hu, S. I., H. L. Drake, and H. G. Wood.** 1982. Synthesis of acetyl coenzyme A from carbon monoxide, methyltetrahydrofolate, and coenzyme A by enzymes from *Clostridium thermoaceticum*. J Bacteriol **149:**440-8.
58. **Hughes, M. N., and R. K. Poole.** 1991. Metal speciation and microbial growth ---- the hard (and soft) facts. J Gen Microbiol **137:**725-734.
59. **Jarrell, K. F., M. Saulnier, and A. Ley.** 1987. Inhibition of methanogenesis in pure cultures by ammonia, fatty acids, and heavy metals, and protection against heavy metal toxicity by sewage sludge. Can J Microbiol **33:**551-4.
60. **Jasper, P., and S. Silver.** 1977. Magnesium transport in microorganisms, p. 7-47. *In* E. D. Weinberg (ed.), Microorganisms and minerals. Marcel Dekker, New York.
61. **Jaun, B.** 1990. Coenzyme F430 from methanogenic bacteria: Oxidation of F430 pentamethyl ester to the Ni(III) form. Helvetica Chimica Acta **73:**2209-2217.
62. **Kamlage, B., and M. Blaut.** 1993. Isolation of a cytochrome-deficient mutant strain of *Sporomusa sphaeroides* not capable of oxidizing methyl groups. J Bacteriol **175:**3043-50.
63. **Keltjens, J. T., J. M. H. Hermans, G. Rijsdijk, C. v. d. Drift, and G. D. Vogels.** 1988. Interconversion of F430 derivatives of methanogenic bacteria. Antonie Van Leeuwenhoek **54:**207-220.
64. **Kirby, T. W., J. R. Lancaster, Jr., and I. Fridovich.** 1981. Isolation and characterization of the iron-containing superoxide dismutase of *Methanobacterium bryantii*. Arch Biochem Biophys **210:**140-8.
65. **Kletzin, A., and M. W. W. Adams.** 1996. Tungsten in biological systems. FEMS Microbiology Reviews **18:**5-63.
66. **Kobayashi, M., and S. Shimizu.** 1999. Cobalt proteins. Eur J Biochem **261:**1-9.
67. **Komeda, H., M. Kobayashi, and S. Shimizu.** 1997. A novel transporter involved in cobalt uptake. PNAS **94:**36-41.
68. **Koster, W.** 2001. ABC transporter-mediated uptake of iron, siderophores, heme and vitamin B12. Res Microbiol **152:**291-301.
69. **Krzycki, J., and J. G. Zeikus.** 1980. Quantification of corrinoids in methanogenic bacteria. Curr Microbiol **3:**243-245.
70. **Lange, S., and G. Fuchs.** 1987. Autotrophic synthesis of activated acetic acid from CO_2 in *Methanobacterium thermoautotrophicum*. Synthesis from tetrahydromethanopterin-bound C1 units and carbon monoxide. Eur J Biochem **163:**147-54.

71. **Lepisto, R., and J. A. Rintala.** 1995. Acetate treatment in 70 degrees C upflow anaerobic sludge-blanket (UASB) reactors: start-up with thermophilic inocula and the kinetics of the UASB sludges. Appl Microbiol Biotechnol **43:**1001-5.
72. **Lettinga, G.** 1995. Anaerobic digestion and wastewater treatment systems. Antonie Van Leeuwenhoek **67:**3-28.
73. **Lettinga, G., W. De Zeeuw, and E. Ouborg.** 1981. Anaerobic treatment of wastes containing methanol and higher alcohols. Water Res **15:**171-182.
74. **Lettinga, G., A. T. van der Geest, S. Hobma, and J. van de Laan.** 1979. Anaerobic treatment of methanolic wastes. Water Res **13:**852-863.
75. **Lisiecki, P., P. Wysocki, and J. Mikucki.** 2000. Occurrence of siderophores in *Enterococci*. Zentralblatt fuer Bakteriologie **289:**807-815.
76. **Ljungdahl, L. G.** 1994. The acetyl-CoA pathway and the chemiosmotic generation of ATP during acetogenesis, p. 63-87. *In* H. L. Drake (ed.), Acetogenesis. Chapman & Hall, New York.
77. **Ljungdahl, L. G., and J. R. Andreesen.** 1978. Formate dehydrogenase, a selenium-tungsten enzyme from *Clostridium thermoaceticum*. Methods Enzymol **53:**360-72.
78. **Lobo, A. L., and S. H. Zinder.** 1990. Nitrogenase in the archaebacterium *Methanosarcina barkeri* 227. J Bacteriol **172:**6789-6796.
79. **Makdessi, K., J. R. Andreesen, and A. Pich.** 2001. Tungstate uptake by a highly specific ABC transporter in *Eubacterium acidaminophilum*. J Biol Chem **276:**24557-24564.
80. **Martinez, J. S., G. P. Zhang, P. D. Holt, H. T. Jung, C. J. Carrano, M. G. Haygood, and A. Butler.** 2000. Self-assembling amphiphilic siderophores from marine bacteria. Science **287:**1245-1247.
81. **Maupin-Furlow, J., J. Rosentel, J. Lee, U. Deppenmeier, R. Gunsalus, and K. Shanmugam.** 1995. Genetic analysis of the modABCD (molybdate transport) operon of *Escherichia coli*. J Bacteriol **177:**4851-4856.
82. **May, H. D., P. S. Patel, and J. G. Ferry.** 1988. Effect of molybdenum and tungsten on synthesis and composition of formate dehydrogenase in *Methanobacterium formicicum*. J Bacteriol **170:**3384-89.
83. **McCarty, P. L., L. Beck, and P. S. Amant.** 1965. Presented at the 24th Purdue Ind. Waste Conference, Purdue Univ. Eng. Ext. Serv., West Lafayette, IN.
84. **Meuer, J., H. C. Kuettner, J. K. Zhang, R. Hedderich, and W. W. Metcalf.** 2002. Genetic analysis of the archaeon *Methanosarcina barkeri* Fusaro reveals a central role for Ech hydrogenase and ferredoxin in methanogenesis and carbon fixation. PNAS **99:**5632-7.
85. **Minami, K., T. Horiyama, M. Tasaki, and Y. Tanimoto.** 1986. Methane production using a bio-reactor packed with pumice stone on an evaporator condensate of kraft pulp mill. J Ferm Technol **64:**523-532.
86. **Mohr, D. H., and C. J. King.** 1985. Identification of polar organic compounds in coal-gasification condensate water by gas-chromatography-mass spectrometry analysis of high-performance liquid chromatography. Environ Sci Technol **19:**929-935.
87. **Muller, V., and G. Gottschalk.** 1994. The sodium ion cycle in acetogenic and methanogenic bacteria: generation and utilization of a primary electrochemical sodium ion gradient, p. 127-156. *In* H. L. Drake (ed.), Acetogenesis. Chapman & Hall, New York.
88. **Nanninga, H. J., and J. C. Gottschal.** 1986. Microbial problems with waste from potato-starch processing. Microbiol Sci **3:**179-82.
89. **Nazina, T. N., A. E. Ivanova, L. P. Kanchaveli, and E. P. Rozanova.** 1987. A New spore-forming thermophilic methylotrophic sulfate reducing bacterium, *Desulfotomaculum kuzetsovii* sp. nov/. Mikrobiologiya **57:**823-827.
90. **Nies, D. H., and S. Silver.** 1989. Metal ion uptake by a plasmid-free metal-sensitive *Alcaligenes eutrophus* strain. J Bacteriol **171:**4073-5.
91. **Nishio, N., T. Kakizono, R. G. Silveira, S. Takemoto, and S. Nagai.** 1992. Nutrient control by the gas evolution in methanogenesis of methanol by *Methanosarcina barkeri*. J Ferm Bioeng **73:**481-485.

92. **Nölling, J., and J. N. Reeve.** 1997. Growth- and substrate-dependent transcription of the formate dehydrogenase (fdhCAB) operon in *Methanobacterium thermoformicicum* Z- 245. J Bacteriol **179:**899-908.
93. **Nozhevnikova, A. N., and V. I. Chudina.** 1984. Morphology of the thermophilic acetate bacterium *Methanothrix thermoacetophila* sp. nov. Mikrobiologiya**:**756-760.
94. **Nyns, E. J.** 1994. A guide to successful industrail implementation of biomethanisation technology in the European Union, successes and guidelines., Report prepared for the European Commision, Directated general for energy, Thermie Programme, DG XVII.
95. **Oleszkiewicz, J. A., and F. K. Sharma.** 1990. Stimulation and inhibition of anaerobic processes by heavy metals - a review. Biol Wastes **31:**45-67.
96. **Paice, M. G., I. D. Reid, R. Bourbonnais, F. S. Archibald, and L. Jurasek.** 1993. Manganese peroxidase, produced by Trametes versicolor during pulp bleaching, demethylates and delignifies kraft pulp. Appl Environ Microbiol **59:**260-265.
97. **Pantskhava, E. S., and V. V. Pchelkina.** 1969. Methane fermentation of methanol by a *Methanobacillus kuzneceovii* culture. Prikladnaya Biokhimiya i Mikrobiologiya **5:**416-20.
98. **Paulo, P. L., B. Jiang, D. Cysneiros, A. J. Stams, and G. Lettinga.** 2004. Effect of cobalt on the anaerobic thermophilic conversion of methanol. Biotechnol Bioeng **85:**434-41.
99. **Paulo, P. L., B. Jiang, S. Rebac, L. H. Pol, and G. Lettinga.** 2001. Thermophilic anaerobic digestion of methanol in UASB reactor. Water Sci Technol **44:**(4)129-36.
100. **Paulo, P. L., B. Jiang, K. Roest, J. B. van Lier, and G. Lettinga.** 2002. Start-up of a thermophilic methanol-fed UASB reactor: change in sludge characteristics. Water Sci Technol **45:**(10)145-50.
101. **Paulo, P. L., A. J. Stams, J. A. Field, C. Dijkema, J. B. van Lier, and G. Lettinga.** 2003. Pathways of methanol conversion in a thermophilic anaerobic (55 degrees C) sludge consortium. Appl Microbiol Biotechnol **63:**307-14.
102. **Phinney, J. T., and K. W. Bruland.** 1994. Uptake of lipophilic organic Cu, Cd, and Pb complexes in the coastal diatom T*halassiosira weissflogii*. Environ Sci Technol **28:**1781-1790.
103. **Plugge, C., M., C. Dijkema, and J. M. Stams Alfons.** 1993. Acetyl-CoA cleavage pathway in a syntrophic propionate oxidizing bacterium growing on fumarate in the absence of methanogens. FEMS Microbiol Lett **110:**71-76.
104. **Ragsdale, S. W.** 2000. Nickel containing CO dehydrogenases and hydrogenases. Subcell Biochem **35:**487-518.
105. **Roest, K., M. Altinbas, P. L. Paulo, H. G. Heilig, A. D. Akkermans, H. Smidt, W. M. de Vos, and A. J. Stams.** 2005. Enrichment and detection of microorganisms involved in direct and indirect methanogenesis from methanol in an anaerobic thermophilic bioreactor. Microb Ecol **0**:1-7.
106. **Roth, J. R., J. G. Lawrence, and T. A. Bobik.** 1996. Cobalamin (coenzyme B12): Synthesis and biological significance. Ann Rev Microbiol **50:**137-181.
107. **Rother, M., and W. W. Metcalf.** 2004. Anaerobic growth of *Methanosarcina acetivorans* C2A on carbon monoxide: an unusual way of life for a methanogenic archaeon. PNAS **101:**16929-34.
108. **Rouviere, P. E., and R. S. Wolfe.** 1988. Novel biochemistry of methanogenesis. J Biol Chem **263:**7913-6.
109. **Savage, M. D., and H. L. Drake.** 1986. Adaptation of the acetogen *Clostridium thermoautotrophicum* to minimal medium. J Bacteriol **165:**315-8.
110. **Scherer, P.** 1989. Vanadium and molybdenum requirement for the fixation of molecular nitrogen by two *Methanosarcina* strains. Achiv Microbiol **151:**44-48.
111. **Scherer, P., H. Lippert, and G. Wolff.** 1983. Composition of the majar elements and trace elements of 10 methanogenic bacteria determined by Inductive Coupled Plasma Emission Spectrometry. Biol Trace Elem Res **5:**183-163.
112. **Schink, B.** 1997. Energetics of syntrophic cooperation in methanogenic degradation. Microbiol Mol Biol Rev **61:**262-280.

113. **Schink, B., and J. G. Zeikus.** 1980. Microbial methanol formation: a majar end production of pectin metabolism. Curr Microbiol **4:**387-390.
114. **Schmidt, J., and B. Ahring.** 1995. Interspecies electron transfer during propionate and butyrate degradation in mesophilic, granular sludge. Appl Environ Microbiol **61:**2765-2767.
115. **Schmitz, R. A., S. P. Albracht, and R. K. Thauer.** 1992. Properties of the tungsten-substituted molybdenum formylmethanofuran dehydrogenase from *Methanobacterium wolfei*. FEBS Lett **309:**78-81.
116. **Schonheit, P., J. Moll, and R. K. Thauer.** 1979. Nickel, cobalt and molybdenum requirement for growth of *Methanobacterium thermoautotrophicum*. Achiv Microbiol**:**105-107.
117. **Shiemke, A. K., J. A. Shelnutt, and R. A. Scott.** 1989. Coordination chemistry of F430. Axial ligation equilibrium between square-planar and bis-aquo species in aqueous solution. J Biol Chem **264:**11236-11245.
118. **Silveira, R. G., T. Kakizono, S. Takemoto, N. Nishio, and S. Nagai.** 1991. Medium optimization by an orthogonal array design for the growth of *Methanosarcina barkeri*. J Ferm Bioeng **72:**20-25.
119. **Silveira, R. G., Y. Nishida, N. Nishio, and S. Nagai.** 1990. Corrinoid production with *Methanosarcina barkeri* in a repeated fed-batch reactor with membrane module. Biotechnol Lett **12:**721-726.
120. **Silveira, R. G., N. Nishio, and S. Nagai.** 1991. Growth characteristics and corrinoid production of *Methanosarcina barkeri* on methanol-acetate medium. J Ferm Bioeng **71:**28-34.
121. **Smith, K. S., and J. G. Ferry.** 2000. Prokaryotic carbonic anhydrases. FEMS Microbiol Rev **24:**335-366.
122. **Stams, A., J. M.** 1994. Metabolic interactions between anaerobic bacteria in methanogenic environments. Antonie Van Leeuwenhoek **66:**271-294.
123. **Steigerwald, V. J., T. D. Pihl, and J. N. Reeve.** 1992. Identification and isolation of the polyferredoxin from *Methanobacterium thermoautotrophicum* strain delta H. PNAS **89:**6929-33.
124. **Stupperich, E., H.-J. Eisinger, and S. Schurr.** 1990. Corrinoids in anaerobic bacteria. FEMS Microbiol Rev **87:**355-360.
125. **Stupperich, E., and R. Konle.** 1993. Corrinoid-dependent methyl transfer reactions are involved in methanol and 3,4-dimethoxybenzoate metabolism by *Sporomusa ovata*. Appl Environ Microbiol **59:**3110-3116.
126. **Stupperich, E., I. Steiner, and H. J. Eisinger.** 1987. Substitution of Co-(5-hydroxybenzimidazolyl)cobamide (factor III) by vitamin B12 in *Methanobacterium thermoautotrophicum*. J Bacteriol **169:**3076-3081.
127. **Thiele, J. H., and J. G. Zeikus.** 1988. Control of interspecies electron flow during anaerobic digestion: significance of formate transfer versus hydrogen transfer during syntrophic methanogenesis in flocs. Appl Environ Microbiol **54:**20-29.
128. **Touzel, J.-P., D. Petroff, and G. Albagnac.** 1985. Isolation and characterisation of a new thermophilic *Methanosarcina*, the strain CHIT55. Syst Appl Microbiol **6:**66-71.
129. **Tsukamoto, T. K., and G. C. Miller.** 1999. Methanol as a carbon source for microbiological treatment of acid mine drainage. Water Res **33:**1365-1370.
130. **van der Meijden, P., C. van der Drift, and G. D. Vogels.** 1984. Methanol conversion in *Eubacterium limosum*. Arch Microbiol **138:**360-364.
131. **Van Leeuwen, H. P.** 1999. Metal speciation dynamics and bioavailability: Inert and labile complexes. Environ Sci Technol **33:**3743-3748.
132. **van Lier, J. B.** 1996. Limitations of thermophilic anaerobic wastewater treatment and the consequences for process design. Antonie Van Leeuwenhoek **69:**1-14.
133. **van Lier, J. B., F. P. van der Zee, N. C. Tan, S. Rebac, and R. Kleerebezem.** 2001. Advances in high-rate anaerobic treatment: staging of reactor systems. Water Sci Technol **44:**(8)15-25.

134. **Venkataramani, E. A., RC; Corbo, P.** 1984. Biological treatment of landfill leachates. Crc Crit Rev Environ Control **14:**333-376.
135. **Wasserfallen, A., J. Nolling, P. Pfister, J. Reeve, and E. Conway de Macario.** 2000. Phylogenetic analysis of 18 thermophilic *Methanobacterium* isolates supports the proposals to create a new genus, *Methanothermobacter* gen. nov., and to reclassify several isolates in three species, *Methanothermobacter thermautotrophicus* comb. nov., *Methanothermobacter wolfeii* comb. nov., and *Methanothermobacter marburgensis* sp. nov. Int J Syst Evol Microbiol **50:**43-53.
136. **Webb, M.** 1970. Interrelationships between the utilization of magnesium and the uptake of other bivalent cations by bacteria. Biochimica et biophysica acta **222:**428-439.
137. **Weijma, J., and A. J. M. Stams.** 2001. Methanol conversion in high-rate anaerobic reactors. Water Sci Technol **44:**(8)7-14.
138. **Weijma, J., A. J. M. Stams, L. W. Hulshoff Pol, and G. Lettinga.** 2000. Thermophilic sulfate reduction and methanogenesis with methanol in a high rate anaerobic reactor. Biotechnol Bioeng **67:**354-363.
139. **Wheatley, A.** 1990. Anaerobic digestion: a waste treatment technology. Elsevier Applied Science, London, UK.
140. **Wiegel, J., B. M., and G. G.** 1981. *Clostridium thermoautotrophicum* species novum, a thermophile producing acetate from molecular hydrogen and carbon dioxide. Curr Microbiol **5:**255-260.
141. **Winter, D. K., and L. G. Ljungdahl.** 1989. PQQ-dependent methanol dehydrogenase from *Clostridium thermoautotrophicum*., p. 35-39. *In* J. A. Jongejan and J. A. Duiine (ed.), PQQ and Quinoproteins. Kluwer Academic Publisher, Dordrecht.
142. **Yang, H. C., and H. L. Drake.** 1990. Differential effects of sodium on hydrogen- and glucose-dependent growth of the acetogenic bacterium *Acetogenium kivui*. Appl Environ Microbiol **56:**81-6.
143. **Zellner, G., and J. Winter.** 1987. Growth promoting effect of tungsten on methanogens and incorporation of tungsten-185 into cells. FEMS Microbiol Lett **40:**81-87.
144. **Zinder, S. H.** 1986. Thermophilic wastewater treatment systems, p. 257-277. *In* T. D. Brock (ed.), Thermophiles: general, molecular and applied biology. Wiley-Interscience, New York.
145. **Zinder, S. H., and R. A. Mah.** 1979. Isolation and characterization of a thermophilic strain of *Methanosarcina* unable to use H_2-CO_2 for methanogenesis. Appl Environ Microbiol **38:**996-1008.

Chapter 2

Methanomethylovorans thermophila sp. nov., a thermophilic methylotrophic methanogen from an anaerobic reactor fed with methanol

B. Jiang, S. N. Parshina, W. van Doesburg,
B. P. Lomans and A. J. M. Stams

Int J Syst Evol Microbiol: 55: 2465-2470

Summary

A novel thermophilic obligate methylotrophic methanogenic archaeon, strain L2FAW^T, was isolated from a thermophilic lab-scale UASB reactor fed with methanol as carbon and energy source. Cells of strain L2FAW^T were non-motile, irregular cocci of 0.7-1.5 μm, occurring mainly single, or in clusters of two or four cells. Cells stained Gram-negative and lysed immediately in 0.1% (w/v) SDS. Growth was inhibited by chloramphenicol and tetracycline, but not by penicillin, bacitracin, spetinomycin, vancomycin and kanamycin. Methanol, and mono-, di- and tri-methylamine were used as substrates, but H_2/CO_2, formate, acetate, propanol, dimethyl sulfide and methanethiol were not. The temperature range for growth was 42-58^{o}C, with an optimum at 50^{o}C. Fastest growth was observed at a salinity below 100 mM NaCl, no growth occurred above 300mM NaCl. The optimal pH for growth was 6.5; growth was observed from pH 5 to 7.5. The G+C content of its genomic DNA was 37.6 mol %. Analysis of the 16S rRNA gene sequence and the partial methyl-CoM reductase gene sequence revealed that the organism was phylogenetically closely related to *Methanomethylovorans hollandica* DMS1^T (98% similarity for 16S rRNA gene and 91% similarity for methyl-CoM reductase gene). The DNA-DNA homology between L2FAW^T and *M. hollandica* DMS1^T was 46%. Based on these results, the novel species *Methanomethylovorans thermophila* strain L2FAW^T is proposed (DSM 17232=ATCC BAA-1173). Genbank accession numbers for the 16S rRNA gene and mcrA gene sequences of *M. thermophila* L2FAW^T are AY672821 and AY672820, respectively.

Key words: *Methanomethylovorans thermophila* sp. nov., methanogen, methylotrophic, thermophilic, methanol.

Main text

The utilization of methylated substrates among methanogenic archaea is almost exclusively restricted to the family of *Methanosarcinaceae*. The only exceptions are members of the genus *Methanosphaera* that can only convert methanol in the presence of H_2. The family of *Methanosarcinaceae* comprises eight genera: *Methanococcoides*, *Methanohalobium*, *Methanohalophilus*, *Methanolobus*, *Methanomethylovorans*, *Methanimicrococcus*, *Methanosalsum*, and *Methanosarcina*. The genus *Methanosarcina* includes the most versatile species among all methanogens capable of using H_2/CO_2, acetate, and methylated compounds (methanol, methylated amines and in some cases methylated sulfides) (Boone *et al.*, 1993). The only member of *Methanimicrococcus*, *M. blatticola* (formerly *Methanomicroccus blatticola*), resembles the *Methanosphaera* species in that it can only utilize methanol (or methaylated amines) by complete reduction in the presence of H_2 (Sprenger *et al.*, 2000). The members of the other six genera are all obligate methylotrophs, utilizing methanol, methylated amines and in some cases methylated sulfides. All species from the genus *Methanomethylovorans* are typical freshwater isolates (indicated by their low salt tolerance), whereas the other obligate methylotrophs are generally regarded as halophiles (Garcia *et al.*, 2000).

The genus of *Methanomethylovorans* was recently described in 1999, representing a group of freshwater species that can utilise methanol, methylated amines, dimethyl sulfide (DMS) and methanethiol (MT) for methanogenesis (Lomans *et al.*, 1999). *Methanomethylovorans hollandica* DMS1^T was isolated from a eutrophic freshwater pond sediment in the Netherlands (Lomans *et al.*, 1999). It is the only validated species in the *Methanomethylovorans* genus. *Methanomethylovorans* sp. strain ZB was isolated from Baldegger Lake in Switzerland (Simankova *et al.*, 2003), where the temperature is only 5-6^{o}C throughout the year. The third species, *Methanomethylovorans victoria* was isolated from the sediment of the eutrophic Lake Victoria in Mwanza of East Africa (Muyodi, 2000). *Methanomethylovorans* species were also detected in samples from rice field soils (Lueders *et al.*, 2001), oil contaminated groundwater (Watanabe *et al.*, 2002), freshwater sediments (Lomans *et al.*, 2001), sludge of an anaerobic baffled reactor treating industrial dye waste (Plumb *et al.*, 2001) and in a bioreactor treating dichloropropan-contaminated wastewater (Schlotelburg *et al.*, 2002). The three *Methanomethylovorans* strains described were all

enriched on either trimethylamine (TMA) or DMS and are all mesophilic (growth above 40^{o}C was not possible). In this paper, the isolation and characterization of a novel thermophilic strain L2FAW^{T} from thermophilic sludge of an anaerobic bioreactor are described. It is proposed that this strain represents a novel methylotrophic species, *Methanomethylovorans thermophila* sp. nov.

Strain L2FAW^{T} was isolated from a methanol degrading enrichment culture, which was obtained from a thermophilic anaerobic reactor (Paulo *et al.*, 2002) and routinely maintained by successive transfers on methanol for about two years in our laboratory. The bicarbonate buffered mineral medium used in this study was prepared as described before (Stams *et al.*, 1993). The gas phase consisted of N_2/CO_2 (80:20 v/v) at a pressure of 170 kPa. Substrates were added from 1 M stock solutions to give a final concentration of 25 mM, unless indicated otherwise. The enrichment culture consisted of only two morphologically distinct microorganisms; a dominant irregular cocci and some short-rods. Attempts to isolate the methanogenic cocci from this enrichment culture by different techniques (petri-dish, roll-tube, deep agar and soft agar) were not successful. The rod-shaped contaminant was not eliminated by serial dilution up to 10^{-8}, even not after the addition of vancomycin. Modifications were made to the deep agar technique, keeping agar medium liquefied after inoculation. Visible colonies developed after two weeks of incubation at 55^{o}C. The well-separated colonies were picked-up by hypodermic needles after cooling the agar at room temperature, and dilution series were made for further purification. The strain isolated from the highest dilution (10^{-8}) was designated as strain L2FAW^{T}.

The colonies of strain L2FAW^{T} in deep liquefied agar were smooth milk-white spheres that reached a diameter of 1 mm after 2 weeks of incubation at 55^{o}C. Cells of strain L2FAW^{T} were non-motile irregular cocci with a diameter of 0.7 to 1.5 μm, occurring mainly single or in clusters consisting of two to four cells (Fig. 1a). Big aggregates, which are typical for *Methanosarcina* species were rarely observed. At the end of the exponential phase, cells became bigger, and eventually lysed (Fig. 1b). During active methanogenesis on methanol, the culture broth gradually turned from light yellow to greenish, which, upon exposure to air, became reddish-brown. This observation indicates that strain L2FAW^{T} produced reduced

forms of corrinoids ([Co-II]-B_{12}), which were oxidized to red corrinoids ([Co-III]-B_{12}) with oxygen as observed for *Methanosarcina barkeri* (Mazumder *et al.*, 1987).

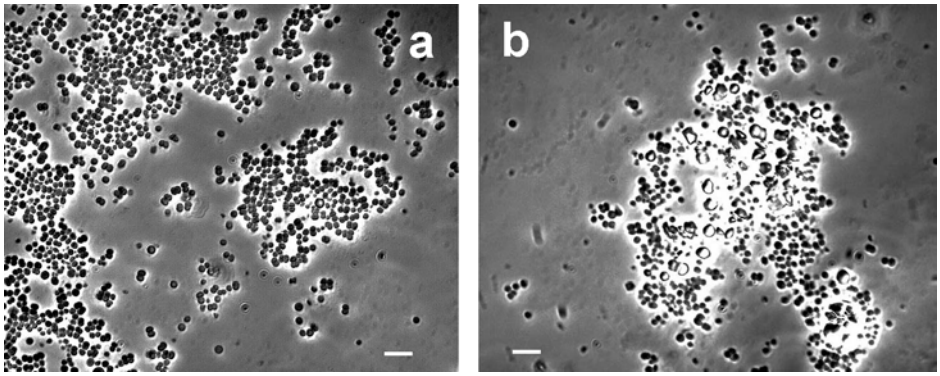


Figure 1. Phase contrast micrograph of strain L2FAW^T grown on methanol. Bar: 10 μm. a: irregular single cocci or clusters of two or four cells in the early exponential phase; b: Larger cells eventually lysed at the end of the exponential phase.

Cells of L2FAW^T stained Gram-negative. They lysed immediately upon the addition of SDS at a concentration of 0.1% (w/v) or Triton X-100 at a concentration of 4% (w/v), but resisted an osmotic stress in demi-water for 20 minutes, indicating that a proteinaceous cell wall was present (Boone & Whitman, 1988). Growth of strain L2FAW^T was inhibited by the addition of chloramphenicol (100 μg ml^{-1}), tetracycline (100 μg ml^{-1}), but not by penicillin (2 mg ml^{-1}), bacitracin (10 μg ml^{-1}), spetinomycin (100 μg ml^{-1}), vancomycin (100 μg ml^{-1}) and kanamycin (100 μg ml^{-1}).

