CO metabolism of *Carboxydothermus hydrogenoformans* and *Archaeoglobus fulgidus*

Anne Meint Henstra

Promotor:

Prof. Dr. Ir. A.J.M. Stams
persoonlijk hoogleraar aan het laboratorium voor microbiologie
Wageningen Universiteit

Promotiecommissie:

Prof. Dr. R. Hedderich
Philipps-Universität Marburg, Duitsland

Prof. Dr. W.R. Hagen
Technische Universiteit Delft

Prof. Dr. L. Dijkhuizen
Rijksuniversiteit Groningen

Prof. Dr. Ir. C.J.N. Buisman
Wageningen Universiteit

Dit onderzoek is uitgevoerd binnen de onderzoekschool WIMEK.

CO metabolism of *Carboxydothermus hydrogenoformans* and *Archaeoglobus fulgidus*

Anne Meint Henstra

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 woensdag 31 mei 2006
des namiddags te half twee in de Aula

A.M. Henstra

CO metabolism of *Carboxydothermus hydrogenoformans* and *Archaeoglobus fulgidus*
Proefschrift Wageningen Universiteit, 2006 – met Nederlandse samenvatting

ISBN: 90-8504-408-1

Voorwoord

Dit proefschrift ligt hier natuurlijk niet zomaar. Daar zijn een aantal jaartjes geploeter en met aanverwante frustraties aan vooraf gegaan. Vooral wanneer het aflopen van het contract nadert is de twijfel sterk. ‘Hoe lang gaat dit nog duren, komt het ooit af?’ Maar het is af, bjusterbaarlik net?

Dat heeft natuurlijk alleen kunnen gebeuren door het geheel aan invloeden om mij heen. Daarom mijn dank en waardering aan iedereen die ik de afgelopen jaren heb mogen ontmoeten, die mijn leven hebben verrijkt, en die mij al dan niet bewust hebben gestimuleerd om het werk dat hier nu op tafel ligt af te ronden. Many thanks to all who came from abroad, who I had the pleasure to meet and work with, and who’s presence enriched my life.

Bedankt,

Anne Meint

aan mijn ouders, sytse, pim

voor het vertrouwen, steun en vrijheid m’n eigen pad te vinden

Contents

General Introduction and Outline

Hydrogen gas is used in various industrial and biotechnological processes. Of current interest is its potential role as a clean fuel used in fuel cells to generate electricity in a future hydrogen economy. Fuel cells are more efficient than conventional internal combustion engines and use of electric power stored in batteries. Additionally, use of fuel cells will lead to a reduction in dispersed emission of CO_2 and harmful exhaust by e.g. automobiles. Therefore a broad potential exist for use of fuel cells in a range of small, portable to large mobile and stationary applications.

Approximately 95 % of all hydrogen gas produced by industry today, is derived from fossil fuels such as coal, oil and natural gas. These fossil resources are first partly oxidized to form a gas mixture that is mainly composed of H_2, CO, CO_2 and H_2S known as synthesis gas. CO is further converted with H_2O in a process called the water-gas shift reaction to H_2 and CO_2. The shift catalysts that are used in the shift reaction are sensitive to contaminants present in the synthesis gas, e.g. hydrogen sulfide, and require frequent regeneration. CO is also converted to H_2 according to the shift reaction by a novel group of strict anaerobic bacteria. Existence of these bacteria and the success of anaerobic biotechnological processes in waste water and flue gas treatment led to the idea of novel process less sensitive to impurities, to replace existing shift catalysts. With the onset of a hydrogen economy with use of H_2 as future fuel in fuel cells another possible advantage of a biotechnological shift process over existing technology surfaces. Low temperature fuel cells, in particular the polymer electrolyte membrane fuel cell (PEM-FC), are extremely sensitive to CO. Conventional shift catalysts are incapable to reach the CO levels that are tolerable, inherent to thermodynamic limitations at the operating temperatures (200 to 400 ^oC). Lower temperatures are beneficial for CO removal and nature provides us with bio-catalysts that are active at these temperatures.

In recent years important advances were made in the field of the physiology of CO metabolizing anaerobes and phototrophs. Until recently it was thought that higher partial pressures of CO mainly inhibited growth of strict anaerobes and that CO only was of importance as key intermediate of the acetyl-CoA pathway. The number of anaerobic micro-organism known to grow actively with CO as energy and carbon source, however, increased rapidly. Chapter 1 provides an overview of CO

metabolizing anaerobes and enzymes of carboxydotrophic hydrogenogenic metabolism. Svetlitchnyi *et al.* (1991) described the isolation and characterization of *Carboxydothermus hydrogenoformans*, a strict anaerobic thermophilic Gram positive bacterium that conserves energy for growth in the conversion of CO with H_2O to H_2 and CO_2. The biological equivalent of the water gas shift reaction described above. Although the reaction is also catalyzed by few purple non-sulfur bacteria, *C. hydrogenoformans* shows high growth rates with CO and is therefore an ideal candidate for further study. No data are available on the capacity of micro-organisms to remove CO from hydrogen rich gas mixtures, while this is of interest to indicate the potential of biotechnological synthesis gas purification. CO thresholds of *C. hydrogenoformans* were studied in Chapter 2 and some data on the rate limiting factors are given in Chapter 3. *C. hydrogenoformans* was described to grow only with CO or pyruvate as substrates. Closer investigation revealed that a whole range of electron donors and acceptors can be used by this bacterium (Chapter 4). CO thus is part of multiple metabolic pathways present in *C. hydrogenoformans*. The recently published genome also encodes multiple CO dehydrogenase genes. To link individual CO dehydrogenases to a particular physiological role, specific CODH staining in native polyacrylamide gels on samples of cells grown at different conditions was performed (Chapter 5). Besides *C. hydrogenoformans*, also the CO metabolism of *Archaeoglobus fulgidus* was studied. It had been speculated by Klenk *et al.* (1997) that *Archaeoglobus fulgidus* could grow with CO in a similar manner as *C. hydrogenoformans*, producing H_2. Instead it was demonstrated that *A. fulgidus* grew homo-acetogenically with CO and also reduced sulfate (Chapter 6). This makes *A. fulgidus* the first true homo-acetogenic autotroph known in the archaeal domain. Hydrogen is not used as a substrate for acetogenesis or sulfate reduction. This creates the possibility to remove trace amounts of CO from synthesis gas, by its selective oxidation through *A. fulgidus*.

The research presented in this thesis was part of a larger STW project that studied the feasibility of a biological alternative for the chemical water gas shift reaction. The project also comprised the technological research carried out by Jan Sipma at the Sub-department of Environmental Technology (Wageningen University), which was presented in the doctoral thesis entitled 'Microbial hydrogenogenic CO conversions: applications in synthesis gas purification and biodesulfurization' (ISBN 90-8504-329-8).

1

Microbial CO conversions with applications in synthesis gas purification and bio-desulfurization

Jan Sipma, Anne M. Henstra, Sofiya N. Parshina, Piet N.L. Lens, Gatze Lettinga and Alfons J.M. Stams

Abstract

Recent advances in the field of microbial physiology demonstrate that carbon monoxide is a readily used substrate by a wide variety of anaerobic micro-organisms, and may be employed in novel biotechnological processes for production of bulk and fine chemicals or in biological treatment of waste streams. Synthesis gas produced from fossil fuels or biomass is rich in hydrogen and carbon monoxide. Conversion of carbon monoxide to hydrogen allows use of synthesis gas in existing hydrogen utilising processes and is interesting in view of a transition from hydrogen production from fossil fuels to sustainable (CO_2-neutral) biomass. The conversion of CO with H_2O to CO_2 and H_2 is catalysed by a rapidly increasing group of micro-organisms. Hydrogen is a preferred electron donor in biotechnological desulfurization of wastewaters and flue gases. Additionally, CO is a good alternative electron donor considered the recent isolation of a CO oxidising, sulfate reducing bacterium. Here we review CO utilisation by various anaerobic micro-organisms and their possible role in biotechnological processes, with a focus on hydrogen production and bio-desulfurization.

Sipma, J., A.M. Henstra, S.N. Parshina, P.N.L. Lens, G. Lettinga and A.J.M. Stams. 2006 Microbial CO conversions with application in synthesis gas purification and biodesulfurization. Critical Reviews in Biotechnology 26:541-547.

1. Introduction

Hydrogen gas has great potential as a future fuel, as the greenhouse gas CO_2 is not produced upon its combustion. The transit from the current unsustainable fossil fuel based economy into a hydrogen economy may be realized gradually, in which fuel cells are the key energy providers both for stationary and mobile applications (81). Fuel cells, such as the low temperature proton exchange membrane fuel cell (PEMFC) and the high temperature solid oxide fuel cell (SOFC), hold the promise to develop into the ultimate universal, efficient, clean and silent devices for converting chemical energy directly into electricity. Progress on both fuel cells (6) and H_2 storage alloys (173) was made and their applications are close to market penetration. These issues are, however, out of the scope of this paper.

Besides the use as a fuel, hydrogen is a potent electron donor for various reductive reactions in biotechnology and in chemical process industry. Processes in which H_2 was shown to be an excellent electron donor include mesophilic and thermophilic sulfate reduction (224,227), denitrification of drinking water (118,212), reductive dehalogenation (67) and catalytic hydrogenation of biomass as green alternative for the production of high demand commodity chemicals, e.g. 1,2-propanediol (41).

Hydrogen is only a true “green” alternative for fossil fuels in case of hydrogen production from biomass fermentation or by extraction of H_2 from water utilizing renewable energy sources like wind, photovoltaics, geothermal energy and biomass. Increasing energy demands can be expected as a result of world population growth and their increased level of development. However, complete transformation from the current fossil fuel based to sustainable energy markets will take considerable time, if even possible at the present mineral fuel consumption in the prosperous world. In the meantime the fossil fuel based energy markets have to be transformed to mitigate the impact of CO_2 released by the use of fossil fuels on the earth’s climate. The production of H_2 gas from fossil fuels will be important in transition to a more sustainable energy production (81). Gasification of fossil fuels and subsequent purification of the produced synthesis gas may yield a highly purified H_2 gas and could prevent the scattered production of CO_2, although the total amount of CO_2 produced will not decrease.

A potential way to decrease overall CO_2 emissions to the earth's atmosphere is CO_2 fixation by carbon sequestration processes. Carbon sequestration would be feasible in case of centralized or large scale H_2 production. The use of fuel cells for generation of electricity with combined CO_2 capturing, concentration and sequestering is in line with current decarbonisation trends (5,81). Several options for sequestration in geological formations have been evaluated, all with different ratings on capacity, costs, storage integrity and technical feasibility. In this respect carbon sequestration in active oil wells, coal beds, depleted oil/gas wells, deep aquifers, mined caverns/salt domes is considered (81). In addition, chemical sequestration could attain more social acceptance for the abatement of CO_2 emissions. However, chemical CO_2 sequestration options are limited. A very promising option of carbon sequestration is mineral carbonation, in which a (pressurized) CO_2 stream is reacted with magnesium or calcium silicate mineral deposits in an exothermic reaction to form geologically stable mineral carbonates. The reaction is part of the natural geological carbon cycle, and can be accelerated through technology. The mineral end products are naturally occurring and harmless. Furthermore, this technology offers a more permanent solution than gas storage, thereby eliminating legacy issues for future (81).

This paper presents an overview of the potential of micro organisms to convert or use carbon monoxide (CO) present in synthesis gas. Besides the presence and activity of these micro organisms, the feasibility of a biological CO converting reactor depends largely on mass transfer rates from the gas to the liquid phase. Thus, reactor design is as important as the inoculum selection. Furthermore, the potential for direct use of CO-rich synthesis gas in biotechnological sulfate reduction without the need for synthesis gas purification is evaluated, as it is believed to enhance its utilization potential considerably.

2. Synthesis gas

2.1 Synthesis gas production and composition

Synthesis gas can be produced by gasification of organic sources, like coal, cokes, oil and natural gas (8,13) as well as from biomass (222) or industrial and municipal solid (organic) wastes (14). Synthesis gas consists mainly of a mixture of H_2, CO and CO_2 and minor amounts of other gasses,

Table 1: Typical composition of synthesis gas derived from various fossil fuel sources.

	Composition vol%, dry basis						
Fossil resource	CO	CO_2	H_2	N_2	CH_4	Other	Ref.
Coke oven gas	5.6	1.4	55.4	4.3	28.4	4.9	13
Water gas	30.0	3.4	31.7	13.1	12.2	9.6	13
Natural gas, steam reforming	15.5	8.1	75.7	0.2	0.5	0	13
Naphta, steam reforming	6.7	15.8	65.9	2.6	6.3	2.7	13
Partial oxidation of heavy fuel oil	47.0	4.3	46.0	1.4	0.3	1.0	225
Coal gasification	59.4	10.0	29.4	0.6	0	0.6	13

e.g. methane, nitrogen and hydrogen sulfide (158). Table 1 presents characteristic compositions of synthesis gas produced from different fossil sources.

Besides the use of synthesis gas as fuel, it is used as a major building block in the synthesis of various chemicals, e.g. in the synthesis of methanol and acetic acid (8,13). Synthesis gas can be obtained by direct gasification, i.e. processes in which oxygen or air is used as the oxidant, as in partial oxidation of coal. These partial oxidation reactions with oxygen are exothermic (8). Indirect gasification employs steam as the oxidant, resulting in an endothermic and often heat transfer limited, but thermodynamically more efficient process (8). The composition of synthesis gas varies greatly depending on its source and the gasification conditions employed. The CO concentration in synthesis gas increases with increased C to H ratio in the hydrocarbon feed, as is illustrated for steam reforming of coal and natural gas (methane) by equations 1 and 2.

$$C(s) + H_2O \rightarrow CO + H_2 \qquad \Delta G^0 = 100\ kJ.mol^{-1\#} \qquad (1)$$

$$CH_4 + H_2O \rightarrow CO + 3\ H_2 \qquad \Delta G^0 = 151\ kJ.mol^{-1\#} \qquad (2)$$

Therefore, steam reforming of natural gas theoretically results in lower CO levels than coal gasification. Process conditions influence synthesis gas composition as well, e.g. by synthesis gas exit temperatures or by the steam-to-feed carbon ratio (8,13).

The gasification of solid waste and biomass is generally more complex due to heterogeneity of the carbon-based materials. For an efficient gasification process certain homogeneity is required (14). Therefore, not all kinds of waste can be gasified as the required pre-treatment makes the process uneconomical. Waste types that are promising for production of synthesis gas include paper mill waste, mixed plastic waste, forest industry waste and agricultural residues (14). A major difference between fossil fuel gasification and biomass gasification is the method usually adopted. With fossil

fuels indirect gasification, with steam is the preferred method. While with biomass usually a gasification method is employed that uses air as the oxidant (14). The high content of nitrogen in the produced synthesis gas results in relative low heating values and classifies such synthesis gas as a low-grade gas. The use of pure oxygen improves the quality of the produced synthesis gas considerably, but it will also increase the costs for production of the synthesis gas. Maschio *et al.* (133) investigated a combined pyrolysis with indirect steam gasification (up to 950°C) of mixed woody biomass. This resulted in a synthesis gas with high concentrations of H_2 (20 – 50%) and CO (15 – 30%), which demonstrates the potential of biomass in the production of high quality synthesis gas. Another interesting method to convert biomass into a gaseous energy carrier is supercritical water gasification. This process directly deals with wet biomass without prior drying and is characterized by high gasification efficiencies at lower temperatures (134). A supercritical fluid is defined as a substance at conditions of temperature and pressure above its vapour-liquid critical point, i.e. for water above 374°C and 22 MPa. At supercritical conditions water shows characteristics between steam and liquid water. Supercritical water in particular has the ability to dissolve materials that are normally insoluble in water or steam. It also seems to promote specific chemical reactions that are beneficial for the gasification process (134).

Analysis of synthesis gas produced in an atmospheric circulating fluidized bed gasifier from ten residual biomass fuels, e.g. demolition wood, verge grass, cacao shells, revealed around 10% CO, 7% H_2 , 16% CO_2, 2.8% CH_4, and N_2 exceeding 60%, as main constituents (222). Within the ten tested samples these concentrations were quite similar, except for the woody fraction of the organic domestic waste, which had a rather low yield in CO and H_2 and consequently contained more N_2 in the synthesis gas (222). Faaij *et al.* (61) derived synthesis gas compositions of comparable biomass sources based on lab-scale fuel reactivity tests and subsequent gasifier model calculations, which are slightly higher in dry gas CO content (16-21%) and H_2 content (around 14%), similar in CO_2 content (16%), but considerably lower in N_2 content (around 47%). In another study, the gasification potential of a broad range of biomass wastes and residues in the Netherlands was evaluated (62), especially waste materials that are available at negative costs, i.e. when land filling or incineration are the treatment routes, could serve as interesting feed stocks for synthesis gas production. Potential waste streams for gasification include waste paper, demolition wood, verge grass, organic domestic waste,

organic rest products from the food industry and sewage sludge (62), waste products from agricultural crops, e.g. sugarcane waste (51) and manure (247). Sewage sludge and manure, however, are difficult materials due to the high nitrogen, sulfur, heavy metal, and high ash content. For use in gasification processes sewage sludge should be mixed with other waste streams to ensure sufficient calorific value of the resulting synthesis gas (61). Nevertheless, sewage sludge is an interesting source for synthesis gas production (141) as the overall sludge production is currently rapidly increasing (238). Despite the numerous research activities in the field of biomass gasification still important bottlenecks have to be overcome before biomass gasification can be economical viable for large-scale synthesis gas and energy production (205). Especially the presence of condensable organic compounds (tars) and the resulting gas composition ensures the need for extensive gas cleaning to allow advanced gas utilization. Currently research is focused at catalytic processes for biomass gasification (205).

2.2 Chemical catalytic water-gas-shift reaction

The major restriction of synthesis gas application is the presence of CO, which can range from as little as 5% to over 50% in case of coal gasification (Table 1). Biological reductive processes utilizing H_2 are generally quite sensitive to CO inhibition, e.g. sulfate reduction with synthesis gas was shown to be hampered by the presence of 5% CO (226). In PEMFC less than 10 ppm CO can be tolerated in order to ensure economical operation of this type of fuel cells, due to CO poisoning of the platinum catalyst (120). Therefore, it is essential to remove CO, preferentially by conversion to hydrogen. This results in a conversion of energy carrier, but not a significant loss of calorific value as the heat of combustion at standard conditions of H_2 and CO is similar at 286 $kJ.mol^{-1}$ and 283 $kJ.mol^{-1}$, respectively (122).

In order to convert synthesis gas into a highly purified H_2 gas, the CO present in synthesis gas is forced to react with water over a metal catalyst, yielding CO_2 and H_2. This reaction is known as the water-gas-shift reaction (reaction 3):

$$CO + H_2O \rightarrow H_2 + CO_2 \qquad \Delta G° = -20\ kJ.mol^{-1\,\#} \qquad (3)$$

The water gas shift reaction is predominantly carried out in a two-stage catalytic process. The first stage, also known as high temperature shift conversion, proceeds between 315 and 510°C using a reduced iron catalyst. At space velocities of 2000 to 4000 h^{-1} exit CO concentrations ranging from 2-

5 % can be obtained. Water to CO ratios of 2 to 4 are used to force the equilibrium towards hydrogen production (8,13). The second stage requires a more active zinc oxide-copper oxide catalyst, which enables operation at lower, thermodynamically more favorable, temperatures. The resulting enriched H_2 gas can have a CO-content as low as 0.2%. The residual CO generally is hydrogenated to methane using a reduced nickel catalyst after removal of bulk carbon dioxide (13).

Catalytic processes are generally easily poisoned by hydrogen sulfide (24). Therefore, a biological alternative, tolerating higher hydrogen sulfide concentrations and operating under far more moderate conditions (pressure, temperature) will become undoubtedly of interest for all industries, which produce or use synthesis gas.

3. Occurrence of carbon monoxide

3.1 Anthropogenic sources of CO

Besides its occurrence as a major component of synthesis gas, carbon monoxide is an abundant atmospheric pollutant generated to a large extent by incomplete combustion of fossil fuels in domestic and industrial processes, e.g. blast furnace gas contains 25% CO, automobile exhaust gas 0.5 to 12%(34). CO occurs in the troposphere at a concentration of 0.1 ppm and in polluted urban areas its concentration has been reported to reach levels of 50-100 ppm (34). Riveros *et al.* (168) measured average CO concentrations of 4 to 5 ppm in the atmosphere of Mexico City in 1992 and 1993. Inside motor vehicles ambient peak CO levels in the late afternoon, as high as 70 ppm have been recorded (9). Furthermore, CO contributes to ground level ozone and indirect greenhouse warming (110).

Natural sources of CO

Naturally formed carbon monoxide can be divided in non-biological (abiotic) and biological (biotic) formation processes, which will be discussed separately in the following sections.

3.2.1 Abiotic sources

Photochemical reactions and thermal decomposition of organic substrates are important abiotic CO generating processes. Generation of CO by light may occur by direct photolysis or via radical

mechanisms with reactive oxygen species, formed by photosensitizing reactions of molecular oxygen, ozone, or water. Photolysis of dissolved organic matter generates CO in oceans and fresh waters (28,37,39,252). CO is formed by oxidation of atmospheric CH_4 by ·OH-radicals. In soil surfaces radical formation is a likely mechanism for CO generation. Abiotic light-independent CO production in soils may occur by auto-oxidation of phenolic compounds and humic acids, which is stimulated by heat, as found by comparing CO formation at 20 and 60ºC (38). One of the major natural sources of CO is its liberation with volcanic gases (206), which may contain 1-2% of CO (211). Numerous other natural sources, most notably the world's oceans, contribute to CO emission to the atmosphere. It has been estimated that, the ambient concentration of CO could be expected to double within 4 to 5 years if it would not be degraded in the atmosphere. The life-time of CO in the atmosphere is about 40 days (95). Hence, CO apparently is not accumulating in the earth's atmosphere since ambient concentrations are remaining essentially constant.

3.2.2 Biotic sources

CO can be formed as a metabolite of microbial metabolism, e.g. heme degradation by aerobic micro organisms (150) and in low amounts by some thermophilic acetogenic bacteria and methanogenic archaea (206). Conrad and Thauer (40) first reported on the production of trace levels of CO under strictly anaerobic conditions. Lupton *et al.* (127) observed the production of small quantities CO by *Desulfovibrio vulgaris* under heterotrophic growth conditions, which was later also found with *Desulfovibrio desulfuricans* (47). Production of trace levels of CO was observed in the off-gas of a laboratory anaerobic digester fed with waste activated sludge (88). In that reactor CO was produced during methanogenesis and subsequently utilized when the substrate concentration was close to depletion. The production of CO was attributed to the presence of CO dehydrogenase, present in many methanogens, as methanogens that lack CO dehydrogenase activity did not produce measurable quantities of CO in batch culture (22). Bae and McCarty (10) observed CO formation after perturbations of methanogenic reactors fed with acetate and formate. Inconsistencies in CO production after substrate perturbations prevented the use of CO as a monitoring tool for the control of anaerobic treatment as proposed by Hickey *et al.* (88).