Strain L2FAW^T could only use methanol, TMA, dimethylamine and methylamine for growth. H_2 was produced in trace amounts (80 to 160 Pa) during growth on methanol. Neither H_2/CO_2 (headspace, 80:20, 170 kPa), $CO/CO_2/N_2$ (headspace, 20:20:60, 170 kPa), formate, 2-propanol nor acetate supported growth, nor were they co-utilized together with methanol. The strain did not utilise a mixture of acetate and hydrogen. The addition of hydrogen in the headspace (170kPa) resulted in a slower growth on methanol. When transferring methanol-grown cultures into TMA-containing medium, a lag phase of about three days was observed. The lag phase became shorter with subsequent transfers on TMA. The same was observed when transferring TMA-grown culture back to methanol. An

increased lag phase was not observed when transferring the culture adapted to one methylated amine, to another methylated amine. These results confirm previous findings, that the enzymes involved in methanol and methylated amines degradation are different and have to be induced (King, 1984). DMS and MT were also tested, but no growth of strain L2FAW^T was observed at the concentrations tested (2mM and 200μM for DMS and MT, respectively). These two substances also could not be co-metabolised during growth on methanol (5 mM) or TMA (5 mM).

Strain L2FAW^T was isolated using mineral medium. Yeast extract or peptone (0.2%, w/v) slightly stimulated growth, but were not necessary. The temperature range for growth was from 42 to 58^{o}C, with an optimum at 50^{o}C. Growth was not observed at 37^{o}C and 60^{o}C. Growth was optimal up to a NaCl concentration of 100 mM. No growth and methane formation was observed at 300 mM NaCl or higher. Strain L2FAW^T could grow at a pH from 5.0 to 7.5. The optimal pH was about 6.5. Remarkably, the growth rate of strain L2FAW^T at pH 5.0 was still relatively high (55% of maximum at pH 6.5), which could be important in the resistance to acidification due to acetate formation by homoacetogens in anaerobic bioreactors (Yamaguchi *et al*., 1989). Addition of extra cobalt stimulated growth of strain L2FAW^T, the optimal concentration of cobalt in the mineral medium was between 0.5 and 2 μM. Further increase of the cobalt concentration was inhibitory. Thus, strain L2FAW^T is a thermophilic, slightly acidophilic, methylotrophic freshwater methanogen. At optimal conditions, the maximal specific growth rate is 0.05 hr^{-1}.

For the phylogenetic analysis, a bead-beating and phenol-chloroform based extraction method was used to extract DNA from strain L2FAW^T (Zoetendal *et al.*, 1998). The 16S rRNA gene was amplified with a GeneAmp PCR System 2400 thermocycler (Perkin-Elmer Cetus, Norwalk, Conn.) using the primer set 7f and 1492r and *Taq* DNA polymerase (Invitrogen, Breda, The Netherlands). The amplified fragment of 16S rRNA gene was cloned into *E. coli* JM109 (Invitrogen, Breda, The Netherlands) using the pGEM-T Easy Vector System (Promega, Madison, WI). Randomly selected recombinant clones were reamplified by PCR with the vector specific primers T7 and Sp6 (Promega, Madison, WI) and sequenced using a sequencing kit (Amersham, Slough, United Kingdom) and T7 and Sp6 IRD800-labelled sequencing primers according to the manufacturer's instructions (MWG-Biotech,

Ebersberg, Germany). The DNA fragment of the methyl coenzyme M reductase (mcrA) gene was sequenced by the primer set of ME1 (5' – GC (AC) ATG CA (AG) AT (ACT) GG (AT) ATG TC –3') and ME2 (5' – TCA T (GT) G C (AG) T AGT T (AGT) G G (AG) T AGT- 3') as described previously (Hales *et al.*, 1996). The partial 16S rRNA gene (1363 bp) and mcrA gene sequence (739 bp) obtained were compared to sequences deposited in Genbank by Blast search tool (Benson *et al.*, 2004). According to the sequence similarity of the 16S rRNA gene, the closest related species of strain L2FAW^T were *Methanomethylovorans hollandica* DMS1^T (98%), *Methanomethylovorans* sp. strain ZB (98%), and *Methanomethylovorans victoria* (97%). Other related methanogenic species were

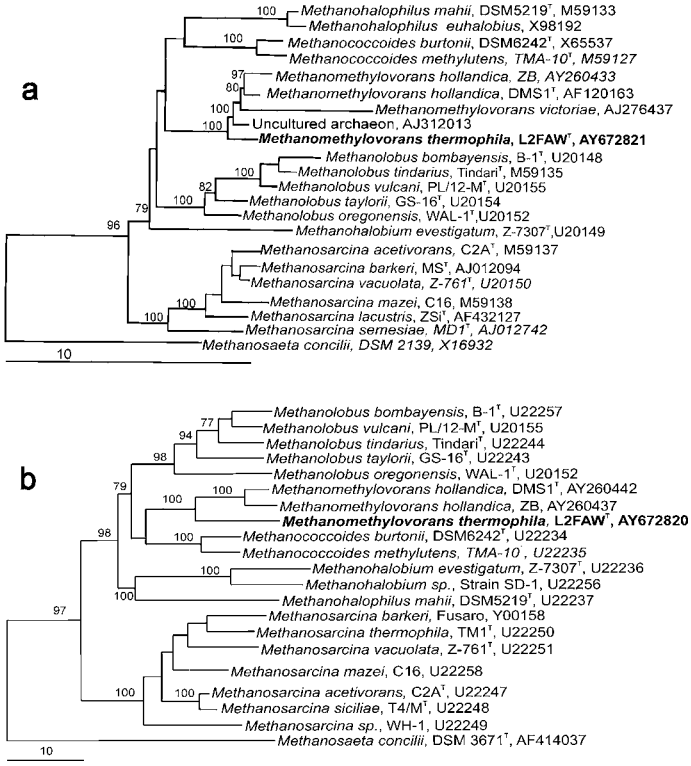


Figure 2. Phylogenetic trees of 16S rRNA gene (a) and mcrA (b) gene sequences showing the relationship between strain L2FAWT and members from *Methanomethylovorans*, *Methanolobus*, *Methanosarcina*, and *Methanococcoides* constructed using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap values for both trees were calculated using neighbour-joining analysis after 1000 replicate data sets and re-evaluated by SeqBoot and DNAPARS implemented in Phylip package (Felsenstein, 1993). *M. concilii* is used as outgroup. The bar indicated represents 10 % sequence divergences.

Methanolobus oregonensis (94%), *Methanolobus taylorii* (94%), *Methanosarcina lacustris* (94%), *Methanosarcina mazei* (93%), *Methanococcoides burtonii* (93%), and *Methanohalophilus euhalobius* (93%). The similarities of mcrA gene sequence with *M. hollandica* and *Methanomethylovorans* sp. ZB were 91% and 90%, respectively. Deduced amino acid similarity between the two methyl-CoM reductases was 88%. The values to other species were below 82%. The 16S rRNA gene and mcrA gene sequences are accessible in Genbank with the accession number of AY672821 and AY672820, respectively.

The sequences from the 16S rRNA gene and mcrA gene of strain L2FAW^T and from other related methanogens were aligned by ClustalV embedded in ARB package (Strunk & Ludwig, 2000). Phylogenetic trees (Fig. 2) were constructed using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap values for the trees with the 16S rRNA gene and mcrA gene sequences were calculated using neighbour-joining analysis after 1000 replicate data sets and re-evaluated by SeqBoot and DNAPARS implemented in Phylip package (Felsenstein, 1993). The whole genomic DNA of strain L2FAW^T was isolated by the method of Marmur (Marmur, 1961). The mol% G+C content of genomic DNA of strain L2FAW^T was 37.6, determined by thermal denaturation using a Pye Unicum SP 1800 spectrophotometer as described by Owen (Owen *et al.*, 1969). DNA-DNA homology between *M. hollandica* DMS1^T and strain L2FAW^T was 46%, determined by using the reassociation method described by De Ley (De Ley *et al.*, 1970).

The 16S rRNA gene and mcrA gene sequence analysis revealed that strain L2FAW^T clustered in *Methanomethylovorans* genus with highest similarity to the only validated species in this genus, *M. hollandica* DMS1^T (DSM15976). Similar to *M. hollandica* DMS1^T, strain L2FAW^T is also a freshwater species, which is a major characteristic of members of the *Methanomethylovorans* genus as compared to the other closest genera, *Methanolobus*, *Methanococcoides*, *Methanohalobium* and *Methanohalophilus* (Lomans *et al.*, 1999). Strain L2FAW^T differs from *M. hollandica* DMS1^T and other isolated *Methanomethylovorans* strains by its cell wall composition, cell morphology, substrate spectum, optimal temperature and its G+C content of genomic DNA (Table 1).

Besides methanol and methylated amines, *M. hollandica* DMS1^T and *Methanomethylovorans* sp. ZB can also utilise DMS and MT for growth (Lomans *et al.*, 1999; Simankova et al., 2003). Utilization of MT and DMS was not reported for *M. victoria*. Strain L2FAW^T, however, cannot grow on DMS and MT nor co-utilise them together with methanol or TMA (Table 1). It is unlikely that strain L2FAW^T lost its ability to degrade DMS and MT due to long term cultivation on methanol, since no methane was produced from the original enrichment culture and from the seed sludge of the UASB reactor when DMS or MT were tested as substrates (results not shown). The inability to use DMS and MT at lower concentrations (200 μM), and even when other reducing agents, such as cysteine or titanium citrate, or when complex medium (addition of yeast extract and peptone, supplement with extra iron and cobalt) were used, indicated that the inability to use DMS or MT is not due to the toxicity of DMS, MT and sulphide on the growth of strain L2FAW^T on DMS and MT or lack of essential nutrients.

The results of bootstrap analysis showed a clear branching of strain L2FAW^T from other *Methanomethylovorans* species and *Methanosarcina* genus. DNA matrix distance analysis of 16S rRNA gene showed the average distance of strain L2FAW^T to all members of genera *Methanolobus*, *Methanococcoides*, *Methanohalobium* were 0.066 ±0.006, 0.073±0.006 and 0.084±0.001, respectively. This value for strain L2FAW^T to the members of *Methanomethylovorans* was 0.022±0.001. Springer *et al* (1995) suggested that, based on phylogenetic analysis of 16S rRNA gene and the mcrA gene sequences from the family of *Methanosarcinaceae*, distance values lower than 0.0214±0.0065 indicated intergeneric relationship whereas values higher than 0.0907±0.0096 represented different genera within this family. Thus, it is clear that strain L2FAW^T is a novel species of the *Methanomethylovorans* genus. We propose *Methanomethylovorans thermophila* for this novel species, and strain L2FAW^T as the type strain.

Only a few thermophiles are described in the family of *Methanosarcinaceae*. Two thermophilic obligated methylotrophs, *Methanohalobium evestigatum* (Zhilina & Zavarzin, 1987) and *Methanosalsum zhilinae* (Mathrani *et al.*, 1988), which is the only species in corresponding genus, are halophiles. The first thermophilic species described in this family, *Methanosarcina thermophila* (Zinder & Mah, 1979) that can utilize methanol, acetate and

H_2/CO_2, is affiliated with other mesophilic species in the genus *Methanosarcin*a. Two other thermophilic strains of *Methanosarcina* have been described, which can utilize acetate and methanol but not H_2/CO_2 (Ollivier *et al.*, 1984; Touzel *et al.*, 1985). *Methanomethylovorans thermophila* strain L2FAW^T described here, is a thermophilic, obligated methylotrophic, freshwater species affiliated with other mesophilic species in the genus *Methanomethylovorans*.

Table 1. Characteristics of *Methanomethylovorans* strains and *Methanococcoides burtonii*

Characteristics	**Strain L2FAW$^{T\infty}$**	***M. hollandica* DMS1^{T*}**	***M. hollandica Strain* ZB†**	***M. victoria*‡**	***M. burtonii*§ DSM6242^T**
Habitat	UASB reactor	eutrophic lake	cold lake	eutrophic lake	cold lake
Cell size (μm)	0.7-1.5	1-1.5	1.0-1.7	0.5-1.2	0.8-1.8
Cell shape	single	aggregates	Clusters (2-4)	Clusters (2-4)	Single/Clusters (up to 4)
SDS sensitivity (w/v)	lysed at 0.1%	not lysed at 1%	n.d.	not lysed at 1%	lysed at 0.1%
Temp. range (opt.T)	42-58^{o}C (50^oC)	12-40^{o}C (37^oC)	1-38^{o}C	n.r.	6-30 (23.4^oC)
NaCl range (mM)	0 to 200	0-300	n.r.	n.r.	200-600
Opt. pH	6.5	6.5-7.0	n.r.	n.r.	7.7
G+C %	37.6	34.4$^{\infty}$	n.r.	n.r.	39.6
Methanol	+	+	+	n.r.	+
Methylated amines	+	+	+	+	+
Dimethyl sulfide	-	+	+	n.r.	-
Methanethiol	-	+	+	n.r.	-

$^{\infty}$: this study; *: Lomans *et al.*, 1999; †: Simankova *et al.*, 2003; ‡: Muyodi, 2000; §: Franzmann *et al.*, 1992. + or – indicates that the growth is positive or negative on this substrate; n.r.: not reported.

Description of *Methanomethylovorans thermophila* sp. nov.

Methanomethylovorans thermophila (Me.tha.no.me.thy.lo'vo.rans. M. L. n. *methanum*, methane; M. L. n. *methylum*, methyl; L. adj. *vorans*, devouring; M. L. n. *Methanomethylovorans*, methane producing, methyl group consuming; L. adj. *thermophila*, ther.mo.phi'la. Gr. adj. *thermos* hot; Gr. adj. *philos* loving; N.L. fem. adj. *thermophila* heat-loving). Cells are irregular, non-motile and coccoid with a diameter of 0.7 to 1.5 μm. Cells occur single or sometimes in clusters of two or four cells. Cells lyse in 0.1% (w/v) SDS. Cells stain Gram negative. Temperature range for growth is from 42 to 58^{o}C with the optimum at 50^{o}C. The strain is slightly acidophilic (pH range 5 to 7.5 with optimum at pH 6.5) and has a low salt tolerance (lower than 0.3 M NaCl). Methanol and methylated amines are the only catabolic and methanogenic substrates. Type strain L2FAW^{T} (DSM 17232=ATCC BAA-1173) was isolated from UASB bioreactor treating paper mill wastewater.

Acknowledgments

We thank P. L. Paulo for providing the sludge sample; F. A. M. de Bok to test DMS and MT utilization. We are grateful to A. M. Lysenko for DNA-DNA homology analysis and G+C content measurement. This work was supported by the Netherlands Technology Foundation STW, applied science division of NWO and the technology program of the Ministry of Economic Affaires of the Dutch government, under the project WWL4928.

References

Benson, D. A., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J. & Wheeler, D. L. (2004). GenBank: update. *Nucleic Acids Res* **32, Database issue**, D23-26.

Boone, D. R. & Whitman, W. B. (1988). Proposal of minimal standards for describing new taxa of methanogenic bacteria. *Int J Syst Bacteriol* **38**, 212-219.

Boone, D. R., Whitman, W. B. & Rouviere, P. (1993). Diversity and taxonomy of methanogens. In *Methanogenesis*, pp. 35-80. Edited by J. G. Ferry. New York: Chapman and Hall.

De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133-142.

Felsenstein, J. (1993). PHYLIP (phylogeny inference package), version 3.5c. Department of Genetics, University of Washington, Seattle, WA, USA.

Franzmann, P. D., Springer, N., Ludwig, W., Conway De Macario, E. & Rohde, M. (1992). A methanogenic archaeon from Ace Lake, Antarctica: *Methanococcoides burtonii* sp. nov. *Syst Appl Microbiol* **15**, 573-581.

Garcia, J.-L., Ollivier, B. & Patel, B. K. C. (2000). Taxonomic, phylogenetic, and ecological diversity of methanogenic Archaea. *Anaerobe* **6**, 205-226.

Hales, B. A., Edwards, C., Ritchie, D. A., Hall, G., Pickup, R. W. & Saunders, J. R. (1996). Isolation and identification of methanogen-specific DNA from blanket bog peat by PCR amplification and sequence analysis. *Appl Environ Microbiol* **62**, 668-675.

King, G. M. (1984). Metabolism of trimethylamine, choline, and glycine betaine by sulfate-reducing and methanogenic bacteria in marine sediments. *Appl Environ Microbiol* **48**, 719-725.

Lomans, B. P., Maas, R., Luderer, R., Op den Camp, H. J. M., Pol, A., van der Drift, C. & Vogels, G. D. (1999). Isolation and characterization of *Methanomethylovorans hollandica* gen. nov., sp. nov., isolated from freshwater sediment, a methylotrophic methanogen able to grow on dimethyl sulfide and methanethiol. *Appl Environ Microbiol* **65**, 3641-3650.

Lomans, B. P., Luderer, R., Steenbakkers, P., Pol, A., van der Drift, C., Vogels, G. D. & Op den Camp, H. J. M. (2001). Microbial populations involved in cycling of dimethyl sulfide and methanethiol in freshwater sediments. *Appl Environ Microbiol* **67**, 1044-1051.

Lueders, T., Chin, K. J., Conrad, R. & Friedrich, M. (2001). Molecular analyses of methyl-coenzyme M reductase alpha-subunit (mcrA) genes in rice field soil and enrichment cultures reveal the methanogenic phenotype of a novel archaeal lineage. *Environ Microbiol* **3**, 194-204.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J Mol Biol* **3**, 208-218.

Mathrani, I. M., Boone, D. R., Mah, R. A., Fox, G. E. & Lau, P. P. (1988). *Methanohalophilus zhilinae* sp. nov., and alkaliphilic, halophilic, methylotrophic methanogen. *Int J Syst Bacteriol* **38**, 139-142**.**

Mazumder, T. K., Nishio, N., Fukuzaki, S. & Nagai, S. (1987). Production of extracellular vitamin B-12 compounds from methanol by *Methanosarcina barkeri*. *Appl Microbiol Biotechnol* **26**, 511-516.

Muyodi, F. J. (2000). Microbiological Analysis of the Waters of Lake Victoria in Relation to the Invasion of the Water Hyacinth, *Eichhornia crassipes* (Mart.) Solms. A Case Study of the Lakeshores of Mwanza Municipality, pp. 158-173. Ph.D. Thesis, University of Dar es Salaam, Tanzania.

Ollivier, B., Lonbardo, A. & Garcia, J. L. (1984). Isolation and Characterization of a New Thermophilic *Methanosarcina* Strain (Strain MP). *Annales de Microbiologie Paris* **135b**, 187-198.

Owen, R. J., Hill, L. R. & Lapage, S. P. (1969). Determination of DNA base compositions from melting profiles in dilute buffers. *Biopolymers* **7**, 503-516.

Paulo, P. L., Jiang, B., Roest, K., van Lier, J. B. & Lettinga, G. (2002). Start-up of a thermophilic methanol-fed UASB reactor: change in sludge characteristics. *Water Sci Technol* **45**, 145-150.

Plumb, J. J., Bell, J. & Stuckey, D. C. (2001). Microbial populations associated with treatment of an industrial dye effluent in an anaerobic baffled reactor. *Appl Environ Microbiol* **67**, 3226-3235.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406-425.

Schlotelburg, C., Von Wintzingerode, C., Hauck, R., Von Wintzingerode, F., Hegemann, W. & Göbel, U. B. (2002). Microbial structure of an anaerobic bioreactor population that continuously dechlorinates 1,2-dichloropropane. *FEMS Microbiol Ecol* **39**, 229-237.

Simankova, M. V., Kotsyurbenko, O. R., Lueders, T., Nozhevnikova, A. N., Wagner, B., Conrad, R. & Friedrich, M. W. (2003). Isolation and characterization of new strains of methanogens from cold terrestrial habitats. *Syst Appl Microbiol* **26**, 312-318.

Sprenger, W. W., van Belzen, M. C., Rosenberg, J., Hackstein, J. H. & Keltjens, J. T. (2000). *Methanomicrococcus blatticola gen.* nov., sp. nov., a methanol- and methylamine-reducing methanogen from the hindgut of the cockroach Periplaneta americana. *Int J Syst Evol Microbiol* **50**, 1989-1999.

Springer, E., Sachs, M. S., Woese, C. R. & Boone, D. R. (1995). Partial gene sequences for the A subunit of methyl-coenzyme M reductase (*mcr*I) as a phylogenetic tool for the family *Methanosarcinaceae*. *Int J Syst Bacteriol* **45**, 554-559.

Stams, A. J. M., van Dijk, J. B., Dijkema, C. & Plugge, C. M. (1993). Growth of syntrophic propionate-oxidizing bacteria with fumarate in the absence of methanogenic bacteria. *Appl Environ Microbiol* **59**, 1114-1119.

Strunk, O. & Ludwig, W. (2000). ARB software package: Technische Universität, München, Germany.

Touzel, J. P., Petroff, D. & Albagnac, G. (1985). Isolation and Characterization of a New Thermophilic *Methanosarcina* Strain Chti-55. *Syst Appl Microbiol* **6**, 66-71.

Watanabe, K., Kodama, Y., Hamamura, N. & Kaku, N. (2002). Diversity, abundance, and activity of archaeal populations in oil-contaminated groundwater accumulated at the bottom of an underground crude oil storage cavity. *Appl Environ Microbiol* **68**, 3899-3907.

Yamaguchi, M., Minami, K., Tanimoto, Y. & Okamura, K. (1989). Effects of Volatile Fatty Acids on Methanogenesis of Methanol and of Pregrowth with Methanol on Acetate Utilization by Methanogens. *J Ferment Bioeng* **68**, 428-432.

Zhilina, T. N. & Zavarzin, G. A. (1987). *Methanohalobium evestigatus*, n. gen., n. sp. - an extremely halophilic methanogenic archaebacterium. *Dokl Akad Nauk SSSR* **293**, 464-468.

Zinder, S. H. & Mah, R. A. (1979). Isolation and characterization of a thermophilic strain of *Methanosarcina* unable to use H_2-CO_2 for methanogenesis. *Appl Environ Microbiol* **38**, 996-1008.

Zoetendal, E. G., Akkermans, A. D. L. & de Vos, W. M. (1998). Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. *Appl Environ Microbiol* **64**, 3854-3859.

Chapter 3

Novel one-carbon metabolism of *Moorella hydrogenoformans* sp. nov., isolated from a methanogenic bioreactor

Bo Jiang, Paula L. Paulo, Anne M. Henstra,
Wim van Doesburg and Alfons J. M. Stams

Submitted to: *Appl. Environ. Microbiol*.

Summary

A thermophilic spore-forming bacterium, strain AMP, was isolated from a thermophilic methanogenic bioreactor that was fed with methanol as sole substrate. Strain AMP was affiliated with the *Moorella* genus based on 16S rDNA sequence analysis, but it had a unique one-carbon metabolism compared with other *Moorella* species and other homoacetogenic species. In pure culture it grew homoacetogenically on methanol, but not on formate and H_2/CO_2. Hydrogenase and formate dehydrogenase activities were detected, but cytochrome *b* was not present in cell-free extracts of strain AMP. This might be the reason for the inability to grow on formate and H_2/CO_2. Growth on formate, but not on H_2/CO_2 was obtained in the presence of thiosulfate as electron acceptor. Unlike other *Moorella* species, strain AMP grew hydrogenogenically on CO, indicating the presence of an energy-conserving hydrogenase. The G+C content of genomic DNA is 57.3 mol%. The DNA-DNA homology showed 75.2±4.7% similarity to *Moorella thermoacetica* DSM 521^T. We propose that strain AMP represents a novel species within the *Moorella* genus. *Moorella hydrogenoformans* was assigned to this novel species with strain AMP^T as its type strain. Genbank accession number of 16S rDNA gene sequence of *M. hydrogenoformans* AMP^T: AY884087.

Introduction

Around 100 species of homoacetogenic bacteria divided over 21 genera in the *Bacillus-Clostridium* subphylum have been described (6, 9). Homoacetogens have the ability to form acetate through the reductive acetyl-CoA pathway. In this pathway CO_2 acts as a sink for reducing equivalents generated in the oxidation of carbohydrates, organic acids, C1 compounds or hydrogen (9). ATP is conserved in this pathway by substrate level phosphorylation in the last step, but ATP has to be invested to activation of formate (formed from CO_2) to formyl-tetrahydrofolate (21). Additional ATP is formed via chemiosmotic processes that involve proton or sodium translocation across the cytoplasmic membrane. Hydrogenase (H_2ase), the bifunctional carbon monoxide dehydrogenase and acetyl-CoA synthase complex (CODH/ACS), formate dehydrogenase (FDH) and corrinoid-containing methyltransferase are key enzymes in the metabolism of homoacetogens.

Moorella is a genus of Gram-positive, sporeforming homoacetogens. This genus consists of four validated species and several undescribed strains. Most *Moorella* species grow on diverse sugars, organic acids, C1 compounds and H_2/CO_2. However, ***M. glycerini*** is not able to grow on methanol, formate and H_2/CO_2 (34). In this report, we describe a novel *Moorella* species. This bacterium grows homoacetogenically on methanol, but is not able to grow homoacetogenically on H_2/CO_2, CO or formate. Remarkably, the bacterium is able to grow hydrogenogenically on CO, a property of facultative anaerobic phototrophs (3, 7, 46), some strictly anaerobic thermophilic anaerobes (36, 38, 39, 42, 43) and an archaeon (37). The novel strain is able to grow on formate, but not on H_2/CO_2, with thiosulfate as electron acceptor. To emphasize the novel physiological features the strain is described as a novel species within the genus *Moorella*, *Moorella hydrogenoformans*.

Material and Methods

Source of microorganisms

Strain AMP, described here, was isolated from a methanol-degrading enrichment culture (27). Strain NJ1, resembled to *Methanothermobacter thermoautotrophicus* ΔH, was isolated from the above mentioned enrichment culture with H_2/CO_2 (reported also in this report). *Moorella thermoacetica* DSM 521 was obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig).

Media and growth conditions

A bicarbonate buffered mineral medium was prepared as described previously (40). Vitamin B_{12} and when indicated, $CoCl_2$ were omitted from this medium. Routine cultivation was carried out in 127-ml serum bottles with 50 ml medium and a N_2/CO_2 (80:20) gas phase at a pressure of 170 kPa. CO or H_2 replaced N_2 when used as substrates. Methanol (40 mM) was used as sole carbon and energy source for enrichment and isolation. Substrates and electron acceptors were added from neutralized sterile stock solutions to a final concentration of 10 mM or an otherwise indicated concentration. For solid media, agar (Difco) was added (8 g/L) to the liquid medium. Yeast extract and peptone (Difco) were amended at concentrations of 0.2 g/L when indicated. Temperature of incubation for enrichment and isolation was 55°C. Other cultivation experiments were performed at 65°C. Strain NJ1 was routinely cultivated in the same medium using H_2/CO_2 as substrate. Coculture experiments were performed by inoculating cultures of strain NJ1 and strain AMP at an inoculum size of 5% each.

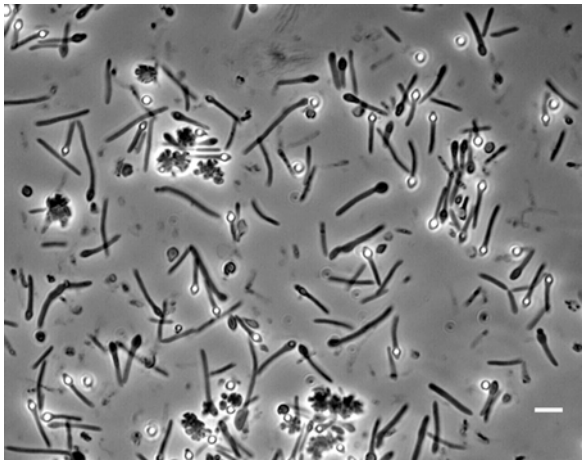


Fig. 1. Phase contrast microscopic picture of strain AMP grown on methanol, showing vegetative cells, sporulating cells and mature spores. Bar indicates 5μm.

Isolation procedure

By using cobalt-deficient media, a methanol-degrading enrichment culture was obtained that consisted of spore-forming bacteria and rod-shaped methanogens (27). The spore-forming bacterium was isolated by repeated serial dilutions in liquid methanol-containing media (with addition of $CoCl_2$) using an autoclaved (121 °C for 1 h.) culture as inoculum. The highest dilution with growth was diluted in soft agar (Difco, 8 g/L). Well-separated colonies were picked up and used as inoculum for serial dilutions in liquid medium with bromoethanesulfonate (Bres, 10 mM) and penicillin G (2mg/ml). The pure culture that was obtained in the highest dilution was designated strain AMP. Purity of the isolate was confirmed by phase contrast microscopy and by incubations in anaerobic Wilkens-Chalgren broth (Oxoid, Basingstoke, UK) amended with thiosulfate and pyruvate.