4. Microbial CO conversion

4.1 CO oxidizing micro organisms

CO is metabolized by a wide variety of micro organisms. A sharp division exists between aerobic and anaerobic species, as they contain fundamentally different enzyme systems for CO biotransformation. Aerobic CO oxidizing bacteria may be divided in two groups, i.e. metabolic, in which CO oxidation provides energy for growth, and co-metabolic, in which CO is used as pseudo-substrate for the enzyme system, but does not provide a nutritional value (34). The latter is observed during aerobic CO oxidation by methane oxidizing bacteria employing the methane monooxygenase complex, which is rather unspecific with respect to its substrate (45,89). Aerobic metabolic CO oxidizing bacteria, also known as carboxydotrophs, use CO as a source of energy, which is oxidized with O_2 as terminal electron acceptor. These bacteria contain a specific CO tolerant cytochrome b1 oxidase and O_2 insensitive Mo-Fe-flavin carbon monoxide dehydrogenase. Aerobic CO oxidation is not discussed in detail in this review, as it has been extensively reviewed elsewhere (139,140,145).

Whereas, aerobic CO oxidation results in the production of CO_2 and biomass in case of an energy-yielding CO metabolism, anaerobic conversion of CO is more complex and can results in the production of a range of valuable compounds. The key enzyme for CO conversions is carbon monoxide dehydrogenase (CODH), which catalyzes the following reversible reaction:

$$CO + H_2O \rightarrow 2\ H^+ + CO_2 + 2\ e^- \qquad (4)$$

In anaerobic micro-organisms, CODH may catalyze CO oxidation coupled to anaerobic respiration, or may help catalyze the synthesis or cleavage of acetyl-CoA as part of the acetyl-CoA synthase / CODH complex (ACS/CODH). As part of the ACS/CODH complex, CODH is involved in a great variety of energy yielding metabolisms (68) with as a central core the acetyl-CoA pathway. However, presence of the acetyl-CoA pathway does not necessarily imply CO utilisation as substrate for growth. Below, the various trophic groups that have been identified in CO metabolism are discussed. An overview of anaerobic micro organisms known to metabolize CO as source of carbon and energy, i.e. carboxydotrophic anaerobes, is presented in Table 2. The phylogenetic tree in Figure 1 demonstrates the wide distribution of CO utilisation among (facultative) anaerobic bacteria. Figure

Table 2: Overview of anaerobic microorganisms from different physiological groups that can utilize carbon monoxide as sole energy and carbon source.

CO oxidizing microorganisms[1]	T_{opt} (°C)	pH	t_d (h)	Max P_{CO} (kPa)	Products formed	Origin	Ref.
Anaerobic (acetogenic) bacteria							
Moorella thermoacetica	55	6.5-6.8	10	72	Acetate, CO_2	Horse faeces	44
Moorella thermoautotrophica	58	6.1	7	214	Acetate, CO_2	Mud and wet soils	175
Clostridium autoethanogenum	37	5.8-6.0	n.r.	100	Acetate, Ethanol, CO_2	Rabbit faeces	1
Oxobacter pfennigii	36-38	7.3	13.9	40	Acetate, n-Butyrate	Rumen of cattle	116
Clostridium ljungdahlii	37	6	3.8	105	Acetate, CO_2	Chicken yard waste	214
Peptostreptococcus productus	37	7	1.5	182	Acetate, CO_2	Sewage sludge digester	125
Acetobacterium woodii	30	6.8	13	n.r.	Acetate, CO_2	Mud	183
Eubacterium limosum	38-39	7.0-7.2	7	152	Acetate, CO_2	Rumen of sheep	182,183
Butyribacterium methylotrophicum	37	6	12-20	120	Acetate, Butyrate, Butanol and Ethanol	Sewage sludge digester	82,128,184
Methanogens							
Methanosarcina barkeri	37	7.4	65	101	$CH_4 + CO_2$	Anaerobic sewage	150
Methanosarcina acetivorans strain C2A	37	7	24	100	Acetate, Formate, CH_4	Marine mud	171
Methanothermobacter thermoautotrophicus	65	7.4	140	45	$CH_4 + CO_2$	Sewage sludge	45
Sulfate reducing bacteria							
Desulfovibrio vulgaris (strain Madison)	37	n.r.	n.r.	<4.5	Via H_2, CO_2, H_2S	Soil	127
Desulfovibrio desulfuricans	37	n.r.	n.r.	<20	Via H_2, CO_2, H_2S	Mud, soil, rumen sheep	47
Desulfosporosinus orientis	35	7	n.r.	<20	H_2S, CO_2	Soil Singapore	111
Desulfotomaculum nigrificans	55	7	n.r.	<20	H_2S, CO_2	Soil	111
Desulfotomaculum kuznetsovii	60	7	n.r.	<70	Acetate, H_2S, CO_2	Underground thermal	156
Desulfotomaculum thermobenzoicum subsp. *thermosyntrophicum*	55	7	n.r.	<70	Acetate, H_2S, CO_2	Methanogenic sludge	156
Desulfotomaculum carboxydivorans	55	7	1.7	180	Via H_2, CO_2, H_2S	Anaerobic granular sludge	157
Elemental sulfur reducing bacteria							
Thermoproteus tenax[2]	88	5.5	n.r.	n.r.	H_2S, CO_2	Solfatarra mud hole	69

[1]Species names according to most recent classification. *Methanobacterium thermoautotrophicum* was renamed as *Methanothermobacter thermoautotrophicus* by Wasserfallen *et al.* (232) The clostridial species *C. thermoaceticum* and *C. thermoautotrophicum* were reclassified into species of a new genus *Moorella* by Collins *et al.* (36). Collins *et al.* (36) also proposed the renaming of *C. pfennigii* into *Oxobacter pfennigii*. *Desulfotomaculum orientis* was recently renamed *Desulfosporosinus orientis* (197).

[2]Only grows in the presence of elemental sulfur as terminal electron acceptor

n.r. not reported

2 provides a combined schematic overview of carbon flow in the metabolisms that employ the acetyl-CoA pathway.

4.1.1 CO conversion by homoacetogens

The reductive acetyl-CoA pathway is essential in acetate formation by homoacetogens (244). In this pathway CO_2 functions as electron acceptor. Two CO_2 molecules are reduced to form the acetyl moiety of acetyl-CoA. Further conversion of acetyl-CoA to acetate is needed for the cell to obtain a net energy gain. In the reductive acetyl-CoA pathway of homo-acetogenic bacteria one ATP is invested to activate formate. This ATP is regained by substrate level phosphorylation in the conversion of acetyl-CoA to acetate. Additional chemiosmotic processes associated with the reduction of CO_2 to the methyl moiety of acetyl-CoA result in a net energy gain (52,93). In heterotrophic homoacetogens the oxidative degradation of organic compounds provides reducing equivalents for the reduction of CO_2. In chemolithoautotrophic homoacetogens the reducing equivalents are usual derived from H_2, but in some of these homoacetogens CO can function both as carbon source and energy substrate.

Table 2 summarizes the homoacetogens capable to grow on CO as sole substrate. However, Table 2 does not summarize all bacteria capable to oxidize CO, as several bacteria found to oxidize CO were either not capable of or not tested to grow on CO as sole substrate. Examples are *Clostridium pasteurianum* capable of CO oxidation at low CO concentrations when growing on glucose (75) or *Clostridium formicoaceticum* growing on fructose (53). Despite that all homoacetogens employ the ACS/CODH enzyme complex, its presence does not imply the ability to oxidize CO when present as the sole substrate. This is illustrated by the CODH activity, measured as a reduction rate of methylviologen, of 13.3 μmol per minute per mg protein in a strain of *Butyribacterium methylotrophicum* adapted for growth on CO as sole substrate, whereas the CODH activity of the Marburg strain of *Butyribacterium methylotrophicum*, unable to grow on CO alone, amounted to 47.2 μmol per minute per mg protein (128). Several homoacetogens were found capable to metabolize CO with stimulation of cell mass production, when grown on metabolic substrates like H_2/CO_2 or glucose, but were unable to grow at the expense of CO alone (220). The inability for growth on CO indicates that despite the ability to oxidize CO, the derived electrons could not be coupled to energy

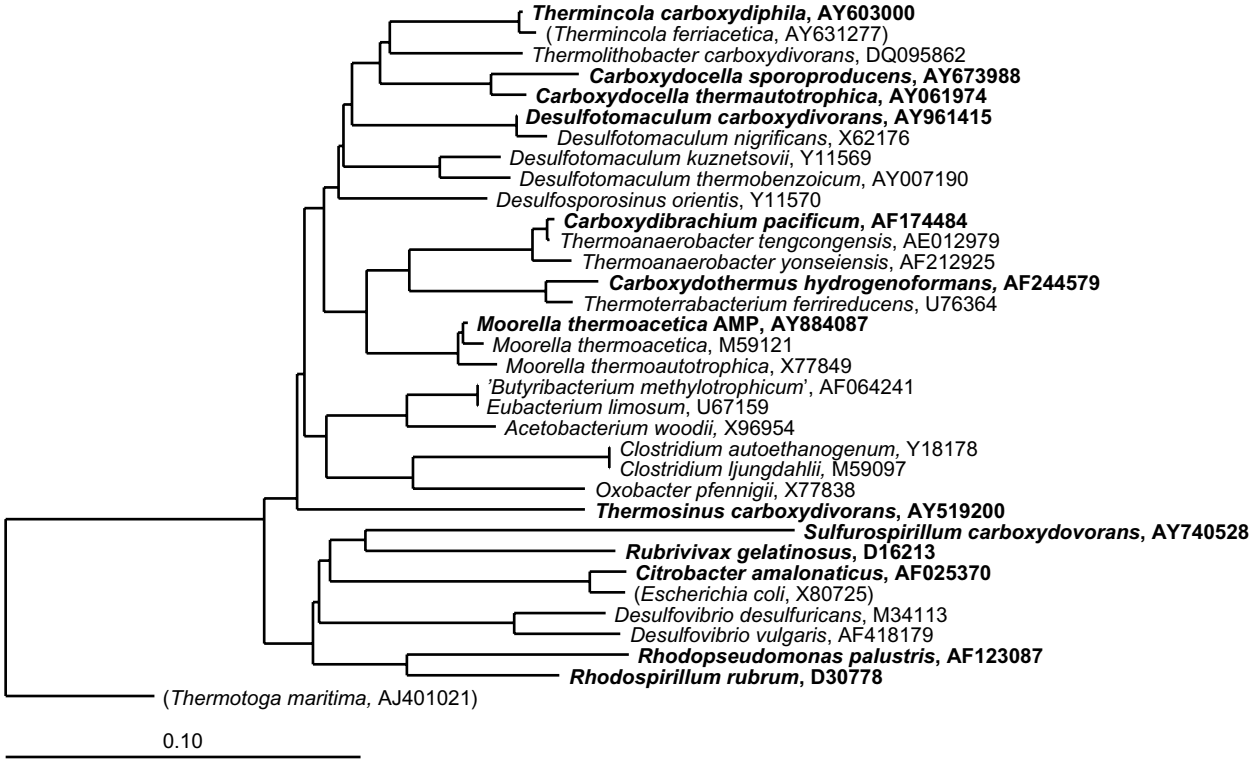

Figure 1:

16S rRNA gene sequence-based phylogenetic tree of (facultative) anaerobic carboxydotrophic bacterial species (Table 2, Table 3). Carboxydotrophic hydrogenogenic bacteria were listed in bold type face. Strains mentioned in Tables 1 and 2 without available 16S rRNA gene sequences were replaced by close relatives: *Rhodopseudomonas palustris* AF123087 replaces strain P4; *Citrobacter amalonaticus* replaces *Citrobacter* strain Y19; *Rubrivivax gelatinosus* D16213 replaces *Rhodopseudomonas* strain 1 (219). In parentheses, strains that were added in support but without reported carboxydotrophic properties; *Thermotoga maritima* (outgroup), *Escherichia coli*, *Thermoincola ferriacetica*. Alignment and phylogenetic analysis of 16S rRNA gene sequences were performed using the ARB software package (126). A phylogenetic tree was constructed using the neighbor joining procedure (172) as implemented in ARB, using a *Bacteria* 50% conservation filter provided with the latest release of the ARB small subunit rRNA database (ssu_jan04_corr). Accession numbers of reference sequences are indicated. The scale bar indicates the branch length that represents 10 % sequence dissimilarity.

conservation as was found for *Thermoanaerobacter kivui* (former *Acetogenium kivui*) during H_2-dependent growth (246).

Many homoacetogens can grow at high CO concentrations (Table 2). The reported values in Table 2 do not indicate that higher CO levels in the gas phase lead to growth inhibition, but merely that higher CO levels were not tested. Nevertheless, homoacetogens are well capable to convert CO not merely to acetate, but to a range of products including ethanol, butyrate and butanol (Table 2).

4.1.2 CO conversion by methanogens

The function of the reductive acetyl-CoA pathway in autotrophic methanogens is mainly assimilatory, although part of the CO_2 reduction steps of the pathway are also employed for methanogenesis (74,171). In acetoclastic methanogens the reverse reaction of acetate formation occurs, i.e. ACS/CODH catalyzes the cleavage of acetyl-CoA (68,145). The carboxyl group of acetyl-CoA is oxidized to CO_2 and the released reduction equivalents are used for further reduction of the methyl group to CH_4.

So far, only three representatives of methanogenic archaea have been found capable to grow with CO as the sole source of energy (Table 2), i.e. *Methanosarcina barkeri*, *Methanosarcina acetivorans* and *Methanothermobacter thermoautotrophicus* (45,150,171). CO acts as an electron donor in the reduction of CO_2 to methane by *Mtb. thermoautotrophicus* (45). O'Brien *et al.* (150) reported the evolution of H_2 during growth of *Msa. barkeri* on CO, when the partial pressure of CO in the gas

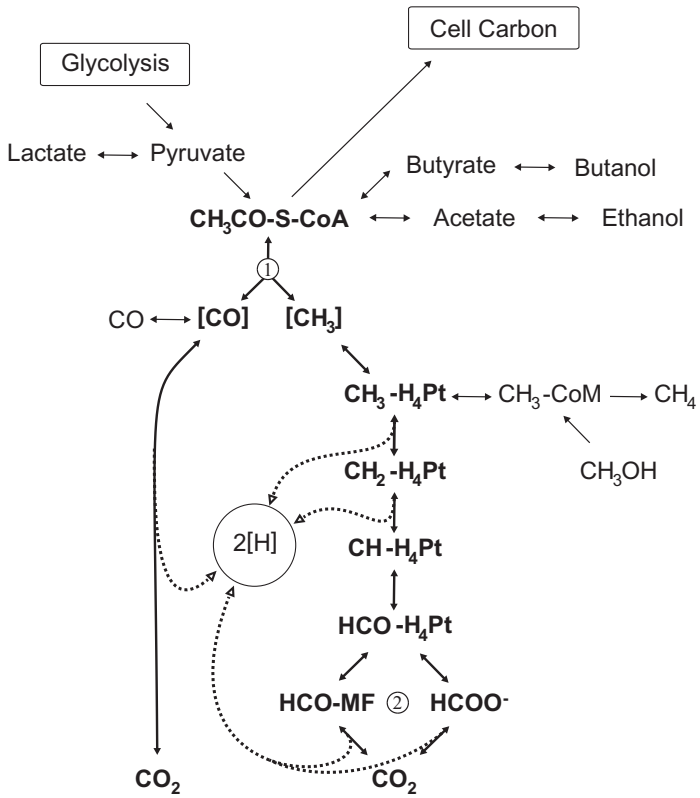

Figure 2:

Schematic representation of carbon flow in the acetyl-CoA pathway (bold letters, thick lines) and associated steps. Dotted lines indicate oxidative/reductive steps of the acetyl-CoA pathway. The bifunctional acetyl-CoA synthase / carbon monoxide dehydrogenase catalyses the step indicated by 1. Step 2, the conversion of CO_2 to HCO-H_4Pt or *vice versa*, generally proceeds through formate in Bacteria and through HCO-MF in Archaea. The acetyl-CoA pathway plays a role in the complete oxidation of organic substrates to CO_2, homo-acetogenesis, acetoclastic methanogenesis, autotrophic methanogenesis, and autotrophic carbon fixation. Abbreviations: Pt, pterin carrier, e.g. tetrahydrofolate, tetrahydromethanopterin or tetrahydrosarcinapterin; MF, methanofuran; HCO-, formyl; CH-, methenyl; CH_2-, methylene; CH_3-, methyl; CoA, coenzyme A; CoM, coenzyme M.

phase exceeded 20 kPa, whereas a net consumption of H_2 occurred below this value. This observation is in line with an early report of Kluyver and Schnellen (113), in which they suggested that CO conversion by methanogens proceeds via an intermediate production of H_2 and CO_2. The most convincing results concerned the liberation of H_2 during growth on CO with *Msa. barkeri* in the presence of a strong alkali solution in the headspace for absorption of the produced CO_2 (113).
Growth rates of *Msa. barkeri* and *Mtb. thermoautotrophicus* on CO are considerably lower compared to growth on H_2 as electron donor (45,150). *Msa. acetivorans* strain C2A exhibited higher growth rates on CO and may therefore be better capable to convert CO (Table 2). *Msa. acetivorans* strain C2A forms acetate, formate and methane during growth on CO, but at increasing CO pressures it reverts to acetogenesis (171). Methanogens are generally more sensitive to elevated levels of CO and growth as well as conversion cease rapidly with increasing CO levels (Table 2). Nevertheless, *Msa. barkeri* could be slowly adapted to an atmosphere of 100% CO (101 kPa) by successive transfers (150).

4.1.3 CO conversion by sulfate reducing bacteria

Despite the large number of sulfate reducing bacteria, reports on their growth with CO or growth in the presence of CO are scarce (Table 2). *Desulfovibrio vulgaris* strain Madison (127), the first sulfate reducer reported to be capable to use CO for sulfate reduction, converts CO to H_2 and CO_2 and subsequently uses the H_2 for sulfate reduction. A similar H_2-dependent sulfate reduction was found with *Desulfotomaculum nigrificans*, *Desulfosporosinus orientis* (111) and *Desulfovibrio desulfuricans* (47) with CO as source of energy. Furthermore, *Desulfovibrio baarsii* used low concentrations of CO (1.5% in the gas phase) as carbon source, but was not tested for its capacity to use CO as sole source of carbon and energy (96). All these sulfate reducing bacteria had a rather limited tolerance for CO, i.e. 4.5% for *Dsv. vulgaris* (127) and up to 20% for *Dsv. nigrificans*, *Dss. orientis* (111) and *Dsv. desulfuricans* (47). The conversion of CO to CO_2 and H_2 probably acts as a CO detoxification pathway, as it did not result in ATP synthesis and did not support growth on CO in the absence of sulfate (127). The CO toxicity is not only the result of an inhibition of hydrogenases by CO, as reported for *Dsv. vulgaris* (127) and *Dsv. desulfuricans* (47), since CO also inhibited sulfate reducers growing on organic substrates (47).

CODH activity is present in many sulfate reducing bacteria, e.g. in *Desulfobacterium autotrophicum* degrading acetate (176). In biochemical tests the presence or absence of CODH activity is used as indicator of the metabolic pathway employed in the complete oxidation of organic compounds to CO_2 or in autotrophic fixation of CO_2. Pathways employed by sulfate reducing bacteria for acetate oxidation or CO_2 fixation are the tricarboxylic acid cycle (TCA) and the acetyl-CoA pathway (176). CODH plays no role in the TCA cycle and is absence in representatives of this pathway, in which organic compounds are completely converted to CO_2. Indicative for the presence of the acetyl-CoA pathway is the absence of the TCA cycle enzyme, 2-oxoglutarate dehydrogenase, and presence of CO dehydrogenase activity (176). However, CO oxidizing activity in cell extracts does not necessarily imply CO utilization (142).

Until recently, CO was generally not considered a suitable electron donor for sulfate reducing bacteria, likely due to the apparent high toxicity of CO. However, it was demonstrated that *Desulfotomaculum kuznetsovii* and *Desulfotomaculum thermobenzoicum* subsp. *thermosyntrophicum* are capable of sulfate reduction with hydrogen in the presence of 50% and 70% CO, respectively. Especially *Dsm. thermobenzoicum* subsp. *thermosyntrophicum* showed a high CO tolerance, although sulfate reduction became inhibited above 50% CO in the gas phase (156). It is likely that CO is the direct electron donor for sulfate reduction in these organisms, although intermittently produced H_2 or acetate might also be considered. Both *Dsm kuznetsovii* and *Dsm thermobenzoicum* subsp. *thermosyntrophicum* are known hydrogenotrophic sulfate reducers (147,161), and both bacteria produced acetate with increasing CO pressures (156) analogous to *Msa acetivorans* C2A (171). Nevertheless, such a high tolerance for CO by any sulfate reducing bacterium has not been reported earlier and it holds a promise for using CO-rich synthesis gas in biotechnological desulfurization. In coculture with a CO tolerant H_2 producing carboxydotroph sulfate reduction in batch culture was even possible with higher CO levels (156).

Furthermore, the recently isolated *Desulfotomaculum carboxydivorans*, strain CO-1-SRB, from a full-scale anaerobic wastewater treatment plant is able to use CO as external electron donor for sulfate reduction. The novel isolate shows rapid growth at 55ºC (generation time 1.7 hours) on pure CO at a CO pressure of 180 kPa. Strain CO-1-SRB converts CO with H_2O in CO_2 and H_2 and can utilize the produced H_2 for sulfate reduction (157).

4.2 Hydrogenogenic microbial CO conversion

A specific group of microorganisms uses CO as a sole source of carbon and energy in the absence of an electron acceptor and form equimolar amounts of CO_2 and H_2, analogous to the water-gas-shift reaction described earlier. Growth is usually not supported by H_2/CO_2. The first observation of hydrogenogenic CO conversion dates back to the 1970s (219). For these microorganisms the terms hydrogenogenic, hydrogenogens and hydrogenogenesis were proposed recently by Svetlichnyi *et al.* (210), referring to the type of metabolism, physiological group, and H_2 formation process, respectively. Over the past decade the number of hydrogenogens rapidly increased (Table 3). Currently this carboxydotrophic hydrogenogenic metabolism is found in three distinct groups of prokaryotes, i.e. mesophilic Gram-negative bacteria, thermophilic Gram-positive bacteria, and thermophilic archaea (Table 3).

4.2.1 Hydrogenogenic mesophilic Gram negative bacteria

Facultative anaerobic bacteria that oxidize CO and evolve H_2 isolated thus far are Gram-negative mesophilic bacteria. Hydrogen is only produced under anaerobic conditions upon CO oxidation. Generally growth rates on CO are low and high levels of CO are inhibitory. Non-sulfur purple bacteria form the predominant part of this group of bacteria. Oxidation of CO coupled to formation of equimolar amounts of H_2 was first discovered with *Rhodopseudomonas gelatinosa* (46,219), later reclassified as *Rhodocyclus gelatinosa* (94) and more recently as *Rubrivivax gelatinosus* (241). Hydrogenogenic CO conversion was also observed with protein extracts of *Rhodospirillum rubrum* S1 (220), although the growth rates of *R. rubrum* S1 were considered too low for elucidating the microbiology of CO metabolism. However recently, Kerby *et al.* (107) demonstrated that *R. rubrum* in fact was capable of a rapid anaerobic growth on CO in the dark, but only after increasing the nickel concentration in the medium. Two different strains of *R. rubrum* revealed different $NiCl_2$ requirements with concentrations exceeding 75 and 600 μM necessary for growth. Besides different nutrient requirements for growth on CO, the presence of CO_2 may have a stimulatory effect on growth and CO conversion, as recently reported for the homoacetogen *Moorella thermoacetica* (55). *R. rubrum* so far is the most studied organism, especially with respect to the properties of its CO dehydrogenase. A major drawback of photosynthetic bacteria in H_2 production from synthesis

gas derived CO is the light requirement for optimal cell growth. *Rhodopseudomonas palustris* P4 (103) was found capable of hydrogenogenic CO conversion when incubated anaerobically in the dark, although growth completely ceased in the absence of light. It was postulated that *Rhodopseudomonas palustris* P4 only obtains maintenance energy from the hydrogenogenic CO conversion (103).