Physiological characterization

Electron donor and electron acceptor utilization by strain AMP was tested in the described mineral medium (with $CoCl_2$) or undefined DSM medium 60 (www.dsmz.de) amended with single and mixed substrates as described in the text. Growth on a substrate was confirmed by substrate consumption, product formation, increase of culture turbidity and subsequent transfers in fresh media.

Optimal growth conditions were tested in bicarbonate buffered media with methanol (40 mM) as substrate. pH values from 5.0 to 9.0 were obtained by the addition of a calculated amount of 1M HCl or 1M NaOH. Cultures were incubated at a temperature range from 37°C to 75^{o}C. To test optimal salt concentrations, NaCl was added to the final concentration of 10 mM to 500 mM. Gram staining was made according to a standard method (13).

Phylogenetic analysis

DNA of strain AMP was extracted as described previously (51). 16S rDNA was amplified using the 7f and 1492r primer set (Invitrogen, Breda, The Netherlands). The pGEM-T Easy vector system was used to clone amplified 16S rDNA fragments into *E. coli* strain JM109 (Promega, Madison, WI). Randomly selected recombinant clones were reamplified by PCR with the vector specific primers T7 and Sp6 (Promega, Madison, WI). Reamplified 16S rDNA fragments were sequenced according to the manufacturer's

instructions (Amersham, Slough, United Kingdom) with IRD800-labelled sequencing primer sets Sp6 and T7 (Promega, Madison, WI), 533f and 1100r (20).

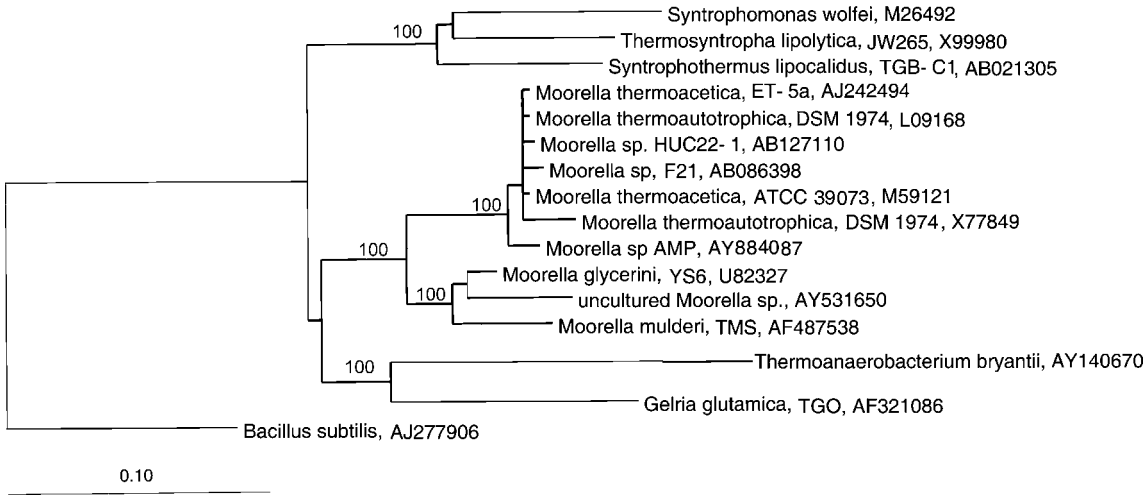


Fig. 2. Phylogenetic tree based on 16S rDNA sequence analysis showing the position of strain AMP within the *Moorella* genus and relationship to selected bacteria using neighbour-joining method (30) embedded in ARB package (41). The bar represents 10 % sequence divergences.

Obtained 16S rDNA sequences (1528 bp) were compared to sequences deposited in the NCBI database (2). 16S rDNA sequences were aligned using ARB software package (41). Phylogenetic trees based on 16S rDNA sequences were constructed using the neighbor-joining method (30). Bootstrap values were calculated using neighbour-joining analysis of 1000 replicate data sets by SeqBoot and subsequent re-evaluation by DNAPARS, both implemented in the Phylip software package (10).

The G+C content of genomic DNA was determined at the identification service of the DSMZ (Braunschweig, Germany). The DNA was isolated according to Visuvanathan et al. (47) and purified as described by Cashion et al. (5). The G+C content was determined by HPLC analysis (24, 44). DNA homology was determined using the reassociation method described by De Ley et al. (8).

Enzyme assays

Cells of strain AMP were cultivated on either methanol or methanol plus CO. Cells were harvested in the late logarithmic phase by centrifugation and cell free extracts were

prepared under anoxic conditions by osmotic shock and sonication using lysozyme as described previously (22). Soluble fractions and membrane fractions were obtained by ultracentrifugation of cell free extracts (15). Carbon monoxide dehydrogenase (CODH), formate dehydrogenase (FDH) and hydrogenase (H_2ase) activities were assayed in 50 mM Tris-HCl (pH 8.5) containing benzylviologen (BV, 5 mM) and dithiothreitol (DTT, 1 mM) at 55°C in a U2010 spectrophotometer (Hitachi, Japan) as previously described (15). H_2-evolution activity (MV-H_2) was assayed in 50 mM MOPS/KOH buffer at pH 7.0 and 2mM DTT with reduced methyl viologen (MV, 2 mM) according to Soboh at al. (35).

Analytical methods

Organic acids were measured by HPLC (40). Gases and alcohols were measured by gas chromatography (1, 14), and nitrate, thiosulfate, sulfate were analysed by a HPLC system equipped with an Ionpac AS9-SC column and ED 40 electrochemical detector (Dionex, Sunnyvale, Calif.) (32). Cell dry weights were analysed as previously described (31). Sulfide was analysed by the method of Trüper and Schlegel (45). To detect the cytochrome *b*, O_2-oxidized membrane fractions were reduced by sodium dithionite, and the reduced-minus-oxidized spectra were recorded with a dual-beam spectrophotometer (Hitachi U2010, Japan) as described by Fröstl et al. (12). Protein was determined according to the Bradford method with bovine serum albumin as a standard (4).

Results

Enrichment and isolation

In previous work we studied the effect of cobalt on methanol conversion in a laboratory scale anaerobic bioreactor operated at 55°C (27, 28). We obtained methanol-degrading enrichment cultures with and without $CoCl_2$ as cobalt source in the medium. Typically, a sarcina-type of methanogen was enriched in cobalt containing media, which can utilize methanol directly for methanogenesis. This methanogen was described recently (16). However, enrichment in the absence of cobalt resulted in a coculture consisting of rod-shaped microorganisms, some of which showed autofluorescence and others that formed spores. That

coculture formed methane as end product from methanol. To obtain the microorganisms in pure culture we continued cultivation of the enrichment culture in cobalt-amended media ($CoCl_2$, 0.5 μM). This resulted in faster growth and a dominance of the spore-forming, rod-shaped microorganism. In that culture, acetate was the main product of methanol utilization instead of methane (27). The spore-forming bacterium, strain AMP, was isolated after repeated dilutions series in liquid media and one dilution series in deep agar. From the same medium, a methanogenic strain was also isolated with H_2/CO_2 as substrate, which designated NJ1.

Morphology and optimal growth conditions

Strain AMP is a rod shaped, Gram-positive, spore-forming bacterium. Cells of strain AMP grown on methanol were 0.4 to 1.2 μm wide, and 5 to 14 μm long. In old cultures, round swollen terminal endospores were observed. Exponentially growing cells were generally longer than sporulating cells (Fig. 1). Cells were shorter and spores were hardly observed when grown on pyruvate or lactate.

Strain AMP grew at a temperature range of 42°C to 75°C with optimal growth between 60°C and 65°C. Growth was observed in a pH range of 5.0 to 8.5 with fastest growth at pH 6.9. Growth rates remained unchanged when NaCl concentrations were below 150 mM. Lower growth rates were observed with NaCl concentrations above 200 mM, while no growth occurred at a NaCl concentration of 400 mM or higher.

Phylogeny of strain AMP

The 16S rDNA gene sequence of strain AMP (1528bp) was sequenced and is accessible under Genbank accession number AY884087. Based on 16S rDNA sequence analysis strain AMP is closely related to *Moorella thermoacetica* strains HUC22-1 (98.7%), ET-5a (98.4%), DSM 521^T (98.3%) and F21 (97.8%), as well as to *Moorella thermoautotrophica* strain DSM1794 (98.2%). Strain AMP was distantly related to other *Moorella* species; *M. glycerini* (94.5%) and *M. mulderi* (91.5%). Neighbor-joining tree based on 16S rDNA sequences indicated that strain AMP fell into the cluster of the genus *Moorella* (Fig. 2). The G-C content of genomic DNA was 57.3 mol%. The DNA-DNA hybridisation showed 75.2±4.7% (duplicate measurements) similarity to *M. thermoacetica* DSM 521^T.

Table 1. Main characteristics of strain AMP related to other *Moorella* species[a].

	Strain AMP	***M. mulderi***	***M. glycerini***	***M. thermo-autotrophica***	***M. thermoactica***
Opt. Temp (°C)	65	65	58	55-58	55-60
Opt. pH	6.9	7.0	6.3	5.7	6.9
G-C% (mol%)	57.3	53.6	54.5	53-55	53-55
Similarity of 16S rDNA, %)	100	91.5	94.5	98.2	98.3
Electron donor					
Glucose	-	+	+	+	+
Fructose	±	+	+	+	+
Pyruvate	+	+	+	+	+
Lactate	+	+	+	+	+
Glycerol	-	-	+	-	-
Methanol	+	+	-	+	+
Formate	±	+	-	+	+
H_2/CO_2	-	+	-	+	+
CO/CO_2	+(to H_2)	na[c]	na[c]	+(to acetate)	+ (to acetate)
Electron acceptor					
Nitrate	-	-	-	+	+
$S_2O_3^{2-}$	+	+	+	+	+
Fumarate	-	-	+	-	-

[a] Characteristics of other *Moorella* species were obtained from (1) and from (11), (49) and (34).

[b] +, - or ± indicates that growth is positive, negative or weak.

[c] not analysed

Substrate utilization

Strain AMP has a different substrate profile than *M. thermoacetica* and *M. thermoautotrophica* (Table 1). It grew on pyruvate, lactate, mannose, methanol (40 mM), vanillate (5 mM), vanillin (5 mM), and CO/CO_2 (headspace, 80:20, 170 kPa). Acetate was the major end product after growth of strain AMP on the mentioned substrates (except on CO/CO_2) with little amount of H_2 and formate formation (Table 2 and data not shown). We did not analyze the formation of aromatic compounds from vanillate and vanillin. The following substrates were tested, but not utilized by strain AMP: H_2/CO_2 (headspace, 80:20,

170kPa), acetate, ethanol, n-propanol, glycerol, glucose (5 mM), melibiose (5 mM), raffinose (2 mM), rhamnose (2 mM), trehalose (5 mM), arabinose, cellobiose, cellulose, galactose, lactose, maltose, xylose, mannitol, melezitose, ribose (5 mM), sorbitol, starch (0.5 g/L), sucrose, benzoate. Similar to other *Moorella* species, thiosulfate was used as electron acceptor by strain AMP. This resulted in the formation of sulfide as major product. Remarkably, formate and fructose only provided measurable growth in the presence of thiosulfate. When methanol or lactate was used as electron donors, nitrate, sulfate, and fumarate were not reduced by strain AMP.

Methanol metabolism of strain AMP

Methanol was readily utilized as energy and carbon source by strain AMP (Fig. 3). The ratio of acetate formed to methanol consumed was 0.74. In the presence of CO or formate, this ratio increased to 1.09 and 1.02, respectively (Table 2). The consumption of 40 mM methanol by strain AMP took 6 days when cobalt was present at a concentration of 0.5 μM; it took more than 16 days to convert the same amount of methanol to acetate when cobalt was not added (Table 3). In that case the residual cobalt concentration was about 0.025 μM. In the presence of *Methanothermobacter* strain NJ1, more methane and less acetate was formed when cobalt was not added (Table 3). The 16S rDNA of strain NJ1 was 99.5% similar to *M. thermoautotrophicus* strain ΔH. Strain NJ1 was not able to grow on methanol, formate or acetate (results not shown). The constructed coculture converted methanol four times faster in the presence of cobalt compared that in the absence of cobalt.

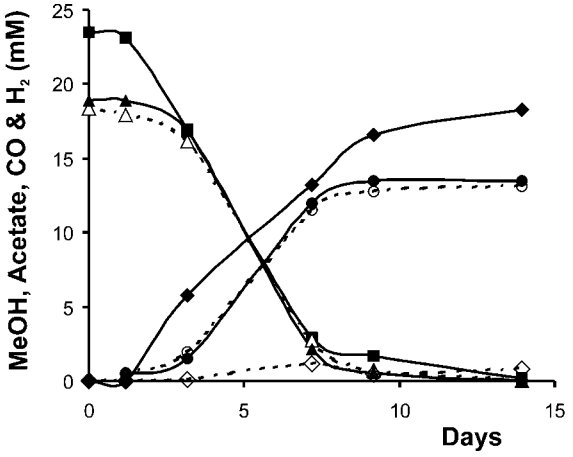


Fig. 3. Conversion of methanol (20 mM) and CO (34 kPa) by strain AMP. Methanol; dashed lines and open symbols. Methanol and CO; solid lines and closed symbols. Symbols: acetate: ○, ●; methanol: Δ, ▲; CO: ■;

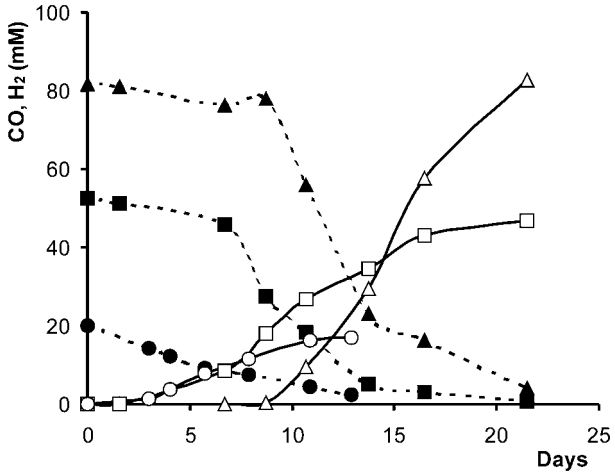


Fig. 4. Conversion of CO (dashed lines, open symbols) by strain AMP coupled to H_2 formation (solid lines and closed symbols) at 20% (●, ○), 50% (■, □) and 80% (▲, Δ) CO.

CO metabolism of strain AMP

Acetate was not the main product when strain AMP was inoculated in a mineral medium with 34 kPa (20%) CO in the headspace. Instead, 18.5 mM H_2 and comparable amount of CO_2 were produced from 19.4 mM CO (Fig 4); acetate formation was not detectable (<0.2 mM). Conversion of CO was considerably slower than conversion of methanol (Figure 3 and 4). With CO as substrate, the optical density of the culture at 600 nm (OD_{600}) increased from 0.025 to 0.086, which indicated that the conversion was coupled to the growth. Chemolithotrophic growth of strain AMP on CO coupled to H_2 evolution was confirmed by subcultivation in 6 successive transfers into fresh mineral medium with CO as sole energy and carbon source. Strain AMP grew also at CO level of 85 kPa (50%) and 136 kPa (80%) and formed H_2 as major product. Acetate was detected, but its concentration was always below 1 mM. Strain AMP showed a faster conversion rate at higher partial pressure of CO, but the lag phase was also longer. Strain AMP could also be subcultured at high CO concentrations, but the lag phase remained long.

As methanol results in acetate formation and CO in hydrogen formation, we investigated mixed substrate utilization by strain AMP. Fig. 3 shows methanol and CO consumption in cultures inoculated with CO adapted cells. Production of acetate and consumption of methanol in the culture with methanol and CO were similar as on methanol alone. In addition, H_2 was produced stoichiometrically from CO. However, if methanol-

grown cells were grown on methanol in the presence of 136 kPa CO, more acetate was formed. In this case less hydrogen was formed from CO (Table 2). CO conversion to H_2 also occurred when cells of strain AMP grew on pyruvate, lactate or formate, and when thiosulfate was present as electron acceptor (Table 2). Higher ratios of acetate produced to substrate consumed were measured when pyruvate or lactate was the substrate together with CO (data not shown and Table 2). Growth rates on methanol or pyruvate in the presence or absence of CO were similar, while the growth rate on lactate in the presence of CO was lower (data not shown).

FDH, H_2-ase, CODH and H_2-evolution activities were detected in cell-free extracts prepared from cells grown on methanol or methanol/CO. FDH levels were not affected by the presence of CO, which remained around 3 U/mg protein. H_2-ase levels decreased from 28 to 9 U/mg protein and CODH levels increased from 60 to 92 U/mg protein when cells grew in the presence of CO. The difference-spectrum of reduced vs. oxidized membranes obtained from cells cultivated with either methanol or methanol/CO showed no absorption peak around 430 and 560 nm, indicating that cytochrome *b* was not present in the cell membrane fraction. Similarly, we could not detect cytochrome *b* peaks in the cytoplasm fractions.

Formate metabolism by strain AMP

Fast growth and formate conversion were observed when strain AMP was inoculated into mineral medium with formate and thiosulfate as substrates (Fig. 5); 3.0 mM sulfide, 2.4 mM H_2 (4750 Pa) and 8.1 mM acetate were formed from 47.5 mM formate in 15 days (Fig. 5 and Table 2). Formate was not consumed when thiosulfate was replaced by sulfate, nitrate or fumarate. In the absence of thiosulfate, H_2 gradually built up to a partial pressure of 2000 Pa in 40 days. Removal of H_2 from the headspace by replacing headspace resulted again in a built up of H_2 back to 2000 Pa, indicating that accumulation of H_2 is inhibitory for the conversion of formate (data not shown).

Discussion

Previously, a syntrophic methanol degrading enrichment culture was obtained by using cobalt limited mineral media (27). An obligate hydrogenotrophic methanogen, strain NJ1 and the methanol-utilizing strain AMP described here were isolated from this enrichment.

This coculture resembled the first described syntrophic methanol-degrading coculture consisting of *Moorella thermoautotrophica* strain Z-99 and *Methanobacterium thermoformicicum* strain Z-245, but this coculture was enriched at a high cobalt concentration of 540 μM (26). In our experiments incubations in cobalt sufficient medium (0.5 μM) resulted in the isolation of a methylotrophic methanogen (16). The effect of cobalt on growth of strain AMP is not exactly clear. We speculate that in the presence of cobalt strain AMP like other homoacetogens employs a corrinoid-containing methyltransferase for methanol degradation. The cofactor-bound methyl group can supply methyl group for acetate synthesis via the acetyl-CoA pathway. It might be that a methanol dehydrogenase is involved in the oxidation of methanol in the absence of cobalt, resulting in the formation of formaldehyde that cannot enter the acetyl-CoA pathway but can be oxidized to CO_2. In *M. thermoautotrophica* a methanol dehydrogenase with pyrroloquinoline quinone as the prosthetic group is present (50), which is an indication that such an enzyme might also exist in strain AMP.

Strain AMP is a homoacetogen as indicated by the product profile, the existence of CODH (9) and its phylogeny. However, unlike many other homoacetogens strain AMP could not grow homoacetogenically on H_2/CO_2 or formate as sole substrates. Growth of *Moorella* and *Sporomusa* species on H_2/CO_2 or formate depends on H_2-ase and FDH, and a membrane electron transport chain that comprises cytochrome *b* for energy conservation (19, 21). H_2-ase and FDH activities were detected in strain AMP, but cytochrome *b* was not found. The absence of cytochrome *b* may be the reason why strain AMP cannot grow homoacetogenically on H_2/CO_2 or formate. A cytochrome *b*-deficient mutant of *Sporomusa sphaeroides* BK824 (17) and *M. thermoacetica* (12) that fails to express cytochrome *b* in the presence of nitrate, were also not able to grow on these substrates as sole energy and carbon sources. However, in *Acetobacterium woodii* and *Thermoanaerobacter kivui*, energy is conserved via a sodium ion gradient, which do not require cytochrome *b* for electron transport (25). It is remarkable that strain AMP cannot grow homoacetogenically on formate, while acetate is formed when grown in the presence of thiosulfate or CO. This may reflect that an energy substrate is required to metabolize formate.

Table 2. The effect of thiosulfate, CO and formate on the conversion of methanol, pyruvate and lactate by strain AMP. Methanol grown culture was used as inoculum for all the incubations.

1st substrate consumed (mM)	2nd substrate consumed (mM)	$S_2O_3^{2-}$ addition (mM)	Biomass mg dry weight/ mol C of 1st substrate	Products (mM)				Recovery		Acetate/ Substrate[c]	
				Acetate	H_2[a]	HS^-	Formate	[H] %[b]	C %[c]		
Methanol (38.7)	-	-	2.84	27.7	0.1	n.d.	1.8	84	98	0.74	(0.75)
Methanol (39.2)	CO (108.3[a,e])	-	3.82	36.2	60.2	n.d.	0.4	93	94	1.09	(1.0)
Methanol (39.8)	-	20	4.77	17.9	0.5	3.2	n.d.	107	76	0.56	(0)
Methanol (39.4)	Formate (48.3)	n.a.	4.82	31.8	1.8	n.d.	n.a.	81	85	1.02	(1.0)
CO (116)	-	-	0.78	1.9	92.0	n.d.	2.0	97	93	0.02	(0.25)
CO (113)	-	20	1.19	0.2	62.9	0.7	3.1	81	99	0.002	(0)
Lactate (16.5)	-	n.a.	3.49	17.4	1.6	n.d.	0.3	104	99	1.23	(1.50)
Lactate (16.4)	CO (34.5)	-	2.68	20.3	14.5	n.d.	4.9	86	97	1.55	(1.67)
Lactate (16.5)	-	20	2.36	17.0	1.3	4.2	0.4	115	104	1.13	(0)
Formate (53.0)	CO (30)	-	1.69	9.9	20.4	n.d.	n.a.	89	79	0.21	(0.25)
Formate (47.4)	-	20	3.56	8.8	2.4	3.0	n.a.	85	105	0.20	(0)

[a] expressed in as mM (mmol/L medium).

[b] electrons produced were calculated using available electrons in the products and substrate based on half reactions.

[c] CO_2 was estimated based on stoichiometry of the reaction.

[d] calculated after correction of biomass formation from corresponding substrate consumed. Theoretic values are in brackets.

[e] CO at around 110 mM and 30 mM equivalent to 136 kPa and 34 kPa, respectively.

-: no addition. n.d.: measured but not detected. n.a.: not applicable.

Strain AMP produced high amounts of H_2 during growth on CO, even when thiosulfate is present as electron acceptor (Fig. 5, Table 2). When CO was the sole substrate H_2 was formed stoichiometrically according to: $CO + H_2O \rightarrow CO_2 + H_2$ ($\Delta G^{o'} = -20$ kJ), indicating carboxydotrophic hydrogenogenic growth of strain AMP. *Carboxydothermus hydrogenoformans* was identified as the first strict anaerobic moderately thermophilic bacterium capable of CO oxidation and H_2 evolution (42). *C. hydrogenoformans* produces H_2 via a monofunctional CODH, an energy conserving hydrogenase (ECH) and a ferredoxin-like protein B that mediates electron transfer between CODH and ECH (33, 35). *M. thermoacetica* also produces high amount of H_2 in the presence of CO during growth on glucose (23). However, in this strain H_2 was not derived from CO oxidation, since in the absence of glucose no H_2 was formed from CO. It was speculated that in *M. thermoacetica* CO just acted as an inhibitor of the CODH-acetyl CoA synthetase (ACS) complex, which resulted in a shift from acetate formation to hydrogen formation (18). It was also concluded that this H_2 production did not result in energy conservation (9, 18). Unlike *M. thermoacetica*, strain AMP is able to couple CO oxidation and H_2 evolution to growth as occurs in other carboxydotrophic H_2-producing anaerobes. H_2 was also produced when strain AMP grew on CO plus lactate, CO plus methanol and CO plus formate (Table 2), suggesting that both bifunctional CODH/ACS complexes and monofunctional CODH/ECH complexes are present in strain AMP. These complexes seem to function simultaneously or separately depending on the substrates that are provided.

The cell morphology, Gram staining, formation of spores and 16S rDNA sequence analysis revealed that strain AMP is a *Moorella* species. It is closely related to *M. thermoacetica* and *M. thermoautotrophica* with 98-99 % similarity based on 16S rDNA sequence analysis. The DNA-DNA similarity of strain AMP and *M. thermoacetica* DSM 521^T is 75.2±4.7%, which is just above the threshold value of 70% for the definition of species according to the recommendation of the ad hoc committee (48). However, strain AMP differs from these two species and *M. mulderi* (1) in its inability to grow fermentatively on glucose, H_2/CO_2, and formate. Similar to *M. mulderi* and *M. glycerini* (34), strain AMP was unable to use nitrate as electron acceptor. In contrast to *M. glycerini*, strain AMP was unable to grow on glycerol and reduce fumarate (Table 1). Like *M. thermoacetica* and *M. thermoautotrophica* strain AMP could grow on CO, but the end product is H_2 rather than

acetate, which is the main end product of the other two *Moorella* species. Carboxydotrophic hydrogenogenic growth is only found in less than ten obligate anaerobic bacteria and one archaeon. Only a few are obligate carboxydotrophic (39, 43), while others can also grow heterotrophically on carbohydrates (29, 36, 38). None of them had been reported to show homoacetogenic growth. Thus, strain AMP is the first homoacetogenic bacterium capable of carboxytrophic hydrogenogenic growth. The G + C content of strain AMP genomic DNA is 57.3 mol%, which is higher than that of the other *Moorella* species (53 to 55 mol%). Although strain AMP clustered in the genus *Moorella* (Fig. 2), it was separated from the sub-cluster of all strains of *M. thermoacetica* and *M. thermoautotrophica* and the sub-cluster of *M. glycerini* and *M. mulderi*. Based on the unique physiological properties, we proposed that strain AMP represent a novel species of the *Moorella* genus. Because of this characteristic hydrogenogenic CO metabolism, we proposed the name of *Moorella hydrogenoformans.*

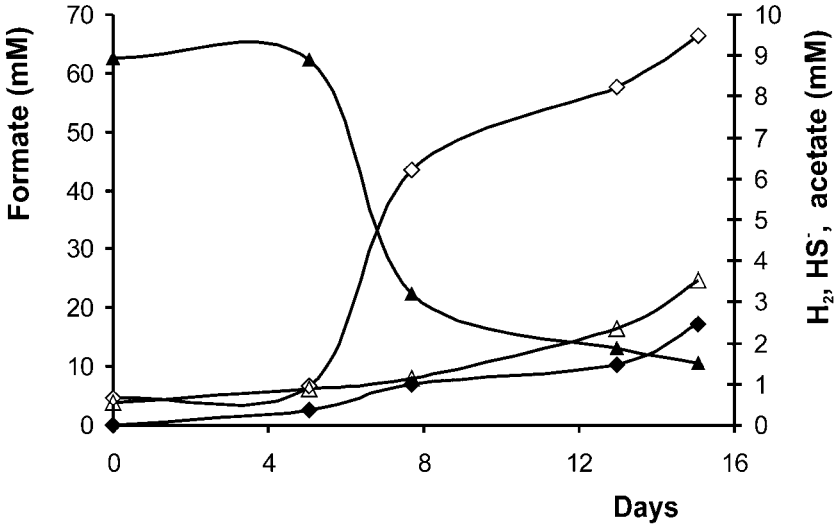


Fig. 5. Formate conversion by strain AMP in the presence of 20 mM thiosulfate as electron acceptor. Symbols: formate: ▲; acetate: ◊; H_2: ♦; HS^-: Δ.

Table 3. The effect of cobalt and *M. methanoautotrphicus* strain NJ1 on methanol conversion by strain AMP.

Micro-organism(s)	Co^{2+} addition	Methanol consumed (mM)	Days to consume	Main products (mM)		
				Acetate	CH_4[a]	H_2[a]
Strain AMP	+	41	5	30	-	0.9
	-	40	16	29	-	4.6
Strain AMP + Strain NJ1	+	42	7	20	6.4	<0.01
	-	42	30	2.2	28	0.4

[a] expressed in mmol/L medium

Description of *Moorella hydrogenoformans* sp. nov.

Moorella hydrogenoformans sp. nov. (hy.dro.ge.no.fo'r.mans. M.L.n. hydrogenium hydrogen, L.adj. formans forming; M.L.adj. hydrogenoformans hydrogen-forming). Cells are gram-positive, long-rods, 0.4-1.2 ×5-14 μm, occurring in single. Forms swollen end spores. Strict anaerobic and thermophilic. Grows fastest at temperature between 60 to 65^{o}C, pH 6.9 and salt concentration below 200 mM with ranges of 42-75^{o}C, pH 5.0 to 8.5 and below 400 mM. Utilizes methanol, pyruvate, lactate, mannose, vanillate and vanillin, forming acetate as the main reduced end product. Grows weakly on fructose. Reduces thiosulfate, but not nitrate, sulfate or fumarate. Does not grow (in the absence of thiosulfate) on H_2/CO_2, formate, glucose, acetate, ethanol, n-propanol, glycerol, melibiose, raffinnose, rhamnose, trehalose, arabinose, cellobiose, cellulose, galactose, lactose, maltose, xylose, mannitol, melezitose, ribose, sorbitol, starch, sucrose and benzoate. Grows hydrogenogenically on 100% CO. G + C content of genomic DNA is 57.3 mol%. Strain AMP^{T} as its type strain. Genbank accession number for the 16S rDNA gene sequence of *M. hydrogenoformans* AMP^{T} is AY884087.

Acknowledgments

We are grateful to S. N. Parshina for fruitful discussions. This work was supported by the Netherlands Technology Foundation STW, applied science division of NWO and the

technology program of the Ministry of Economic Affaires of the Dutch government, under the project WWL4928.

References

1. **Balk, M., J. Weijma, M. W. Friedrich, and A. J. M. Stams.** 2003. Methanol utilization by a novel thermophilic homoacetogenic bacterium, *Moorella mulderi* sp. nov., isolated from a bioreactor. Arch. Microbiol. **179:**315-320.
2. **Benson, D. A., I. Karsch-Mizrachi, D. J. Lipman, J. Ostell, and D. L. Wheeler.** 2004. GenBank: update. Nucleic. Acids. Res. **32 Database issue:**D23-26.
3. **Bonam, D., L. Lehman, G. P. Roberts, and P. W. Ludden.** 1989. Regulation of carbon monoxide dehydrogenase and hydrogenase in *Rhodospirillum rubrum*: effects of CO and oxygen on synthesis and activity. J. Bacteriol. **171:**3102-3107.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248-254.
5. **Cashion, P., M. A. Holder-Franklin, J. McCully, and M. Franklin.** 1977. A rapid method for the base ratio determination of bacterial DNA. Anal. Biochem. **81:**461-466.
6. **Collins, M., P. Lawson, A. Willems, J. Cordoba, J. Fernandez-Garayzabal, P. Garcia, J. Cai, H. Hippe, and J. Farrow.** 1994. The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. Int. J. Syst. Bacteriol. **44:**812-826.
7. **Dashekvicz, M. P., and R. L. Uffen.** 1979. Identification of a carbon monoxide metabolizing bacterium as a strain of *Rhodopseudomonas gelatinosa* (Molisch) van Niel. Int. J. Syst. Bacteriol. **29:**145-148.
8. **De Ley, J., H. Cattoir, and A. Reynaerts.** 1970. The quantitative measurement of DNA hybridization from renaturation rates. Eur. J. Biochem. **12:**133-142.
9. **Drake, H. L., K. Küsel, and C. Matthies.** 2002. Ecological consequences of the phylogenetic and physiological diversities of acetogens. Antonie Van Leeuwenhoek **81:**203-213.
10. **Felsenstein, J.** 1989. PHYLIP - Phylogeny inference package (Version 3.2). Cladistics **5:**164-166.
11. **Fontaine, F. E., W. H. Peterson, E. McCoy, and M. J. Johnson.** 1942. A new type of glucose fermentation by *Clostridium thermoaceticum* nov. sp. J. Bacteriol. **43:**701-715.
12. **Fröstl, J. M., C. Seifritz, and H. L. Drake.** 1996. Effect of nitrate on the autotrophic metabolism of the acetogens *Clostridium thermoautotrophicum* and *Clostridium thermoaceticum*. J. Bacteriol. **178:**4597-4603.

13. **Gerhardt, P., R. G. E. Murry, W. A. Wood, and N. R. Krieg.** 1994. p. 31-32. *In* P. Gerhardt, R. G. E. Murry, W. A. Wood, and N. R. Krieg (ed.), Methods for general and molecular bacteriology. American Society for Microbiology, Washington, DC.
14. **Henstra, A. M., and A. J. M. Stams.** 2004. Novel physiological features of *Carboxydothermus hydrogenoformans* and *Thermoterrabacterium ferrireducens*. Appl. Environ. Microbiol. **70:**7236-7240.
15. **Hugenholtz, J., D. M. Ivey, and L. G. Ljungdahl.** 1987. Carbon monoxide-driven electron transport in *Clostridium thermoautotrophicum* membranes. J. Bacteriol. **169:**5845-5847.
16. **Jiang, B., S. N. Parshina, W. van Doesburg, B. P. Lomans, and A. J. M. Stams.** 2005. *Methanomethylovorans thermophila* sp. nov., a thermophilic methylotrophic methanogen from an anaerobic reactor fed with methanol. Int. J. Syst. Evol. Microbiol.**:**Papers in Press, doi:10.1099/ijs.0.63818-0.
17. **Kamlage, B., and M. Blaut.** 1993. Isolation of a cytochrome-deficient mutant strain of *Sporomusa sphaeroides* not capable of oxidizing methyl groups. J. Bacteriol. **175:**3043-3050.
18. **Kellum, R., and H. L. Drake.** 1984. Effects of cultivation gas phase on hydrogenase of the acetogen *Clostridium thermoaceticum*. J. Bacteriol. **160:**466-469.
19. **Kuhner, C. H., C. Frank, A. Griesshammer, M. Schmittroth, G. Acker, A. Gossner, and H. L. Drake.** 1997. *Sporomusa silvacetica* sp, nov., an acetogenic bacterium isolated from aggregated forest soil. Int. J. Syst. Bacteriol. **47:**352-358.
20. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115-175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic Acid Techniques in Bacterial Systematics. John Wiley and Sons, New York.
21. **Ljungdahl, L. G.** 1994. The acetyl-CoA pathway and the chemiosmotic generation of ATP during acetogenesis, p. 63-87. *In* H. L. Drake (ed.), Acetogenesis. Chapman & Hall, New York.
22. **Lundie, L. L., and H. L. Drake.** 1984. Development of a minimally defined medium for the acetogen *Clostridium thermoaceticum*. J. Bacteriol. **159:**700-703.
23. **Martin, D. R., L. L. Lundie, R. Kellum, and H. L. Drake.** 1983. Carbon monoxide-dependent evolution of hydrogen by the homoacetate-fermenting bacterium *Clostridium thermoaceticum*. Curr. Microbiol. **8:**337-340.
24. **Mesbah, M., and W. B. Whitman.** 1989. Measurement of deoxyguanosine/thymidine ratios in complex mixtures by high-performance liquid chromatography for determination of the mole percentage guanine + cytosine of DNA. J. Chromatogr. **479:**297-306.
25. **Muller, V., and G. Gottschalk.** 1994. The sodium ion cycle in acetogenic and methanogenic bacteria: generation and utilization of a primary electrochemical sodium ion gradient, p. 127-156. *In* H. L. Drake (ed.), Acetogenesis. Chapman & Hall, New York.
26. **Pantskhava, E. S., and V. V. Pchelkina.** 1969. Methane fermentation of methanol by a *Methanobacillus kuzneceovii* culture. Prikladnaya Biokhimiya i Mikrobiologiya **5:**416-420.

27. **Paulo, P. L., B. Jiang, D. Cysneiros, A. J. M. Stams, and G. Lettinga.** 2004. Effect of cobalt on the anaerobic thermophilic conversion of methanol. Biotechnol. Bioeng. **85:**434-441.
28. **Paulo, P. L., B. Jiang, K. Roest, J. B. van Lier, and G. Lettinga.** 2002. Start-up of a thermophilic methanol-fed UASB reactor: change in sludge characteristics. Water Sci Technol **45:**145-150.
29. **Pusheva, M. A., and T. G. Sokolova.** 1995. Distribution of CO-dehydrogenase activity in anaerobic thermophilic carboxydotrophic bacterium *Carboxydothermus hydrogenoformans* grown at the expense of CO or pyruvate. Mikrobiologiya **64:**581-586.
30. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406-425.
31. **Savage, M. D., and H. L. Drake.** 1986. Adaptation of the acetogen *Clostridium thermoautotrophicum* to minimal medium. J. Bacteriol. **165:**315-318.
32. **Scholten, J. C., and A. J. M. Stams.** 1995. The effect of sulfate and nitrate on methane formation in a freshwater sediment. Antonie Van Leeuwenhoek **68:**309-315.
33. **Shelver, D., R. L. Kerby, Y. He, and G. P. Roberts.** 1997. CooA, a CO-sensing transcription factor from *Rhodospirillum rubrum*, is a CO-binding heme protein. Proc. Natl. Acad. Sci. U. S. A. **94:**11216-11220.
34. **Slobodkin, A., A. L. Reysenbach, F. Mayer, and J. Wiegel.** 1997. Isolation and characterization of the homoacetogenic thermophilic bacterium *Moorella glycerini* sp. nov. Int. J. Syst. Bacteriol. **47:**969-974.
35. **Soboh, B., D. Linder, and R. Hedderich.** 2002. Purification and catalytic properties of a CO-oxidizing: H_2-evolving enzyme complex from *Carboxydothermus hydrogenoformans*. Eur. J. Biochem. **269:**5712-5721.
36. **Sokolova, T. G., J. M. Gonzalez, N. A. Kostrikina, N. A. Chernyh, T. P. Tourova, C. Kato, E. A. Bonch-Osmolovskaya, and F. T. Robb.** 2001. *Carboxydobrachium pacificum* gen. nov., sp. nov., a new anaerobic, thermophilic, CO-utilizing marine bacterium from Okinawa Trough. Int. J. Syst. Evol. Microbiol. **51:**141-149.
37. **Sokolova, T. G., C. Jeanthon, N. A. Kostrikina, N. A. Chernyh, A. V. Lebedinsky, E. Stackebrandt, and E. A. Bonch-Osmolovskaya.** 2004. The first evidence of anaerobic CO oxidation coupled with H_2 production by a hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent. Extremophiles **8:**317-323.
38. **Sokolova, T. G., N. A. Kostrikina, N. A. Chernyh, T. V. Slepova, E. A. Bonch-Osmolovskaya, J. M. Gonzalez, and F. T. Robb.** 2004. *Thermosinus carboxydivorans* gen. nov., sp. nov., a new anaerobic, thermophilic, carbon-monoxide-oxidizing, hydrogenogenic bacterium from a hot pool of Yellowstone National Park. Int. J. Syst. Evol. Microbiol. **54:**2353-2359.

39. **Sokolova, T. G., N. A. Kostrikina, N. A. Chernyh, T. P. Tourova, T. V. Kolganova, and E. A. Bonch-Osmolovskaya.** 2002. *Carboxydocella thermautotrophica* gen. nov., sp. nov., a novel anaerobic, CO-utilizing thermophile from a kamchatkan hot spring. Int. J. Syst. Evol. Microbiol. **52:**1961-1967.
40. **Stams, A. J. M., J. B. Van Dijk, C. Dijkema, and C. M. Plugge.** 1993. Growth of syntrophic propionate-oxidizing bacteria with fumarate in the absence of methanogenic bacteria. Appl. Environ. Microbiol. **59:**1114-1119.
41. **Strunk, O., and W. Ludwig.** 2000. ARB software package, 2.5b ed. Techniciche Universitat, Munchen, German.
42. **Svetlichny, V. A., T. G. Sokolova, M. Gerhardt, M. Ringpfeil, N. A. Kostrikina, and G. A. Zavarzin.** 1991. *Carboxydothermus hydrogenoformans* gen. nov., sp. nov., a CO-utilizing thermophilic anaerobic bacterium from hydrothermal environments of Kunashir Island. Syst. Appl. Microbiol. **14:**254-260.
43. **Svetlichny, V. A., T. G. Sokolova, N. A. Kostrikina, and A. M. Lysenko.** 1994. *Carboxydothermus restrictus* sp. nov. a new thermophilic anaerobic carboxydotrophic bacterium. Mikrobiologiya **63:**523-528.
44. **Tamaoka, J., and K. Komagata.** 1984. Determination of DNA base composition by reversed-phase high-performance liquid chromatography. FEMS Microbiol. Lett. **25:**125-128.
45. **Trüper, H. G., and H. G. Schlegel.** 1964. Suphur metabolism in Thiorhodaceae. I. Quantitative measurements on growing cells of *Chromatium okenii*. Antonie Van Leeuwenhoek **30:**225-238.
46. **Uffen, R. L.** 1976. Anaerobic growth of a *Rhodopseudomonas* species in the dark with carbon monoxide as sole carbon and energy substrate. Proc. Natl. Acad. Sci. U. S. A. **73:**3298-3302.
47. **Visuvanathan, S., M. T. Moss, J. L. Stanford, J. Hermon-Taylor, and J. J. McFadden.** 1989. Simple enzymic method for isolation of DNA from diverse bacteria. J. Microbiol. Methods **10:**59-64.
48. **Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, M. I. Krichevsky, L. H. Moore, W. E. C. Moore, R. G. E. Murray, E. Stachebrandt, M. P. Starr, and H. G. Truper.** 1987. Report of the *ad hoc* committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. **37:**463-464.
49. **Wiegel, J., M. Braun, and G. Gottschalk.** 1981. *Clostridium thermoautotrophicum* species novum, a thermophile producing acetate from molecular hydrogen and carbon dioxide. Curr. Microbiol. **5:**255-260.
50. **Winters D. K., and L. G. Ljungdahl.** 1989. PQQ-dependent methanol dehydrogenase from *Clostridium thermoautotrophicum*., p. 35-39. *In* J. A. Jongejan and J. A. Duine (ed.), PQQ and Quinoproteins. Kluwer Academic Publisher, Dordrecht.

51. **Zoetendal, E. G., A. D. L. Akkermans, and W. M. De Vos.** 1998. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl. Environ. Microbiol. **64:**3854-3859.

Chapter 4

Syntrophic growth on formate of a coculture of *Moorella hydrogenoformans* and *Methanothermobacter* sp. NJ1

Jan Dolfing, Bo Jiang, Anne Meint Henstra,
Caroline M. Plugge and Alfons J. M. Stams

Prepared for submission

Syntrophic associations of acetogenic bacteria and methanogenic archaea operate at the limits of what is thermodynamically possible[1]. Key is the interspecies transfer of electrons with formate and hydrogen as substrates for the methanogens[2]. Contrary requirements of acetogens and methanogens for growth sustaining conditions keep the formate and hydrogen concentrations low and within a narrow range[3]. A niche for organisms growing on the conversion of formate to hydrogen - or vice versa - is therefore unlikely. Here we report growth on formate by a syntrophic consortium of a *Moorella* species with a hydrogen-consuming *Methanothermobacter* species. In pure culture neither *M. hydrogenoformans* nor the methanogen can grow on formate. The co-culture grows slowly with an estimated doubling time of about 17 days and reaches cell densities of 7.5×10^7 per ml with 60 mM formate. Based on genomic data we hypothesize that the *Moorella* sp. conserves energy from the mineralization of formate via a special formate-hydrogen-lyase system encompassing an energy conserving hydrogenase. These results imply the existence of a previously unrecognized microbial guild.

The possibility that life exists on mars and other extraterrestrial bodies has triggered a search for the limits of life on earth[4-6]. Much attention is thereby paid to the environmental conditions that limit microbial growth and activity, such as high salt concentrations, high pressure, high and low pH, high and low temperatures, and combinations thereof[6-12]. Less attention is being given to the thermodynamic limits of microbial life. These limits are approached in methanogenic environments, where syntrophic associations of acetogenic bacteria and methanogenic archaea obtain energy for growth from catalyzing pathways that operate close to thermodynamic equilibrium ($\Delta G' \sim 0$ kJ/mol.)[1,2]. Methanogenic ecosystems are generally schematized as four different functional groups (or guilds) of bacteria and archaea. Primary fermenters convert complex material into substrates for a second group of fermenters, also known as acetogens, which obligately depend on two groups of methanogens, one that uses hydrogen and formate, and another that uses acetate[2,3]. For thermodynamic reasons growth of the acetogens is sustainable only through the removal of their waste products by the methanogens. Hydrogen is the main electron carrier in such syntrophic associations, but formate is important too, especially in associations where electron fluxes are high[13-15]. It seems logical that formate and hydrogen are in thermodynamic equilibrium[16,17], but this is not always the case. Measurements in a shallow methanogenic aquifer in Denmark have indicated a potential energy gain of 5 to 10 kJ/mol per electron for the conversion of formate to H_2 and bicarbonate[18]. This implies a previously unrecognized niche for organisms that are able to catalyze this reaction.

Cleavage of formate to H_2 and bicarbonate has been described by using a coculture of *Desulvibrio vulgaris* JJ and *Methanobacterium bryantii*[19], but it has never been shown that this can be coupled to growth. Here we describe for the first time that a bacterium is able to grow on the conversion of formate to H_2 and bicarbonate provided that hydrogen is consumed by a methanogen. The bacterium was obtained in a study on the effect of cobalt on methanol conversion in a bioreactor operated at 55^{o}C. In the presence of cobalt a novel methanogen was enriched. In the absence of cobalt methanol consumption was sluggish. Under these conditions a methanol-degrading co-culture was enriched consisting of a *Moorella* species, strain AMP, and a *Methanothermobacter* species, strain NJ1[20]. Pure cultures of these two microorganisms were obtained in media to which cobalt had been added. Based on 16S rRNA sequence analysis strain AMP was closely related to *Moorella thermoacetica* and *Moorella thermoautotrophica*. However, it had the special property of growth on CO with H_2 rather than acetate as end product. This led us to propose a novel species, *Moorella*

hydrogenoformans. Strain NJ1 was a hydrogen utilizing methanogen; its 16S rRNA was 99.5% similar to *Methanothermobacter thermoautotrophicus* strain ΔH.

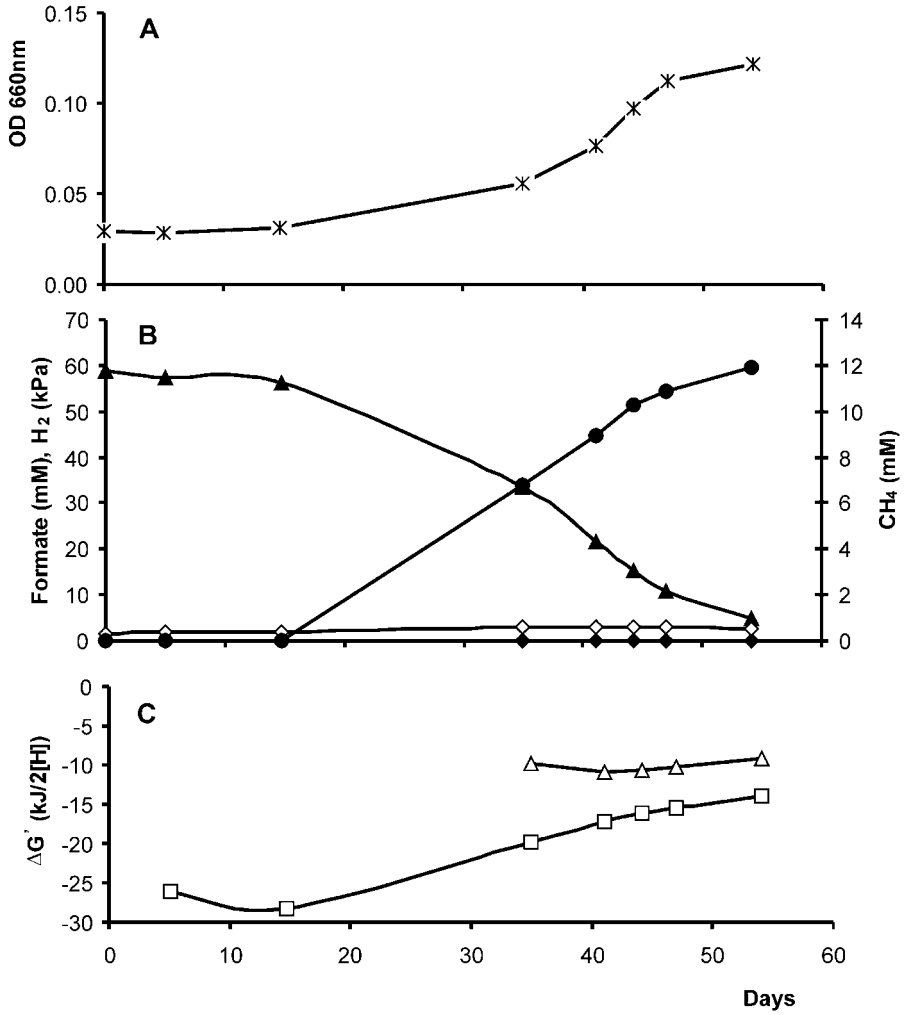
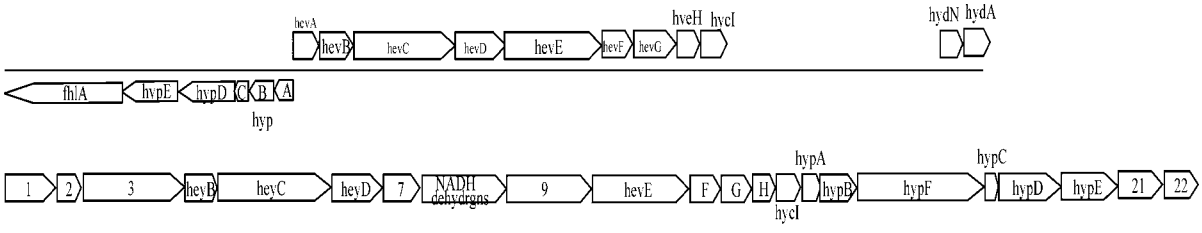


Figure 1 | Syntrophic growth on formate by a co-culture of *Moorella hydrogenoformans* strain AMP and *Methanothermobacter thermoautotrophicus* strain NJ1. Symbols: ▲: formate; ●: methane; ◊: acetate; ♦: H_2 in Panel B; □: Gibbs free energy changes for H_2 formation from formate, and Δ: Gibbs free energy changes for methane formation in Panel C.

M. hydrogenoformans grew on formate when thiosulfate was added as electron acceptor, but did not grow when thiosulfate was replaced by sulfate, nitrate or fumarate[20]. In the absence of thiosulfate, H_2 gradually accumulated to a partial pressure of 2000 Pa. Removal of H_2 from the headspace via flushing again resulted in accumulation of H_2. These observations indicate that accumulation of H_2 was inhibitory to the conversion of formate.

Therefore we tested whether *Methanothermobacter* strain NJ1 could serve as alternative electron acceptor. A co-culture of *M. hydrogenoformans* and *Methanothermobacter* sp. NJ1 grew on formate as sole carbon and energy substrate (Figure 1A and B), while the individual pure cultures did not grow in formate-containing media (data not shown). In the coculture 0.61±0.04 mmol CH_4 was produced by consumption of 2.71±0.13 mmol formate within 60 days, which is consistent with stoichiometric reaction of 4 $HCOO^-$ + H_2O + H^+→ CH_4 + 3HCO_3^-, resulting a total cell number increase from 9±2.8×10^6 to 7.5±1.4×10^7 cells/ml and estimated doubling time of 17±1 days. In the co-culture, hydrogen levels were between 10 and 150 Pa. Under these conditions the Gibbs free energy changes in the coculture ranged between -14 and -28 kJ/mol H_2 for *Moorella* and around –11 kJ/mol H_2 for the methanogen (Figure 1C). Apparently, both microorganisms are metabolizing close to the thermodynamic limits, as found by Jackson & McInerney for syntrophic degradation of butyrate and benzoate[1]. Taken together these data indicate that interspecies hydrogen transfer is essential to sustain growth of the co-culture and that *M. hydrogenoformans* can grow by the conversion of formate to H_2 and bicarbonate when the hydrogen concentration is kept low.

Figure 2 | **Comparative structures of formate hydrogenlyase genes in *E. coli* K12[23] (upper) and *Moorella thermoacetica* (bottom).** Predicted functions of genes in contig302 of draft genome sequence of *Moorella thermoacetica* were obtained using The Integrated Microbial Genomes (IMG) System[27] with permission of US Department of Energy Joint Genome Institute. Genes with no homologues in *E. coli* are predicted as 1: Formate/Nitrate transporter; 2: Nitrate reductate; 3: Molybdopterin oxidoreductase:Molydopterin dinucleotide-binding region; 7: NADH-ubiquinone oxidoreductase, chain 4L; 9: NADH dehydrogenase (quinone); 21: Formate dehydrogenase, subunit FdhD; 22: Molybdopterin-guanine dinucleotide biosynthesis protein A.



M. hydrogenoformans is able to grow in pure culture on carbon monoxide[20]. Carbon monoxide is converted into H_2 and CO_2 in a similar fashion as described for *Carboxydothermus hydrogenoformans* and several other gram-positive bacteria[21]. The energetics of CO conversion (CO + $2H_2O$ → H_2+ HCO_3^- + H^+; $\Delta G^{o'}$= -15.4 kJ at 25°C and

$\Delta G^{o'}$= -12.4 kJ at 65°C) is more favorable than the energetics of formate conversion ($HCOO^-$ + H_2O → H_2 + HCO_3^-; $\Delta G^{o'}$= 1.1 kJ at 25°C and $\Delta G^{o'}$= -1.6 kJ at 65°C). Thus CO can be removed to low concentrations, even if the partial pressure of H_2 is high, especially when the bicarbonate concentration is kept low (Henstra, unpublished results). In *Carboxydothermus hydrogenoformans* there is compelling evidence that the organism conserves energy via a novel carbon-monoxide-hydrogen-lyase. The key for energy conservation is that the hydrogenase part of the enzyme complex is located at the cytoplasmic site of the cell membrane[22]. We propose that *M. hydrogenoformans* conserves energy from the formate-hydrogen-lyase reaction via an analogous enzyme complex with an energy conserving hydrogenase located at the cytoplasmic side of the cell membrane. Support for this hypothesis was obtained via an analysis of the genome of *Moorella thermoacetica*. This is the closest relative of *Moorella hydrogenoformans* with 98 % homology at the 16S rRNA level, and its genome sequence has recently become available (http://www.jgi.doe.gov/). In *M. thermoacetica* the genes encoding for the constituents of the presumed energy conserving formate-hydrogen-lyase complex are all located in one open reading frame with 22 genes (Figure 2). It has high similarity with formate-hydrogen-lyase complex in *E. coli*, which generates energy by production of H_2 from formate oxidation[23]. But, it is never reported that *E. coli* can grow on formate as sole energy source by this life style. Since we could not find similar formate-hydrogen-lyase complex genes in the draft genome of *Desulfovibrio vulgaris*, the mechanism of syntrophic formate oxidation by this organism remains unclear. Based on these findings it would seem prudent to also test *M. thermoacetica* for its ability to grow syntrophically on formate, but this was pointless because *M. thermoacetica* can grow on formate in pure culture by virtue if its ability to convert formate into acetate ($4HCOO^- + H^+$ → $CH_3COO^- + 2HCO_3^-$; $\Delta G^{o'}$= -99.7 kJ at 25°C and $\Delta G^{o'}$= -95.2 kJ at 65°C). Indeed *M. thermoacetica* is the model organism with which the pathway for the formation of acetate from hydrogen and formate was elucidated first.

This research describes the construction of an anaerobic microbial consortium that grows on a simple substrate, which from a biochemical and thermodynamic point of view would not seem to be an obvious substrate for syntrophic growth. In nature formate oxidizers have to compete with methanogens that can directly convert formate to methane, and have more energy available than the organisms that convert formate to hydrogen. The detour via hydrogen calls for the introduction of a new microbial guild consisting of bacteria that sponge

on substrates that can also be used by methanogens. Syntrophic acetate oxidizers are other members of this guild[24]. Anaerobic sediments are the habitats where these organisms find their niche[25].

ACKNOWLEDGEMENTS

These sequence data of *Moorella thermoacetica, Carboxydothermus hydrogenoformans* and *Desulfovibrio vulgaris* were produced by the US Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/) and The Institute for Genomic Research (http://www.tigr.org), respectively.

METHODS

Strains of *Moorella hydrogenoformans* sp. AMP and *Methanothermobacter* sp. NJ1were isolated as described previously[20] and maintained routinely on methanol and H_2/CO_2, respectively. Media and growth conditions were as described previously[20]. For the coculure study, pre-grown pure culture of *Moorella hydrogenoformas* on methanol and *Methanothermobacter* sp. strain NJ1 on H_2/CO_2 were inoculated (10%, inoculate size) into fresh mineral medium with 60 mM formate as sole energy source. Subculture was made after formate was consumed until a stable coculture was obtained. Growth yield was determined by optical density increase and cell number counting with a Burke-Turk counting chamber. Formate and acetate were measured with a HPLC as described elsewhere[26]. Methane and hydrogen were measured by gas chromatograph at the conditions indicated before [21].

Thermodynamic calculations were after Amend and Shock[25] using the Nernst equation to correct Gibbs energy values for the actual temperature, and the Van't Hoff equation to correct the free energy of formation of reactants and products. The comparative analysis of genome sequences were performed using The Integrated Microbial Genomes (IMG) System[27], which is available from the US Department of Energy Joint Genome Institute at website of http://www.jgi.doe.gov.