Besides these phototrophic strains only one non-phototrophic gram negative facultative anaerobe was described so far capable to convert CO to H_2, viz. *Citrobacter* strain Y19, isolated from an activated sludge plant (104). As the growth of this organism under anaerobic conditions compared to aerobic conditions is low, a two step cultivation of the biomass for biotechnological application was proposed, i.e. an aerobic growth phase followed by an anaerobic CO conversion phase (104). Nevertheless, separation of growth and bioconversion complicates the reactor operation considerably.

4.2.2 Obligate anaerobic thermophilic hydrogenogenic bacteria

A rapidly increasing group of carboxydotrophic hydrogenogenic prokaryotes is formed by strict anaerobic thermophiles that belong to the *Bacillus/Clostridium* subphylum of bacteria (Table 3). Conversion of CO to H_2 at elevated temperatures has been observed in freshwater as well as marine environments with temperatures ranging from 40 to 85 °C and pH between 5.5 and 8.5 (17,206). Hydrogenogenic carboxydotrophy was also found in sludges of anaerobic wastewater treatment plants incubated at moderately thermophilic (55ºC) conditions (186). From one of the tested anaerobic sludges the previously mentioned *Dsm. carboxidivorans* was isolated, which exhibited hydrogenogenic and sulfate reducing growth with CO (157).

All isolated species are capable of chemolithotrophic growth on CO. So far, there exists no evidence of growth inhibition by high levels of CO. Some isolates also grow by fermentation or anaerobic respiration. Because of simultaneous H_2 production and acceptor reduction it is unknown whether CO is a direct electron donor in anaerobic respiration or H_2 acts as an intermediate. *Carboxydothermus hydrogenoformans* was originally described as obligate carboxydotroph (207). Later it was found capable to ferment pyruvate to acetate (208), and more recently that it could respire anaerobically CO as electron donor with different electron acceptors, e.g. iron, nitrate and quinones (87). *Carboxydobrachium pacificum* (reclassified as *Caldanaerobacterium subterraneus* subsp. *pacificus*)

is the only marine carboxydotrophic bacterial species described so far (64,192). It was isolated from a submarine hydrothermal vent. Besides on CO, it also grows organotrophically on several mono- and disaccharides, cellulose and starch. External electron acceptors were not tested. *Thermincola carboxydophila* grows up to pH 9.5 and thus is an alkali-tolerant, moderate thermophilic, obligately hydrogenogenic carboxydotroph that required acetate as carbon source for growth (194). *Thermosinus carboxydivorans* is the only species with a Gram negative cell wall in this group of thermophilic carboxydotrophs that also clusters in the *Bacillus/Clostridium* phylum (191).

4.2.3 Hydrogenogenic CO converting archaea

So far, *Thermococcus* strain AM4 is the only representative of the carboxydotrophic hydrogenogenic archaea, isolated from a hydrothermal vent on the east pacific rise (193). Other tested *Thermococcus* species did not exhibit chemolithotrophic growth with CO (193). The genome sequence of *Archaeoglobus fulgidus* contains CO dehydrogenase genes highly similar to those of *C. hydrogenoformans* and *R. rubrum* (79). This led to the suggestion that this organism may be another candidate for carboxydotrophic hydrogenogenic growth (112).

4.3 Recently discovered anaerobic CO respirations

The possible role of CO as electron donor in anaerobic respiration has received little attention until recently. Since, the standard electrode potential of the CO/CO_2 couple (E°' -520 mV) is lower than that of the H_2/H^+ couple (E°' -414 mV), CO can theoretically replace H_2 in anaerobic respiration. However, the number of species known to use CO in anaerobic respiration is still limited. *M. thermoacetica* can grow chemolithotrophically with CO as electron donor and nitrate as electron acceptor (55,73). Furthermore, *Thermosinus carboxydivorans* reduces ferric iron and selenite with CO as electron donor (191), and *C. hydrogenoformans* reduces fumarate and 9,10-antraquinone-2,6-disulfonate (AQDS) with CO as electron donor (87). *C. hydrogenoformans* reduces nitrate, thiosulfate, sulfur, and sulfite with lactate as electron donor, but according to Henstra and Stams (87) CO might be able to serve as electron donor as well. However, *Thermosinus carboxydivorans* and *C. hydrogenoformans* form H_2 from CO, which might be the actual electron donor for these reductions. In contrast, *Thermoterrabacterium ferrireducens* does not form hydrogen with CO, but

Table 3: Hydrogenogenic CO converting microorganisms.

Microorganism[1]	T_{Opt} (°C)	pH opt	t_d(h)	max P_{CO} (kPa) [2]	Origin	Ref.
Phototrophs						
Rubrivivax gelatinosus	34	6.7-69	6.7	101	Lake and pond sediments	46,219
Rhodopseudomonas palustris P4	30	n.r.	23	101	Anaerobic sludge digester	103
Rhodospirillum rubrum	30	6.8	8.4	101	Brakish ditch	107
Facultative anaerobes						
Citrobacter sp Y19	30-40	5.5-7.5	8.3	505	Wastewater sludge digester	104,105
Obligate anaerobes						
Carboxydothermus hydrogenoformans	70-72	6.8-7.0	2	101	Hot swamp, Kunashir Island	207
Carboxydothermus restrictus	70	7	8.3	101	Terrestrial hot vent	208
Carboxydibrachium pacificus[1]	70	6.8-7.1	7.1	110	Submarine hot vent	192
Carboxydocella thermoautotrophica	58	7	1.1	101	Terrestrial hot vent	195
Thermincola carboxydiphila	55	8	1.3	101	Hot spring, Baikal lake	194
Thermosinus carboxydivorans	60	6.8-7.0	1.2	100	Hot pool, Yellowstone Park	191
Desulfotomaculum carboxydivorans	55	6.8-7.0	1.7	180	Anaerobic granular sludge	157
Archaea						
Thermococcus strain AM4	82	6.8	n.r.	101	Submarine hot vent	193

[1] Species names according to most recent classification. *Rubrivivax gelatinosus* was formerly classified as *Rhodopseudomonas gelatinosus* as well as *Rhodocyclus gelatinosus* (241). *Carboxydobrachium pacificum* was re-classified as *Caldanaerobacter subterraneus* subsp. *pacificus* (64,192).

[2] Maximal CO concentration tested, at which CO oxidation proceeds and therefore does not necessarily represent the maximal tolerance of CO for a specific microorganism

[3] Growth was only observed in the light, but no H_2 production was measured in the light; H_2 production could be maintained for prolonged periods when light was eliminated at the late growth phase

[4] The generation time of *R. rubrum* decreased with increasing P_{CO} and the lowest observed generation time was 4.8 hours when initially 25 kPa CO was present

[5] Growth and CO conversion to H_2 occurred at 50 kPa, but growth rates were decreasing with increasing P_{CO} (at P_{CO} = 50 kPa, Td = 8.3 h). H_2 production activity expressed in mmol/g cell.h decreased as well with increasing P_{CO}, although at a P_{CO} between 30 and 50 kPa H_2 production activity was similar.

n.r. not reported

is able to reduce AQDS and fumarate with CO. Besides AQDS *Thermoterrabacterium ferrireducens* may reduce Fe(III), nitrate, sulfite, thiosulfate and sulfur with CO as well, as it does with H_2 or lactate (87,188). *Sulfurospirillum carboxydovorans* was shown to reduce elemental sulfur, DMSO, and thiosulfate with CO, out of a large range of electron acceptors. However, growth was slow (t_d = 50 h) with a 100% CO gas phase. Interestingly, perchloroethylene (PCE) was reduced with pyruvate

by this organism, but CO was not tested. Nevertheless, this opens the possibility to dechlorinate environmental pollutants with CO (100).

The exact range of microorganisms capable to use CO is still unclear, as CO utilization is rarely tested in growth studies. Although CO may initially inhibit growth, adaptation to CO can occur after long term incubation or multiple transfers with increasing CO levels (150,171). Furthermore, growth on CO may require different nutrients or concentrations(107). Even habitats, in which CO does not seem to be present, may contain CO oxidizing microorganisms, as shown with different anaerobic bioreactor sludges (186). The ability of CO oxidation to CO_2 seems ubiquitously present in nature (68).

4.4 Key enzymes involved in CO conversion to H_2

Anaerobic carboxydotrophic hydrogenogenic microorganisms conserve energy by oxidation of CO to CO_2 coupled to reduction of protons to H_2. These reactions are catalyzed by CO dehydrogenase and hydrogenase, respectively. These two enzymes must conserve energy in a yet unknown energy conserving mechanism as they do not fit in classical substrate level phosphorylation (SLP) and electron transfer phosphorylation (ETP) theories (84). The Gibbs free energy of the reaction under standard physiological conditions is –20 kJ.mol^{-1} (Table 4), insufficient to be directly coupled to ATP formation via SLP (ATP $\rightarrow$ ADP+Pi; ΔG^{o} = -31.8 kJ.mol^{-1}). In ETP an electrochemical proton gradient, i.e. a proton motive force (pmf) is generated over the cytoplasmic membrane by coupling electron transfer to proton membrane-translocation. Electrons released by oxidation of an electron donor are transferred via a chain of electron carriers to a terminal electron acceptor. E.g. quinones accept electrons and protons at the cytoplasmic side of the membrane and release protons at the periplasmic side of the membrane and transfer electrons to the next component of the chain, thus facilitating proton translocation. However, standard electrode potentials of known quinones fall outside the CO_2/CO (E°' -520 mV) and H^+/H_2 (E°' -414 mV) redox couple (84,215). It is unlikely that an electron transfer chain composed of quinones and cytochromes facilitates proton translocation between CODH and hydrogenase. Instead it has been proposed that CODH and an unknown type of hydrogenase form one membrane associated enzyme complex that facilitates CO oxidation, proton reduction and proton translocation (59,84,210). A short overview of CODH and hydrogenase is presented.

4.4.1 Carbon monoxide dehydrogenases (CODH)

CO dehydrogenases (CODHs) of anaerobic prokaryotes are nickel containing, O_2 sensitive enzymes and are either monofunctional or bifunctional (123). Monofunctional CODHs catalyze the oxidation of CO, which is part of the energy metabolism and linked to reduction of protons by the energy converting hydrogenases (ECH) or to the reduction of various other electron acceptors in anaerobic respiration. Recent publications reported about the reduction of sulfate, nitrate, fumarate, Fe(III) and selenate with CO (55,73,87,156,191). A single microbial species may contain multiple CODHs, each with its own physiological function (123). E.g. five CODH genes were found in the genome of *C. hydrogenoformans* and possible functions were discussed (245). Bifunctional CODHs catalyze the synthesis of acetyl-CoA or its decarbonylation beside the oxidation of CO. These CODHs form a complex with acetyl-CoA synthase (ACS) and are denominated ACS/CODH. Synthesis of acetyl-CoA by ACS/CODH is a key step in carbon fixation of autotrophic anaerobic prokaryotes, in acetate formation by homoacetogens and in acetate metabolism in sulfate reducing bacteria and methanogenic archaea. Decarbonylation of acetyl-CoA via the oxidative acetyl-CoA pathway allows for complete oxidation of organic substrates to CO_2 by heterotrophic methanogens and sulfate reducers (68,244). The biochemistry of ACS/CODH catalysed acetyl-CoA synthesis has been extensively reviewed recently by Ragsdale (164) and Lindahl (123). Here we focus on the phylogeny and metabolic roles of the mono- and bifunctional CODHs. ACS/CODH complexes are composed of multiple subunits that together comprise a CODH and a corrinoid-iron-sulfur protein (CoFeSP). While CODH delivers the carboxyl group of acetyl-CoA, CoFeSP carries the methyl group. Archaeal ACS/CODH are typically composed of 5 subunits (α, β, γ, δ, ε) which γ-δ form the CoFeSP moiety, α-β contain CODH activity, and ε interacts with the acetyl group of acetyl-CoA. Bacterial ACS/CODH are formed by two autonomous proteins composed of a total of 4 different subunits. CODH functionality is present in a protein of α_2-β_2 configuration and a γ-δ heterodimer forms CoFeSP. Recently, however, it was demonstrated that *C. hydrogenoformans* contains a monomeric ACS unit (209). Monofunctional CODH are α_2 homodimeric proteins. Note that in literature the α subunits of archaeal ACS/CODH and the monofunctional CODHs that contain CODH activity are similar to the β subunit of bacterial ACS/CODH, while the α subunit of bacterial ACS/CODH is similar to the β subunit of archaeal ACS/CODH (123).

The phylogeny of the CODH subunits with CO oxidizing activity shows that essentially two types of Ni-CODH exist (124). Monofunctional CODH are relatively closely related to the CODH subunit of bacterial ACS/CODH, while α subunits of archaeal ACS/CODH are more distinct. Archaeal type CODH is exclusively found in archaea as part of the bifunctional ACS/CODH. Bacterial type CODH is found in bacteria both as monofunctional and bifunctional enzymes and in archaea as monofunctional CODH.

The presence of multiple CODH genes in one organism is explained by the different metabolic functions that are associated with CODH (123), e.g. the genome of the extremely thermophilic sulfate reducing archaeon *A. fulgidus* contains three open reading frames that code for putative CODHs (Klenk *et al.*, 1997). Two have the archaeal α-ε configuration (AF1100/1101, AF2397/2398), while the third (AF1849) is homologous to the CODH of *R. rubrum* (*CooS*) and *C. hydrogenoformans* (79). *A. fulgidus* contains only one gene copy of the γ (AF0376), δ (AF0377) and β (AF0379) subunits, besides the two α-ε open reading frames. It is known that *A. fulgidus* completely oxidizes lactate to CO_2 with sulfate as electron acceptor and grows autotropically on H_2, CO_2 and thiosulfate (231). The presence of bacterial type CODH homologous to *cooS* indicates the possibility that this organism can evolve H_2 with CO as a substrate (112).

The monofunctional CODHs of *R. rubrum* (CooS) and *C. hydrogenoformans* involved in H_2 evolution are functionally associated with the iron-sulfur protein CooF, which contains 4 [4Fe-4S] clusters (79,106). CooF mediates electron transfer from CooS to ECH (Fig.3)(189,210). Although, CO can serve as electron donor for the reduction of external electron acceptors in anaerobic respiration (87,191), no data are available that indicate the nature of the physiological electron acceptor of the CODH. H_2 may act as intermediate if ECH is present. However, *Thermoterrabacterium ferrireducens*

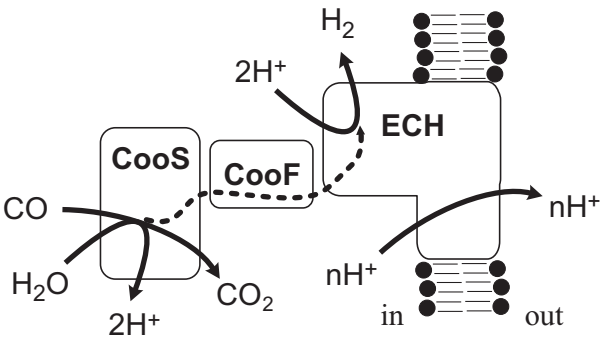

Figure 3:

Simplified scheme of CO oxidation, electron transfer (dotted line), H_2 evolution and proton translocation as proposed for the CO oxidizing:H_2 evolving enzyme complex of *C. hydrogenoformans* (after Hedderich 2004)(84).

does not evolve H_2 in the presence of CO but can reduce various electron acceptors (87). The sulfate reducing bacteria, *Dsm. kuznetsovii* and *Dsm. thermobenzoicum* subsp. *thermosyntrophicum* reduce sulfate with CO as electron donor apparently without intermediate production of H_2 (156). Therefore an intermediate electron acceptor must be present for CODH to transfer electrons to the electron transfer chain.

4.4.2 Hydrogenases

Hydrogenases catalyze the reduction of protons to H_2 or the reverse reaction according to:

$$2H^+ + 2e^- \rightarrow H_2 \qquad (5)$$

The physiological function of hydrogenases is generally restricted to one of these directions and is referred to as hydrogen uptake hydrogenases or hydrogen evolving hydrogenases. Recently the classification and phylogeny of hydrogenases was reviewed by Vignais *et al.* (229). Three classes of hydrogenases are recognized based on phylogeny and metal content (also transition-metal content or H_2-activating site content). The first and largest class is formed by the [NiFe]-hydrogenases. The second class, [FeFe]-hydrogenases only contain Fe in their active site, while the third class is formed by hydrogenases that until recently were named 'metal-free' hydrogenases (15). The latter class was discovered in methanogens, where they catalyze the reduction of F_{420} with H_2 in complex with methylenetetrahydromenthanopterin dehydrogenase under nickel-deprived conditions (2,3,250). Now they are referred to as iron-sulfur cluster free hydrogenases, since the presence of a novel light sensitive iron-coordinating cluster in this type of hydrogenases was found (29,129,185).

Awareness that a unique subclass of [NiFe]-hydrogenases exists has grown in the past decade. These hydrogenases were indicated as energy converting hydrogenases (ECH), after their capacity to couple proton translocation to the reduction of protons or oxidation of molecular H_2, and were recently reviewed by Hedderich *et al.* (84). A limited sequence similarity and a deviating enzyme topology mark the main differences between ECH and other [NiFe]-hydrogenases. The basic structure of [NiFe]-hydrogenases comprises two subunits, called the hydrogenase large and the hydrogenase small subunit. ECH large and small subunit share little sequence similarity with other [NiFe]-hydrogenases apart from the metal binding centres. ECH are membrane bound enzyme complexes

composed of a minimum of 6 subunits of which two are integral membrane proteins. ECH play a key role in energy generation in the carboxydotrophic hydrogenogenic metabolism (84).

As mentioned, ECH couple oxidation of H_2 or reduction of protons to translocation of protons over the cytoplasmic membrane. The electrochemical gradient of protons over the membrane is generally referred to as proton motive force (pmf) and is the driving force for ATP synthesis. Translocation of protons by ECH may generate or dissipate the pmf, depending on its direction. Oxidation of H_2 is linked to a dissipation of the pmf and plays a role in reversed electron transfer (138). For example, dissipation of the pmf drives the endergonic reduction of a low potential ferredoxin with H_2 in the archaeon *Msa. barkeri*. A reaction that is thermodynamically unfavorable, especially if the low physiological concentrations of 5 ppm H_2 are considered (16,137). Reduction of protons to H_2 is coupled to the generation of a pmf, which in turn drives ATP synthesis. The number of suitable electron donors for reduction of the protons is limited by the relatively low electrode potential of the H^+/H_2 couple (E°' -414 mV). The CO_2/CO couple (E°' -520 mV) is sufficiently low to drive proton translocation by ECH, but also formate or reduced ferredoxin generated by pyruvate:ferredoxin oxidoreductase in fermentative metabolisms serve as electron donors for ECH (11,84,174,190). Reduced ferredoxin may donate electrons directly to ECH while CODH and formate dehydrogenase form a complex with ECH. In these complexes a ferredoxin like subunit facilitates electron transfer. In the carboxydotrophic hydrogenogenic metabolism ECH together with CODH play an important role as described for *C. hydrogenoformans* and *R. rubrum* (71,72,84). These organisms contain similar enzymatic systems that catalyze the conversion of CO into H_2. Genes that code for the involved enzymes are arranged in two gene clusters that share high sequence similarity between both organisms. One cluster comprises the genes for the 6 subunits of ECH, the other cluster encodes for a CODH and CooF. One functional CO oxidizing H_2-evolving complex is formed with these subunits, as was shown for *C. hydrogenoformans* (189). Coupling of proton translocation to oxidation of CO by *C. hydrogenoformans* and *R. rubrum* enables them to use CO as sole source of energy and to grow with the formation of H_2.

5. Biotechnological applications of CO converting microorganisms

Interest in applying suitable biocatalytic processes for the production of useful chemicals from CO or synthesis gas has led to various studies into production of alternative compounds, e.g. methane, acetate, butyrate and other organic compounds (248). The conversion of CO to ethanol and butanol was demonstrated for *Butyribacterium methylotrophicum* (184) and for a recently isolated *Clostridium* species strain P7 (165). Studies employing methyl viologen and other viologen dyes, as inhibitors of methanogenesis, resulted in the production of formate by *Msa. barkeri*(135) and methanol by *M. thermoacetica* (239). Lapidus *et al.* (119) showed that cell free extracts of *Dsv. desulfuricans* can produce methanol, ethanol, acetic acid and C_8-C_{24} paraffins from CO and H_2 at elevated pressures. Incubations of different inocula with mixtures of H_2/CO/CO_2, revealed the presence of various acids, ranging from acetic to caproic acid, but only acetic acid and butyric acid were observed in high quantities (121). Table 4 summarizes thermodynamically possible synthesis gas fermentations, either from CO or H_2/CO.

Besides the use of growing cells, also purified enzymes of CO converting organisms, especially CODH, might offer interesting potentials in biotechnology (68). Purified CODH could be used in biofilters for cleaning the air in underground car parks or in biosensors for CO detectors (34). Recently, a CODH from *M. thermoacetica* was found to catalyze the reduction of 2,4,6-trinitrotoluene (TNT), an important chemical explosive commonly present in soil of military training sites (92). CODH can be applied in dechlorination as well as in the reductive carboxylation of phenols (68).

With the increasing discovery of fast growing anaerobic isolates capable of hydrogenogenic CO conversion (Table 3) together with the current interest in H_2, a biological alternative for the chemical water-gas-shift reaction might represent one of the most interesting applications. In the next sections the use of hydrogenogenic bacteria for the purification of synthesis gas will be discussed, as well as the application of CO converting organisms in biological desulfurization.

5.1 Biotechnological synthesis gas purification

The actual biotechnological application of hydrogenogenic CO converting bacteria as an alternative for the currently employed chemical water gas shift reaction has received little attention so far.

Bredwell *et al.* (24) and Vega *et al.* (228) studied the design of bioreactors for coal synthesis gas fermentations. They did not study the hydrogenogenic conversion of CO, but conversions of synthesis gas into methane, acetate, ethanol and butanol. Wolfrum and Watt (243) investigated the application of bioreactors for the production of H_2 from CO, by employing *Rubrivivax gelatinosus* strain CBS (131) in a trickle-bed bioreactor. A maximal CO conversion of 74% in continuous experiments was found. This research aimed to estimate design parameters rather than to optimize H_2 production. Recently, an enhanced integrated hydrogen production system was proposed, including biomass gasification, solid oxide and proton exchange fuel cells, biological hydrogenogenic CO conversion by *Rubrivivax gelatinosus* strain CBS and CO_2 sequestering by the same organism when growing in the light (136). A limitation of using phototrophic organisms in a bioreactor obviously is that their growth under anaerobic conditions in the dark is usually low compared to growth in the light and light-dark cycles may be required to sustain a sufficiently high population density in the reactor. However, with the discovery of several anaerobic microorganisms lately, showing even higher growth rates on CO alone (Table 3), the selection of another micro organism could be considered.

The challenge in designing bioreactors for the commercial conversion of sparingly soluble gaseous substrates is to enhance its mass transfer from the gas phase to the microorganisms that are present in the liquid phase (24,228). This has been the subject of many studies in biological treatment of waste gas and their results can be beneficially applied for the purification of synthesis gas. The fundamental difference in biological conversion of waste gas compared with synthesis gas, which is rather a purification than pollution mitigation, is the concentration of the compound that requires conversion. Whereas, the concentration of the pollutants in biological waste gas treatment usually is in the lower ppm range, the concentration of CO in synthesis gas may exceed 50% in coal gasification (Table 1).