1. Jackson, B. E. & McInerney, M. J. Anaerobic microbial metabolism can proceed close to thermodynamic limits. **415**, 454-456 (2002).
2. Schink, B. & Stams, A. J. M. in *The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community, 3rd edition, release 3.0.* (Springer Verlag, New York, 2004).
3. Dolfing, J. in *Biological of Anaerobic Microorganisms* (ed. Zehnder, A. J. B.) 417-468 (Wiley-Interscience, New York, 1988).
4. Nealson, K. H. The limits of life on Earth and searching for life on Mars. *J Geophys. Res.* **102**, 23675-23686 (1997).
5. Newman, D. K. & Banfield, J. F. Geomicrobiology: How molecular-scale interactions underpin biogeochemical systems. *Science* **296**, 1071-1077 (2002).
6. Bakermans, C., Souza-Egipsy, V., Nealson, K. H., Tsapin, A. I. & Gilichinsky, D. A. Reproduction and metabolism at -10°C of bacteria isolated from Siberian permafrost. *Environ. Microbiol.* **5**, 321-326 (2003).
7. Walker, J. J., Spear, J. R. & Pace, N. R. Geobiology of a microbiol endolithic community in the Yellowstone geothermal environment. *Nature* **434**, 1011-1014 (2005).
8. Gaidos, E. J., Kirschvink, J. L. & Nealson, K. H. Life in ice-covered oceans. *Science* **284**, 1631-1633 (1999).
9. Navarro-González, R. et al. Mars-Like Soils in the Atacama Desert, Chile, and the Dry Limit of Microbial Life. *Science* **302**, 1018-1021 (2003).
10. Kashefi, K. & Lovley, D. R. Extending the upper temperature limit for life. *Science* **301**, 934 (2003).
11. Kasting, J. F. & Siefert, J. L. Life and the evolution of Earth's atmosphere. *Science* **296**, 1066-1068 (2002).
12. Edwards, K. J., Bond, P. L., Gihring, T. M. & Banfield, J. F. An Archaeal iron-oxidizing extreme acidophile important in acid mine drainage. *Science* **287**, 1796-1799 (2000).
13. Thiele, J. H. & Zeikus, J. G. Control of Interspecies Electron Flow during Anaerobic Digestion: Significance of Formate Transfer versus Hydrogen Transfer during Syntrophic Methanogenesis in Flocs. *Appl. Environ. Microbiol.* **54**, 20-29 (1988).
14. Boone, D. R. & R. L. Johnson, a. Y. L. Diffusion of the Interspecies Electron Carriers H_2 and Formate in Methanogenic Ecosystems and Its Implications in the Measurement of Km for H_2 or Formate Uptake. *Appl. Environ. Microbiol.* **55**, 1735-1741 (1989).
15. de Bok, F. A. M., Plugge, C. M. & Stams, A. J. M. Interspecies electron transfer in methanogenic propionate degrading consortia. *Water Res.* **38**, 1368-1375 (2004).
16. Lueders, T. & Friedrich, M. Archaeal population dynamics during sequential reduction processes in rice field soil. *Appl. Environ. Microbiol.* **66**, 2732-2742 (2000).

17. Wu, W.-M., Hickey, R. F. & Zeikus, J. G. Characterization of metabolic performance of methanogenic granules treating brewery wastewater: Role of sulfate-reducing bacteria. *Appl. Environ. Microbiol.* **57**, 3438-3449 (1991).
18. Hansen, L. K., Jakobsen, R. & Postma, D. Methagnogenesis in a shallow sandy aquifer, Rømø, Denmark. *Geochim. Cosmochim. Acta* **65**, 2925-2935 (2001).
19. Guyot, J.-P. & Brauman, A. Methane production from formate by syntrophic association of *Methanobacterium bryantii* and *Desulfovibrio vulgaris* JJ. *Appl. Environ. Microbiol.* **52**, 1436-1437 (1986).
20. Jiang, B., Paulo, P. L., Henstra, A. M., van Doesburg, W. & Stams, A. J. M. Novel one-carbon metabolism of *Moorella hydrogenoformans* sp. nov., isolated from a methanogenic bioreactor. *Appl. Environ. Microbiol.* (submitted).
21. Henstra, A. M. & Stams, A. J. Novel physiological features of *Carboxydothermus hydrogenoformans* and *Thermoterrabacterium ferrireducens*. *Appl. Environ. Microbiol.* **70**, 7236-40 (2004).
22. Hedderich, R. Energy-converting [NiFe] hydrogenases from archaea and extremophiles: ancestors of complex I. *J Bioenerg. Biomembr.* **36**, 65-75 (2004).
23. Sauter, M., Bohm, R. & Bock, A. Mutational analysis of the operon (hyc) determining hydrogenase 3 formation in *Escherichia coli*. *Mol. Microbiol.* **6**, 1523-32 (1992).
24. Zinder, S. H. & Koch, M. Non-aceticlastic methanogenesis from acetate: Acetate oxidation by a thermophilic syntrophic coculture. *Archives of Microbiology* **138**, 263-272 (1984).
25. Amend, J. P. & Shock, E. L. Energetics of overall metabolic reactions of thermophilic and hyperthermophilic Archaea and Bacteria. *FEMS Microbiol. Rev.* **25**, 175-243 (2001).
26. Stams, A., J. M., Van Dijk Johan, B., Dijkema, C. & Plugge Caroline, M. Growth of syntrophic propionate-oxidizing bacteria with fumarate in the absence of methanogenic bacteria. *Appl. Environ. Microbiol.* **59**, 1114-1119 (1993).
27. Markowitz, V. et al. The Integrated Microbial Genomes (IMG) System. *Nucleic Acids Res.*, special database issue, in press (2006).

Chapter 5

Competitive uptake of cobalt and nickel by *Methanosarcina barkeri* and the effect of competitive uptake on growth on methanol

Summary

The effect of cobalt and nickel on the corrinoid and F430 content and on growth of *Methanosarcina barkeri* on methanol was studied. The cells were grown in media containing 1 mM sulfide and 200 μM EDTA. Thus metal precipitation was kept minimal, enabling direct determination of the cell metal content. Cobalt and nickel limitation was achieved and competition between cobalt and nickel uptake was observed. Uptake efficiency of cobalt was high at low cobalt concentration and decreased when the cobalt concentration in the medium was increased. Corrinoid and F430 content correlated positively with the cell content of the corresponding metal, but incorporation in the corrinoid and F430 was significant less at low cell metal contents, ranging from 35% to 80% for corrinoid and 5% to 15% for F430.

Keywords: Nickel, Cobalt, Methanogenesis, Speciation, Bioavailability, competition uptake.

Introduction

Trace elements, such as cobalt, nickel and iron stimulate anaerobic treatment of different types of wastewater [27,31,35]. Metal availability in wastewater, however, can be severely lowered by precipitation with sulfide [5,17]. On the other hand, metal uptake is also affected antagonistically by other essential metals with similar chemical properties and that are taken up by common transporters. For instance, the uptake of tungsten is antagonistically affected by the presence of molybdenum (or vice versa) in many methanogenic archaea and bacteria [23]. Therefore, for optimal metal dosing, it is important to understand the competition between different metals, as well as the chemical speciation of each individual essential metal.

Anaerobic conversion of methanol containing wastewater is stimulated by the addition of cobalt [2,12,26,28]. Under mesophilic conditions, methanol is mainly degraded by *Methanosarcina*-like methanogens, which convert methanol to methane and CO_2 [11]. The importance of cobalt and nickel for anaerobic treatment is related to the metabolic pathways of anaerobic bacteria and methanogenic archaea [39,41]. Cobalt is present mainly in corrinoid cofactors of methyltransferase that are involved in all kinds of methyl transfer reactions [37,41]. Nickel is present in hydrogenases and carbon monoxide dehydrogenate [15], and in the unique cofactor F430 present in the methyl-Come reductase that catalyzes the last step of methane formation in methanogens [8,20]. It was estimated that more than 90% of the cell cobalt of *Methanosarcina barkeri* [25] and nearly 70% of the cell nickel of *Methanobacterium thermoautotrophicum* are present in the form of corrinoid and F430, respectively [8]. Both cofactors have similar tetrapyrrole structure and are derived from dihydrosiromhydrochlorin [18,40]. The insertion of cobalt in biosynthesis of corrinoid is carried out by a cobaltochelatase, called CbiX [4]. It is still not clear how nickel is inserted, but it is likely that a similar protein as CbiX plays a role [4]. Cobalt and nickel are taken up by certain transporters: a non-specific low affinity magnesium-transporter and a high-affinity specific transporter [10,15]. There is competition between cobalt and nickel [7,10,24]. All uptake studies only focused on metal uptake or relevant enzyme activities, but not on the growth of microorganisms. The aim of the present study is to quantify cobalt and nickel uptake, and to relate that to the cofactor content and methanogenic activity of *Methanosarcina barkeri*.

Material and methods

Methanogen and Medium composition

Methanosarcina barkeri (DSM 800) was obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany).

A bicarbonate buffered mineral medium as described elsewhere [36] was used with following modifications: 1) vitamin B_{12} was omitted; 2) 5 times more trace element solution was added, which contained no iron, cobalt, nickel and EDTA; 3) Ni(II) and Co(II) were added as chloride salts, EDTA was added to a total concentration of 200 μM as indicated in Table 1; 4) Fe(II) was added as $Fe(NH_4)_2(SO_4)_2.6H_2O$ to a final concentration of 50 μM. After preparation and sterilization, the medium was left to equilibrate for at least 24 hours before inoculation. All experiments were carried out in glass serum bottles that were soaked in 4 M HNO_3 and rinsed with de-mineralized water. One-liter bottles containing 300 ml medium and a gas phase of N_2-CO_2 (170kPa, 80:20, vol/vol) were used. Under these conditions the pH was 7.0 ±0.2.

Pure culture studies

Pure culture studies with *M. barkeri* (DSM 800) were carried out using above described media at the conditions indicated in Table 1. Prior to the experiments, three successive subcultures were made in a medium containing 0.1 μM of both nickel and cobalt and 200 μM EDTA. The inoculum size was 10%. Growth was followed by measuring pressure increase in time using a Chrompack Flow-rate-Pressure meter (Chrompack B.V., the Netherlands). Cumulative methane formation was recalculated based on the stoichiometric reaction of methane formation from methanol ($4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$). Growth rate was estimated from the methanol degradation curve, since preliminary experiments showed it correlates with the increase of biomass. In the late exponential phase, cells were harvested by centrifugation (32000 g; 20 minutes) and washed with 0.02 M EDTA (pH 7) and centrifuged again. The respective cell pellets were used for determination of dry weight, cofactor content and cell metal content. All experiments and sample analysis were done in four replicates.

Cofactor content measurement

Corrinoid and factor F430 were analyzed by HPLC according to previous methods [21,38], but with following modifications. An amount of 0.5 g (wet weight) of cell pellet was suspended in phosphate buffer (50 mM, pH 7.5). The cell pellet was re-suspended in acetate buffer (0.5 M, pH 5.0) with 1% KCN. The cofactors were extracted by autoclaving the cell suspension at 121^{o}C for 20 minutes. Cell debris and denatured protein were separated by centrifugation. The cell pellet was resuspended in the same buffer and centrifuged. The resulting supernatants of each sample were pooled and loaded onto C18 reverse phase sep-Pak cartridge (Waters Chromatography, Millipore). The loaded column was washed with distilled water and bound cofactors were eluted by 80% methanol. This fraction was dried by flash evaporation and the residue was dissolved in 1 ml distilled water. HPLC analysis was performed with a Chromosampler C18 column (300 x 3 mm), eluted with a methanol gradient in 0.1% acetate from 20 to 49 % for 20 minutes, 60% for the next 5 minutes and 20% for the last 7 minutes. Corrinoid and factor F_{430} were recorded at 540 nm and 430 nm, respectively.

Metal content analysis

Cell and sludge metal contents were determined after microwave digestion (Milestone Ethos E, Monroe, CT, USA) in a mixture of HNO_3 (65%) and HCl (37%) at volume ratio of 1:3 as described by Zandvoort [42]. Digested samples were diluted 10 times before ICP-MS determination. Metal concentrations were determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Perkin Elmer ELAN6000) according to the manufacture's standard procedure.

Other analytical methods

Methanol concentration was measured by gas chromatography using a Chrompack gas chromatograph (Model CP9000) equipped with Sil5 CB column (25 m x 0.32 mm) and flame ionization detector at 300^{o}C. The column temperature was 50^{o}C and the injection port temperature was 250^{o}C. The carrier gas was N_2 saturated with formic acid. For dry weight determination, cells were resuspended after centrifugation in 2 ml H_2O and transferred to dry aluminum cup of known weight. After drying in a stove at 103^{o}C for 24 hr, the filled cups were left to cool down in an exsiccator and weighted.

Results

Growth of Methanosarcina barkeri

The effect of cobalt on the growth of a pure culture of *M. barkeri* was studied (Fig.1, Table 1). Growth rate of *M. barkeri* increased with cobalt concentration in the medium up to 0.5 μM (batch 1 to 4), in which nickel and EDTA concentration were kept at 5 and 200 μM, respectively. Fastest growth, 0.61±0.02 day^{-1}, was observed at a cobalt concentration of 0.5 μM (batch 4). A slightly lower growth rate was calculated (batch 5) when cobalt concentration was 5 μM. This suggests that an inhibitory effect of cobalt was higher at a concentration of 5 uM. Growth yield, however, was not affected by the cobalt concentrations in the medium, which was averaged around 4.2 g cell dry weight/mol methanol consumed. The somewhat higher yield in batch 7 can be attributed to metal precipitates, since EDTA was not present.

Table 1. Growth rate, growth yield and cell cobalt, cell nickel, corrinoid and F430 content in cells of *M. barkeri* grown in media with varying cobalt, nickel and EDTA concentrations.

Batch	Media (μM)			Growth rate (/day)	Yield (g DW/mol methanol)	Concentration (nmol/g cell dry weight)			
	Co^{2+}	Ni^{2+}	EDTA			Cell Co^{2+}	Cell Ni^{2+}	Corrinoid	F430
1	0.02	5	200	0.14±0.04	4.14±0.09	58±3	3759±238	14±5	112±45
2	0.05	5	200	0.26±0.01	4.66±0.48	91±7	4667±156	58±9	174±15
3	0.1	5	200	0.35±0.06	4.26±0.16	124±5	3301±1.2	60±12	194±9
4	0.5	5	200	0.61±0.02	4.05±0.04	473±27	1696±125	403±43	223±28
5	5	5	200	0.56±0.01	3.99±0.15	961±56	701±32	859±51	100±14
6	5	0.05	200	0.28±0.02	4.00±0.38	2082±100	115±4	1998±135	5.4±0.7
7	5	5	0	0.52±0.01	4.84±0.29	4486±410	4496±452	242±24	43±6
8	0.1	0.1	200	n.m.	n.m.	n.m.	n.m.	65±10	18±2

n.m.: not measured.
DW: dry weight
Data and standard deviation were from four replicate experiments.

Lowering the nickel concentration from 5 μM to 0.05 μM (Fig. 1 and Table 1) decreased the growth rate by a factor 2 (from 0.56 day^{-1} to 0.28 day^{-1}). However, the growth yield was still the same as for the other cultures.

Cobalt and nickel content in M. barkeri

Cobalt and nickel content in the cells of *M. barkeri* grown on methanol were measured to assess the growth-dependent uptake of cobalt (Table 1). Cell cobalt content increased from 58±3 to 473±27 nmol/g cell dry weight when increasing the cobalt concentration from 0.02 to 0.5 μM. A further increase of the cobalt concentration from 0.5 μM to 5 μM, resulted in a doubled cell cobalt content (from 473±27 to 961±56 nmol/g cell dry weight). On the other hand, cell nickel content decreased substantially with the increase of cobalt concentration in the medium with one exception (Batch 2).

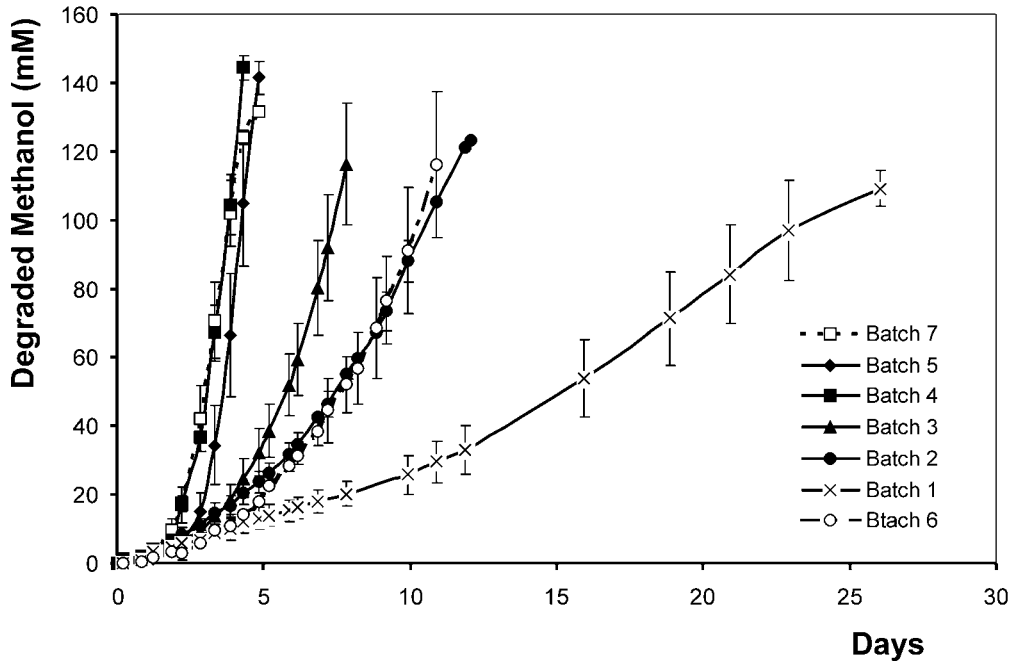


Fig. 1 Methanol degradation by *M. barkeri* in the medium with varying cobalt, nickel and EDTA concentrations. Growth conditions were as stated in Table 1. Data and standard deviation were from four replicate experiments.

Cells of *M. barkeri* grown at a nickel concentration of 0.05 μM and 5 μM cobalt (Batch 6) had a remarkably high cobalt content of 2082±100 nmol /g cell dry weight. This value was twice as that in the cells grown with both 5 μM nickel and cobalt added to the medium. By contrast, cells grown under these conditions had the lowest nickel content, which was only 115±4 nmol /g cell dry weight.

To elucidate the uptake efficiency, the proportion of cobalt or nickel recovered inside cells of *M. barkeri* to the total cobalt or nickel present in the medium was calculated (Fig 2). Almost 80% of the total amount of cobalt was taken up by the cells at cobalt concentrations below 0.05 μM. Upon a further increase of the cobalt in the media, cobalt uptake efficiency

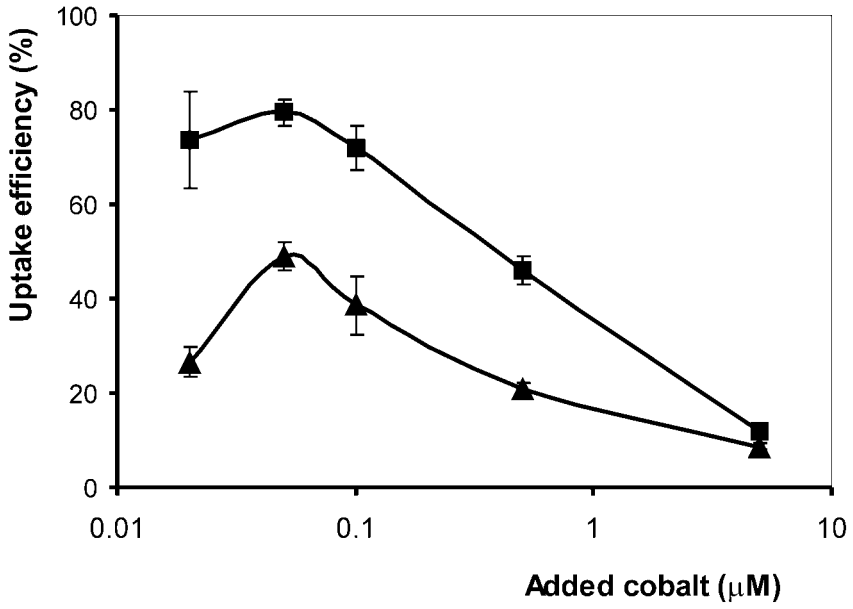


Fig. 2 Cobalt (■)and nickel (▲) uptake efficiency of *M. barkeri* grown in media with varying cobalt concentrations.

decreased significantly from 80% to 12% (at cobalt concentration of 5 μM). Nickel uptake efficiency had doubled when the cobalt concentration increased from 0.02 to 0.05 μM in the media. A decreased nickel uptake efficiency was observed with further increased cobalt concentration, although the nickel concentration was kept at 5 μM. When both cobalt and nickel concentrations were at the same concentration of 5 μM, cobalt and nickel uptake efficiencies were similar (12% for cobalt and 8.6% for nickel). Cells grown under nickel limited conditions took up almost all nickel added to the medium (Batch 6). A doubled cobalt uptake efficiency under this condition was also observed in comparison with the medium in which the nickel concentration was 5 μM (24% vs 12%).

Cell corrinoid and F430 contents in M. barkeri

The corrinoid content increased with increasing cobalt concentration in the media thus following the pattern of the cell cobalt content. Remarkably, F430 content increased also with the increase of cobalt concentration up to 0.5 μM. However, at a cobalt concentration of 5 μM, F430 content was less than 50% of its maximum measured at 0.5 μM cobalt (Table 1).

The highest corrinoid content, 1998±135 nmol/g cell dry weight, was measured in cells grown in the media with 0.05 μM nickel (batch 6). This value was twice as high as in cells grown with 5 μM nickel and 5 μM cobalt (batch 5, 859±51 nmol/g cell dry weight). F430 content in these cells, however, was more than 18 times lower compared with corresponding cells in batch 5 (5.4±0.7 vs 100±14 nmol/g cell dry weight).

Fig. 3 depicts the ratio of cell metals (cobalt and nickel) that was present in the form of corrinoid and F430, respectively. It is clear that a relatively higher amount of the cobalt and nickel that was taken up by cells of M. barkeri was incorporated into corrinoid and F430,

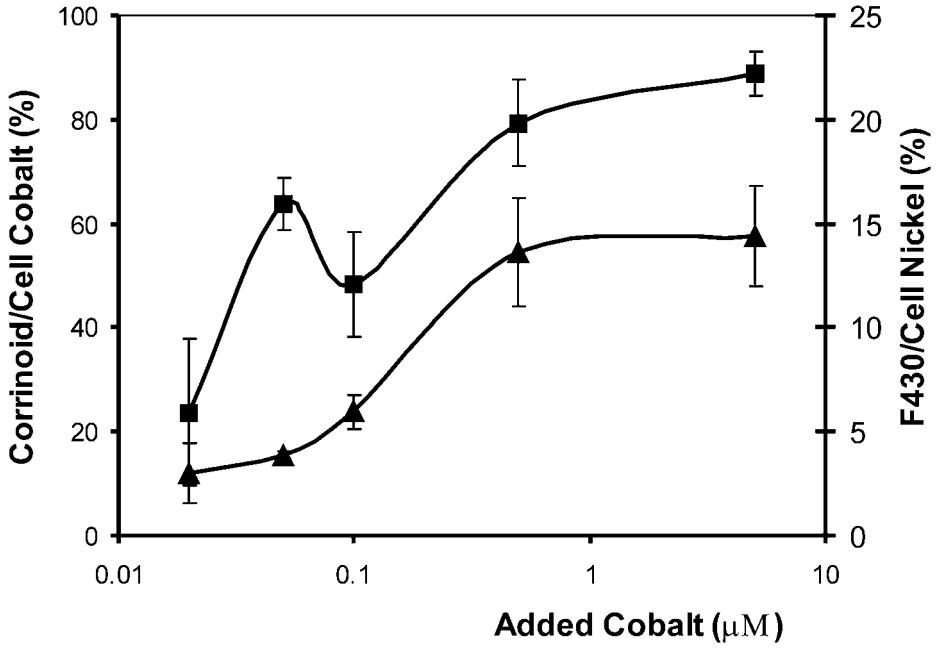


Fig. 3 Ratio of corrinoid-cobalt to cell cobalt content (■) and F430-nickel to cell nickel content (▲) in *M. barkeri* grown in media of varying cobalt concentrations.

respectively, when the cobalt concentration increased in the medium. More than 85% of the cobalt was present as corrinoid which may reflect that cobalt is only present in corrinoid in anaerobes [16]. On the other hand, the maximal F430-nickel was 15% of the nickel that had been taken up. This value was observed at 5 μM nickel and 5 μM cobalt. Nickel is not only present in F430, but also in other enzymes, like hydrogenase and carbon monoxide dehydrogenase [15,16].

Discussion

M. barkeri grew at a similar rate in the presence or the absence of EDTA (Batch 5 and 7). This indicates that such an EDTA concentration is not toxic for this microorganism. The optimal cobalt concentration was around 0.5 μM, which is consistent with what was found before [12]. The increased growth rate could be related to an increased free cobalt concentration as was found in another study [19]. This stimulated the uptake of cobalt, which resulted in higher cell cobalt content, higher corrinoid content and faster growth.

A slightly inhibitory effect was observed at cobalt concentration of 5 μM, which is far lower than 0.8 mM, which was found to inhibit methanogenesis [2]. In that study, no EDTA was present. Obviously, the presence of EDTA in our study as metal chelating compound increased metal bioavailability, therefore inhibitory cobalt concentration became lower. Metal ligands, such as humic substances, EDTA, NTA and citric acid, have been proven to increase the bio-availability of trace metals, and thus stimulate growth of microorganisms [3,29] and the performance of methanogenic bioreactors [14]. Yeast extract, besides its role as source of vitamins, improves metal availability, likely due to the presence of complexing compounds [13]. Our results of corrinoid and F430 contents in cells grown in the absence of EDTA were around three times lower than those grown in the presence of EDTA, although it had little effect on the growth rate of *M. barkeri* on methanol (Batch 5 vs Batch 7 in Table 1). This result clearly showed that bioavailablity of cobalt and nickel is influenced by the presence of EDTA.

Cell cobalt and nickel contents in cells of *M. barkeri* grown in the absence of EDTA likely were overestimated, due to co-sedimentation of cells and metal precipitate (Batch 7,

Table1). In the presence of EDTA, cobalt and nickel were present in soluble EDTA complexes, thus preventing metal sulfide precipitation that may bias the metal content analysis. Reduction of the sulfide concentration in the medium to 0.3 mM to avoid metal precipitation allowed a better analysis of cell cobalt and nickel contents in methanogenic archaea [30]; 0.17-2 μmol cobalt/g cell dry weight and 1.1-3 μmol nickel/g cell dry weight were determined in different methanogenic species grown on different substrates [30]. Cells of *M. barkeri* grown at similar cobalt and nickel concentrations in the presence of EDTA in our study (Batch 5) had comparable cobalt and nickel contents as found in the above study.

Cells of *M. barkeri* grew slowly at cobalt concentrations below 0.1 μM. They also had profoundly lower cell cobalt and corrinoid contents. Apparently, they were limited by the availability and uptake of cobalt. Little is known about cobalt and nickel transport systems in methanogens. An uptake system for nickel was described in *Methanothrix concilii*, which has an affinity of K_m = 91 μM and uptake capacity of 23 nmol/min/mg dry weight [1]**.** *Methanobacterium bryantii* transported nickel against a concentration gradient by a high-affinity system (K_m = 3.1 μM) [20]. Magnesium, calcium, or manganese had little influence on nickel uptake in this methanogen; cobalt, however, did compete when its concentration was 10 times higher than nickel [1,20]. The best studied specific cobalt and nickel transporters were in *E. coli* and other microorganisms [10]. One of the transporters, Nik, is an ABC transporter with relative high affinity (K_m < 0.1 μM) [6]. Another one, HoxN, is an integral membrane permease with extremely high affinity (K_m = 10 nM) but a low capacity of 1.5 pmol Ni^{2+}/min/mg protein [7]. Both nickel transporters were found to be also involved in cobalt uptake [10]. Specific high affinity cobalt permeases were found in *Rhodococcus rhodochrous* [24] and *Schizosaccharomyces pombe* [9]. Cobalt uptake activity is inhibited by the presence of nickel, although other metals such as manganese, iron and copper had no effect [9,24]. Our findings that cell nickel content decreased with increasing cobalt concentration in the medium and that cell cobalt increased with decreasing nickel concentration in the medium suggest that such competition of uptake of cobalt and nickel also occurs in *M. barkeri*. We speculate that cobalt is transported by a high affinity and low capacity transporter at low cobalt concentration (below 0.1 μM) and by another lower affinity but higher capacity transporter at high cobalt concentration (above 0.1 μM). The slower growth rate observed at lower cobalt concentration seemed to be caused by this lower capacity transporter. Since such a transporter has a high affinity, it is still operational at an extremely

low concentration. Therefore, the cobalt uptake efficiency is much higher (up to 80%, Fig.2) at lower cobalt concentrations than at high cobalt concentrations. It is also a reflection of the regulation of transcription of different transporter genes under cobalt limited conditions, in order to fulfill its high cobalt requirement, as was found for the Nik transporter [6]. Uptake efficiency decreased at high cobalt concentration, and the similar values (12% for cobalt and 8.6% for nickel) at 5 μM cobalt and nickel might indicate that they share the same transporter(s).

Corrinoid and F430 are important cofactors in methanogens [39]. *M. barkeri* has been studied as a source for Vitamin B12 production using methanol as substrate [25,32-34]. A corrinoid of 8.5 mg/l was produced at 40 μM cobalt and 250 mM methanol [34], which is just twice as much as achieved at 5 μM cobalt and 200 mM methanol. It is not known yet why *M. barkeri* synthesizes so much corrinoids, but corrinoid content in the cells had a clear relation with growth rates when cobalt is the growth-limiting factor (Table 1). This is in accordance with previous findings that both the methanogenic activity and the corrinoid content increased by the addition of cobalt (8.4 μM), resulting in a fast growth rate of 0.6 /day [22]. Therefore, corrinoid and F430 content can be an indicator of metal limitation of bioreactors. It was found that both performance of a lab-scale reactor and the specific methanogenic activity of the sludge from this reactor on methanol increased significantly even after 44 days bioreactor operation on methanol without the addition of both cobalt and nickel [43]. Cofactor content analysis in the sludge samples indicated that the corrinoid and the F430 content increased 8 times and 1.5 times, although the sludge severely loses its cobalt and nickel content [43]. Metal content data showed this sludge had similar cobalt and nickel content [43]. The other sludge used in the same study under same conditions, which showed decrease of methanogenic activity and both cofactor content after 50 days operation on methanol, had much higher (4 times) nickel content than cobalt content. We speculate that competitive uptake of cobalt and nickel could be one of the reasons for the different responses of these two reactors. Therefore, in practice, it is also important to pay attention to the possibility of one metal at high concentration might suppress the uptake of another in optimization of trace metal dosing to bioreactors.

Acknowledgments

We would like to thank S. W. Kengen for providing the F430 standards and kind assistance in the analysis of corrinoid and F430 and M. H. Zandvoort for providing the sludge samples. This work was supported by the Netherlands Technology Foundation STW, applied science division of NWO and the technology program of the Ministry of Economic Affaires of the Dutch government, under the project WWL4928.

References

1. Baudet, C., Sprott, G.D. and Patel, G.B. (1988) Adsorption and uptake of nickel in *Methanothrix-concilii*. Arch Microbiol 1988; 150, 338-342.
2. Bhattacharya, S.K., Uberoi, V., Madura, R.L. and Haghighi-Podeh, M.R. (1995) Effect of cobalt on methanogenesis. Environ Technol 16, 271-278.
3. Bretler, G. and Marison Ian, W. (1996) A medium optimization strategy for improvement of growth and methane production by *Methanobacterium thermoautotrophicum*. J Biotechnol 50, 201-212.
4. Brindley, A.A., Raux, E., Leech, H.K., Schubert, H.L. and Warren, M.J. (2003) A story of chelatase evolution: identification and characterization of a small 13-15-kDa "ancestral" cobaltochelatase (CbiXS) in the archaea. J Biol Chem 278, 22388-95.
5. Callander, I.J. and Barford, J.P. (1983) Precipatation, chelation, andthe avilability of methals as nutrients in anaerobic digestion. I. Methodology. Biotechnol Bioeng 25, 1947-1957.
6. de Pina, K., Navarro, C., McWalter, L., Boxer, D.H., Price, N.C., Kelly, S.M., Mandrand-Berthelot, M.A. and Wu, L.F. (1995) Purification and characterization of the periplasmic nickel-binding protein NikA of *Escherichia coli* K12. Environ Technol 227, 857-65.
7. Degen, O., Kobayashi, M., Shimizu, S. and Eitinger, T. (1999) Selective transport of divalent cations by transition metal permeases: the *Alcaligenes eutrophus* HoxN and the *Rhodococcus rhodochrous* NhlF. Arch Microbiol 171, 139-45.
8. Diekert, G., Klee, B. and Thauer, R.K. (1980) Nickel, a component of factor F430 from *Methanobacterium thermoautotrophicum*. Arch Microbiol 124, 103-6.
9. Eitinger, T., Degen, O., Bohnke, U. and Muller, M. (2000) Nic1p, a relative of bacterial transition metal permeases in *Schizosaccharomyces* pombe, provides nickel ion for urease biosynthesis. J Biol Chem 275, 18029-33.
10. Eitinger, T. and Mandrand-Berthelot, M.A. (2000) Nickel transport systems in microorganisms. Arch Microbiol 173, 1-9.

11. Florencio, L., Field, J.A. and Lettinga, G. (1994) Importance of cobalt for individual trophic groups in an anaerobic methanol-degrading consortium. Appl Environ Microbiol 60, 227-34.
12. Florencio, L., Jenicek, P., Field, J.A. and Lettinga, G. (1993) Effect of cobalt on the anaerobic degradation of methanol. J Ferment Bioeng 75, 368-374.
13. Gonzalez-Gil, G., Jansen, S., Zandvoort, M.H. and van Leeuwen, H.P. (2003) Effect of yeast extract on speciation and bioavailability of nickel and cobalt in anaerobic bioreactors. Biotechnol Bioeng 82, 134-42.
14. Hartung, H.A. (1992) Stimulation of anaerobic digestion with peat humic substance. Sci Total Environ 113, 17-33.
15. Hausinger, R.P. (1987) Nickel utilization by microorganisms. Microb Rev 51, 22-42.
16. Holliger, C., Schraa, G., Stupperich, E., Stams, A.J. and Zehnder, A.J. (1992) Evidence for the involvement of corrinoids and factor F430 in the reductive dechlorination of 1,2-dichloroethane by *Methanosarcina barkeri*. J Bacteriol 174, 4427-34.
17. Hughes, M.N. and Poole, R.K. (1991) Metal speciation and microbial growth - The hard (and soft) facts. J Gen Microbiol 137, 725-734.
18. Hungerer, C., Weiss, D.S., Thauer, R.K. and Jahn, D. (1996) The hemA gene encoding glutamyl-tRNA reductase from the archaeon *Methanobacterium thermoautotrophicum* strain Marburg. Bioorg Med Chem 4, 1089-95.
19. Jansen, S. (2004) The impact of Co and Ni speciation on methanogenesis in sulfidic media. Speciation and bioavailability of cobalt and nickel in anaerobic wastewater treatment. PhD Thesis. Wageningen University. Wageningen, the Netherlands.
20. Jarrell, K.F. and Sprott, G.D. (1982) Nickel transport in *Methanobacterium bryantii*. J Bacteriol 151, 1195-203.
21. Kengen, S.W., Daas, P.J., Duits, E.F., Keltjens, J.T., van der Drift, C. and Vogels, G.D. (1992) Isolation of a 5-hydroxybenzimidazolyl cobamide-containing enzyme involved in the methyltetrahydromethanopterin: coenzyme M methyltransferase reaction in *Methanobacterium thermoautotrophicum*. Biochim Biophys Acta 1118, 249-60.
22. Kida, K., Shigematsu, T., Kijima, J., Numaguchi, M., Mochinaga, Y., Abe, N. and Morimura, S. (2001) Influence of Ni^{2+} and Co^{2+} on methanogenic activity and the amounts of coenzymes involved in methanogenesis. J Biosci Bioeng 91, 590-5.
23. Kletzin, A. and Adams, M.W.W. (1996) Tungsten in biological systems. Environ Technol 18, 5-63.
24. Komeda, H., Kobayashi, M. and Shimizu, S. (1997) A novel transporter involved in cobalt uptake. PNAS 94, 36-41.
25. Mazumder, T.K., Nishio, N., Fukuzki, S. and Nagai, S. (1987) Production of extracellular vitamin B12 compounds from methanol by *Methanosarcina barkeri*. Appl Microbiol Biotechnol 26, 511-516.

26. Patidar, S.K. and Tare, V. (2004) Influence of nutrients on biomass evolution in an upflow anaerobic sludge blanket reactor degrading sulfate-laden organics. Water Environ Res 76, 2620-2627.
27. Puhakka, J.A., Salkinoja-Salonen, M., Ferguson, J.F. and Benjamin, M.M. (1990) Effect of molybdate ions on methanation of simulated and natural waste-waters. Appl Microbiol Biotechnol 32, 494-498.
28. Ram, M.S., Singh, L., Suryanarayana, M.V.S. and Alam, S.I. (2000) Effect of iron, nickel and cobalt on bacterial activity and dynamics during anaerobic oxidation of organic matter. Water Air Soil Pollut 117, 305-312.
29. Reid, G., Soboh, F., Bruce, A.W. and Mittelman, M. (1998) Effect of nutrient composition on the in vitro growth of urogenital lactobacilli and uropathogens. Can J Microbiol 44, 866-871.
30. Scherer, P., Lippert, H. and Wolff, G. (1983) Composition of the majar elements and trace elements of 10 methanogenic bacteria determined by inductive coupled plasma emission spectrometry. Biol Trace Elem Res 5, 183-163.
31. Shen, C.F., Kosaric, N. and Blaszczyk, R. (1993) The effect of selected heavy metals (Ni, Co and Fe) on anaerobic granules and their extracellular polymeric substance (EPS). Water Res 27, 25-33.
32. Silveira, R.G., Kakizono, T., Takemoto, S., Nishio, N. and Nagai, S. (1991) Medium optimization by an orthogonal array design for the growth of *Methanosarcina barkeri*. J Ferment Bioeng 72, 20-25.
33. Silveira, R.G., Nishida, Y., Nishio, N. and Nagai, S. (1990) Corrinoid production with *Methanosarcina barkeri* in a repeated fed-batch reactor with membrane module. Biotechnol Lett 12, 721-726.
34. Silveira, R.G., Nishio, N. and Nagai, S. (1991) Growth characteristics and corrinoid production of *Methanosarcina barkeri* on methanol-acetate medium. J Ferment Bioeng 71, 28-34.
35. Speece, R.E., Parkin, G.F. and Gallagher, D. (1983) Nickel stimulation of anaerobic digestion. Water Res 17, 677-683.
36. Stams, A., J. M., Van Dijk Johan, B., Dijkema, C. and Plugge Caroline, M. (1993) Growth of syntrophic propionate-oxidizing bacteria with fumarate in the absence of methanogenic bacteria. Appl Environ Microbiol 59, 1114-1119.
37. Stupperich, E. (1993) Recent advances in elucidation of biological corrinoid functions. Environ Technol 12, 349-65.
38. Stupperich, E., Steiner, I. and Ruhlemann, M. (1986) Isolation and analysis of bacterial cobamides by high-performance liquid chromatography. Anal Biochem 155, 365-70.
39. Thauer, R.K. (1998) Biochemistry of methanogenesis: a tribute to Marjory Stephenson. 1998 Marjory Stephenson Prize Lecture. Microbiology 144 (Pt 9), 2377-406.
40. Thauer, R.K. and Bonacker, L.G. (1994) Biosynthesis of coenzyme F430, a nickel porphinoid involved in methanogenesis. Ciba Found Symp 180, 210-22; discussion 222-7.

41. van der Meijden, P., van der Drift, C. and Vogels, G.D. (1984) Methanol conversion in *Eubacterium limosum*. Arch Microbiol 138, 360-364.
42. Zandvoort, M.H., Gieteling, J., Lettinga, G. and Lens, P.N. (2004) Stimulation of methanol degradation in UASB reactors: in situ versus pre-loading cobalt on anaerobic granular sludge. Biotechnol Bioeng 87, 897-904.
43. Zandvoort, M.H., van Hullebusch, E.D., Peerbolte, A., Golubnic, S., Lettinga, G. and Lens, P.N. (2005) Influence of pH shocks on trace metal dynamics and performance of methanol fed granular sludge bioreactors. Biodegradation 16, 549-67.

Chapter 6

Effect of tungsten and molybdenum on growth of a syntrophic coculture of *Syntrophobacter fumaroxidans* and *Methanospirillum hungatei*

Bo Jiang, Frank A. M. de Bok, Chingling Tsai,
Caroline M. Plugge and Alfons J. M. Stams

Submitted to: *Archives of Microbiology*

Summary

The trace elements tungsten and molybdenum play an essential role in the growth of anaerobic microorganisms. Depletion of tungsten and/or molybdenum in the media did not affect axenic growth of *Syntrophobacter fumaroxidans* on propionate + fumarate, indicating under these conditions this organism does not have a high tungsten or molybdenum requirement. However, growth of *Methanospirillum hungatei* on either formate or hydrogen and carbon dioxide required tungsten, and molybdenum can replace tungsten to some extent. Growth of the *Syntrophobacter-Methanospirillum* coculture on propionate is significantly affected by the addition of these two metals. Measurement of enzyme levels in cell extracts of syntrophically grown cells indicated that the levels of hydrogenase and formate dehydrogenase activity were correlated with the methane formation rates by the cocultures, which suggests both hydrogen and formate play important role in syntrophic propionate oxidation.

Keywords: *Syntrophobacter fumaroxidans*; *Methanospirillum hungate*; Propionate oxidation; Tungsten; Molybdenum; Syntrophy.

Introduction

Tungsten (W) and molybdenum (Mo) are essential trace elements for growth of many anaerobic microorganisms (Kletzin and Adams 1996). Several redox-enzymes depend on these metals in obligate anaerobes. Examples of enzymes which contain either W or Mo are: formate dehydrogenase (FDH), which is required for energy conservation and carbon assimilation in anaerobic bacteria and archaea (Ljungdahl and Andreesen 1978; Ferry 1999), formylmethanofuran dehydrogenase (FMDH), which reduces carbon dioxide (CO_2) and couples the formyl group to methanofuran in methanogens (Hochheimer et al. 1998), a variety of aldehyde oxidizing enzymes and acetylene hydratase (Kletzin and Adams 1996). Since the chemical and catalytic properties of these two metals are very similar, some enzymes contain either Mo or W, and some microorganisms even contain analogues of enzymes containing either W or Mo, depending on the growth conditions (Bertram et al. 1994; Hochheimer et al. 1998; Stewart et al. 2000; Brondino et al. 2004). However, most microorganisms just possess one type of enzyme, in which the W and Mo can not effectively replace each other (Jones and Stadtman 1977; Van Bruggen et al. 1986; Zindel et al. 1988; Afshar et al. 1998), and in some cases, they even have antagonistic effects (Ljungdahl and Andreesen 1978; Zellner and Winter 1987; May et al. 1988). It was hypothesized that mesophilic microorganisms prefer to incorporate Mo into their enzymes, while thermophilic microorganisms prefer W (Kletzin and Adams 1996).

We were interested in the effect of W and Mo on growth of *Syntrophobacter fumaroxidans*. This anaerobic bacterium oxidizes propionate via the methyl-malonyl-CoA pathway and is able to dispose of electrons by either fumarate or sulfate reduction (Plugge et al. 1993; Stams et al. 1993; Harmsen et al. 1998). Without these electron acceptors, *S. fumaroxidans* reduces protons to hydrogen (H_2) and bicarbonate to formate by means of hydrogenase (H_2ase) and FDH (Stams and Dong 1995). H_2 and formate formation coupled to propionate oxidation are endergonic under standard conditions. Therefore, the levels of H_2 and/or formate have to be kept low in order to make the conversion energetically feasible (Stams 1994; Schink 1997). Low H_2 and formate levels are created by methanogenic partners that grow on H_2 and CO_2 and/or formate. Interspecies electron transfer in syntrophic propionate oxidizing consortia has been studied, but it is still unclear whether hydrogen or formate is the preferred electron carrier (Stams 1994; Schink 1997; de Bok et al. 2004). *Syntrophobacter* species only oxidize propionate together with methanogens that utilize both

H_2 and formate, such as *Methanospirillum hungatei* and *Methanobacterium formicicum*, not with *Methanobrevibacter* strains that only utilize H_2 (Dong et al. 1994). Therefore, besides H_2, formate was also considered to play a role in syntrophic propionate oxidation (Thiele and Zeikus 1988; Boone et al. 1989; Stams and Dong 1995). Biochemical evidence was obtained as well (Dong and Stams 1995; de Bok et al. 2002a). Both microorganisms possess H_2ases and FDHs, of which the expression levels are much higher during syntrophic growth on propionate compared to the pure cultures (de Bok et al. 2002a), and two W-containing FDHs were purified from *S. fumaroxidans* (de Bok et al. 2003). We hypothesized that when interspecies formate-transfer is essential, syntrophic growth of the *Syntrophobacter-Methanospirillum* coculture should be affected by the level of W and/or Mo. In this study we investigated the importance of W and Mo for syntrophic and axenic growth of *S. fumaroxidans* and *M. hungatei*, and determined the FDH and H_2ase levels in cell extracts.

Materials and methods

Growth conditions

Syntrophobacter fumaroxidans (DSM10017) and *Methanospirillum hungatei* (DSM864) were routinely cultured at 37°C in 120-ml serum flasks with 50 ml of medium and a gas phase of 172 KPa N_2/CO_2 or H_2/CO_2 (80:20, vol/vol) as described previously (Stams et al. 1993). W and Mo were added as sodium tungstate and sodium molybdate. Propionate, fumarate and formate (all sodium salts) were added from 1 M sterile stock solutions as indicated in the text. *M. hungatei* cultures were amended with 2 mM of acetate as a carbon source, and these cultures were shaken at 150 rpm to prevent gas diffusion limitation. To minimize metal contamination, glassware was soaked in a 4 M nitric acid solution for three days; butyl rubber stoppers were soaked overnight and washed thoroughly with nano-pure water before use. All solutions and media were prepared with ultra-pure water. All chemicals and vitamins were of the highest grade.

Cell separation and fractionation

The cells were harvested in the late exponential phase by centrifugation under anoxic conditions as described previously (de Bok et al. 2002b). Syntrophically grown cells of *S.*

fumaroxidans and *M. hungatei* were separated by Percoll gradient centrifugation as described before (de Bok et al. 2002b). Separated cells were suspended in buffer A (50mM Tris-HCl, pH 8.0, 100 μM sodium dithionite). Cell free extract was obtained by sonication and cell debris was removed by centrifugation at 16,000 × *g* (de Bok et al. 2002b).

Enzyme activities

Enzyme activities were routinely measured at 37°C in N_2-flushed or H_2-flushed cuvettes containing 1 ml of 50 mM Tris-HCl and closed with butyl-rubber stoppers. Formate and H_2 oxidation rates were recorded at 578 nm as described by others (Boonstra et al. 1975; Odom and Peck 1981), with 1 mM benzyl viologen (BV^+, ε = 8.65 mM^{-1} cm^{-1}) as electron acceptor. Protein was determined according to Bradford with bovine serum albumin as a standard (Bradford 1976).

Analytical methods

Growth was followed by determination of the optical density at 660 nm or by following the methane production. It was assumed that methane formation is linearly correlated to growth. CH_4 and H_2 were determined on a Chrompack 9001 gas chromatograph with a PlotFused silica column (30 m by 0.53 mm) packed with Molsieve 5A (DF=15 μm). The temperature of the column, injection port and thermal conductivity detector were 50, 60 and 130ºC, respectively. Argon was used as carrier gas (20 ml min^{-1}). Metal content of the medium was measured by inductively coupled plasma mass spectrometry (ICP-MS, Elan 6000, Perkin-Elmer).

Results and discussion

Effects of W and Mo on the axenic growth of S. fumaroxidans

S. fumaroxidans is able to ferment fumarate, and it can also couple the oxidation of propionate, H_2 or formate to reduction of fumarate or sulfate (Stams et al. 1993; Harmsen et al. 1998). FDH and H_2ase are expressed constitutively on all these substrates and substrate combinations (de Bok et al. 2002b). A culture of *S. fumaroxidans* growing on propionate (20 mM) and fumarate (60 mM) was used to study the effects of W and Mo on the axenic growth

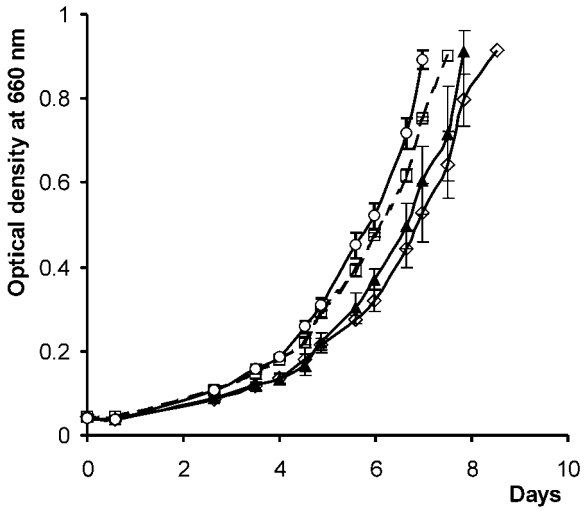


Fig. 1 Axenic growth of *S. fumaroxidans* on propionate (20mM) and fumarate (60mM) after six transfers in medium with W and Mo (▲), W only (○), Mo only (◊) or without W and Mo (□). Data are the mean of duplicate measurements.

of *S. fumaroxidans*. The strain was adapted to each condition by routinely subculturing in fresh medium for six transfers. Growth rates and maximal optical densities were not affected by the absence or presence of W and/or Mo (Table 1, Fig. 1). The average growth rate in each of the different media was around 0.21 day^{-1}, which is in the same range as observed previously (Stams et al. 1993). ICP-MS analysis of our basal medium without W and Mo revealed that the residual W and Mo concentrations were below 55 pM and 10 pM, respectively, indicating that chemical contamination as a source of W or Mo in our basal medium was kept to a minimum. Depletion of W to such level, completely inhibited growth of *Pyrobaculum aerophilum* (Afshar et al. 1998). Therefore, our results suggest that *S. fumaroxidans* does not require W and Mo for axenic growth on propionate and fumarate.

Eubacterium acidaminophilum (Granderath 1993). However, unlike these two microorganisms, the FDH level in *S. fumaroxidans* was significantly lower in medium with both W and Mo, as compared to medium with only W. Similar results were obtained for the W- and Mo- containing FDHs in *Desulfovibrio alaskensis* (Brondino et al. 2004) and dimethyl sulfoxide reductase (DMSO) in *Rhodobacter capsulatus* (Stewart et al. 2000). The explanation for this is that although Mo and W can be used by *D. alaskensis* and *R. capsulatus* indiscriminately, there might be a competition for the binding site between the two metals, for which Mo might have a higher affinity, even though its catalytic activity is less efficient than

with W. Therefore, when Mo is not present, W is incorporated in enzymes that prefer Mo, resulting in a higher specific activity. However, the two FDHs from *S. fumaroxidans* that were characterised previously appeared to be W-containing enzymes although both metals were present in the growth medium (de Bok et al. 2003). So if this would be true for the *S. fumaroxidans* FDHs, it would imply that part of the total FDH activity in the cells grown on propionate plus fumarate represents additional FDH(s). Indeed, quantification of the different FDHs in *S. fumaroxidans* suggested that it contains a third FDH (de Bok et al. 2003). In our study, the difference in FDH-level between cells grown in medium without W and Mo and in medium with only Mo was also 2.5 U mg^{-1}, which could not be addressed to one of the W-containing-FDHs (de Bok et al. 2003). Therefore, the most likely explanation for our observations is that *S. fumaroxidans* possesses a third constitutively expressed FDH of which the activity increases when Mo is substituted by W. This then would imply that substitution of Mo for W in the two well-studied FDHs does not produce active FDHs. This would be in contrast with the *M. thermoacetica* FDH, which could also incorporate Mo into its active site (Durfor et al. 1983). To confirm our ideas the third FDH of *S. fumaroxidans* would need to be purified from selected media. Although FDH activities in *S. fumaroxidans* strongly depend on either W or Mo, the H_2ase levels in *S. fumaroxidans* grown on propionate plus fumarate were not significantly affected by the absence of W and Mo.

Effects of W and Mo on the axenic growth of M. hungatei

M. hungatei utilizes H_2 and/or formate produced by *S. fumaroxidans* when co-cultured on propionate (Dong et al. 1994). Therefore, the influence of W and Mo on the growth of *M. hungatei* on H_2/CO_2 or formate was also assessed to understand the role of these metals in syntrophic propionate oxidation. The composition of the media used to assess the effects of Mo and W on *M. hungatei* was similar as described above for *S. fumaroxidans*, except that acetate (2 mM) was added as extra carbon source. The cultures of *M. hungatei* were adapted to growth on either H_2/CO_2 (172 kPa, 80:20, vol/vol) or formate (120 mM) as substrate for at least three subsequent transfers in the corresponding media. Growth was followed by measuring methane (CH_4) formation.

Table 1 Growth rates and specific formate dehydrogenase (FDH) and hydrogenase (H_2ase) activities in cell extracts of *S. fumaroxidans* (grown on propionate plus fumarate) and *M. hungatei* (grown on formate or H_2/CO_2) after adaptation to growth in media with or without W and/or Mo.

Medium	I	II	III	IV
WO_4^{2-} (μM)	0	0	0.1	0.1
MoO_4^{2-} (μM)	0	0.1	0	0.1
***S. fumaroxidans* (propionate/fumarate)**				
FDH (U mg^{-1})	2.5±0.1	5.0±0.5	25.3±0.1.3	14.5±0.4
H_2ase (U mg^{-1})	15.6±0.4	9.1±0.4	7.4±0.9	17.1±0.1
Growth rate (day^{-1})	0.21±0.03	0.19±0.01	0.22±0.01	0.21±0.02
***M. hungatei* (formate)**				
FDH ((U mg^{-1})	0.3±0.1	1.1±0.1	7.3±0.4	4.8±0.8
H_2ase (U mg^{-1})	14.5±2.1	9.2±1.2	5.6±0.1	2.5±0.1
Growth rate (day^{-1})	0.11±0.02	0.41±0.03	0.33±0.05	0.41±0.01
***M. hungatei* (H_2/CO_2)**				
FDH (U mg^{-1})	0.006±0.0	3.2±0.1	18±0.4	25±0.5
H_2ase (U mg^{-1})	0.8±0.1	1.3±0.1	1.7±0.2	1.7±0.2
Growth rate (day^{-1})	0.40±0.16[a]	0.60±0.07	0.76±0.06	0.87±0.08

[a] Growth ceased after a short exponential phase, from which the growth rate was calculated.

[b] Growth rates are the mean of triplicate measurements and enzyme activities are duplicate measurements.