In case of biological waste gas treatment a vast experience in reactor design already is available, from which a future commercial biological hydrogen production from synthesis gas may benefit. In their design, biological waste gas treatment reactors may be divided in 4 basic concepts, i.e. biofilters (70), biotrickling filters (42,160), bioscrubbers (178,223), and membrane bioreactors (60,166). Bioscrubbers likely are not suited for hydrogenogenic CO conversion in synthesis gas, as these are conventionally used to transfer the pollution to a liquid, which subsequently is converted in a separate

reactor and only functions well for water soluble pollutants. Biofilters and biotrickling filters represent more practical options, whereas membrane bioreactors are still in their infancy. The use of biotrickling filters has received attention in waste gas treatment as they are relatively easy to control, with respect to nutrient supply and pH. Biotrickling filters are known to be susceptible for clogging due to excess growth of biomass (160). Ariga *et al.* (7) already pointed out the potential advantages of using honeycomb-monolith bioreactors for bioconversions of gaseous substrates due to their low pressure drop and thin liquid layers. However, this concept has not yet found practical applications in biological gas treatment, although the working principle is quite similar to that of biotrickling filters, i.e. thin liquid layer to enhance mass transfer from the gas phase to the microorganisms. Also the concept of a 'dry' granular activated carbon biobed inoculated with strain *Mycobacterium* E3 was investigated for ethene removal from a synthetic waste gas (49). This study showed that under relative dry conditions the removal rate could be increased with a factor 2.25. This study shows that operational modifications of a conventional biofilter could be beneficial for increasing mass transfer, although supply of nutrients and desired pH value are even more difficult to control than in conventional biofilters.

Table 4: Summary of reported reactions with CO and H_2/CO, characteristic for synthesis gas fermentations.

Product	Reaction	$\Delta G^{\circ'}$ kJ/mol CO[#]
From CO		
formate	$CO + H_2O \rightarrow HCOO^- + H^+$	-16
acetate	$4\ CO + 2\ H_2O \rightarrow CH_3COO^- + H^+ + 2\ CO_2$	-44
butyrate	$10\ CO + 4\ H_2O \rightarrow CH_3(CH_2)_2COO^- + H^+ + 6\ CO_2$	-44
ethanol	$6\ CO + 3\ H_2O \rightarrow CH_3CH_2OH + 4\ CO_2$	-37
n-butanol	$12\ CO + 5\ H_2O \rightarrow CH_3(CH_2)_3OH + 8\ CO_2$	-40
hydrogen	$CO + H_2O \rightarrow H_2 + CO_2$	-20
methane	$4\ CO + 2\ H_2O \rightarrow CH_4 + 3\ CO_2$	-53
From H_2/CO		
acetate	$2\ CO + 2\ H_2 \rightarrow CH_3COO^- + H^+$	-67
butyrate	$4\ CO + 6\ H_2 \rightarrow CH_3(CH_2)_2COO^- + H^+ + 2\ H_2O$	-80
methanol	$CO + 2\ H_2 \rightarrow CH_3OH$	-39
ethanol	$2\ CO + 4\ H_2 \rightarrow CH_3CH_2OH + H_2O$	-72
n-butanol	$4\ CO + 8\ H_2 \rightarrow CH_3(CH_2)_3OH + 3\ H_2O$	-81
methane	$CO + 3\ H_2 \rightarrow CH_4 + H_2O$	-151

[#] Standard Gibbs free energy changes (273.15 K; 101.325 kPa) were calculated at pH 7 using the thermodynamic data set compiled by Amend and Shock (4).

5.2 Product requirements for synthesis gas purification and product upgrading

The application potential of biological synthesis gas purification depends first of all on the capabilities of the selected micro organism. When designing a synthesis gas purification system the most important factor is the required product specification, especially with respect to the permitted remaining CO levels, which will depend on the application of the produced H_2 gas. For application in low temperature PEMFC, the purification requirement is to the level of maximally 100 ppm CO (120), and preferably even less than 10 ppm (120,153). For other applications, e.g. when H_2 is used in chemical or biological reductive processes, the requirements may be less stringent.

Nevertheless, for most synthesis gas purification processes, the separation of H_2 from the exhaust gas of the bioreactor, which will generally contain compounds such as CO_2, possibly CH_4 and unconverted CO, will be required. In that case the overall selectivity of the H_2 generation plant, including physical-chemical gas separation techniques such as the use of H_2 selective membranes or pressure swing adsorption (63,114) ultimately determines the application potential of the product. The post-treatment method selected will depend on both the achieved exit CO concentrations as well as the product requirements.

Due to the poor energetics of the water gas shift reaction the achievement of sufficiently low CO concentrations in the product gas of the bioreactor may be problematic, especially as at low CO concentrations the driving forces for mass transfer of CO into the liquid phase are low. Therefore, another option is to maintain higher CO concentrations in the hydrogenogenic bioreactor, which are expected to be highly advantageous as it enhances the driving force for mass transfer of the gaseous CO to the hydrogenogenic bacteria. To achieve this, a combined biological and physico/chemical process could be considered in which the CO is biologically converted to H_2 and a high grade H_2 could be derived by physical-chemical gas separation techniques. As separation technique usually the so called pressure swing adsorption (PSA) is preferred when high purity gases are required (114). This PSA comprises a system with fixed beds of different molecular sieves that selectively adsorb gasses at relatively high pressures (500-5000 kPa; Air Liquide technical specifications) and subsequently the adsorbed gases are desorbed at low pressures as an offgas stream. In this manner the CO content in the gas could be maintained at relatively high levels by recycling the remainder of CO and removing CO_2 and H_2 separately.

The removal of CO_2 from the product gas, e.g. by physical absorption in cold methanol, as in the rectisol wash (90), offers the possibility for selective CO_2 sequestration and geological storage (76), thus preventing its emission to the atmosphere. Liberation of high purity CO_2 could be even useful for reuse in chemical synthesis of e.g. methanol (163) or in greenhouse horticulture, which nowadays consumes large quantities of natural gas for the production of CO_2. In this respect greenhouse horticulture represents an interesting application for CO_2.

5.3 Utilization of CO containing synthesis gas for biological desulfurization

Any biological H_2 production process may suffer from the simultaneous occurrence of H_2 consumption processes, i.e. especially when using mixed populations as is the case with anaerobic granular sludge. Successful application of biological H_2 production implies the need to minimize undesired H_2 consumption. The prevention of H_2 consumption by methanogens in practice may be difficult and it may depends on the microbial populations present whether or not a specific inhibition method, e.g. using chemical inhibitors (33,152,180) or pretreatment of the sludge prior to inoculation (32,151), is sufficiently effective. Little is known about the possibility to prevent H_2 consumption by homoacetogens, which may turn out to be even more difficult to suppress once methanogenesis is eliminated. For practical applications, therefore, H_2 production combined with a desired H_2 consumption process, like H_2 utilization for biological sulfate reduction, might result in maximal utilization of H_2 by the desired processes, provided that the sulfate reducers can out-compete the other H_2 consuming microorganisms. Kinetical growth characteristics of sulfate reducers generally are superior to that of methanogens with H_2 as electron donor, while also the thermodynamics of sulfate reduction are more favorable (154,221), and consequently sulfate reducers in principle should be capable to out-compete methanogens. However, it should be understood that the overall result of the competition also depends on various other factors, e.g. pH, temperature, medium composition, immobilization characteristics, substrate limitations inside biomass aggregates and the bacterial composition of the seed sludge (221). Results of various reactor studies revealed that hydrogenotrophic sulfate reducers usually become dominant over hydrogenotrophic methanogens at temperatures around 35ºC (224,230). However, according to Van Houten *et al.* (227) at elevated temperatures (55ºC) the outcome of this competition could not be predicted.

Sulfate- or sulfite-rich wastewaters with a low organic matter pollution are generated e.g. in galvanic processes, in the detoxification of metal-contaminated soils, in the mining of heavy metals and coal, and in waste streams generated by flue gas desulfurization (101). In order to enable the biological desulfurization of these wastewaters a suitable electron donor needs to be supplied in the treatment process. The mining of heavy metals and coal is responsible for the largest quantity of sulfur-rich inorganic wastewater known as acid mine drainage (AMD)(102). This AMD contains high concentrations of dissolved metals and its temperature may vary from 5 to 50ºC (12,18,218). Biological treatment of AMD is generally performed mesophilically. Treatment of AMD primarily aims at acid consumption in order to neutralize the pH (101). This can be achieved by biological sulfate reduction, because herewith protons are consumed and a weak acid is produced (H_2S) and subsequently insoluble sulfide precipitates are formed with many heavy metals present in AMD (102). Besides several so called "passive" biological remediation processes, e.g. aerobic wetlands, permeable reactive barriers and packed bed iron-oxidation bioreactors (102), there is an increasing interest in "active" high-rate biological sulfate reduction processes for remediation of AMD in off-line sulfidogenic bioreactors. The main advantages of the latter processes are that they are more predictable and allow better control. Additionally, they enable the selective recovery of metals, and reach lower effluent sulfate concentrations (102). High-rate sulfate reduction processes are successfully applied for remediation of zinc-polluted groundwater at the Budelco zinc refinery in the Netherlands and the selective copper recovery at the Kennecott Bingham Canyon copper mine in Utah (19). Initially ethanol was used as electron donor at the Budelco zinc refinery, but was later replaced by H_2 and CO_2 as energy and carbon source (19). Although high sulfate elimination rates (12 g $S.L^{-1}.d^{-1}$) were achieved with ethanol (50), the main drawback of its use is the formation of acetate due to incomplete oxidation (146). Hydrogen is a more economical electron donor for sulfate reduction and it results in the formation of less biomass (19).

Scrubbing water from flue gas treatment represents another typical example of a sulfur-rich inorganic wastewater. Stringent legislation enacted to prevent or minimize acid deposition from sulfur compounds present in flue gas requires desulfurization of flue gas. SO_2 in flue gas was reported to account for approximately 90% of the anthropogenic SO_2 emissions in 1989 (25). The release of relative high amounts of SO_x leads to the formation of H_2SO_4, which ultimately results in acid

deposition and subsequently damages the ecosystem (31,221). The intensive use of fossil fuels will remain an important source of anthropogenic SO_2 emissions. As the natural reserves of coal exceed those of oil or natural gas reserves considerably, the use of coal likely will increase in the near future (179). One approach to reduce the SO_x contents of flue gas is the gasification of coal to a sulfur-free synthesis gas prior to its combustion. However, end-of-pipe technologies will remain an important measure to prevent SO_x emissions to the atmosphere till the time coal gasification will be implemented on a large scale.

The production of gypsum, a widely applied physical-chemical end-of-pipe technology for SO_x removal from flue gas, results in a voluminous waste product, with little reuse potential due to contamination with heavy metals (221). Biological flue-gas-desulfurization is an attractive alternative method, because of its low operational costs and the fact that relatively pure (>98%) and reusable elemental sulfur is produced, without the need for further wastewater treatment (97). In this biotechnological process the SO_x, mainly present as SO_2, is dissolved in a slightly alkaline solution to form sulfites and minor amounts of sulfate (97). The sulfite and sulfate containing scrubbing water is subsequently subjected to an anaerobic sulfate reduction process, where the oxidized S-compounds are converted into sulfides. In the next step, the sulfide is partially oxidized to elemental sulfur under oxygen limitation (27,98,117) and the insoluble sulfur than can be separated from the water phase (97). Flue-gas-desulfurization scrubbing wastewaters generally are liberated at high temperatures (± 55ºC), because besides SO_2 also heat is transferred to the scrubbing solution. Treatment with (moderate) thermophilic microorganisms is therefore preferred.

Over the past decade a lot of research was undertaken to find cheap and efficient electron donors for the biological treatment of inorganic sulfate-rich wastewaters. Many types of organic waste materials have been considered as potential substrate for the sulfate reducing bacteria, e.g. molasses and whey (66,234), tannery effluents (20), dried algal biomass (21), solid waste materials (30) and sheep and poultry manures (78). Despite, the fact that these organic substrates have been shown to stimulate sulfate reduction, their use in high-rate bioreactors is rather complicated due to their complexity. Van Houten and Lettinga (225) already pointed out that organic waste materials are less suitable electron donors, as their use would result in an additional pollution of the wastewater and this would require a supplementary treatment. Since the reported sulfate reduction rates with organic waste

materials were rather low, these authors considered the use of cheap bulk chemicals like methanol, ethanol, H_2 gas, or synthesis gas more appropriate. Ethanol (213,240), methanol (80,233-235) and hydrogen (35,224) all have been successfully applied in biological sulfate reduction studies. According to a cost estimate made by Van Houten and Lettinga (225) concerning the use of ethanol and hydrogen as electron donors for sulfate reduction, the use of ethanol would be cheaper for small scale installation (<5 $kmol.h^{-1}$), whereas H_2 would be cheaper at larger installations, assuming the use of a high purity H_2. Synthesis gas, a mix of H_2 and CO, is cheaper than pure hydrogen and is widely available as by-product of coal burners. Even low-grade coal (rich in S deposits) can be used safely as the sulfur compounds can be treated within the process as well (57).

The utilization of synthesis gas for sulfate reduction processes so far received little attention in research, and to our knowledge only three publications appeared (56,57,226). Van Houten *et al.* (226) achieved a sulfate reduction rate in the range of 6-8 g $SO_4^{2-}.(L.d)^{-1}$, with a feed gas containing maximally 20% CO. Du Preez and Maree (56) reported a sulfate reduction rate of 2.4 g $SO_4^{2-}.(L.d)^{-1}$ with pure CO as the feed gas. Both groups of researchers speculate on the occurrence of a hydrogenogenic CO conversion, although their results did not support that. Van Houten *et al.* (226) observed that the total H_2 consumption was insufficient to account for the total amount of sulfate reduced and acetate produced. The direct conversion of CO to acetate or conversion of CO and H_2 to acetate were not taken into account and could explain the non-stoichiometric utilization of H_2 (Table 4). Du Preez and Maree (56) observed a two-fold higher sulfate reduction rate with pure CO compared to a 90:10 H_2/CO mixture and therefore it is unlikely that H_2 acts as an intermediate electron donor in sulfate reduction when the reactor was fed with CO. In case of hydrogenotrophic sulfate reduction it is expected that sulfate reduction rates are higher since an inhibiting effect of elevated CO concentrations on sulfate reduction is absent (56). The results might be explained by a direct reduction of sulfate with CO or by an intermediate involvement of homoacetogens. Due to the fact that the number of mesophilic homoacetogens capable of CO conversion is rather large and many of these organisms are commonly encountered in anaerobic environments, acetate most probably served as the actual electron donor for the sulfate reduction.

6. Conclusions

Biological synthesis gas conversions have a wide range of potential interesting applications, e.g. in the production of a wide-range of valuable chemicals. With the discovery of anaerobic hydrogenogenic microorganisms capable of removing carbon monoxide with concomitant H_2 production, the biotechnological potential of synthesis gas utilization has drastically increased.

The isolation of an increasing number of hydrogenogens (Table 3) shows that biotechnological purification of synthesis gas to a pure H_2 gas seems within reach as an attractive alternative to the catalytic processes nowadays employed. Nevertheless, more research is required to assess the practical applicability of this biological water gas shift reaction. Especially research into the biotechnological utilization of these hydrogenogens has to be performed. The development of a successful biotechnological gas water shift production method, not only depends on the microorganisms and biological reactor, but also on the product requirements and thus its economic value.

Successful application of H_2-rich synthesis gas in biodesulfurization, without the need for prior purification, requires tolerance for the presence of CO. Furthermore, it would be most beneficial to the overall sulfate reduction process when CO within the synthesis gas is used for sulfate reduction as well. Thus, both toxicity and potential metabolic use of CO as electron donor are important factors governing the utilization potential of CO-rich synthesis gas for biotechnological sulfate reduction. In that respect, the recent discovery of more CO tolerant sulfate reducing bacteria indicates that CO-rich synthesis gas may represent an interesting and cheap electron donor for biodesulfurization.

7. Acknowledgments

The authors are deeply grateful to Dr. Hauke Smidt and Dr. Bo Jiang for construction of the phylogenetic tree. This research was financially supported by a grant from the Technology Foundation STW (grant STW-WBC 5280), applied science division of NWO, the Netherlands, and Shell Global Solutions (Amsterdam, the Netherlands) and Paques B.V., Balk, the Netherlands.

2

Ultra deep shift catalysis by *Carboxydothermus hydrogenoformans*

Anne M. Henstra and Alfons J.M. Stams

Abstract

The catalytic conversion of CO with H_2O to CO_2 and H_2 is a step in the industrial production of H_2. This reaction is also catalysed by *Carboxydothermus hydrogenoformans*, but at much lower temperatures. Lower temperatures favor CO removal and are thus of potential interest for H_2 utilizing, CO sensitive applications. Here we demonstrate that cultures of *Carboxydothermus hydrogenoformans* reach CO levels below 2 ppm. The reaction is at these levels near thermodynamic equilibrium. It is anticipated that formation of acetate is necessary to obtain the observed CO thresholds.

submitted

Introduction

The application of low temperature fuel cells requires highly purified hydrogen gas that contains less than 10 ppm CO (199). A novel group of thermophilic strict anaerobic bacteria holds a promise in fuel cell grade hydrogen gas production. These bacteria conserve energy for growth from the water gas shift reaction, i.e. the conversion of CO and H_2O to H_2 and CO_2 (191,207). Shift catalysis is applied in the purification of hydrogen rich synthesis gas obtained by steam reforming of natural gas or gasification of coal (8), but current chemical technology cannot reach required threshold levels (65,204). Removal of CO below desired threshold levels in a single catalytic step, ultra deep shift catalysis, is thermodynamically feasible at the relatively low temperature of the biological shift (148). Here we demonstrate that cultures of *Carboxydothermus hydrogenoformans* reach CO levels below 2 ppm. A novel biotechnological process to replace existing chemical technology for CO removal is proposed.

Microbial growth by conversion of CO to H_2 and CO_2 was first shown (219) in 1976 for a *Rhodopseudomonas* sp. This activity was exclusively ascribed to a few purple-non sulfur bacteria, until in 1991 the first strict anaerobic, Gram positive, thermophilic bacterium *C. hydrogenoformans* was described that performed this reaction (207). While many strict anaerobes exist that grow with the products H_2 and CO_2 and form e.g. acetate or methane, this activity is absent in *C. hydrogenoformans* (87,207). Thus, *C. hydrogenoformans* may be appropriate to purify CO-containing H_2-rich synthesis gas in a biotechnological process. *C. hydrogenoformans* grows relatively fast (t_d = 2h)(207) by the conversion of CO according to Reaction 1.

$$CO\ (g) + H_2O\ (l) \rightarrow CO_2\ (g) + H_2\ (g) \quad \Delta G = -23.4\ kJ\ mol^{-1},\ P = 1\ atm\ and\ 70°C \qquad (1)$$

CO is oxidised by carbon monoxide dehydrogenase (CODH). Two CODHs of *C. hydrogenoformans* were isolated and characterised (210). The crystal structure of CODH2 was resolved (54). CODH1 was shown to be part of a CO oxidising:H_2 evolving enzyme complex (189). The hydrogenase is expected to conserve energy in chemiosmotic ion gradient, by proton translocation. Thus, an

elementary and possibly ancient respiratory chain is present in *C. hydrogenoformans* (84). The amount of free energy needed by an organism to translocate one mole of protons over the cytoplasmic membrane is considered the biological minimum energy quantum (177). This minimum energy quantum ranges between -8 and -20 kJ per mole of protons (91). Removal of CO to desired levels will bring the metabolism of *C. hydrogenoformans* close to thermodynamic limits.

Methods

Final P_{CO} levels of the hydrogenogenic CO metabolism of *C. hydrogenoformans* was studied in batch cultures with and without a CO_2-trap. *C. hydrogenoformans* (DSM6008) was obtained from the German culture collection (DSMZ, Braunschweig) and cultured in 585-mL serum bottles with 200 mL MOPS (20 mM) buffered medium (87) and a 100 % CO gas phase at 65 °C and 200 rpm (1" stroke Innova 44 incubator, New Brunswick Scientific). Bottles contained a glass reaction tube with either 5 mL water or 5 mL 10 M NaOH. CO, H_2, CO_2, organic acids and growth of *C. hydrogenoformans* were analysed as described (87). Gas and liquid samples were taken with intervals of 180 minutes until 15.5 h. Beyond 15.5 h of cultivation samples were only analysed for trace levels of CO (P_{CO} < 800 Pa) on a GC2010 (Shimadzu, Japan) fitted with methanizer (MTN-1, Shimadzu) and FID. Gas samples of 100 µl were withdrawn from cultures with a glass syringe and allowed to equilibrate with atmospheric pressure just before injection in the GC. Gibbs free energy changes were calculated with observed gas partial pressures and tabulated data for 70 °C (4). Partial pressures of CO_2 and H_2 at 15.5 h were used for calculation of ΔG where only trace CO was measured.