The importance of W and Mo in methanogenesis has been recognized for a long time. The stimulating effect of both elements was found to be related to either the FDH levels in methanogens growing on formate, such as *M. formicicum* and *Methanococcus vannielii* (Jones and Stadtman 1981; May et al. 1988), or to the FMDH level in methanogens growing on H_2/CO_2 such as *Methanothermobacter wolfei* and *Methanothermobacter marburgensis* (Bertram et al. 1994; Hochheimer et al. 1998). Growth of *M. formicicum* and *M. vannielii* on H_2/CO_2 was not affected by Mo or W when H_2/CO_2 was substrate instead of formate, as FDH is not required for autotrophic growth (Jones and Stadtman 1981; May et al. 1988). Similar to these two methanogens, *M. hungatei* grows on both H_2/CO_2 and formate. Therefore, one can expect that growth on formate but not on H_2/CO_2 is influenced by W or Mo. Indeed, presence of either W or Mo stimulated growth of *M. hungatei* on formate (Fig. 2a, Table 1). The highest growth rates (0.41 day^{-1}) were observed when Mo was present in the medium (medium II and IV). In the absence of Mo but presence of W the growth rate was slightly lower (0.33

day^{-1}). When both W and Mo were absent, the growth rate was only 0.11 day^{-1}. The highest FDH-level was detected when only W was present in the medium and it was substantially lower when W was absent, suggesting the presence of W-containing-FDH(s) in *M. hungatei*. Apparently, the growth rate of *M. hungatei* was directly related to the FDH activity, as both FDH level and growth rate increased about 4 times when Mo was added in the medium. But, further increase of FDH level by addition of W did not further increase growth rate,

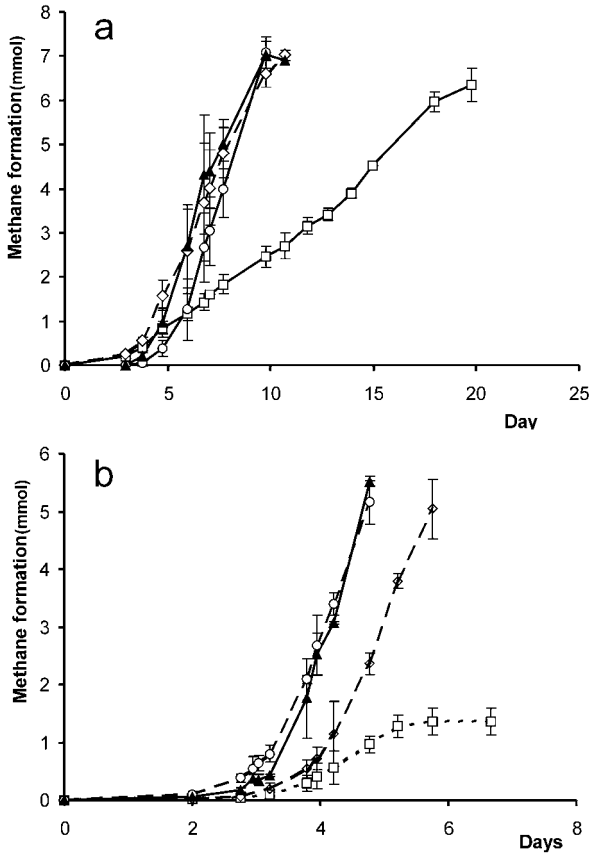


Fig. 2 Growth as determined from methane production of *M. hungatei* on formate (a) or on H_2/CO_2 (b) in the media with W and Mo (▲), W only (○), Mo only (◊) or without W and Mo (□). Data are the mean of triplicate measurements.

indicating that something other than FDH is limiting. These results are more difficult to interpret since we do not know if the *M. hungatei* FDH(s) contain W and methanogens also contain FMDH that is also Mo or W dependent (Bertram et al. 1994; Kletzin and Adams 1996; Hochheimer et al. 1998). However, all FDHs from mesophilic methanogens studied so far contain Mo. If we assume that also the *M. hungatei* FDH(s) contains Mo, our results suggest that W stimulates the production of Mo-containing-FDH and that W effectively substitutes Mo in *M. hungatei* FDH, resulting in an enzyme with an even higher activity.

However, we do not exclude the possibility that *M. hungatei* possesses a W-containing-FDH, and that perhaps W-containing-FDHs are much more common than we think.

Growth of *M. hungatei* on H_2/CO_2 was also stimulated by W and Mo (Fig. 2b, Table 1). The highest growth rates of *M. hungatei* on H_2/CO_2 (0.87 & 0.76 day^{-1}) were measured in the cultures grown in the presence of W (Medium III & IV). Growth rates, FDH and H_2ase levels were substantially lower in the media without W (Medium I & II). When both W and Mo were absent the culture eventually ceased to grow after a short exponential growth phase. The FDH level in these cells was significant almost negligible, which suggests that FDH is also required for growth on H_2/CO_2. There are some reports that FDH is needed for biosynthesis in some methanogens during growth on H_2/CO_2 (Tanner et al. 1989; White 1997). Interestingly, even *fdh* gene expression was found to be controlled by the presence of H_2 and not by the presence of formate in *Methanococcus maripaludis* (Wood et al. 2003). A stimulating effect of W on methanogens growing on H_2/CO_2 has also been demonstrated for *Methanocorpusculum parvum* and *Methanoplanus endosymbiosus* (Van Bruggen et al. 1986; Zellner and Winter 1987). Clearly this might also be true for *M. hungatei*, as increased FDH levels were observed when it grew on H_2/CO_2. The effects of W and Mo on growth of *M. hungatei* could be also related to the FMDH-level, as it was speculated by Kletzin and Adams (Kletzin and Adams 1996). Then, the most likely explanation for our results is that *M. hungatei* contains a W-FMDH in which W can be substituted by Mo, or that is contains a W-FMDH as well as a Mo-FMDHs as was found for *M. wolfei* and *M. thermoautotrophicus* (Bertram et al. 1994; Hochheimer et al. 1998).

Effects of W and Mo on co-cultures of S. fumaroxidans and M. hungatei

To study if W and Mo are required for syntrophic growth on propionate, co-cultures of *S. fumaroxidans* and *M. hungatei* were cultivated on propionate (30mM) in media with or without Mo and W. Cells were successfully separated by percoll gradient centrifugation. The FDH and H_2ase activities in the cell-free extracts of syntrophically grown *S. fumaroxidans* and *M. hungatei* cells are presented in Table 2. Maximum methane formation rates in these co-cultures were calculated from the methane production curves (Fig. 3).

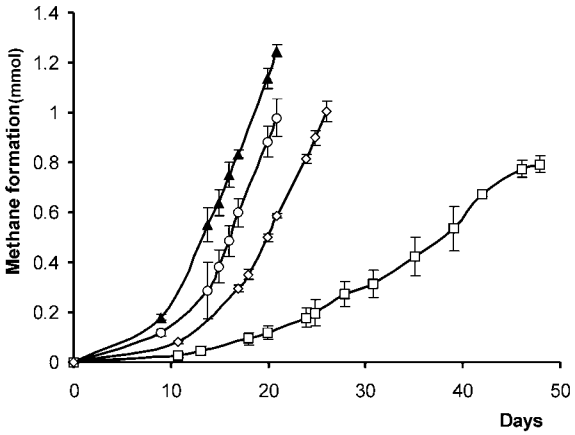


Fig. 3 Syntrophic propionate oxidation as determined from methane production by a coculture of *S. fumaroxidans* and *M. hungatei* in the media with W and Mo (▲), W only (○), Mo only (◊) or without W and Mo (□). Data are the mean of triplicate measurements.

Table 2. The effects of W and Mo on the CH_4 formation rates and specific formate dehydrogenase (FDH) and hydrogenase (H_2ase) activities in the cell extracts of *S. fumaroxidans* and *M. hungatei* grown syntrophically on propionate (30 mM). Cells of *S. fumaroxidans* and *M. hungatei* were separated by percoll gradient centrifugation.

Medium	I	II	III	IV
WO_4^{2-} (µM)	0	0	0.1	0.1
MoO_4^{2-} (µM)	0	0.1	0	0.1
S. fumaroxidans				
FDH (U mg^{-1})	5.5±0.4	19.5±1.1	102±3.1	95±0.9
H_2ase (U mg^{-1})	8.2±0.1	9.5±0.7	9.9±0.6	12.7±1.5
M. hungatei				
FDH (U mg^{-1})	0.02±0.02	2.2±0.3	25.5±1.2	44±2.1
H_2ase (U mg^{-1})	0.6±0.1	0.8±0.1	1.8±0.2	3.2±0.4
CH_4 formation rate[a] (nmol min^{-1} ml^{-1})	0.13±0.02	0.41±0.03	0.57±0.04	0.59±0.02

[a] CH_4 formation rates are the mean of triplicate measurements and enzyme activities are duplicate measurements.

Also in syntrophically grown cells of *S. fumaroxidans* and *M. hungatei* the highest FDH levels were detected when W was present in the medium (medium III and IV). These

specific activities were much higher than those in axenically grown cells of *S. fumaroxidans* and *M. hungatei* on formate, consistent with findings reported previously (de Bok et al. 2002a). In W-depleted media (medium I and II), FDH levels were significantly lower in both organisms (Table 2). The highest methane formation rate, 0.59 nmol min^{-1} ml^{-1}, was measured in medium with both W and Mo, comparable to the rate measured by Dong and Stams (Dong and Stams 1995). The rate was more or less the same (0.57 nmol min^{-1} ml^{-1}) when Mo was limiting, but it decreased to 0.41 nmol min^{-1} ml^{-1} in W-depleted medium (medium II). In the medium without W and Mo the methane formation rate was only 0.13 nmol min^{-1} ml^{-1}, which is at least due to the inhibition of *M. hungatei* by W- and Mo limitation as we demonstrated in our experiments with axenic cultures. The FDH-level in *M. hungatei* under these conditions was 0.02 U mg^{-1}, which is almost negligible compared to its level in medium without any limitations, considering that the efficiency of percoll separation with these co-cultures is not 100% (de Bok et al. 2002a), and assuming this activity could be attributed to residual FDH from *S. fumaroxidans* cells in the percoll separated *M. hungatei* cells. Therefore we can state that *S. fumaroxidans* and *M. hungatei* are able to grow syntrophically with H_2 as the only interspecies electron carrier. Whether the lower rate can be attributed to the absence of interspecies formate transfer alone is not clear, because we do not know how much the *M. hungatei* FMDH is affected by W- and Mo-limitation in the co-cultures, since in this case there is also competition for residual W and Mo between the individual organisms. However, if we assume that W is required for CO_2-reduction in *S. fumaroxidans* both for disposal of electrons as hypothesized by de Bok and co-workers (de Bok et al. 2003), and for CO_2 fixation for biomass synthesis (Plugge et al. 1993), and that the *M. hungatei* FMDH is a Mo-enzyme (or a W-enzyme in which Mo can replace W effectively) the contribution of formate to interspecies electron transfer is perhaps not that high. The CH_4-formation rate in medium with Mo was only ~1/3 less than compared to the rate in medium containing both metals. Also here we have to be careful with our conclusions, since CO_2 reductase activity has not been demonstrated for any of the Mo-containing FDHs so far, while most W-containing FDHs did reduce CO_2 in vitro (de Bok et al. 2003). So if in there is no residual CO_2-reductase activity in *S. fumaroxidans* in W-limited medium, apparently interspecies H_2 transfer prevails in this situation, but it will need further investigation to confirm this. However, H_2 is assumed to be the preferred electron carrier in dense aggregated biomass whereas formate is more favourable in suspended cultures (Boone et al. 1989; Schmidt and Ahring 1995). Limitation of W and Mo may also affect the aggregation behaviour of organisms that depend on syntrophic relationships, although such effect has never been reported. Floc formation was observed in

syntrophic co-cultures of *S. fumaroxidans* and *M. hungatei* (de Bok et al. 2002a), and in our experiments we noticed that the formation of aggregates was more evident in cultures without W than in cultures with W (results not shown).

Acknowledgments

This work was supported by the Netherlands Technology Foundation STW, Applied Science Division of NWO and the Technology Program of the Ministry of Economic Affaires of the Dutch government, under the project WWL.4928 and 349-5619. The investigations were also supported by the Research Council for Earth and Life Sciences (ALW) with financial aid from the Netherlands Organization for Scientific Research (NWO).

References

Afshar S, Schröder I, Kim C, Monbouquette HG (1998) Effect of tungstate on nitrate reduction by the hyperthermophilic archaeon *Pyrobaculum aerophilum*. Appl Environ Microbiol 64:3004-3008

Andreesen JR, Ljungdahl LG (1973) Formate dehydrogenase of *Clostridium thermoaceticum*: incorporation of selenium 75, and the effects of selenite, molybdate, and tungstate on the enzyme. J Bacteriol 116:867-873

Bertram PA, Schmitz RA, Linder D, Thauer RK (1994) Tungstate can substitute for molybdate in sustaining growth of *Methanobacterium thermoautotrophicum*. Identification and characterization of a tungsten isoenzyme of formylmethanofuran dehydrogenase. Arch Microbiol 161:220-228

Boone DR, Johnson RL, Liu Y (1989) Diffusion of the interspecies electron carriers H_2 and formate in methanogenic ecosystems and its implications in the measurement of K_m for H_2 or formate uptake. Appl Environ Microbiol 55:1735-1741

Boonstra J, Huttunen MT, Konings WN (1975) Anaerobic transport in *Escheichia coli* membrane vesicles. J Biol Chem 250:6792-6798

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72:248-254

Brondino CD et al. (2004) Incorporation of either molybdenum or tungsten into formate dehydrogenase from *Desulfovibrio alaskensis* NCIMB 13491; EPR assignment of the proximal iron-sulfur cluster to the pterin cofactor in formate dehydrogenases from sulfate-reducing bacteria. J Biol Inorg Chem 9:145-151

de Bok FAM et al. (2003) Two W-containing formate dehydrogenases (CO_2-reductases) involved in syntrophic propionate oxidation by *Syntrophobacter fumaroxidans*. Eur J Biochem 270:2476-2485

de Bok FAM, Luijten MLGC, Stams AJM (2002a) Biochemical evidence for formate transfer in syntrophic propionate-oxidizing cocultures of *Syntrophobacter fumaroxidans* and *Methanospirillum hungatei*. Appl Environ Microbiol 68:4247-4252

de Bok FAM, Plugge CM, Stams AJM (2004) Interspecies electron transfer in methanogenic propionate degrading consortia. Water Res 38:1368-1375

de Bok FAM, Roze EHA, Stams AJM (2002b) Hydrogenases and formate dehydrogenases of *Syntrophobacter fumaroxidans*. Antonie van leeuwenhoek 81:283-291

Dong X, Plugge CM, Stams AJM (1994) Anaerobic degradation of propionate by a mesophilic acetogenic bacterium in coculture and triculture with different methanogens. Appl Environ Microbiol 60:2834-2838

Dong X, Stams AJM (1995) Evidence for H_2 and formate formation during syntrophic butyrate and propionate degradation. Anaerobe 1:35-39

Durfor CN, Wetherbee PJ, Deaton JC, Solomon EI (1983) Characterization and spectroscopic properties of reduced Mo and W formate dehydrogenase from *Clostridium thermoaceticum*. Biochem Biophys Res Commun 115:61-67

Ferry JG (1999) Enzymology of one-carbon metabolism in methanogenic pathways. FEMS Microbiol Rev 23:13-38

Granderath K (1993) Charakterisierung der Formiat Dehydrogenase und Aldehyd Dehydrogenase als Wolframhaltige Enzyme von *Eubacterium acidaminophilum*. In. PhD thesis. University of Gottingen, German, Gottingen

Harmsen HJM, Van Kuijk BLM, Plugge CM, Akkermans ADL, De Vos WM, Stams AJM (1998) *Syntrophobacter fumaroxidans* sp. nov., a syntrophic propionate-degrading sulfate-reducing bacterium. Int J Syst Bacteriol 48:1383-1387

Hochheimer A, Hedderich R, Thauer RK (1998) The formylmethanofuran dehydrogenase isoenzymes in *Methanobacterium wolfei* and *Methanobacterium thermoautotrophicum*: induction of the molybdenum isoenzyme by molybdate and constitutive synthesis of the tungsten isoenzyme. Arch Microbiol 170:389-393

Jones JB, Stadtman TC (1977) *Methanococcus vannielii*: culture and effects of selenium and tungsten on growth. J Bacteriol 130:1404-1406

Jones JB, Stadtman TC (1981) Selenium-dependent and selenium-independent formate dehydrogenases of *Methanococcus vannielii*. Separation of the two forms and characterization of the purified selenium-independent form. J Biol Chem 256:656-663.

Kletzin A, Adams MWW (1996) Tungsten in biological systems. FEMS Microbiol Rev 18:5-63

Ljungdahl LG, Andreesen JR (1978) Formate dehydrogenase, a selenium-tungsten enzyme from *Clostridium thermoaceticum*. Methods Enzymol 53:360-372

May HD, Patel PS, Ferry JG (1988) Effect of molybdenum and tungsten on synthesis and composition of formate dehydrogenase in *Methanobacterium formicicum*. J Bacteriol 170:3384-3389

Odom JM, Peck HD, Jr. (1981) Localization of dehydrogenases, reductases, and electron transfer components in the sulfate-reducing bacterium *Desulfovibrio gigas*. J Bacteriol 147:161-169

Plugge C, M., Dijkema C, Stams AJM (1993) Acetyl-CoA cleavage pathway in a syntrophic propionate oxidizing bacterium growing on fumarate in the absence of methanogens. FEMS Microbiol Lett 110:71-76

Schink B (1997) Energetics of syntrophic cooperation in methanogenic degradation. Microbiol Mol Biol Rev 61:262-280

Schmidt JE, Ahring BK (1995) Interspecies Electron Transfer during Propionate and Butyrate Degradation in Mesophilic, Granular Sludge. Appl Environ Microbiol 61:2765-2767

Stams AJM (1994) Metabolic interactions between anaerobic bacteria in methanogenic environments. Antonie van Leeuwenhoek 66:271-294

Stams AJM, Dong X (1995) Role of formate and hydrogen in the degradation of propionate and butyrate by defined suspended cocultures of acetogenic and methanogenic bacteria. Antonie van Leeuwenhoek 68:281-284

Stams AJM, Van Dijk JB, Dijkema C, Plugge CM (1993) Growth of syntrophic propionate-oxidizing bacteria with fumarate in the absence of methanogenic bacteria. Appl Environ Microbiol 59:1114-1119

Stewart LJ, Bailey S, Bennett B, Charnock JM, Garner CD, McAlpine AS (2000) Dimethylsulfoxide reductase: an enzyme capable of catalysis with either molybdenum or tungsten at the active site. J Mol Biol 299:593-600

Tanner RS, McInerney MJ, Nagle DP, Jr (1989) Formate Auxotroph of *Methanobacterium-Thermoautotrophicum* Marburg. J Bacteriol 171:6534-6538

Thiele JH, Zeikus JG (1988) Control of Interspecies Electron Flow during Anaerobic Digestion: Significance of Formate Transfer versus Hydrogen Transfer during Syntrophic Methanogenesis in Flocs. Appl Environ Microbiol 54:20-29

Van Bruggen JJA, Zwart KB, Hermans GF, van Hove EM, Stum CK, Vogels GD (1986) Isolation and characterization of *Methanoplanus endosymbiosus* sp. nov., an endosymbiont of the marine sapropelic ciliate *Metopus contortus* Quennerstedt. Arch Microbiol 144:367-374

White R (1997) Purine biosynthesis in the domain Archaea without folates or modified folates. J Bacteriol 174:3374-3377

Wood GE, Haydock AK, Leigh JA (2003) Function and regulation of the formate dehydrogenase genes of the methanogenic archaeon *Methanococcus maripaludis*. J Bacteriol 185:2548-2554

Zellner G, Winter J (1987) Growth promoting effect of tungsten on methanogens and incorporation of tungsten-185 into cells. FEMS Microbiol Lett 40:81-87

Zindel U, Freudenberg W, Rieth M, Andreesen JR, Schnell J, Widdel F (1988) *Eubacterium acidaminophilum* sp. nov., a versatile amino acid-degrading anaerobe producing or utilizing H_2 or formate. Description and enzymatic studies. Arch Microbiol 150:254-266

Chapter 7

Summary and concluding remarks

Anaerobic treatment of wastewater is a proven technology that has several advantages. The successful operation of an anaerobic wastewater treatment plant depends on well functioning and balanced microbial communities in the sludge of bioreactors, which consists of fermentative bacteria, homoacetogens and methanogens. Trace metals are extremely important for high-rate bioreactors, due to their roles in key enzymes or cofactors of metabolic pathways, e.g., cobalt in corrinoid of methyltransferases of methanogens and homoacetogens, nickel in F430 of methyl-CoM reductase in methanogens and carbon monoxide dehydrogenase in methanogens and homoacetogens, tungsten and molybdenum in formate dehydrogenase of methanogens and homoacetogens. Addition of these metals in the medium or influent of bioreactors showed the stimulation of growth of microorganisms and an enhanced performance of the wastewater treatment system.

However, proper dosing of these metals as nutrient is a great challenge, since dosing of a metal at a high concentration is toxic for growth of microorganisms and dosing of a specific metal may lead to an out-competition of one group of microorganisms by another. For instance, cobalt dosing caused acidification of a bioreactor under mesophilic conditions, when methanol is the main pollutant (4). Therefore, it is important to get a better understanding of the microorganisms in sludge and their requirement of metals as nutrients for certain physiological functions. The objective of part of the research described in this thesis was to understand the microbial aspects of methanogenesis, and the role of metals on the metabolism of methanogenic consortia, in order to apply the obtained knowledge to optimize trace metal dosing in anaerobic bioreactors, using methanol as model substrate and cobalt as model metal nutrient.

Methanol is converted syntrophically under thermophilic conditions

As mentioned above, acetate accumulation in the effluent is a problem in the treatment of methanolic wastewater under mesophilic conditions, and caused deterioration of the performance of the bioreactor (4). However, in our previous study, no significant acetate accumulation was observed in a lab-scale thermophilic bioreactor treating synthetic methanol-containing wastewater (7). It was demonstrated, by using specific inhibitors and nuclear magnetic resonance spectroscopy, that besides direct conversion of methanol to methane by methylotrophic methanogens, indirect conversion via H_2 by homoacetogens and hydrogenotrophic methanogens plays an important role (7, 8). This is different from methanol

conversion under mesophilic conditions, which is mainly carried out by *Methanosarcina* species (2). Using 16S rRNA based molecular approaches, it was found that *Methanothermobacter*- and *Methanomethylovorans*-related methanogens, and *Moorella*-related homoacetogens are dominant species in this thermophilic sludge (10). Therefore, the first part of this thesis focused on the isolation and characterization of the dominant microorganisms that play a role in the thermophilic conversion of methanol.

In the research described in **Chapter 2**, a novel methanogen, *Methanomethylovorans (Mmv) thermophila* strain L2FAW, was isolated from this sludge and characterized as the second thermophilic, obligated methylotrophic, fresh water species (fully-characterized so far) after *Methanosarcina (Ms) thermophila*. *Mmv. thermophila* grows on methanol with a maximal specific growth rate of 0.05 hr^{-1}, which is similar to the growth rate of *Ms. thermophila*. The strain was enriched and isolated by using cobalt-containing medium. The growth on methanol is stimulated by the addition of cobalt; the optimal cobalt concentration is 0.5 to 2 μM. Cobalt is important for direct methanol conversion by this methanogen. Unlike *Ms. thermophila*, *Mmv. thermophila* cannot grow on acetate, H_2/CO_2. Therefore, this strain is specialized in methanol conversion in this sludge.

By using a cobalt-deficient medium, a methanol-degrading culture was enriched from the sludge sample, which contained mainly spore-forming bacteria and long-rod shaped H_2-consuming methanogens. Methane is the end product in this enrichment culture when cobalt was absent in the medium (**Chapter 3)**. The spore-forming bacterium is characterized as a novel homoacetogen, *Moorella hydrogenoformans*. In pure culture this homoacetogenic bacterium grows on methanol with acetate as main final product, and H_2 and formate as minor end products. In a coculture with the H_2-consuming methanogen (characterized as *Methanothermobacter* sp NJ1), it converted methanol partially to methane and partially to acetate. Remarkably, the dominant end product in this coculture seems to be dependent on the presence of cobalt. If cobalt is present, acetate is the main product; otherwise it is methane (Table 3, **Chapter 3**). This is consistent with the findings of our previous research, in which acetate accumulation from methanol by an enrichment culture (from which these two strains were isolated) increases with an increase of the cobalt concentration in the medium (9).

Removal of H_2 is important for methanol conversion

It is known that most intermediates of the anaerobic digestion process, like ethanol, butyrate, propionate, are only converted when the H_2 partial pressure is kept low by H_2-consuming methanogens (11). The results described in **Chapter 3** showed that methanol can be converted syntrophically by *Moorella hydrogenoformans*, in a similar manner as other homoacetogens (1, 6). *Moorella hydrogenoformans* grown on methanol produced a substantial amount of H_2, especially when cobalt is absent from the medium, but acetate is the main end-product. However, when a hydrogenotrophic methanogen is present in the culture, methane is the main product, and less than 10% of methanol is converted to acetate. Obviously, the presence of the methanogen, combined with the absence of cobalt, led to a change in the metabolic route of methanol conversion by homoacetogens.

The mechanism of syntrophic methanol conversion is not clear. *Moorella hydrogenoformans* can grow on carbon monoxide with the formation of H_2, indicating that an energy converting hydrogenase is present in this homoacetogen. This enzyme was found to exist in many microorganisms, like *E. coli, Methanosarcina barkeri, Pyrococcus furiosus* and carboxydotrophic hydrogenogens (3). In *E. coli*, this enzyme complexes with formate dehydrogenase to form a formate hydrogenlyase that catalyses the conversion of formate to H_2 and CO_2. This conversion is coupled to the translocation of protons (5). In **Chapter 4**, we demonstrated that *Moorella hydrogenoformans* can grow on formate syntrophically with a H_2-consuming methanogen, *Methanothermobacter* NJ1. It may have a similar gene cluster of formate hydrogenlyase as found in *Moorella thermoacetica*. Based on above results and the finding by Winter and Ljungdahl (12) that a methanol dehydrogenase might be involved in the oxidation of methanol to formaldehyde or formate, we speculate that the release of reducing equivalents as H_2 by this hydrogenase during methanol oxidation might be a possible mechanism of energy conservation. Under normal growth conditions, homoacetogens obtain energy by formation of acetate. Only under certain conditions, like (in our case) limitation of cobalt, and the presence of methanogens, methanol conversion to H_2 and CO_2 becomes the dominant process.

Conversion of methanol to H_2 and CO_2 is thermodynamically unfavorable. Therefore, interspecies electron transfer plays a crucial role in this type of methanol conversion. Electrons are normally transferred as H_2, but formate transfer was proposed as a possible

mechanism as well (11). The importance of H_2 and formate transfer in syntrophic interaction using propionate oxidation by *Syntrophobacter fumaroxidans* and *Methanospirillum hungatei* as a model was studied in **Chapter 6.** It is found that the methane formation rate from propionate by this coculture correlates with either hydrogenase or formate dehydrogenase activity levels in both microorganisms. The FDH activity in cells of *M. hungatei* grown in molybdenum and tungsten deficient medium is negligible and in *S. fumaroxidans* cells the FDH activity is low. However, methane is still formed by this coculture at relative high rate (20% of that by the coculture grown in non-deficient medium). These results indicated that interspecies H_2 transfer is possible as the only mechanism, but that formate transfer might result in a higher rate of propionate oxidation.

Cobalt can regulate the route of methanol conversion

Cobalt is essential for the metabolism of methanol, because of its role in corrinoid dependent methyltransferase in the methanogenic and the acetyl-CoA pathways. The importance of cobalt can be seen in the Fig 1. The presence (or amendment) of cobalt resulted a fast methanol degradation and methane formation by a thermophilic enrichment culture. On the other hand, when the culture was grown in the absence of cobalt methanol was still converted to methane after long lag phase and at a much slower rate. Starting from these cultures, *Mvv. thermophila* was isolated from an enrichment culture by using cobalt sufficient

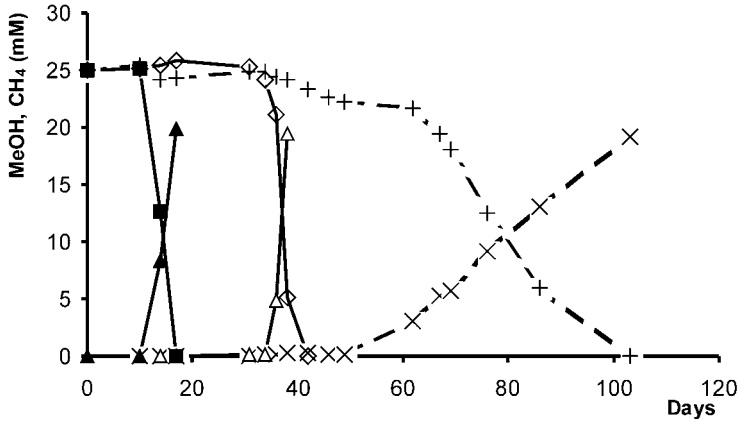


Fig. 1 The importance of cobalt on the methanol degradation and methane formation by a thermophilic enrichment culture. Symbols: ■: methanol; ▲: methane. Solid lines: culture grown in the presence of 0.5 μM cobalt; Dot lines: culture grown in the absence of cobalt in the first 33rd days and amended with 0.5 μM cobalt at the 33rd day; Dashed lines: culture grown in the absence of cobalt.

medium (**Chapter 2**). On the other hand, a syntrophic coculture was enriched by using cobalt deficient medium, from which *M. hydrogenoformans* and *Methanothermobacter* sp. NJ1 were isolated (**Chapter 3**). Optimal cobalt concentration for *Mvv. thermophila* was around 0.5 to 2 μM (**Chapter 2**). At suboptimal conditions, the growth rate of *Mmv thermophila* is linearly related to the cobalt concentration in the medium (data not show). Although we did not test the optimal cobalt concentration for *M. hydrogenoformans*, its requirement for cobalt in pure culture seems to be high. However, the requirement of cobalt by a coculture of *M. hydrogenoformans* and *Methanothermobacter* sp. NJ1 seems to be low. In fact, addition of cobalt stimulated the formation of acetate (**Chapter 3**, and (9)). The optimal cobalt concentration for syntrophic methanol conversion by an enrichment culture was around 0.1 μM; at this concentration little acetate accumulated (9). Therefore, cobalt plays a role in the regulation of the pathway of methanol conversion in this coculture: to methanol oxidation to H_2 and CO_2 at low cobalt concentration, or to direct methane formation or acetate formation at a high cobalt concentration. This can be used to explain why acetate always accumulates in the effluent of anaerobic bioreactors treating methanolic wastewater upon the addition of cobalt.