Results and Discussion

C. hydrogenoformans served as model organism in an empirical approach to test the ability of carboxydotrophic hydrogenogenic bacteria to obtain CO levels below 10 ppm. Growth and CO conversion in batch cultures of *C. hydrogenoformans* are presented in Figure 1. CO is converted to stoichiometric amounts of CO_2 and H_2. The OD at 660 nm increased to 0.113, indicating growth of *C. hydrogenoformans.* Growth stopped after 9.5 h, while CO conversion continued. At this stage approximately 7.5 % (2.5 mmol) of the initial CO remained and 17.5 mmol CO_2 and 27.1 mmol H_2

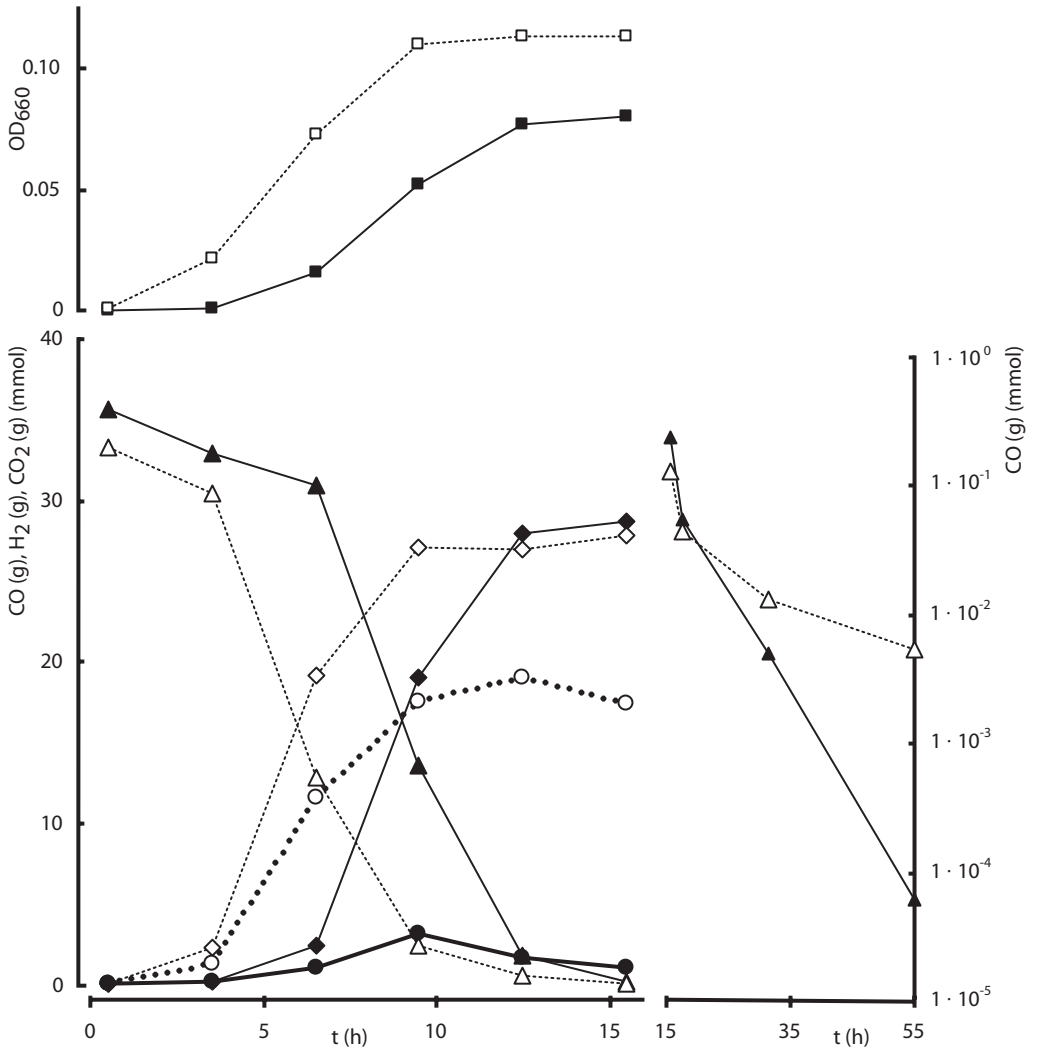

Figure 1:

Growth (squares) and conversion of CO (triangles) to H_2 (diamonds) and CO_2 (circles) by *C. hydrogenoformans* batch cultures equiped with CO_2-trap (closed symbols) and without CO_2-trap (open symbols). *C. hydrogenoformans* was cultured in 585 mL bottles with 200 mL medium at 65 °C.

were detected in the gas phase. The pH decreased from pH 6.8 to 6.0. CO levels at t = 55 h amounted to 117 ppm. With the observed gas concentrations, a ΔG of -16 kJ mol^{-1} was calculated for Reaction 1 (ΔG_{R1}) at the moment that growth stopped (9.5 h) and of +1.4 kJ mol^{-1} at the end of the experiment (t = 55 h)(Table 1). The ΔG_{R1} value of +1.4 kJ mol^{-1} could indicate that not the conversion of CO to H_2 but another metabolic reaction is used by *C. hydrogenoformans*. In support of this idea is the detection of acetate (3.8 mM) in samples obtained at the end of the experiment.

The CO concentration of 117 ppm at the end of the experiment is approximately one order of magnitude below the threshold mentioned for the chemical shift reaction, but still one order of magnitude above the target of 10 ppm. Removal of CO_2 or H_2 may result in a lower final CO threshold. Removal of H_2 requires special selective membranes, while CO_2 is more easily removed by physicochemical techniques. Therefore, *C. hydrogenoformans* cultures were equipped with an alkaline CO_2 trap, a glass reaction tube with 5 ml 10 M NaOH. Incubations with CO_2-trap showed similar substrate consumption and product formation rates, but the lag phase was longer (Fig.1). The pH decreased from pH 6.8 to 6.6. CO_2 accumulated to an intermediate maximum of 3.2 mmol at 9.5 h, compared to 12 mmol for cultures without CO_2 trap at 6.5 h. This indicates that the CO_2-trap was effective. In cultures with CO_2 trap the OD increase stopped around 12.5 h of incubation. At 12.5 h of incubation approximately 5 % (1.9 mmol) of the original amount of CO was present and 28 mmol H_2 and 1.7 mmol CO_2, corresponding to a ΔG_{R1} of -22 kJ mol^{-1} (Table 1). After 55 h of cultivation CO levels had dropped below detectable levels of 2 ppm. With partial pressure for CO that corresponded to 2 ppm a ΔG_{R1} of +6.2 kJ mol^{-1} was calculated. No acetate was detected in the cultures with CO_2 trap.

Our results with *C. hydrogenoformans* demonstrate that CO levels in hydrogen-rich gas below reported threshold levels of existing chemical technology can be achieved. The CO levels of <2 ppm (with CO_2 trap) and 107 ppm (without CO_2 trap) are the actual detected CO levels in the cultures. Final ΔG_{R1} values that were calculated for cultures without and with CO_2 trap were +1.4 kJ mol^{-1} and +6.2 kJ mol^{-1}, respectively. For calculation of the ΔG_{R1} values for 17.5, 31.5, and 55 h only the CO concentrations were measured and CO_2 and H_2 concentrations were assumed to be equal to the concentrations measured at 15.5 h. This assumption is not completely correct. In cultures with trap, CO_2 levels had decreased to undetectable levels ($P_{CO2} < 350$ Pa) after the experiment was stopped. If a P_{CO2} of 350 Pa is used in the calculation of a ΔG_{R1} of -3 kJ mol^{-1} was calculated for the cultures with CO_2 trap at t = 55 h (Table 1). Together with the ΔG_{R1} of +1.4 kJ mol^{-1} for cultures without CO_2 trap, it appears that CO can be removed to thermodynamic equilibrium ($\Delta G_{R1} = 0$) by *C. hydrogenoformans*. The theory of the biological minimum energy quantum states that it is not possible for a micro-organism to conserve energy by this reaction under these conditions.

Table 1: Observed partial pressures and calculated Gibbs free energy change of CO conversion by *C. hydrogenoformans* in batch culture.

culture	t (h)	H_2 (Pa)	CO (Pa)	CO_2 (Pa)	Acetate (mM)	ΔG_{R1} §	ΔG_{R2} §
- CO_2 trap	0.5	$6.0 \cdot 10^2$	$2.4 \cdot 10^5$	$1.1 \cdot 10^3$	0.2*	-53	-52
	9.5	$2.0 \cdot 10^5$	$1.8 \cdot 10^4$	$1.3 \cdot 10^5$		-16	
	55	$2.0 \cdot 10^5$	$3.9 \cdot 10^1$	$1.3 \cdot 10^5$	3.8	+1	-18
+ CO_2 trap	0.5	$2.2 \cdot 10^2$	$2.6 \cdot 10^5$	$8.4 \cdot 10^2$	0.2	-57	-52
	12.5	$2.0 \cdot 10^5$	$1.4 \cdot 10^4$	$1.2 \cdot 10^4$		-22	
	55	$2.1 \cdot 10^5$	$0.4 \cdot 10^0$	$3.5 \cdot 10^2$	0.2	-3	-16

*underlined - detection limits that were used in calculation of free energy
§ΔG_{R1} and ΔG_{R2} – Calculated Gibbs free energy change (kJ mol CO^{-1}) for Reaction 1 and Reaction 2, respectively, at 70 °C (4).

Instead, formation of acetate from CO (Reaction 2) may be used by the micro-organism to conserve energy. This conversion may determine the final CO threshold that can be reached.

$$4\ CO\ (g) + 2\ H_2O\ (l) \rightarrow CH_3COOH\ (aq) + 2\ CO_2\ (g) \qquad \Delta G = -36.9\ kJ\ mol^{-1},\ P = 1\ atm,\ 70°C \qquad (2)$$

The Gibbs free energy change of Reaction 2 for standard pressures at 70 °C is -36.9 kJ per mole CO, and more exergonic than Reaction 1 (-23.3 kJ mol^{-1}), although, they are comparable under initial experimental conditions (Table 1). Initially, CO is converted to H_2 only, and not to acetate. The detected acetate indicates that the energy metabolism may switch to acetogenesis. If it is assumed that the switch occurs at a fixed ΔG for Reaction 1, then the lack of acetate in cultures with CO_2 trap can be explained. To produce the 4 mM acetate present in cultures without CO_2 trap, 16 mmol L^{-1} CO is needed. At 9.5 h of cultivation 2.5 mmol CO remained in cultures without trap. The ΔG_{R1} at this point of time (-16 kJ mol^{-1}) corresponded to t = 15.5 h for cultures with CO_2 trap (ΔG_{R1} = -17 kJ mol^{-1}) at which only 0.2 mmol CO remained. This amount of CO is not sufficient for formation of detectable levels of acetate (approx. 0.2 mM). For Reaction 2 a ΔG_{R2} of -16 kJ per mole CO can be calculated, using 0.2 mM acetate, 2 ppm CO and 350 Pa CO_2 at t = 55h in cultures with CO_2 trap (Table 1). Irrespective of the responsible reaction, *C. hydrogenoformans* can bring the CO levels down to very low levels.

Removal of CO below 10 ppm by *C. hydrogenoformans* makes this micro-organism an attractive catalyst in a biotechnological process for cost effective production of high grade hydrogen gas suitable for e.g. polymeric electrolyte membrane fuel cells (PEM-FC). Biotechnology that employs anaerobic micro-organisms in waste water purification and flue gas treatment has proven to be simple and reliable (99). We expect that a biological shift process is tolerant to contaminants in feed synthesis gas, like H_2S, which is even present in the growth media of the bacterium. This allows the use of lower grade but cheap feed stocks as oil, coal or biomass to produce H_2. Batch culture studies that we performed (data not shown) revealed that except at very low biomass density, gas/liquid mass transfer is rate limiting. Suitable bioreactor concepts therefore require optimal gas/liquid mass transfer characteristics, e.g. biotrickling-filter reactors or the novel monolith bioreactor (58,159). As growth is minimal at low P_{CO} it may be necessary to convert CO in two stages. An initial stage aimed at biocatalyst generation (growth) that consequently has CO thresholds 10 to 100 fold higher than desired. The second stage should obtain desired thresholds levels, possibly in a batch or plug-flow mode. Between both stages CO_2 may be removed, if necessary to obtain the desired CO threshold. Thermodynamic calculation that considers a level of 10 parts CO per million H_2 and the assumption that thermodynamic equilibrium ($\Delta G_{R1} = 0$ kJ mol^{-1}) is reached, indicates that P_{CO2} of approximately 4000 Pa is allowed. Development of PEM-FC membranes with CO tolerance up to several hundred ppm might even make CO_2 removal in the biotechnological process less stringent (83,155), while also these CO thresholds are beyond the reach of chemical shift (65).

Acknowledgements

The authors are grateful to Dr. Yutaka Nakashimada for discussions regarding experimental setup. This work was financially supported by grant WBC.5280 of the Dutch Technology Foundation STW (Utrecht, The Netherlands).

3

Mass transfer limitation in batch cultures of *Carboxydothermus hydrogenoformans*

Anne M. Henstra and Alfons J.M. Stams

Abstract

Batch cultures of *Carboxydothermus hydrogenoformans* grown with a CO gas phase reached CO thresholds below 10 ppm after 55 h of incubation. It is not known what limits the biological conversion of CO to H_2. Here we studied whether gas/liquid mass transfer or biomass activity limited the rate of CO conversion. The maximum conversion rates doubled and the time needed for complete conversion shortened by at least a factor 8, when *C. hydrogenoformans* was incubated under experimental conditions that enhanced gas/liquid mass transfer. From this we conclude that the biological activity is not the main bottleneck in hydrogenogenic CO conversion by *C. hydrogenoformans*.

submitted

Introduction

Carboxydothermus hydrogenoformans converts CO with H_2O to CO_2 and H_2. A reaction, that is of interest for a tentative biotechnological process to produce H_2 gas from synthesis gas with low remaining CO pressures. It has been demonstrated that batch cultures of *C. hydrogenoformans* could remove CO from the gas phase to thresholds below 2 ppm, a level of CO that is below the 10 ppm threshold that is required for low temperature fuel cells. However, the time that was needed to get to these thresholds was relative long (Chapter 2). Biomass activity or gas/liquid mass transfer could be limiting the rate of the CO conversion. Gas/liquid mass transfer is an important factor in the conversion, since the substrate CO and the products, H_2 and CO_2, are gaseous compounds. Here we demonstrate that mass transfer is indeed limiting CO conversion by *C. hydrogenoformans* in standard microbial incubations. If mass transfer limitation is not considered in physiological studies with CO, the maximum conversion rates and specific activity of the biomass can be adversely affected.

Materials and methods

Carboxydothermus hydrogenoformans (DSM6008) was cultivated in butyl-rubber stoppered 585 mL serum flasks that contained 200 mL Mops buffered medium. Batch cultures were incubated at 65 ^{o}C in an Innova 44 rotary shaker incubator with 1" stroke, at 0 or 200 rpm (New Brunswick Scientific, Edison -NJ), with bottles in up-right position, or lying horizontally (when indicated). Pressure in the bottles was measured by a GMSD3,5BAE pressure probe (Greisinger, Regenstauf Germany) and recorded every 10 minutes. The medium composition was as described (Chapter 2). The initial gas phase was composed of 140 kPa CO. The gas phase was changed to fresh CO during cultivation according to the following procedure. Serum bottles were taken from the incubator and applied to a vacuum until a pressure below 15 kPa was reached, then CO was added to approximately 140 or 170 kPa and placed back in incubator. To compensate the loss of sulfide by gas phase exchange, 1 % sodium sulfide was added to the medium from a 40 mM stock solution. After the gas phase exchange flasks were allowed to heat up before the shaker was started.

Results

C. hydrogenoformans was cultured with a 100% CO gas phase at different shaking regimes to investigate if the conversion rate was limited by gas/liquid mass transfer or by biomass activity. The pressure inside the serum flasks was recorded as indicator of CO conversion activity (Fig.1). During incubation the gas phase was exchanged with CO for 3 times. The incubation started with uninoculated serum flasks that were allowed to heat up before inoculation (2% v/v) and start of the shaker (200 rpm). After inoculation a lag phase of approximately 10 h was observed before the pressure increased exponentially for 4 h to a maximum rate of 0.33 kPa min^{-1}. The conversion rate decreased gradually during further incubation (Fig.1 frame 1). 22 h after inoculation the gas phase of the culture was changed (ca. 160 kPa CO) and allowed to heat up before the shaker (200 rpm) was started. The culture showed no exponential increase in pressure, the rate of conversion only decreased starting with the highest rate of 0.75 kPa min^{-1} (Fig.1 frame 2). After approximately 5 h the gas phase was changed (to 180 kPa CO) and incubated for almost 13 h without shaking. The pressure increased with 20 kPa during these 13 h, compared to an increase in pressure of 85 kPa during the 12 h of the first incubation (Fig.1 frame 3). After the 13 h of incubation the shaker was started (200 rpm) and a similar increase in pressure was observed as before (Fig.1 frame 4). After 5.5 h the gas phase was changed (to 180 kPa) and the culture was shaken at 200 rpm in horizontal orientation. In 100 minutes of incubation the pressure increased approximately with 125 kPa, where after the pressure increase stopped (Fig.1 frame 5). The rate at which the pressure raised, increased in the first 30-40 minutes. The maximum attained rate was 1.68 kPa min^{-1}. Interestingly the conversion of this last incubation seemed complete as the final pressure did not increase anymore, in contrast to the former incubations.

Discussion

Conversion of CO with H_2O to H_2 and CO_2 results in an increase of pressure in cultures of *Carboxydothermus hydrogenoformans*. Pressure change gives thus an indication of the CO conversion rate. Pressure increase was monitored to investigate if the rate was limited by biomass activity or by gas/liquid mass transfer in batch incubations. Culture serum flasks were shaken at 200 rpm in up-right and in horizontal position. The latter condition resulted in more vigorous mixing of gas and

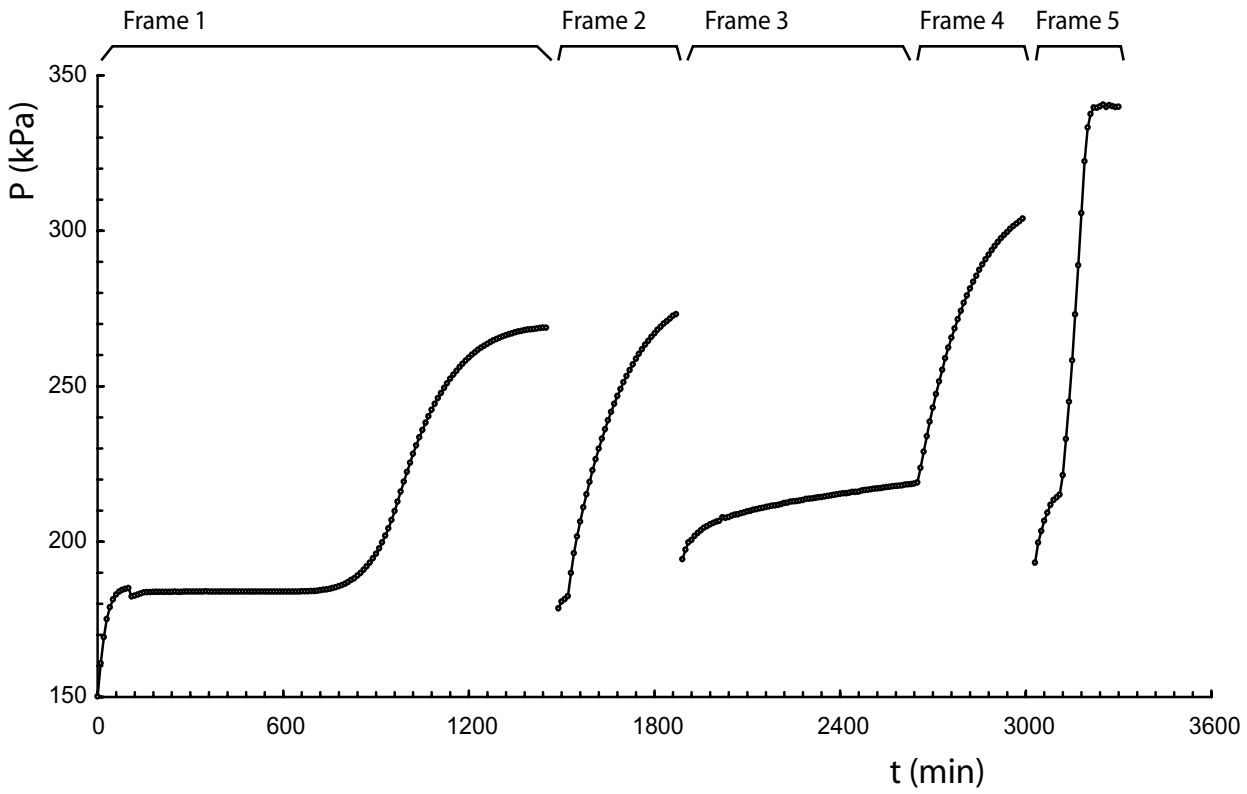

Figure 1:

Effect of shaking regime on the pressure increase in batch incubations of *Carboxydothermus hydrogenoformans* with CO as substrate. Serum flasks of 585 mL contained 200 mL medium and were incubated at 200 rpm in up-right position (frame 1,2,4), at 0 rpm in up-right position (frame 3), or at 200 rpm in lying position (frame 5). The gas phase was exchanged with fresh CO between frames 1 and 2 (to approx. 160 kPa at room temperature), frames 2 and 3 (to approx. 180 kPa), and frames 4 and 5 (to approx. 180 kPa). Cultures were allowed to heat up before the shaker was started.

liquid phases. The maximum rates of pressure increase differed by a factor 2.2. However, the time needed for complete conversion differed at least by a factor 8. This factor is obtained by linear extrapolation (by 0.08 kPa min^{-1}) of the pressure of the incubation in frame 4 to the final pressure (340 kPa) of the incubation in frame 5. More vigorous shaking seems therefore especially beneficial for obtaining low CO thresholds in relative short time.

Apparently biomass is only limiting the CO conversion rates in case of the low biomass densities that are present just after inoculation. This is indicated by the exponential increase in pressure that is observed after the initial lag phase (Fig.1 frame 1). Gas/liquid mass transfer becomes the limiting

factor in CO conversion after sufficient growth of biomass. This is confirmed by the lack of an exponential increase of pressure after replenishing the gas phase with CO in the grown culture (Fig.1 frame 2). Only in case of vigorous shaking there was also an initial increase in conversion rate (Fig.1 frame 5). However in that case, it can not be concluded whether biomass activity or mass transfer is limiting the conversion. An increase in pressure can also be caused by a release of CO_2 from the medium, either by acidification of the medium through CO_2 formation or due to a relative high CO_2 concentration still present in the medium after the gas phase exchange.

Gas/liquid mass transfer limitation in batch cultures of *C. hydrogenoformans* could adversely affect analysis of specific CO conversion activity of the biomass. Only at low biomass concentrations an accurate measurement of specific CO converting activity can be obtained. These biomass concentrations are too low to be accurately measured by optical density (data not shown) and alternatives should be considered. It was found by Svetlitchnyi *et al.* (2001) that the specific CO oxidizing activity in cell free extracts of *C. hydrogenoformans* cells grown in 50 L cultures with continuous supply of CO increased from 20 U mg protein^{-1} to 1800 U mg protein^{-1}. Therefore, dilutions of full grown cultures seem best for analysis of the specific CO conversion activity of the cells.

In Chapter 2 it was demonstrated that it was possible to obtain CO thresholds below the 10 ppm that is required for polymer electrolyte membrane fuel cells. However, a long time period was necessary to obtain these levels and it could be questioned whether the rate of CO conversion was sufficient for an attractive biotechnological process. Here it was demonstrated that in the applied incubation method gas/liquid mass transfer is limiting. If gas/liquid mass transfer is improved, higher conversion rates are possible. Especially the time needed to obtain low CO thresholds is shortened. Whether the CO threshold levels are also reached this fast remains to be confirmed by trace level CO analysis. Based on the results obtained here we recommend that special attention is paid to mass transfer limitations in physiological study with CO as substrate.

4

Novel physiological features of *Carboxydothermus hydrogenoformans* and *Thermoterrabacterium ferrireducens*.

Anne M. Henstra and Alfons J.M. Stams

Abstract

Carboxydothermus hydrogenoformans is able to grow by conversion of CO to H_2 and CO_2. Besides CO, only pyruvate was described to serve as an energy source. Based on 16S rRNA gene sequence similarity *C. hydrogenoformans* is closely related to *Thermoterrabacterium ferrireducens*. *T. ferrireducens* is like *C. hydrogenoformans* a gram positive, thermophilic, strict anaerobic bacterium. However, it is capable of using various electron donors and acceptors for growth. Growth of *C. hydrogenoformans* with multiple electron donors and acceptors was tested. *C. hydrogenoformans* oxidized formate, lactate, glycerol, CO and H_2 with 9,10-anthraquinone-2,6-disulfonate as electron acceptor. Sulfite, thiosulfate, sulfur, nitrate and fumarate were reduced with lactate as electron donor. *T. ferrireducens* oxidized CO with 9,10-anthraquinone-2,6-disulfonate as electron acceptor, but did not produce H_2 from CO. In contrast to what was published before *T. ferrireducens* was able to grow on lactate with sulfite, sulfur, and nitrate as electron acceptor.

Henstra, A.M. and A.J.M. Stams. 2004. Novel physiological features of *Carboxydothermus hydrogenoformans* and *Thermoterrabacterium ferrireducens*. Applied and Environmental Microbiology 70:7236-7240.

Introduction

Carboxydothermus hydrogenoformans was isolated from hydrothermal fresh water springs on Kunashir island (Russian Kurils). The bacterium grows by the conversion of $CO + H_2O$ to $H_2 + CO_2$ (77,207). An obligate dependence on CO for its growth was argued since no other substrates were shown to support growth. Volcanic exhalations, decomposition of plant material and interspecies CO transfer are possible sources of CO (207). Growth on CO is fast. Doubling times of 120 min are attained, despite the low standard Gibbs free energy of this reaction (-20 kJ/mol CO).

Several studies have addressed biochemical aspects of *C. hydrogenoformans*. Two carbon monoxide dehydrogenases (CODHs) and a CO-oxidizing / H_2-evolving enzyme complex have been purified and characterized (189,210). The first crystal structure of a nickel-CODH was solved for CODH2 obtained from *C. hydrogenoformans* (54). In addition, whole genome sequencing of *C. hydrogenoformans* is in progress (The Institute for Genomic Research, Rockville, U.S.; Centre of Marine Biotechnology, Baltimore, U.S.).

A limitation in further studies on *C. hydrogenoformans* is its restricted substrate use; only pyruvate and CO were reported to support growth of this bacterium (208). However, indications for a wider substrate range exist. *C. hydrogenoformans* and *Thermoterrabacterium ferrireducens* share a phylogenetic close relationship. An identity of more than 98% between 16S-rRNA gene sequences of *T. ferrireducens* (U76363) and *C. hydrogenoformans* can be deduced from the published phylogenetic tree (192). *T. ferrireducens* was isolated from Yellowstone National Park and is described to grow by fermentation of pyruvate, lactate, glycerate, glycerol and 1,2 propanediol. Glycerol and H_2 also support growth with fumarate, 9,10-anthraquinone-2,6-disulfonate (AQDS), ferric iron, or thiosulfate as terminal electron acceptor (188). These substrate combinations have not been tested for *C. hydrogenoformans*.

Here we demonstrate that *C. hydrogenoformans* is capable of anaerobic respiration in a similar fashion as *T. ferrireducens*. In addition we show that *T. ferrireducens* is able to use CO as electron donor for AQDS and fumarate reduction.

Materials and Methods

Organisms

C. hydrogenoformans (DSM 6008) and *T. ferrireducens* (DSM 11255) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany).

Medium composition and cultivation

Both micro-organisms were cultivated under strictly anaerobic conditions in a basal carbonate buffered medium composed as described by Stams *et al.* (198). However, 1.5 g l^{-1} $NaHCO_3$, 0.2 g l^{-1} $Na_2S \cdot 9$ H_2O, 0.1 g l^{-1} yeast extract and 2 µmol l^{-1} $NiCl_2$ were used instead of reported concentrations, Na_2S concentrations were lowered to 0.04 g l^{-1} when AQDS was present in the growth medium. Butyl rubber stoppered bottles of 120 ml contained 50 ml medium. AQDS (8.25 g l^{-1}) or sulfur (2 g l^{-1}) were added to bottles as solids prior to sterilization. Bottles were autoclaved for 25' at 121 °C. Sulfur containing bottles were autoclaved at 110 °C for 35'. Gas phases were pressurized to 170 kPa and were composed of 20 % CO_2 and either 80 % of N_2, H_2 or CO. The following compounds were added to the bottles from separately autoclaved stock solutions. Formate, methanol, acetate, lactate (DL), glycerol and sulfate were used in concentrations of 20 mM, unless stated otherwise. Sulfite, thiosulfate, nitrate and fumarate were used in concentrations of 10 mM, unless stated otherwise. Cultures with a H_2/CO_2 or CO/CO_2 gas phase were incubated at 60 °C and shaken at 100 rpm. Cultures with a N_2/CO_2 gas phase were incubated, non-shaken, at 65 °C.

Analytical methods

H_2, CO, N_2O and CO_2 were analysed by gas chromatography on a Chrompack CP9001 gas chromatograph fitted with a TCD detector. The injector and detector temperatures were 60 °C and 130 °C, respectively. CO_2 was analyzed using a Poraplot Q column (Chrompack, 25*0.53, df = 20 µm). Helium was the carrier gas at a flow rate of 18 ml min^{-1} and the oven temperature was 45 °C. H_2 and CO were analyzed using a molsieve column (Chrompack, 30*0.53, df = 15 µm), argon at a flow rate of 10 ml min^{-1} was the carrier gas. The oven temperature was 50 °C.

Organic acids, glycerol and methanol were analysed by HPLC using a Polyspher OA HY column (300-6.5mm, Merck, Darmstadt, Germany) and RI SE-61 refractive index detector (Shodex, Tokyo Japan) as described previously (181). Sulfate, thiosulfate, nitrate and nitrite were analysed by HPLC using an Ionpac AS9-SC column (Dionex, Sunnyvale, USA) and ED 40 electrochemical detector (Dionex, Sunnyvale, USA) as described previously (181). Sulfide was analysed according to the modified colorimetric method of Pachmeyer (217). Ammonium was determined using a variation on the Berthelot reaction (167). Reduction of AQDS was indicated by visual colour change of the growth medium.

Selective media and denaturing gradient gel electrophoresis

Purity of the cultures was examined by phase contrast microscopy, selective media and denaturing gradient gel electrophoresis (DGGE). *C. hydrogenoformans* and *T. ferrireducens* were transferred 5 times on the medium with lactate and a N_2/CO_2 gas phase. Subsequently the strains were transferred to media supplemented with glucose, sucrose, lactate, or lactate + nitrate, media with a CO/CO_2 gas phase, either with or without fumarate, and media with a H_2/CO_2 gas phase. Both strains were also tested with the control medium of Svetlichny *et al.* (207) containing 20 mM glucose, 5 g l^{-1} yeast extract, 2 g l^{-1} peptone and a N_2/CO_2 atmosphere.

DGGE analysis of 16S rDNA was performed by amplification of the V6 - V8 region by PCR on DNA isolated from cultures of *T. ferrireducens* and *C. hydrogenoformans* grown with different substrates. DNA was isolated from 5 to 10 ml culture suspension as described by Zoetendal *et al.* (251), using bead-beat cell disruption, phenol-chloroform extraction and ethanol precipitation. Isolated cells from the cultures grown with CO alone were treated with lysozyme for 1 hr at 37 °C before cell disruption. PCR and DGGE were performed as described by Heilig *et al.* (86) with primers F0968GC and R1401 (149). A 40% - 60% denaturing gradient gel was used for DGGE.

Results and Discussion

The close phylogenetic relationship of *C. hydrogenoformans* and *T. ferrireducens* indicates a wider spectrum of substrates used for growth by *C. hydrogenoformans* than CO and pyruvate alone.

Table 1: Anaerobic respiration of selected electron donors and acceptors by *C. hydrogenoformans* and *T. ferrireducens*.

	C. hydrogenoformans	*T. ferrireducens*	
		actual	expected*
donors, with AQDS as acceptor			
formate	+	+	-
methanol	-	-	-
acetate	-	-	-
lactate	+	+	+
glycerol	+	+	+
H_2	+	+	+
CO	+	+	nr#
acceptors, with lactate as donor			
sulfate	-	-	-
sulfite	+	+	-
thiosulfate	+	+	+
sulfur	+	+	-
nitrate	+	+	-
fumarate	+	+	+

* expected results based on the data reported by Slobodkin *et al.* 1997; # not reported

Especially anaerobic respiration is of interest as *T. ferrireducens* uses various electron donors and acceptors as energy source while no data on anaerobic respiration by *C. hydrogenoformans* is documented.

The utilization of different electron donors and acceptors as for growth by *C. hydrogenoformans* was tested in batch cultures in a direct comparison with *T. ferrireducens*. Formate, methanol, acetate, glycerol, H_2 and CO were tested as electron donor with AQDS as terminal electron acceptor. Sulfate, sulfite, thiosulfate, sulfur, nitrate, and fumarate were tested as electron acceptor with lactate as electron donor. Use of substrates was qualified using multiple approaches. AQDS reduction results in bright orange colour of the cultures and can be used to score conversion. Other substrates (except sulfur) and their products were analysed after 3 and 14 days of cultivation. Furthermore, microscopic observations were used to indicate increase in numbers of cells. Table 1 lists the results on the use of the different substrates in anaerobic respiration by *C. hydrogenoformans* and *T. ferrireducens*. The expected results for *T. ferrireducens* based on published data (188) are included as well. The range of substrates used by *C. hydrogenoformans* was identical to that of *T. ferrireducens*. All tested

substrates were used by *C. hydrogenoformans* and *T. ferrireducens* except methanol, acetate and sulfate (Table 1). Differences with reported data and specific observations during experimentation prompted us to perform more detailed studies. These are discussed below.

CO had a different fate in the metabolism of *C. hydrogenoformans* and *T. ferrireducens*. Both micro-organisms reduced AQDS with CO as electron donor. However, H_2 was detected in cultures of *C. hydrogenoformans* as well, while H_2 was not detected in cultures of *T. ferrireducens*. Additional experiments with CO as a sole substrate of carbon and energy were performed with *T. ferrireducens* to check for presence of the carboxydotrophic hydrogenogenic activity. No growth or H_2 formation was observed in incubations of *T. ferrireducens* with gas phases that contained CO in the range of 5% up to 80%. These results indicate that the capacity to perform the characteristic metabolism of *C. hydrogenoformans*, which accumulates H_2 with CO as substrate, is absent in *T. ferrireducens*.

Growth of *C. hydrogenoformans* and *T. ferrireducens* with CO as electron donor was studied in more detail in 200 ml cultures with fumarate (50 mM) as electron acceptor. Bottles with 200 ml medium and a 385 ml CO/CO_2 gas phase were inoculated with *C. hydrogenoformans* or *T. ferrireducens*. Reduction of fumarate and formation of H_2 occurred simultaneously in cultures of *C. hydrogenoformans* (Fig.1A, C). No H_2 was formed in cultures of *T. ferrireducens* (Fig.1B). Simultaneous fumarate reduction and hydrogen production resulted in a higher consumption rate of CO for *C. hydrogenoformans* (Fig.1A) than *T. ferrireducens* (Fig.1B). Intermediate accumulation of malate was observed in cultures of *C. hydrogenoformans* (Fig.1C) and *T. ferrireducens* (Fig.1D). Changes in fumarate, succinate and malate concentrations were similar for *C. hydrogenoformans* (Fig.1C) and *T. ferrireducens* (Fig.1D). Succinate was formed at a constant rate of similar magnitude (0.3 mmol h^{-1}) by cultures of both micro-organisms. Biomass development (Fig.1A, B) was also similar. The mass balance of CO oxidation (114.3 mmol/l medium) for *C. hydrogenoformans* cultures was 92 % complete with 56.6 mM fumarate reduced and 48.7 mmol/l medium H_2 formed. In *T. ferrireducens* cultures some what more fumarate was reduced (56.2 mM) than can be accounted for by CO oxidation (51.6 mM).

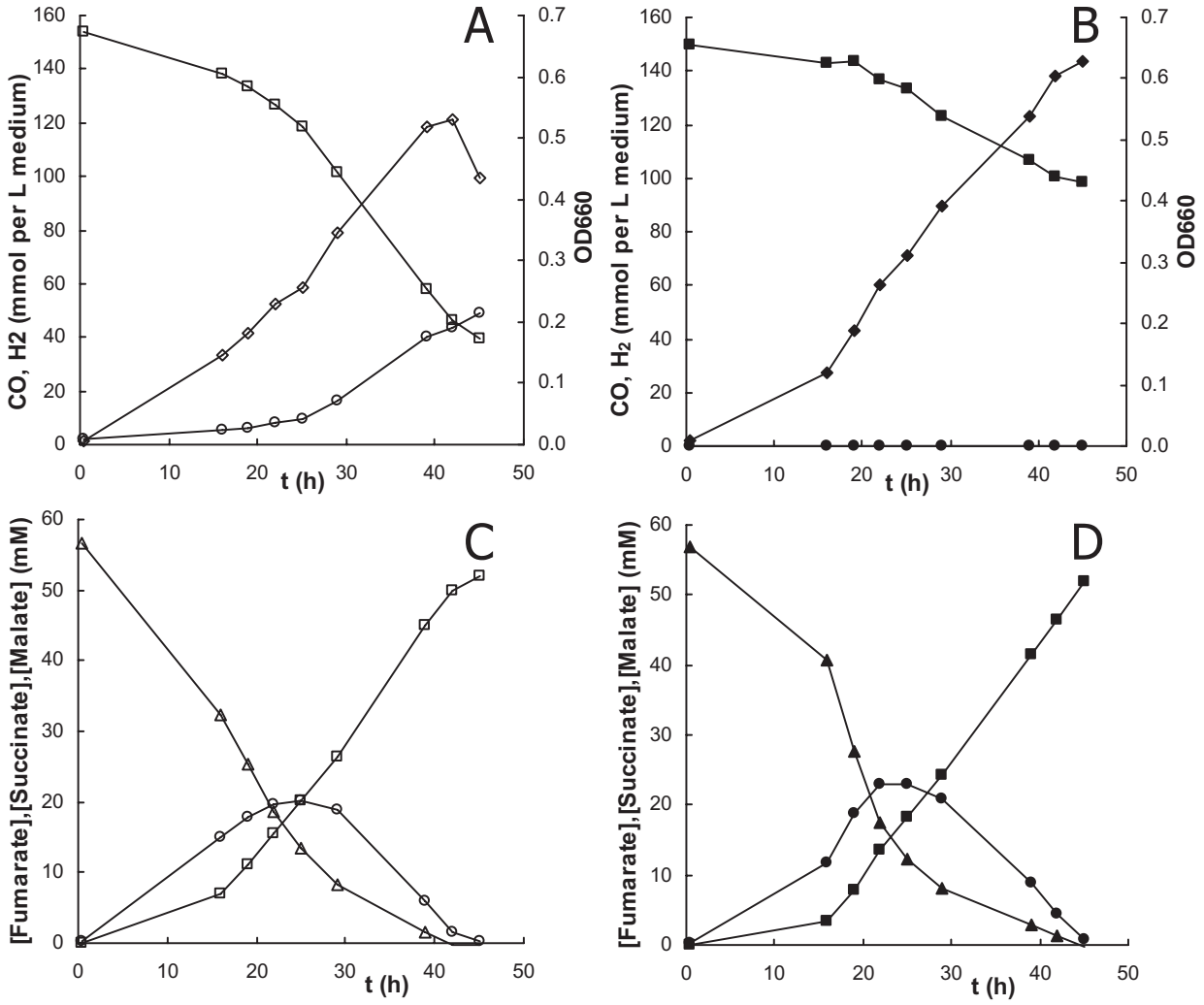

Figure 1:

Reduction of fumarate to succinate with carbon monoxide as electron donor. *C. hydrogenoformans* (open symbols) and *T. ferrireducens* (closed symbols) were cultivated in 585 ml bottles with 200 ml medium. Plotted are the average data of duplicate incubations. A, B Development of biomass represented by optical density at 660 nm (OD660, diamonds), quantities of CO (squares) and H_2 (circles) present per liter medium during incubation of *C. hydrogenoformans* (A) and *T. ferrireducens* (B). C, D Concentrations (mM) of fumarate (triangles), succinate (squares), and malate (circles) during incubation of *C. hydrogenoformans* (C) and *T. ferrireducens* (D).

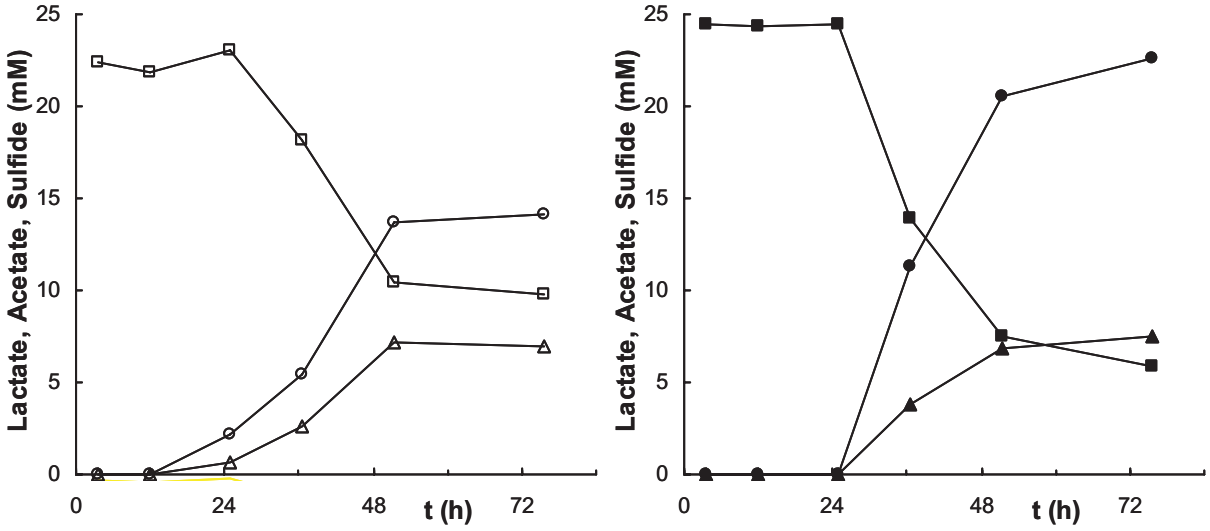

Figure 2:

Reduction of sulfite (10 mM) with lactate (20 mM) as electron donor by pure cultures of *C. hydrogenoformans* (open symbols) and *T. ferrireducens* (closed symbols). Lactate (squares) was oxidized to acetate (circles), and sulfite (not analysed) was reduced to sulfide (triangles).

In contrast to the results of Slobodkin *et al*. (1997), sulfite was reduced by *T. ferrireducens* in our experiments. In that study potentially toxic concentrations of sulfite (20 mM) were used. *C. hydrogenoformans* and *T. ferrireducens* were inoculated in medium with 2 mM, 5 mM, 10 mM and 20 mM sulfite and lactate (20 mM) as electron donor to test this hypothesis. Formation of sulfide was detected in cultures of *C. hydrogenoformans* and *T. ferrireducens* started with 2 mM, 5 mM, and 10 mM of sulfite, but not in cultures started with 20 mM sulfite. *C. hydrogenoformans* cultures with 10 mM sulfite produced 6.9 mM sulfide in 76 h of cultivation (Fig.2). 12.6 mM lactate was consumed and 14.1 mM acetate was produced. The formation of 6.9 mM sulfide requires oxidation of 10.35 mM lactate. If homoacetegenesis is considered, then the remaining consumed lactate (2.25 mM) balances with 90 % of the surplus of acetate formed (3.75 mM). A functional acetyl-CoA synthase, essential for homoacetogenesis, is present in *C. hydrogenoformans* (209). Cultures of *T. ferrireducens* formed 7.5 mM sulfide and 22.6 mM acetate (Fig.2). 18.6 lactate was consumed. 97 % of surplus acetate formed (11.35 mM) can be accounted for if homoacetogenic oxidation of lactate (7.35 mM) is considered. Cultures with 5 mM sulfite obtained the highest turbidity, whereas cultures with 20 mM did not show visible growth. These results validate that *C. hydrogenoformans* and *T. ferrireducens* do grow with sulfite as electron acceptor.

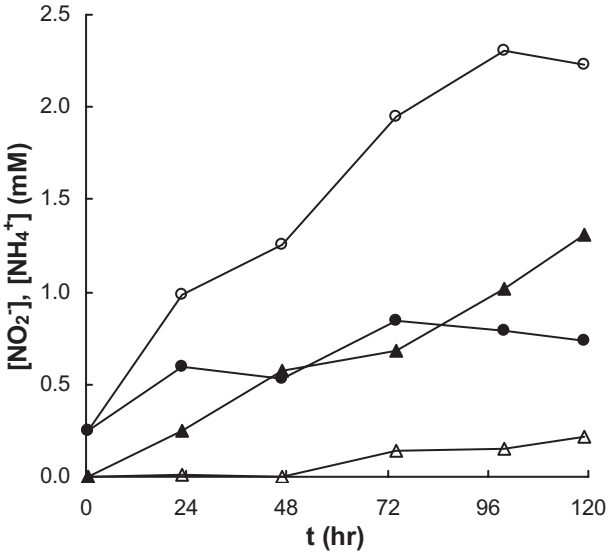

Figure 3:

Reduction of nitrate to nitrite (triangles) and ammonium (circles) by pure cultures of *C. hydrogenoformans* (open symbols) and *T. ferrireducens* (closed symbols). Nitrate was added after 0.5, 23, 47, 74, and 100 hours in aliquots corresponding with a final concentration of 0.5 mM. Lactate (5 mM) served as electron donor.

In our experiments thiosulfate was reduced to sulfide by *T. ferrireducens* whereas sulfur was formed in experiments of Slobodkin *et al*. (1997). Sulfur was not reduced in the latter study. *Thermoanaerobacter* species are reported to produce either sulfide or sulfur from thiosulfate depending on medium composition and growth stage (109,115). *Carboxydothermus* and *Thermoterrabacterium* are closely related to the genus *Thermoanaerobacter* (192). Growth conditions may determine the product of thiosulfate reduction by *T. ferrireducens* as well. The difference observed for sulfur in our experiments and those of Slobodkin may be explained similarly.

Two pathways are known for dissimilatory nitrate reduction; denitrification, which yields N_2 and nitrate ammonification, which yields ammonium (216). *C. hydrogenoformans* and *T. ferrireducens* both reduced nitrate (10 mM) with lactate (20 mM) as electron donor to nitrite. Additional experiments were performed to determine whether further reduction of nitrite does occur.

Cultures were started with lower nitrate concentrations in these experiments since the resazurin turned pink upon nitrate reduction in cultures with initial nitrate concentrations of 10 mM. This indicates relative high, possibly unfavourable, redox conditions of the medium. During experiments with 2 mM nitrate slight increases of ammonium were detected in cultures of *C. hydrogenoformans.* However, when extra nitrate was added to these cultures no further reduction was observed (data not shown). Because of poor reproducibility of ammonium formation and the absence of ammonium

formation in cultures of *T. ferrireducens*, nitrate concentrations were further decreased. In experiments where 0.5 mM of nitrate was added in 24 h intervals increased ammonium concentrations were detected in cultures of both *C. hydrogenoformans* and *T. ferrireducens* (Fig.3). Cultures of *C. hydrogenoformans* converted nitrate completely to ammonium in the first 48 h of cultivation. After 48 h residual nitrite accumulated to a final 0.2 mM at 120 h. Nitrate was not fully converted to ammonium in cultures of *T. ferrireducens*. Ammonium and nitrite accumulated simultaneously during the first 74 h in these cultures, resulting in lower final concentrations of ammonium compared to cultures of *C. hydrogenoformans* (Fig.3). Based on these results we conclude that nitrate can be reduced to ammonium by *C. hydrogenoformans* and *T. ferrireducens*, provided that nitrite concentrations remain low. Conversion of nitrate to ammonium is apparently slower in *T. ferrireducens* than *C. hydrogenoformans*.

Phase contrast microscopy, selective culturing and DGGE of 16S rDNA were applied to check the purity of used cultures. Microscopical observations and the growth on selective media did not reveal the presence of contaminants. *C. hydrogenoformans* and *T. ferrireducens* share a similar rod shaped morphology. Contamination of *T. ferrireducens* cultures with *C. hydrogenoformans* was excluded by the inability of these cultures to form H_2 from CO. Figure 4 shows the result of DGGE analysis of amplified 16S rDNA sequences from cultures of *C. hydrogenoformans* and *T. ferrireducens* grown with different substrates. It is expected that abundant contaminants lead to additional bands in the DGGE gel. Lanes 1 to 4 contain PCR samples of *C. hydrogenoformans* cultures; one band is visible for each tested condition. Lanes 6 to 8 contain PCR samples of *T. ferrireducens* cultures. Each growth condition shows two bands, which corresponds to the number of 16S rRNA genes of *T. ferrireducens* in the Genbank database (accession numbers U76363 and U76364). Both bands vary equally in intensity over the different growth conditions, which is not expected in case of contaminants. These results confirmed purity of the utilized cultures of *C. hydrogenoformans* and *T. ferrireducens* and excludes cross contamination of *C. hydrogenoformans* cultures with *T. ferrireducens*.