Cobalt availability can be affected by nickel

Mostly, cobalt and nickel share uptake transporters due to their similar chemical properties. However, these metals have different role in the metabolic pathways of methanogenic archaea and homoacetogens. Precipitation of cobalt and nickel by sulfide can severely influence their bioavailablity. The effect of cobalt and nickel on the corrinoid and F430 content and on growth of *Methanosarcina barkeri* on methanol was studied in **Chapter 5**. Metal sulfide precipitation was prevented by the addition of EDTA to the media. This enabled the direct determination of the cell metal content. Stimulation of uptake of cobalt and nickel by addition of EDTA was demonstrated by the increase of the corrinoid and F430 content. A direct link of cell cobalt content and corrinoid content with growth rate at sub-optimal cobalt concentration was found. Limitation of nickel on growth on methanol was observed, when cobalt was added in excess. Most interestingly, competitive uptake of cobalt and nickel was demonstrated. This is of importance for the optimization of trace metal dosage for anaerobic treatment process, as was discussed in **Chapter 5**.

Implications for practice

The results obtained from the research described in this thesis can be applied to optimize metal dosage of anaerobic bioreactors in practice.

A. It is extremely important to understand the dominant bacterial population of the sludge and the corresponding biodegradation pathways. For example, the requirement of cobalt for the direct methanol conversion is about 10 times higher than that for syntrophic methanol conversion via hydrogen (**Chapter 2, 3**, and (9)). Overdosing of cobalt to stimulate the direct methanol conversion may cause acetate accumulation by homoacetogenic bacteria (**Chapter 3)**.

B. It is also important to maintain balanced consortia in the sludge, especially the H_2-consuming methanogens, by means of dosing of metals like nickel, tungsten and molybdenum. Maintaining a low H_2 partial pressure is essential for the degradation of intermediates of anaerobic digestion. Syntrophic methanol degradation also needs an efficient removal of H_2 (**Chapter 3, 4 & 5**). Uncoupled H_2 removal and formation may result in an accumulation of acetate.

C. Due to competitive uptake and antagonistic effect of different metals, e.g. cobalt and nickel (Chapter 5), tungsten and molybdenum (Chapter 6), balanced dosing of all metals required is essential to maintain a high biological activity. Overdosing of a metal may not only cause toxicity of this metal, but also inhibit the uptake of another essential metal.

Reference

1. **Balk, M., J. Weijma, M. W. Friedrich, and A. J. Stams.** 2003. Methanol utilization by a novel thermophilic homoacetogenic bacterium, *Moorella mulderi* sp. nov., isolated from a bioreactor. Arch Microbiol **179:**315-20.
2. **Florencio, L., J. A. Field, and G. Lettinga.** 1994. Importance of cobalt for individual trophic groups in an anaerobic methanol-degrading consortium. Appl Environ Microbiol **60:**227-34.
3. **Hedderich, R.** 2004. Energy-converting [NiFe] hydrogenases from archaea and extremophiles: ancestors of complex I. J Bioenerg Biomembr **36:**65-75.
4. **Lettinga, G., W. De Zeeuw, and E. Ouborg.** 1981. Anaerobic treatment of wastes containing methanol and higher alcohols. Water Res **15:**171-182.
5. **Meuer, J., H. C. Kuettner, J. K. Zhang, R. Hedderich, and W. W. Metcalf.** 2002. Genetic analysis of the archaeon *Methanosarcina barkeri* Fusaro reveals a central role for Ech hydrogenase and ferredoxin in methanogenesis and carbon fixation. PNAS **99:**5632-7.

6. **Pantskhava, E. S., and V. V. Pchelkina.** 1969. Methane fermentation of methanol by a *Methanobacillus kuzneceovii* culture. Prikladnaya Biokhimiya i Mikrobiologiya **5:**416-20.
7. **Paulo, P. L., B. Jiang, S. Rebac, L. H. Pol, and G. Lettinga.** 2001. Thermophilic anaerobic digestion of methanol in UASB reactor. Water Sci Technol **44:**(4)129-36.
8. **Paulo, P. L., A. J. Stams, J. A. Field, C. Dijkema, J. B. van Lier, and G. Lettinga.** 2003. Pathways of methanol conversion in a thermophilic anaerobic (55 degrees C) sludge consortium. Appl Microbiol Biotechnol **63:**307-14.
9. **Paulo, P. L., M. V. Vallero, R. H. Trevino, G. Lettinga, and P. N. Lens.** 2004. Thermophilic (55 degrees C) conversion of methanol in methanogenic-UASB reactors: influence of sulphate on methanol degradation and competition. J Biotechnol **111:**79-88.
10. **Roest, K., M. Altinbas, P. L. Paulo, H. G. Heilig, A. D. Akkermans, H. Smidt, W. M. de Vos, and A. J. Stams.** 2005. Enrichment and Detection of Microorganisms Involved in Direct and Indirect Methanogenesis from Methanol in an Anaerobic Thermophilic Bioreactor. Microb Ecol **0:**1-7.
11. **Stams, A., J. M.** 1994. Metabolic interactions between anaerobic bacteria in methanogenic environments. Antonie Van Leeuwenhoek **66:**271-294.
12. **Winter, D. K., and L. G. Ljungdahl.** 1989. PQQ-dependent methanol dehydrogenase from *Clostridium thermoautotrophicum*., p. 35-39. *In* J. A. Jongejan and J. A. Duiine (ed.), PQQ and Quinoproteins. Kluwer Academic Publisher, Dordrecht.

Samenvatting

Anaërobe biologische afvalwaterzuivering is een bewezen technologie met belangrijke voordelen. Voor het optimaal functioneren is een anaërobe afvalwaterzuiveringsinstallatie afhankelijk van gebalanceerde microbiële consortia in het reactor slib. Het gaat hierbij om fermentatieve bacteriën, homoacetogenen en methanogenen. Spore-elementen zijn zeer belangrijk voor hoge omzettingssnelheden in bioreactoren, doordat zij deel uitmaken van enzymen en co-factoren met een centrale rol in de metabole afbraak routes. Kobalt is essentieel in het corrinoid van methyl-transferases van methanogenen en homo-acetogenen. Nikkel is essentieel voor de werking van koolmonoxide dehydrogenases en in F430 als co-factor voor methyl-CoM reductase van methanogenen. Wolfraam en molybdeen zijn veel voorkomend in de actieve centra van formiaat dehydrogenases. Toevoeging van deze metalen aan het medium of het influent van bioreactoren zorgt voor een stimulatie van de groei van biomassa en een verhoogde activiteit.

Het toevoegen van de spore-elementen in de juiste dosering is echter een grote uitdaging, doordat hoge concentraties van spore-elementen remmend kunnen werken op de groei van de micro-organismen. Bovendien kan de dosering van een specifiek element tot overheersing van een bepaalde groep van micro-organismen leiden. Kobalt dosering kan bijvoorbeeld leiden tot een verzuring van de bioreactor door vorming van acetaat, zoals beschreven is voor een mesofiele bioreactor met methanol als belangrijkste verontreiniging in het afvalwater (4). Een goed inzicht van de samenstelling van de microbiële populatie van het reactorslib en de spore- elementen behoefte van de micro-organismen is daarom van groot belang. Deel van het hier beschreven onderzoek was gericht op het beter begrijpen van de microbiële achtergrond van methaanvorming uit methanol als substraat en de rol van spore-elementen, in het bijzonder kobalt, met als doel op de methanogene consortia, om tot een betere spore-elementendosering in de praktijk te komen.

Methanol wordt syntroof omgezet onder thermofiele condities

Zoals genoemd is acetaatvorming een probleem in de behandeling van afvalwater in mesofiele reactoren. Het zorgt voor een afname van de activiteit van de bioreactor (4). Acetaatvorming werd niet waargenomen onder thermofiele condities in lab-schaal reactoren en artificieel methanol bevattend afvalwater (7). Met NMR en het gebruik van specifieke remmers werd aangetoond dat naast de directe omzetting van methanol door methylotrofe methanogenen, methanol vooral ook indirect werd omgezet via H_2 naar methaan door

homoacetogenen en waterstof consumerende methanogenen (7,8). Dit verschilt van de omzetting onder mesofiele condities waar methylotrofe methanogenen van het genus *Methanosarcina* voor de omzetting van methanol zorgen (2). Met behulp van op 16S rRNA gebaseerde moleculaire technieken werd gevonden dat methanogenen uit de genera *Methanothermobacter* en *Methanomethylovorans*, en homoacetogenen uit het genus *Moorella*, dominant aanwezig waren in het thermofiele slib (10). De isolatie en karakterisatie van deze dominante micro-organismen is beschreven in het eerste deel van dit proefschrift.

Hoofdstuk 2 beschrijft de isolatie en karakterisatie van een tweede bekende thermofiele, obligaat methylotrofe, zoetwater methanogeen, *Methanomethylovorans (Mmv) thermophila* stam L2FAW, na *Methanosarcina* (*Ms*) *thermophila*. *Mmv. thermophila* groeit op methanol met een maximale specifieke groeisnelheid van 0.05 h^{-1}, hetgeen overeenkomt met de groeisnelheid van *Ms. thermophila*. *Mmv. thermophila* werd geïsoleerd uit een ophopingscultuur waarin kobalt aanwezig was. Een optimale kobaltconcentratie voor groei van 0.5 – 2.0 μM werd vastgesteld. Kobalt is belangrijk voor de directe methanol omzetting door deze methylotrofe methanogeen. In tegenstelling tot *Ms. thermophila*, kan *Mmv. thermophila* niet groeien met acetaat of H_2/CO_2 als substraat. *Mmv. thermophila* is dus gespecialiseerd in methanol omzetting in het bestudeerde reactor slib.

Wanneer kobalt afwezig was in het medium werd een ophopingscultuur verkregen die methaan vormde uit methanol en voornamelijk bestond uit sporenvormende bacteriën en lange staafvormige H_2-consumerende methanogenen **(Hoofdstuk 3)**. De sporenvormende bacterie werd geïsoleerd en gekarakteriseerd als een nieuwe homoacetogeen, *Moorella hydrogenoformans*. In reincultuur vormt deze bacterie voornamelijk acetaat uit methanol, en kleine hoeveelheden formiaat en H_2. De H_2-consumerende methanogeen werd gekarakteriseerd als een *Methanothermobacter* stam NJ1. De cocultuur van deze methanogeen en de homoacetogeen, vormde deels methaan en deels acetaat met methanol als substraat. De kobaltconcentratie bleek van grote invloed te zijn op de verhouding van gevormd methaan en acetaat. In aanwezigheid van kobalt werd voornamelijk acetaat gevormd, terwijl voornamelijk methaan wordt gevormd als kobalt afwezig is (Tabel 3, **Hoofdstuk 3**). Dit komt overeen met resultaten van eerder onderzoek waarin accumulatie van acetaat correleerde met de kobaltconcentratie in de ophopingscultuur waaruit beide organismen werden geïsoleerd (9).

Verwijdering van H_2 is van belang voor methanolomzetting.

Het is bekend dat een lage waterstofspanning essentieel is voor de afbraak van belangrijke intermediairen, zoals ethanol, butyraat en propionaat in de anaërobe afbraak. Een lage waterstofspanning wordt bereikt met waterstof-consumerende methanogene archaea (11). De resultaten die zijn beschreven in **Hoofdstuk 3** laten zien dat methanol omgezet kan worden door *Moorella hydrogenoformans*, in syntrofe samenwerking met een methanogeen (1, 6). Met methanol als substraat wordt vooral acetaat gevormd door reincultures van *Moorella hydrogenoformans*, maar ook een substantieel deel waterstof wordt gevormd, vooral als kobalt wordt weggelaten uit het medium. Als een methanogeen aanwezig is in hetzelfde medium dan wordt voornamelijk methaan gevormd; minder dan 10% van de methanol wordt dan nog omgezet in acetaat. Het is duidelijk dat de aanwezigheid van een methanogeen en de afwezigheid van kobalt in het medium leidt tot een verandering van de metabole route voor methanol omzetting door homoacetogenen.

Het is onduidelijk waar in de syntrofe afbraak van methanol door *Moorella hydrogenoformans* energie, nodig voor groei wordt geconserveerd. Mogelijk speelt een energie conserverende hydrogenase hierin een rol. *Moorella hydrogenoformans* kan CO met H_2O omzetten in H_2 en CO_2, een teken dat een energie conserverende hydrogenase aanwezig is in dit organisme (3). Dit type hydrogenase is aanwezig in meerdere micro-organismen, zoals *Escherichia coli*, *Methanosarcina barkeri*, *Pyrococcus furiosus*, *Rhodospirillum rubrum* en *Carboxydothermus hydrogenoformans*, waarin het energie conserveert door middel van de oxidatie van verschillende substraten. In *E. coli* vormt de energie conserverende hydrogenase een enzym complex, bekend als het formiaat-waterstof lyase, met een formiaat dehydrogenase, dat de omzetting van formiaat naar H_2 en CO_2 katalyseert. Energie uit deze omzetting wordt opgeslagen door middel van een gelijktijdige translocatie van protonen over de celmembraan (5). In **Hoofdstuk 4** hebben we laten zien dat *Moorella hydrogenoformans* kan groeien met formiaat als substraat en een waterstof consumerende methanogeen, *Methanothermobacter* NJ1, als syntrofe partner. *Moorella hydrogenoformans* beschikt mogelijk over een zelfde formiaat-waterstof lyase gencluster zoals gevonden in het genoom van *Moorella thermoacetica*. Gebaseerd op dit en op de bevindingen van Winter en Ljungdahl (12) dat zeer waarschijnlijk een methanol dehydrogenase betrokken is bij de omzetting van methanol tot formaldehyde of formiaat, lijkt het waarschijnlijk dat een formiaat-waterstof

lyase verantwoordelijk is voor de vorming van waterstof en conservering van energie bij de omzetting van methanol door *Moorella hydrogenoformans*. Onder standaard condities halen homoacetogene bacteriën energie uit de vorming van acetaat. Wanneer de beschikbaarheid van kobalt gelimiteerd is en methanogenen aanwezig zijn, wordt de vorming van H_2 en CO_2 door de homoacetogeen een dominant proces.

De omzetting van methanol in H_2 en CO_2 is thermodynamisch ongunstig als de producten ophopen. Daarom is interspecies elektronentransport cruciaal voor deze omzetting. De overdracht vindt vaak plaats via H_2, maar ook de overdracht via formiaat is voorgesteld als mogelijk mechanisme (11). De syntrofe afbraak van propionaat door *Syntrophobacter fumaroxidans* en *Methanospirillum hungatei* werd bestudeerd als model voor H_2 en formiaat overdracht in **Hoofdstuk 6**. Methaan vorming door deze cocultuur uit propionaat correleerde met hydrogenase- of met formiaat dehydrogenase activiteit van beide organismen. De formiaat dehydrogenase activiteit in cellen van *M. hungatei* die waren gekweekt in medium zonder molybdeen en wolfraam, was verwaarloosbaar en zeer laag in vergelijking met cellen van *S. fumaroxidans*. Deze cocultuur vormde toch methaan met relatief hoge snelheid (20% ten opzichte van een cocultuur met standaard molybdeen en wolfraam). Deze resultaten laten zien dat elektronenoverdracht met H_2 mogelijk is als enige mechanisme, maar dat formiaatoverdracht mogelijk leidt tot een hogere afbraaksnelheid van propionaat.

Kobalt reguleert de route van methanolomzetting

Kobalt is essentieel voor de afbraak van methanol, omdat het onmisbaar is voor de werking van het corrinoidafhankelijke methyltransferase, dat een belangrijke rol speelt in methanogene en acetogene metabole routes. Het belang van kobalt blijkt uit Figuur 1. Aanwezigheid van kobalt, of latere toevoeging van kobalt aan medium zonder kobalt, resulteert in een snelle afbraak van methanol en de vorming van methaan, door een thermofiele ophopingscultuur. Zonder kobalt vindt ook methanol afbraak en methaanvorming plaats, maar met duidelijk langere lag-fase en met lagere snelheid. Uit de ophopingscultuur met kobalt werd *Mmv. thermophila* geïsoleerd **(Hoofdstuk 2)**. Uit dezelfde ophopingscultuur, zonder kobalt, werden *Moorella hydrogenoformans* en *Methanothermobacter* sp. NJ1 geïsoleerd **(Hoofdstuk 3)**. De optimale kobaltconcentratie voor *Mmv. thermophila* lag rond de 0.5 – 2.0 μM **(Hoofdstuk 2)**. Bij suboptimale kobaltconcentraties is de groeisnelheid van *Mmv. thermophila* lineair daaraan gekoppeld (data niet gegeven). Hoewel de optimale

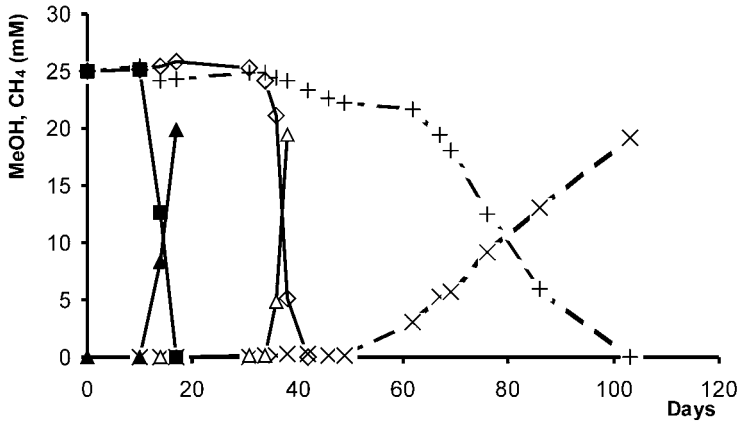


Fig. 1 Het effect van kobalt op methanolafbraak (■,+) en methaanvorming (▲,×) door een thermofiele ophopingscultuur. Kobalt was aanwezig in een concentratie van 0.5 μM (dichte symbolen), werd toegevoegd na 33 dagen (open symbolen), of was afwezig (stippel lijn).

kobaltconcentratie voor *Moorella hydrogenoformans* niet is getest, lijkt deze hoog te zijn voor een reincultuur. Daarentegen is voor een methaanvormende cocultuur van *Moorella hydrogenoformans* met *Methanothermobacter* stam NJ1 de vereiste kobaltconcentratie laag. Sterker nog, toevoeging van kobalt aan de cocultuur verhoogde de acetaatvorming **(Hoofdstuk 3)**(9). De optimale kobaltconcentratie voor syntrofe methanolomzetting tot methaan door de ophopingscultuur lag rond de 0.1 μM; bij deze concentratie werd weinig acetaat gevormd (9). Hieruit blijkt dat kobalt een belangrijke rol speelt in de regulatie van het mechanisme achter de methanolomzetting door deze ophopingscultuur. Bij lage kobaltconcentraties wordt methanol omgezet tot H_2 en CO_2, waaruit vervolgens methaan gevormd wordt. Bij hoge kobaltconcentraties wordt acetaat gevormd. Dit verklaart waarom na toevoeging van kobalt, acetaat ophoopt in het effluent van anaërobe bioreactoren die methanol bevattend afvalwater verwerken.

Kobalt beschikbaarheid wordt beïnvloed door nikkel.

Over het algemeen worden kobalt en nikkel vanwege hun vergelijkbare chemische eigenschappen door dezelfde membraantransporteiwitten opgenomen in de cel. Het neerslaan van nikkel en kobalt als sulfide precipitaten kan hun bio-beschikbaarheid sterk beïnvloeden. Beide elementen hebben elk hun eigen rol in de metabole routes van methanogene archaea en

homoacetogene bacteriën. Het effect van kobalt en nikkel op het corrinoid en F430 gehalte van cellen, en op de groei van *Methanosarcina barkeri* met methanol is bestudeerd in **Hoofdstuk 5**. Door toevoegen van EDTA aan het medium werd het neerslaan van metaalsulfideprecipitaten verhinderd. Daardoor was het mogelijk het metaal gehalte van cellen te bepalen. De stimulerende invloed van EDTA op kobalt en nikkel opname door cellen bleek uit een hogere corrinoid en F430 gehalte. Verder bleek een direct verband te bestaan tussen kobalt en corrinoid gehalte in cellen die gekweekt werden bij suboptimale kobaltconcentraties. Nikkel beschikbaarheid werd limiterend voor groei bij een overmatige kobalt dosering aan het medium. De opname van kobalt en nikkel bleek competitief. Deze waarneming is van belang voor een juiste dosering van spore-elementen aan anaërobe bioreactoren **(Hoofdstuk 5)**.

Toepassing in de praktijk

De bevindingen uit deze studie zijn van belang voor een optimale spore-element dosering aan anaërobe bioreactoren in de praktijk.

A. Kennis van de dominante bacteriële populatie en afbraakroutes in het reactorslib is zeer belangrijk. Bijvoorbeeld, de kobaltconcentratie nodig voor directe methanolomzetting naar methaan, is een factor 10 hoger dan voor syntrofe methanolomzetting via waterstof **(Hoofdstuk 2, 3)**(9). Een hogere dosering van kobalt om directe methaanvorming te stimuleren kan ook leiden tot acetaatvorming door homoacetogene bacteriën **(Hoofdstuk 3)**.

B. Het is belangrijk om de consortia van methanogenen en homoacetogenen in het reactorslib in balans te houden, en vooral die van de H_2-consumerende methanogenen, door middel van de dosering van nikkel, wolfraam en molybdeen. Het handhaven van een lage waterstofspanning is essentieel voor een volledige afbraak van de intermediairen die worden gevormd in de anaërobe afbraak. Een lage waterstofspanning is ook essentieel voor de syntrofe afbraak van methanol **(Hoofdstuk 3, 4, 5)**. Een onbalans in de reactorpopulatie kan leiden tot een hogere waterstofspanning en daardoor tot acetaatvorming.

C. Door competitieve opname tussen, en antagonistische van, de verschillende spore-elementen, b.v. kobalt en nikkel (Hoofdstuk 5) of wolfraam en molybdeen (Hoofdstuk 6), is een uitgebalanceerde dosering van alle spore-elementen noodzakelijk voor een hoge biologische activiteit. De overdosering van een enkel element kan niet alleen de toxiciteit door dit element, maar ook de verhindering van de opname van een ander essentieel element tot gevolg hebben.

Referenties

1. **Balk, M., J. Weijma, M. W. Friedrich, and A. J. Stams.** 2003. Methanol utilization by a novel thermophilic homoacetogenic bacterium, *Moorella mulderi* sp. nov., isolated from a bioreactor. Arch Microbiol **179:**315-20.
2. **Florencio, L., J. A. Field, and G. Lettinga.** 1994. Importance of cobalt for individual trophic groups in an anaerobic methanol-degrading consortium. Appl Environ Microbiol **60:**227-34.
3. **Hedderich, R.** 2004. Energy-converting [NiFe] hydrogenases from archaea and extremophiles: ancestors of complex I. J Bioenerg Biomembr **36:**65-75.
4. **Lettinga, G., W. De Zeeuw, and E. Ouborg.** 1981. Anaerobic treatment of wastes containing methanol and higher alcohols. Water Res **15:**171-182.
5. **Meuer, J., H. C. Kuettner, J. K. Zhang, R. Hedderich, and W. W. Metcalf.** 2002. Genetic analysis of the archaeon *Methanosarcina barkeri* Fusaro reveals a central role for Ech hydrogenase and ferredoxin in methanogenesis and carbon fixation. PNAS **99:**5632-7.
6. **Pantskhava, E. S., and V. V. Pchelkina.** 1969. Methane fermentation of methanol by a *Methanobacillus kuzneceovii* culture. Prikladnaya Biokhimiya i Mikrobiologiya **5:**416-20.
7. **Paulo, P. L., B. Jiang, S. Rebac, L. H. Pol, and G. Lettinga.** 2001. Thermophilic anaerobic digestion of methanol in UASB reactor. Water Sci Technol **44:**(4)129-36.
8. **Paulo, P. L., A. J. Stams, J. A. Field, C. Dijkema, J. B. van Lier, and G. Lettinga.** 2003. Pathways of methanol conversion in a thermophilic anaerobic (55 degrees C) sludge consortium. Appl Microbiol Biotechnol **63:**307-14.
9. **Paulo, P. L., M. V. Vallero, R. H. Trevino, G. Lettinga, and P. N. Lens.** 2004. Thermophilic (55 degrees C) conversion of methanol in methanogenic-UASB reactors: influence of sulphate on methanol degradation and competition. J Biotechnol **111:**79-88.
10. **Roest, K., M. Altinbas, P. L. Paulo, H. G. Heilig, A. D. Akkermans, H. Smidt, W. M. de Vos, and A. J. Stams.** 2005. Enrichment and Detection of Microorganisms Involved in Direct and Indirect Methanogenesis from Methanol in an Anaerobic Thermophilic Bioreactor. Microb Ecol **0:**1-7.
11. **Stams, A., J. M.** 1994. Metabolic interactions between anaerobic bacteria in methanogenic environments. Antonie Van Leeuwenhoek **66:**271-294.
12. **Winter, D. K., and L. G. Ljungdahl.** 1989. PQQ-dependent methanol dehydrogenase from *Clostridium thermoautotrophicum*., p. 35-39. *In* J. A. Jongejan and J. A. Duiine (ed.), PQQ and Quinoproteins. Kluwer Academic Publisher, Dordrecht.

Acknowledgements

. The thesis would not be here without the help of many people, who made their contribution in one way or another. First I would like to thank my promoter and supervisor: Prof. Alfons J. M. Stams,. Dear Fons, thanks for your inspiration and encouragement during my research. Your enthusiast for the research makes the topic even more interesting. The insightful discussion with you brings my attention from the phenomena to the mechanism behind. My special thanks also go to Dr. Caroline Plugge. Caroline, I'm grateful to your kind help in the lab, especially the "first lesson" to prepare the anaerobic medium. Working with you in the lab is also a learning process for me.

My thesis is a result of "syntrophic" cooperation with many people. I'd like to thank all of my co-authors for their efforts to make this thesis as it is now. Sonja, the "competitive" isolation of *Methanomethylovorans thermophila* made this work specially interesting. Bart, your critical comments and quick review of my manuscript have help a lot to get it published with only a few minor modifications. Anne-Meint, the finding of carboxydotrophic hydrogenogenic growth of *Moorella* strain AMP has "forced" us to look more carefully into the cycle of carbon monoxide. Wim D., thanks for your assistance of the molecular work of 16S rRNA analysis, and Mahmut for your assistance in the construction of phylogeny trees. Jan Dolfing, your special interest in the limit of life on formate and your enthusiasm to take the lead in writing Chapter 4 has made the subject now very popular in the Micfys group. Frank, you help me to learn how to work in an "oxygen-free" environment. And your insightful interpretation of the experiment results on syntrophic propionate oxidation is valuable to this research. I also would like to Chingling, for your help in participating part of the research.

This thesis is also part of the "trace element project" with close "syntrophic" cooperation with two fellow PhDs and other researchers. Marcel Zandvoort, I'm grateful for the fruitful discussion with you and for allowing me to access the sludge samples from your work. Stefan Jansen, many thanks for your help with the votammetry analysis and our cooperation in the study of the competitive uptake, which was described in Chapter 5. My special thanks goes also to Paula Pauli, my supervisor during my MSc. study. Working

together with you on Environmental Technology and Microbiology is a valuable experience during my stay in Wageningen.

I'm also grateful to the supervision team of the "trace metal project". Prof. Gatze Lettinga has suggested the trace metal research as the topic of my MSc thesis. Your broad overview of this topic has been an inspiration for me. After my MSc. study in Environmental Technology, Dr. Look Hulfhoff-Pol has introduced me to continue the Ph.D. research closely related to the environmental technology in practice. Many thanks to Herman van Leeuwen and Piet Lens for your constructive comments on many issues to keep this research on track.

Working in the Lab of Microbiology is a valuable time for me. I have received a lot of help from many people from Micfys group and other groups of the Lab. It is always helpful to share ideas and experiences with the others. Besides those mentioned above, my thanks go to Arthur, Judith, Bernd, Melike, Heleen, Miriam, Sander, Wout, John, Nico, Kees, and many others.

In the end, I would like to thank everyone in my family for their love and support. I also would like to thank all my friends in Wageningen and in China. Thanks for any help and accompany. Your friendship is precious for me and will be cherished always.

Hartstikke Bedankt!

Bo Jiang

Dit onderzoek is gesteund door de Technologiestichting STW als onderdeel van NWO en het technologieprogramma van Economische Zaken, en Paques Natural Soltions, Balk , The Nederlands.

This research was supported by the Technology Foundation STW, applied science division of NWO and the technology program of the Ministry of Economic Affairs, and Paques Natural Soltions, Balk , The Netherlands.