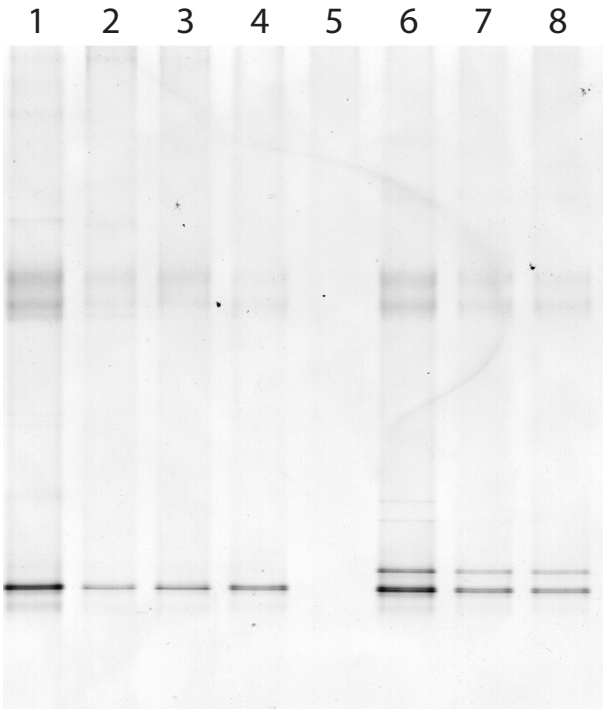

Figure 4:

DGGE of PCR-amplified V6 - V8 region of 16S rDNA from cultures of *C. hydrogenoformans* (Lanes 1 - 4) and *T. ferrireducens* (Lanes 6 - 8) grown with different substrates. Substrates were CO (lane 1), CO + nitrate (lane 2), CO + fumarate (lane 6), lactate + nitrate (lane 3, 7), and lactate alone (lane 4, 8).

Evidence for close phylogenetic relation between *C. hydrogenoformans* and *T. ferrireducens* based on 16S rDNA similarity exists (192). This similarity prompted us to re-examine the metabolic capacities of *C. hydrogenoformans*. Pyruvate and CO were the only substrates reported to support growth of *C. hydrogenoformans*. *T. ferrireducens* on the other hand grows with multiple electron donors and acceptors. The presented results show that *C. hydrogenoformans* can grow with formate, lactate, glycerol, H_2, CO, AQDS, sulfite, thiosulfate, sulfur, nitrate and fumarate. The comparable metabolisms of *C. hydrogenoformans* and *T. ferrireducens* with these substrates and in incubations with CO as electron donor and fumarate as electron acceptor is consistent with the close phylogenetic relation between both micro-organisms. The results also indicate that *C. hydrogenoformans* is not solely dependent on the presence of CO to survive in its habitat.

Acknowledgements

This work was financially supported by grant WBC.5280 of the Dutch Technology Foundation STW (Utrecht, The Netherlands). The authors thank Dr. Sofia Parshina for discussion and assistance.

5

Physiological function of the CO dehydrogenases of *Carboxydothermus hydrogenoformans*

Anne M. Henstra and Alfons J.M. Stams

Abstract

Five carbon monoxide dehydrogenase genes are encoded in the genome of *Carboxydothermus hydrogenoformans*. It is expected that each of these carbon monoxide dehydrogenases (CODHs) has a dedicated physiological function. Here it was attempted to assign a function to each of the CODHs through cultivation of *C. hydrogenoformans* under different conditions and investigate selective expression of the CODHs. CODH expression profiles were obtained by native CODH stained polyacrylamide gel electrophoresis (PAGE). The complexity of CODH profiles of *C. hydrogenoformans* prevented direct linkage of CODHs with function. However, specific CO oxidizing activity of CFE samples and CODH profiles confirm that CODH are differentially expressed over the various tested growth conditions. Additionally, the results indicate that in nitrate amended cultures a yet uncharacterised CODH may be present. Recently it was deduced from genome context analysis that a second uncharacterised CODH may be expressed under micro-aerophilic conditions. Novel proteomic techniques likely will provide better perspectives on differences in expression over conditions. Although a separation of protein in a native PAGE dimension may be considered if the comparable sizes of the multiple CODH monomers.

Introduction

Strict anaerobic micro-organisms may contain multiple nickel containing carbon monoxide dehydrogenase (CODH) encoding genes. It has been suggested that each CODH present has its own dedicated physiological function (123). CODH catalyses the oxidation of CO according to reaction 1, or the reverse of that reaction.

$$CO + H_2O \rightarrow CO_2 + 2\ H^+ + 2\ e^- \qquad (1)$$

Both oxidation of CO to CO_2 and reduction of CO_2 to CO by CODH can be part of the acetyl-CoA pathway (244). The acetyl-CoA pathway is employed by micro-organisms in different pathways which result in energy conservation and in autotrophic carbon fixation (187). Dependent on the direction of the pathway it can act as a source or a sink of electrons. Homoacetogenic prokaryotes reduce 2 CO_2 molecules to acetate via the acetyl-CoA pathway. One CO_2 molecule is reduced to the methyl level, while the second CO_2 is reduced by CODH to CO which ends up in the carboxyl moiety of acetate. CO, the methyl group and coenzyme-A are joined by acetyl-CoA synthase (ACS) (123,164). CODH, that reduces CO_2 to CO, forms an enzyme complex with ACS (ACS/CODH). Beside the acetyl-CoA pathway, CODH may catalyse the oxidation of CO in anaerobic respiration with different electron acceptors (187). *Rhodospirillum rubrum* and *Carboxydothermus hydrogenoformans* contain a specialised multi-enzyme complex that couples oxidation of CO to CO_2 with reduction of protons to H_2 (59,106,189). Thus five physiological functions for CODH can be deduced. i) Reduction of CO_2 to CO in the acetyl-CoA pathway for autotrophic growth. ii) Reduction of CO_2 to CO in the acetyl-CoA pathway in homoacetogenesis. iii) CO oxidation to CO_2 in acetyl-CoA pathway in acetate oxidation. iv) oxidation of CO to CO_2 in anaerobic respiration. v) oxidation of CO to CO_2 in specialised complexes with energy conserving hydrogenases. Note that i and ii are similar reactions that might be catalysed by the same CODH. CODH for anaerobic respiration with CO is likely only expressed when CO is present in the gas phase, while CODH in acetyl-CoA pathway does not dependent on free CO. So for each physiological function a specific CODH may be present, but not all functions are necessarily represented in one organism.

Table 1: Details of CODHs present in *Carboxydothermus hydrogenoformans*

name	proposed function	locus tag	size (aa)*	size (kDa)	references
CODH1	energy conservation	CHY_1824	636	67.5*	189,210,245
CODH2	NADPH generation	CHY_0085	636	66.8*	210,245
CODH3	carbon fixation	CHY_1221		73.3#	209,245
CODH4	oxygen stress response	CHY_0736	633	68.1*	245
CODH5	none proposed	CHY_0034	629	68.1*	245

* Entrez Genomes (NCBI), # Svetlitchnyi *et al.* (2004)

Whole genome sequence data of *Carboxydothermus hydrogenoformans* revealed that this strict anaerobic thermophilic gram positive bacterium contains 5 CODH encoding genes (245). The genomic context and especially biochemical work on three of these CODHs, give indications about their physiological role (Table 1). CODH1 is part of a CO oxidising, H_2 evolving enzyme complex that is proposed to build a proton motive force by proton translocation (84,189,210). CODH2 is a membrane associated enzyme and assumed to function in NADPH generation for assimilatory purposes. This was concluded from the finding that NADPH generation rates in cell free extracts increased after addition of purified CODH2 (210). CODH3 is a monomeric acetyl-CoA synthase / carbon monoxide dehydrogenase (ACS/CODH) that is involved in autotrophic carbon fixation via acetyl-CoA pathway (209). CODH3 is located in an operon with genes encoding for enzymes that are part of the ACS/ CODH complex (245). It has been reported by Svetlitchnyi *et. al.* (2004) that if CO is present in the gas phase, ACS is a free monomeric enzyme and in the absence of CO in the gas phase ACS forms a complex with CODH3. Apparently, if free CO is present in the gas phase, CODH3 expression is repressed. Functions of CODH4 and CODH5 remain speculative as no biochemical studies have been presented for these CODHs. For CODH4 a function in oxidative stress response was proposed based on the genomic context that contained a rubrerythrin encoding gene downstream of the cooS-IV gene (245). Rubrerythrin of *Pyrococcus furiosus* was demonstrated to reduce hydrogen peroxide (236). For CODH5 no apparent function was deduced from the gene context (245).

Two genes that are functionally associated with CooS are CooA and CooF. Their presence in the genomic context of the CooS genes add to the insight of the function of CODHs. CooA is a CO sensor that is related to the CRP/FNR family of transcription regulators (169). Presence of CooA in a gene cluster that includes CooS, could indicate up regulation of CooS, in the case that CO is

present in the gas phase. CooA is present in clusters of CODH1 and CODH2 (245). CooF is a ferredoxin-like protein that likely is involved in electron transfer from CODH to an electron accepting enzyme complex. It is proposed for CODH1 that CooF transfers electrons to the hydrogenase (84,190). CooF is present in the genomic context of CODH1, CODH2 and CODH4 (245).

To get a better understanding of the possible function of the different CODHs of *C. hydrogenoformans*, physiological and biochemical data are needed. Here, we evaluate native polyacrylamide gel electrophoresis, combined with CODH activity staining, as technique to study the function of the different CODHs (48). Different growth conditions may be imposed to evoke selective expression of one or more CODHs. It is anticipated that it is possible to visualize differences in individual CODH expression by native PAGE and deduce a physiological role for each CODH. As *C. hydrogenoformans* is capable of growth with a wide variety of substrates (87), it is possible to apply different conditions. *C. hydrogenoformans* was described to grow chemolithoautotrophically with CO, while producing H_2 (207). Additionally, it can grow with pyruvate and by anaerobic respiration with sulfite, thiosulfate, sulfur, nitrate and fumarate as electron acceptors and CO, H_2, lactate, glycerol, pyruvate and formate as electron donors (87). Advantages of native CODH stained PAGE are that relative small amounts of biomass are needed for study. *Thermoterrabacterium ferrireducens* resembles *C. hydrogenoformans* physiologically and phylogenetically, and was included in the study (87,188).

Materials and methods

Organisms and cultivation

Carboxydothermus hydrogenoformans (DSM6008) and *Thermoterrabacterium ferrireducens* (DSM11255) were obtained from the German culture collection (DSMZ, Braunschweig) and cultured in butyl-rubber stoppered serum bottles with bicarbonate buffered medium, at 65 °C, without shaking. Medium was prepared as described, with minor modifications (87). Sodium salts of nitrate (10 mM), fumarate (50 mM), formate (50 mM) or pyruvate (50 mM) were dissolved in medium to give the indicated concentrations, where after the pH was adjusted to pH 7. Gas phases (170 kPa at room temperature) were composed of either 80:20 N_2/CO_2, H_2/CO_2, or CO/CO_2. After these preparations,

serum bottles were autoclaved for 25' at 121 °C. Before inoculation the medium was reduced with 2% v/v of a separately autoclaved stock solution of 40 mM sodium sulfide and 75 g L^{-1} sodium bicarbonate. Finally, vitamins, calcium chloride, ammonium chloride, magnesium chloride and sodium chloride, were added from a separate sterile stock solution as described (87).

Preparation of cell free extract

Cultures, cell suspensions and cellular extracts were handled under anoxic conditions in an anaerobic glove box with N_2/H_2 (96:4) atmosphere that was continuously recirculated over a palladium catalyst. Cells were harvested by centrifugation (25000 g) of cultures for 60' at 4 °C in airtight 250 mL centrifugation tubes and washed by resuspending the cell pellet in buffer A (50 mM Tris.HCl pH 7, 1 mM dithiotreitol) and centrifugation for 10' at room temperature in 2 mL eppendorf tubes. The obtained cell pellets were stored at -20 °C, and defrosted before sonication. Cell free extract (CFE) was obtained as supernatant after centrifugation of the sonified cell suspension for 20' at RT. Protein content of CFE was analysed according to Bradford (23).

Enzyme activity

CODH activity of CFE samples was analysed by measuring methyl viologen (MV, ε_{578} = 9.7 mM^{-1} cm^{-1})(210) reduction rate at 578 nm in an U-2010 spectrophotometer (Hitatchi). CO-saturated assay buffer B (50 mM Mops or Tris.HCL pH 7, 20 mM MV, 2 mM DTT) was incubated at 5' at 65 °C in CO flushed 1 mL stoppered quarts cuvettes (Hellma), before addition of sample. Substrate independent MV reducing activity was analysed in N_2 flushed cuvettes, in N_2-saturated assay buffer B.

Native polyacrylamide gel electrophoresis and activity staining

Native polyacrylamide gel electrophoresis (PAGE) and CO oxidizing-activity staining were performed to visualize CODH expression of cells grown with different substrates. Aerobically prepared polyacrylamide gels (6.5% w/v; 30:0.8 acrylamide/bisacrylamide) were placed in an anaerobic glove box, 24 h prior to electrophoresis. Electrophoresis buffer C (3 g L^{-1} Tris, 14.4 g L^{-1} glycine). After electrophoresis, gels were transferred to anaerobic containers that contained staining buffer (50 mM Tris.HCl pH 8) reduced with 0.2 % v/v freshly prepared dithionite solution (200 mM). Containers

were sealed airtight and the gas phase was flushed with 100% N_2 gas to remove H_2, included with the glove box atmosphere. CO oxidizing-activity staining was initiated by addition of 1 % v/v triphenyltetrazoliumchloride solution (100 mM) and flushing the container gently with CO gas. The staining process was accelerated optionally, by placing the containers at elevated temperature and was stopped by exposing the contents to air (approximately 80:20 N_2/O_2). A similar approach was used for staining of hydrogenases, except that H_2 was used instead of CO and flushing with N_2 was skipped.

Results

Anaerobic micro-organisms may contain multiple CODH, each with a dedicated metabolic role (123). The genome of *C. hydrogenoformans* contains five CODH encoding genes (245). *C. hydrogenoformans* was cultured with different substrates as energy and carbon source to explore the possibility to selective expression of the CODH genes present. Substrates were selected to impose autotrophic growth conditions with CO or CO and H_2 as electron donor and an inorganic electron acceptor (nitrate). Similar, heterotrophic, conditions were imposed with fumarate as electron acceptor. Additionally *C. hydrogenoformans* was grown with CO in the presence of pyruvate or formate and with pyruvate alone. The substrates used for the different growth conditions are listed in Table 2.

Table 2: Protein concentration, CO oxidation activity and further details of CFE derived from *C. hydrogenoformans* grown with different substrates.

			CFE	
substrates	culture volume	color of cell suspension	protein mg/ml	activity* U/mg
CO	800	black	4.2	82
CO + nitrate	800	grey	11	21
CO + fumarate	600	brown/grey	18	43
CO + pyruvate	600	orange/red	50	225
CO + formate	800	black	5.6	185
H_2 + nitrate	800	black/grey	12	3.0
H_2 + fumarate	400	lightgrey/greenish	4.5	4.4
Pyruvate	800	red/brown	23	7.6

* One unit is defned as 1 µmol CO oxidized min^{-1} with 20 mM methyl viologen as electron acceptor at pH 7.

C. hydrogenoformans was grown in medium with the different substrates present in volumes aimed to obtain a minimum wet weight yield of 200 mg cells. Protein content and CO oxidizing activity of prepared CFE were analysed and results are listed in Table 2. The obtained wet weight yields varied from approximately 100 mg to more than 1 gram. Wet weight cell yields were relatively high for pyruvate grown cells, while yields for autotrophically grown cells were relative low, which is reflected in the protein content of prepared CFEs. Cell pellets had typical colours, orange-red to reddish brown for pyruvate grown cells, greyish for cells grown in presence of nitrate or fumarate, and black for cells grown with CO alone or with formate. Protein contents of prepared CFE ranged from 4 mg mL^{-1} for CO grown cells to 50 mg mL^{-1} for cells grown with CO and pyruvate (Table 2). The specific CO oxidizing activity of CFE samples ranged from 3 μmol min^{-1} mg $protein^{-1}$ (U mg^{-1}) for H_2/NO_3^- grown cells to 225 U mg^{-1} for CO/pyruvate grown cells.

CODH activity stained native PAGE was used to visualize CODH. Therefore PAGE gels were loaded with samples normalized for CO oxidizing activity and normalized for protein content. Gels loaded with samples normalized for protein content would allow to distinguish absolute differences in CODH content in cells grown under different conditions. Before loading of CFE on the gel, samples were diluted to 1 mg protein mL^{-1}, of these dilutions, 15 μL were loaded on gel that is presented in Figure 1A. Activity normalized PAGE would allow to distinguish between relative changes in expression levels of the different CODHs present. Preliminary experiments (data not shown) showed that loading of 0.5 to 1 U CO oxidizing activity on polyacrylamide gels resulted in acceptable activity staining levels. The activity in CFE samples of H_2 grown cells was insufficient to load 1 Unit activity, instead 0.4 U was loaded for these samples. The resulting CO oxidizing activity stained PAGE gel is presented in Figure 1B. The lane with CO/pyruvate sample was overloaded, while the adjacent lane loaded with CO/formate sample was stained with much lower intensity than expected, caused by a clogged well that prevented loading of sample. Expression profiles obtained for fumarate grown cells, with CO or H_2 as electron donor, show sharpest bands. Between both conditions, i.e. CO/fumarate and H_2/fumarate, differences in profiles are visible. These lanes are presented separately in Figure 2 to illustrate more clearly the differences. Nitrate amended cultures show a CODH band that is not present in other lanes. Besides CODH staining, the same samples

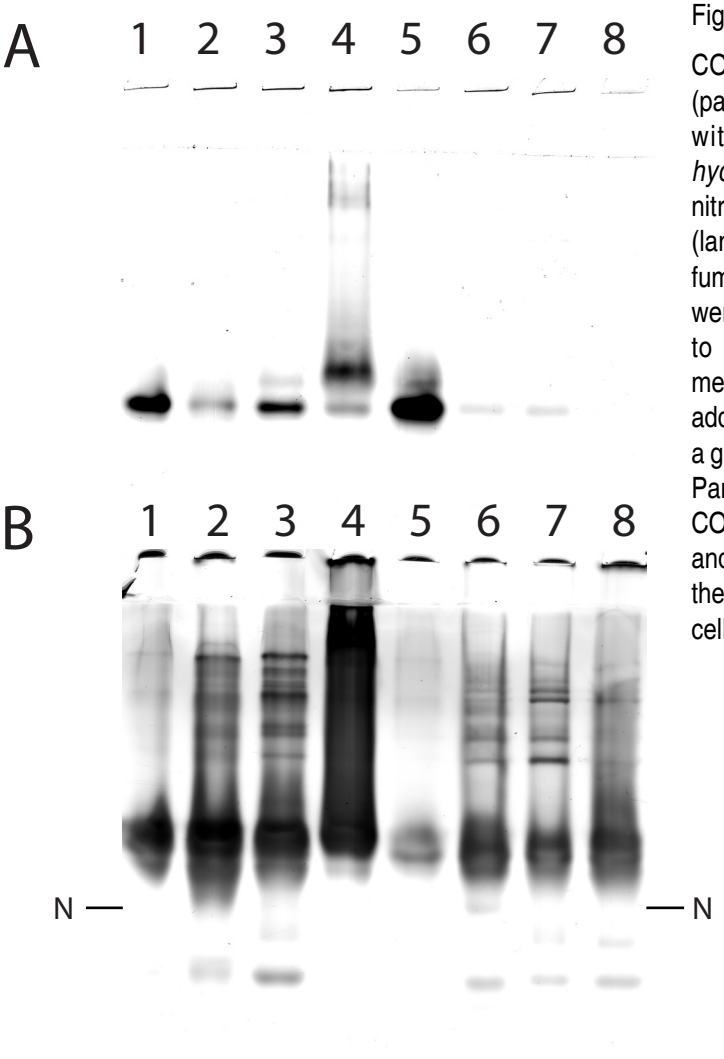

Figure 1:

CO-dehydrogenase (panel A and B) and H_2-ase (panel C) activity stained native PAGE gels loaded with CFE samples obtained from *C. hydrogenoformans* grown with CO (lane 1), CO/nitrate (lane 2), CO/fumarate (lane 3), CO/pyruvate (lane 4), CO/formate (lane 5), H_2/nitrate (lane 6), H_2/fumarate (lane 7), and N_2/pyruvate (lane 8). Enzymes were stained by CODH mediated reduction of TPTC to its insoluble red colored formazan, or H_2-ase mediated reduction of TPTC. Methyl viologen was added as intermediate electron carrier. Panel A shows a gel that was loaded with 10 mg protein per sample. Panel B and C show gels that were loaded with 1U CO oxidizing activity per sample, except for lanes 6 and 7 that were loaded with 0.4 U activity. N indicates the migration distence for bands that are specific for cells grown with nitrate.

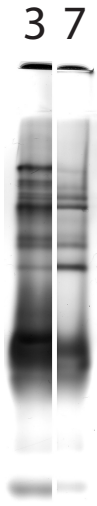

Figure 2:

Direct comparison of CODH profiles of *C. hydrogenoformans* grown with CO + fumarate and H_2 + fumarate; lanes 3 and 7 from Figure 1B.

were used in hydrogenase stained native PAGE. Instead of CO, H_2 was flushed through the anaerobic containers used in staining procedure. The resulting gel is shown in Figure 1C.

In addition to the above presented expression studies, CODH expression of *C. hydrogenoformans* was compared with that of *T. ferrireducens* by native PAGE and CO oxidising activity staining. This experiment, performed with CFE of CO/fumarate grown *C. hydrogenoformans* and *T. ferrireducens* cells, was part of trials to optimise the activity staining protocol (data not presented). Part of the optimisation experiments was the effect of addition of Triton X100 to gels and to samples in an attempt to reduce the amount of smearing often observed for *C. hydrogenoformans* CFE samples. Figure 3 shows lanes of gels with and without Triton X100 added, loaded with CFE samples obtained from CO/fumarate grown cells. For triton treated gels and samples one band is visible at migration distance X while two bands are visible for untreated gels and samples. Additionally, *T. ferrireducens* samples show sharper CODH profiles than *C. hydrogenoformans* samples.

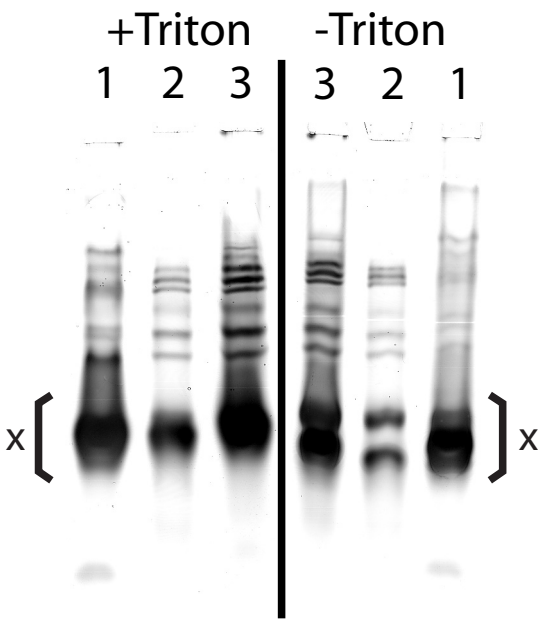

Figure 3:

CO-dehydrogenase profiles of CFE samples derived from *C. hydrogenoformans* (lanes 1) and *T. ferrireducens* (lanes 2 and 3) grown with CO + fumarate in activity stained native PAGE gels. The left panel shows lanes of the resulting gel if Triton X100 was added to gel and samples. The right panel shows lanes of a gel loaded with the same samples but without Triton X100 added, on a gel without Triton X100. The loaded samples contained 1 mg mL^{-1} protein (lanes 1 and 3), or 0.5 mg mL^{-1} protein. At migration distance X one band is observed for triton treated samples and two bands for untreated samples.

Discussion

Multiple CODH genes are encoded in the genome of *C. hydrogenoformans* (245). It has been proposed that each of the multiple CODHs present in one organism, may have a dedicated function in the metabolism (123). Based on the genomic context and reports on separately characterised CODH, functions were proposed for most of the CODHs (189,209,210). The proposed function for CODH4 and CODH5 remained speculative (245). Here we explored the suitability of native PAGE combined with CO oxidizing activity staining as a method to improve insight in CODH expression and function in addition to available biochemical, physiological and genomic data.

The theory that different CODHs present in one organism have a dedicated physiological function, allows the thought that the genes for these CODH are differentially expressed depending on the applied growth conditions. Therefore, *C. hydrogenoformans* was grown under autotrophic and heterotrophic conditions, in the presence and absence of CO (Table 2). Specific CO oxidizing activities of the obtained CFE for these growth conditions differed markedly, with the largest difference of almost 2 orders of magnitude between CFEs of CO/pyruvate grown cells and H_2/NO_3^- grown cells. The obtained specific CO oxidizing activity in CFE of CO grown cells also differed with those reported earlier for the isolation and characterization of CODH1 and CODH2 of *C. hydrogenoformans* (210). The CFE's, used in the isolation of CODH1 and CODH2, had a specific activity of 1,169 U/mg at pH 8, while the activities in our CFEs were only 82 U mg^{-1} at pH 7. Although the pH of activity measurements differed, the large difference is more likely caused by differences in the cultivation technique. It was described by Svetlitchnyi *et al.* (2001) that activity increased from 20 U mg^{-1} to 1800 U mg^{-1} during cultivation on CO. The major difference in the cultivation techniques is the continuous supply of CO used by Svetlitchnyi *et al.* and the fixed amount of CO added in our experiments. The limited amount of CO available for growth, and consequently the short cultivation time, may have led to the lower specific activity in the obtained CFE. As cultivation time and growth rate determine the specific CO oxidizing activity and as yield data are lacking, the conclusions that can be drawn from the data in Table 2 are limited. However, a marked difference in specific CO oxidizing activity exists between cultures supplied with CO and cultures grown without CO. It is likely that presence of CO in the gas phase stimulated expression of CODH through the transcriptional

regulator CooA. Also CFE of CO/formate grown cells contains considerably higher CO oxidizing activity than CFE of CO-only grown cells, while the protein content of both CFE's was comparable.

The obtained CFE samples were analysed by activity stained native PAGE. Figure 1A and 1B present CODH stained PAGE gels that were loaded with CFE samples with equal amounts of protein or equal amounts of CODH oxidizing activity, respectively. It is clear that the obtained results presented in Figure 1B are not optimal. CODH staining is weak in CFE samples obtained from CO grown cells, missing in CO/formate grown cells and excessive in CO/pyruvate grown cells. Improved PAGE results may be expected if cell suspensions of comparable density are used for preparation of CFEs. The obtained CODH profiles are of considerable complexity (Fig.1B). The number of bands in the lanes loaded with CFE sample obtained under these conditions, exceeds that of what can be expected based on the number of CODH genes encoded in the genome. The multitude of bands is likely caused by complexation of specific CODHs with proteins that are part of the functional enzyme complexes present in the cell. At least two different CODH complexes can be expected. The CO-oxidizing:H_2-evolving enzyme complex that comprises CODH1 and is composed of 8 different subunits (84,189), of which two are integral-membrane subunits, and the ACS/CODH complex that is composed of CODH3 and acsB (209), but also might comprise acsC and acsD that together form the CoFeS-protein (245). In fact, since size differences of the individual CODH are small, the different CODH bands that are observed are more likely complexes of CODH with other proteins than individual CODH.

Less complex profiles were observed for hydrogenase activity stained gels (Fig.1C), which proofs that the native staining technique is suitable to study redox enzymes. The differences between CO and H_2 grown cells are pronounced. CFE samples of CO grown cells showed a hydrogenase band at the interface of the stacking and running gel, that was absent for H_2 grown cells. The membrane bound hydrogenase of the CODH oxidising:H_2 evolving enzyme complex is a likely candidate for this band. H_2 grown cells contained two additional hydrogenases when compared to cells grown in similar conditions with CO. Expression of these hydrogenases is likely repressed in the absence of H_2, since they are not present when grown on CO or N_2/pyruvate. One CO tolerant hydrogenase is

present for all conditions, except for CO and CO/pyruvate, and possibly involved in the reduction of external electron acceptors, although no electron acceptors were added in case of CO/formate and N_2/pyruvate grown cells.

CODH profiles of *T. ferrireducens* CFE samples showed much sharper bands and thus appears more suitable to study CODHs with the tested technique than *C. hydrogenoformans* (Fig.3). The possible reason for the sharper bands is the apparent absence of a CO-oxidising:H_2-evolving complex in *T. ferrireducens* (87). Nonetheless, a considerable number of CODH bands is visible in native PAGE gels of *T. ferrireducens* CFE samples. Two dominant CODH bands are shared by *C. hydrogenoformans* and *T. ferrireducens*. Above these two dominant bands, 8 CODH bands are visible. Six of these bands seem organised in two triplets and might arise from one CODH that forms a protein complex that is composed of multiple subunits, with each band of lower migration containing additional subunits. A candidate for these bands is the CODH that is part of the ACS/CODH complex, which is very likely present in *T. ferrireducens.* The results with *T. ferrireducens* demonstrate that complexity of the CODH profiles depends on the species and study of CODH with this technique in *T. ferrireducens* may be less ambiguous than in *C. hydrogenoformans*.

Although the CODH profiles of *C. hydrogenoformans* samples are rather complex and the staining results are suboptimal, the gels do show that the CODH profiles, and thus the expression of the CODHs changes. The difference is best visible between CODH profiles of CO/fumarate and H_2/fumarate grown cells (Fig.2). In CFE samples of H_2/fumarate grown cells another CODH-protein complex is more pronounced than in CO/fumarate grown samples. It is likely that expression of the CO-oxidising:H_2-evolving complex is repressed in H_2/fumarate grown cells. Possibly the bands arise from the ACS/CODH complex, which is remarkable since fumarate and succinate, potential organic carbon sources, are present.

Samples of cells grown with CO/pyruvate show a CODH profile that differs from all other loaded samples, in the gel loaded with equal amounts of protein (Fig.1A). The CODH band at shorter migration distance has higher intensity than the other band in CO/pyruvate CFE samples, while the

opposite is true for cells grown at the other conditions. Although it is tempting to speculate that both bands arise from different CODHs, results with Triton X100 treated gels and samples indicate something different (Fig.3). In these gels, loaded with CO/fumarate grown *C. hydrogenoformans* and *T. ferrireducens* CFE samples, a single band is visible if Triton X100 was used, while two bands were visible in the absence of Triton X100. It is likely that Triton X100 breaks the interaction of a CODH-protein complex responsible for the band of shorter migration distance, with one CODH band resulting. Possibly the protein that forms the complex with the CODH is the ferredoxin-like protein CooF. The higher intensity of the CODH band in CO/pyruvate grown cells could be caused by a higher ferredoxin content in these samples. As is listed in Table 2, the cell suspension of CO/pyruvate grown cells had a orange/red colour, indicative of a high ferredoxin content.

A unique weak band is visible in native CODH stained gels, in lanes loaded with CFE samples of H_2/NO_3^- and, less clear, CO/NO_3^- grown cells (Fig.1B). Possibly one of the yet uncharacterised CODH can be isolated from *C. hydrogenoformans* cells grown in the presence of nitrate. CODH and nitrate reduction are not completely unrelated. It has been reported that the CODH of *Clostridium thermoaceticum* (currently known as *Moorella thermoacetica*) and *Clostridium acetobutylicum* catalyze the reduction of nitro-groups of trinitrotoluene (TNT)(92). Furthermore, CODH shares sequence similarity with the prismane or hybrid cluster protein (HCP). Recently, the HCP of *Escherichia coli* was shown to catalyse reduction of hydroxylamine to ammonia (242), the last step of dissimilatory ammonification of nitrate, which is also present in *C. hydrogenoformans* which contains a gene for a putative HCP (CHY_1816). Possibly a CODH of *C. hydrogenoformans* might have a role in nitrate metabolism, or HCP also has CODH reducing activity.

Wu *et al.* (2005) speculated on a role in oxidative stress response for CODH4, as part of a multi-subunit complex that also comprises a rubrerythrin subunit, a NAD/FAD-dependent oxidoreductase, and a CooF. The rubrerythrin was expected to catalyse H_2O_2 reduction with electrons supplied by CODH4. The CooF and the NAD/FAD dependent oxidoreductase were speculated to act as intermediate electron carriers. The group of NAD/FAD dependent oxidoreductases is diverse, which prevents unambiguous prediction of its substrate specificity. The group comprises NADH-oxidases,

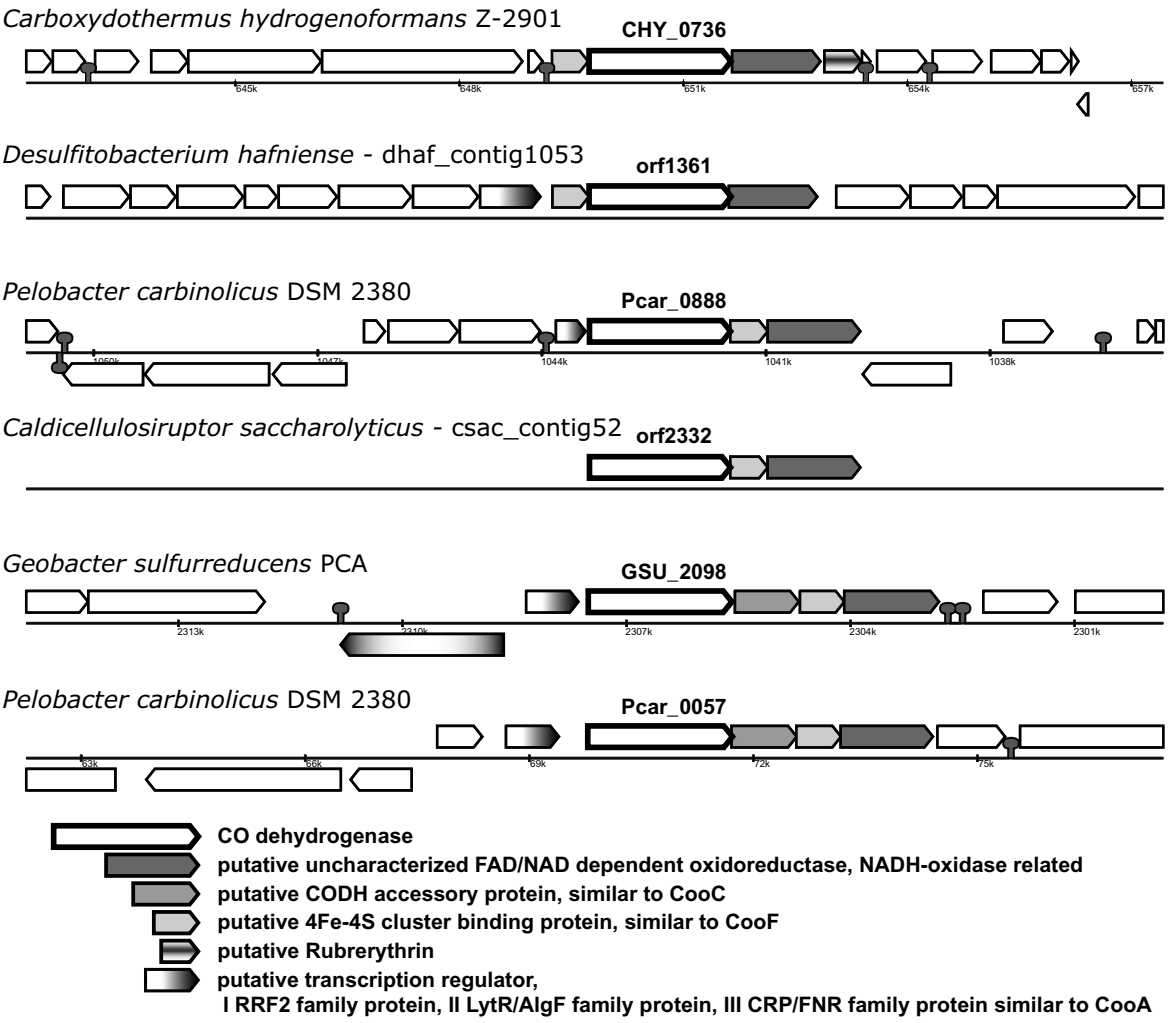

Figure 4:

Genomic context of *cooS-IV* of *C. hydrogenoformans* and similar contexts of a gene encoding a carbon monoxide dehydrogenase and putative NADH-oxidase in *Desulfitobacterium hafniense*, *Pelobacter carbinolicus*, *Caldicellilosiruptor saccharolyticus*, and *Geobacter sulfureducens*. Linear genome maps of completed genomes were obtained through the Microbial Genome Viewer (108). Linear genome maps of draft genome sequences (*D. hafniense* and *C. saccharolyticus*) were obtained through the Integrated Microbial Genomes system (132) and modelled after the genome maps of the completed genomes.

NADH peroxidases, nitrate reductases, nitrite reductases, glutathione reductase, and more. A role as NADH-oxidase would fit with the expected activity of the rubrerythrin. NADH oxidases reduce O_2 to H_2O_2 or directly to H_2O, depending upon the type. Detoxification of O_2 could then be catalysed by the NAD/FAD dependent oxidoreductase and the rubrerythrin in two reductive steps, with CODH4 providing the electrons and CooF acting as intermediate electron carrier. In *Desulfitobacterium hafniense*, *Pelobacter carbinolicus*, *Caldicellilosiruptor saccharolyticus* and *Geobacter sulfurreducens* also a CODH in genomic context with a NAD/FAD dependent oxidoreductase and putative CooF are present (Fig.4). A catalase/peroxidase gene is present upstream of the gene cluster in *G. sulfurreducens*, an indication of possible role in oxidative stress response. A possible role for a CODH in oxidative stress response is remarkable, as CODH are known to be very sensitive towards O_2.

The obtained results demonstrate that by growth of *C. hydrogenoformans* with different substrates different CODH expression profiles can be obtained. Possibly a yet uncharacterised CODH is selectively expressed in nitrate amended cultures. Biomass for study of this CODH might best be obtained with H_2 as electron donor, instead of CO, for nitrate reduction to suppress CO induced CODHs. Growth in micro-aerophilic conditions might result in the expression of CODH4. With the genome sequence of *C. hydrogenoformans* available, CODH expression can be more appropriately studied with novel proteomic and transcriptomic techniques using DNA micro arrays and denaturing 2D gel electrophoresis with peptide mass fingerprinting. A native PAGE dimension may be considered if the comparable sizes of the CODH subunits prevent detailed analysis in denaturing PAGE (Table 1).

6

Archaeoglobus fulgidus couples CO oxidation to sulfate reduction and acetogenesis with intermediate formate accumulation.

Anne M. Henstra, Cor Dijkema and Alfons J.M. Stams

Abstract

Presence of *cooF* and *cooS* in the genome of *Archaeoglobus fulgidus* VC16 suggests hydrogenogenic growth of this archaeon with carbon monoxide as a substrate. However, growth of *A. fulgidus* with CO was never confirmed. Here we demonstrate the successful adaptation of *A. fulgidus* to CO. CO served as electron donor for the reduction of sulfate to sulfide, but acetate was formed as well. In the absence of sulfate, *A. fulgidus* grew homoacetogenically. At either growth condition intermediate formate formation was observed, but hydrogen was never detected as product. A pathway is proposed that explains the putative physiological role of CooS and CooF in *A. fulgidus*. Electrons released in the oxidation of CO to CO_2 by CooS are transferred via CooF to formylmethanofuran dehydrogenase that reduces CO_2 to form formyl-methanofuran. Formate accumulation remains unexplained by available genome data as is also the case for *Methanosarcina* sp. Based on ^{1}H-NMR measurements it is hypothesised that CO_2 and not CO is the precursor of formate. Formate might be formed by hydrolysis of formyl-MFR and consumed by oxidation through a formate dehydrogenase. Hydrolysis of formyl-MFR to formate requires the activity of an unrecognised enzyme complex in archaea similar to that found in *Methylobacterium extorquens.* The results extend insight in the C1 metabolism of *A. fulgidus* and *Methanosarcina sp.* In addition, CO utilisation by *A. fulgidus* may lead to the identification of novel signal transduction pathways with relevance to medical sciences, and lead to novel biotechnological processes with application in hydrogen gas production.

submitted

Introduction

Carbon monoxide utilisation by anaerobic micro-organisms is generally linked to the presence of the multifunctional acetyl-CoA pathway in which carbon monoxide dehydrogenase (CODH) and CO play a central role (244). CO utilisation in e.g. homoacetogenic, methanogenic and sulfate reducing micro-organisms is thus explained (68). However, some micro-organisms may contain a CODH that is unrelated to the acetyl-CoA pathway. *Rhodospirillum rubrum* is devoid of a functional acetyl-CoA pathway but couples the oxidation of CO to the reduction of protons and forms H_2 (107). A similar carboxydotrophic hydrogenogenic metabolism was found in the strict anaerobic thermophile *Carboxydothermus hydrogenoformans* (207). Recent investigations demonstrated that beside the reduction of protons, CO can be used as electron donor in reduction of a wide range of electron acceptors, like reduction of sulfate, sulfur, DMSO, thiosulfate, nitrate, ferric iron, selenite, fumarate, and 9,10-anthraquinone-2,6-disulfonate (55,87,100,130,157,191-193,195,208). Whether a CODH of the acetyl-CoA pathway or a dedicated CODH is involved in these anaerobic respirations is not known.

From biochemical perspective CODHs are divided in a bifunctional and a monofunctional group (123,164). Bifunctional CODHs catalyse the synthesis or cleavage of acetyl-CoA and the oxidation of CO to CO_2. Monofunctional CODHs only catalyse the oxidation of CO. Bifunctional CODHs are part of the acetyl-CoA synthase / carbon monoxide dehydrogenase (ACS/CODH) enzyme complex. Archaeal and bacterial ACS/CODHs differ slightly in configuration. In bacteria the ACS/CODH is composed of two autonomous proteins, an α_2-β_2 CODH and a cobalt-iron-sulfur protein (CoFeSP). Archaeal ACS/CODHs are composed of 5 different subunits (α, β, γ, δ, ε), of which α-β contain CODH activity and γ-δ contain the cobalt iron sulfur centre (123,164). The genome sequence of *A. fulgidus* contains two α-ε gene pairs and one set of genes that encode β, γ, δ subunits (112). Based on phylogeny, the CODH in ACS/CODHs of archaea and bacteria form two separate clusters (124). The monofunctional CODHs, which are present in bacteria and archaea, fall within the phylogenetic cluster of the bifunctional CODHs found in bacteria (124). CO oxidation in the hydrogenogenic metabolism of *C. hydrogenoformans* and *R. rubrum* is catalysed by a monofunctional CODH (59,210). These CODHs are referred to as CooS, after the gene present in *R. rubrum* (79,106). Presence of CooS is associated with CooF, a ferredoxin like protein. For *R. rubrum* it was shown that CooF is essential in electron transfer from CooS to hydrogenase (59,84,189,210).

Presence of *cooS* and *cooF* in *A. fulgidus* led to the suggestion that a carboxydotrophic hydrogenogenic metabolism is present in this organism (112). However, genes that encode the energy conserving hydrogenase similar to *R. rubrum* CO induced hydrogenase are absent (72,85,112). Instead, sulfate may be reduced by *A. fulgidus* with CO as electron donor. Reduction of sulfate with CO is not uncommon in sulfate reducing bacteria (47). At elevated CO levels (P_{CO} > 20 kPa), CO tolerant sulfate reducing bacteria start to produce acetate with CO instead of reducing sulfate to sulfide (156). So far no reports on CO utilisation by *A. fulgidus* are available. Possibilities for CO utilising pathways are the reduction of sulfate with CO as electron donor, the conversion of CO to H_2, and acetogenesis with CO. Here we demonstrate growth of *A. fulgidus* with CO as a substrate. CO oxidation in *A. fulgidus* is linked to sulfate reduction and to acetate formation with intermediate formate accumulation. ^{1}H-NMR spectroscopy was employed to analyse the fate of carbon in ^{13}CO grown cultures. Possible physiological roles for CooS and CooF are proposed and a pathway for formate formation is discussed. The available data suggest existence of an unrecognised energy conserving reaction through hydrolysis of formyl-methanofuran to formate. CO utilisation by *A. fulgidus* has further potential relevance to medical sciences and biotechnological applications.

Materials and Methods

Organism

Archaeoglobus fulgidus strain VC-16 (DSM 4304) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany).

Medium composition

A. fulgidus was cultivated under strictly anaerobic conditions in a bicarbonate buffered medium. The medium contained 0.14 g L^{-1} K_2HPO_4, 0.14 g L^{-1} $CaCl_2 \cdot 2\ H_2O$, 0.25 g L^{-1} NH_4Cl, 2.75 g L^{-1} $MgCl_2 \cdot 6\ H_2O$, 3.45 g L^{-1} $MgSO_4 \cdot 7\ H_2O$, 0.33 g L^{-1} KCl, 18 g L^{-1} NaCl, and 0.5 g L^{-1} yeast extract. Resazurin and trace elements were added from separately prepared stock solutions as described (198). After boiling, the medium was cooled to room temperature under a N_2 flow and dispensed in serum bottles that were closed with butyl rubber stoppers. Gas phases were changed to 170 kPa 80:20 N_2/CO_2 or CO/CO_2. Bottles of 120 ml contained 50 ml medium and bottles of 585 ml contained 200 ml medium. The bottles were autoclaved for 25' at 121 °C. Sodium sulfide and sodium bicarbonate

were added from a separately autoclaved stock solution to a final concentration of 1.5 g L^{-1} $NaHCO_3$ and 0.8 mM Na_2S, prior to inoculation. Cultures were incubated in an Innova 44 incubator (New Brunswick Scientific) with 1" stroke, at 130 rpm and 80 °C. In contrast to DSMZ medium 399, the medium contained Na_2WO_4 (0.1 μM), but not Al and Cu as trace minerals. Gas phases with 2, 5, 10, 20 and 40 kPa CO were created by addition of CO with a gastight syringe to bottles with a N_2/CO_2 gas phase prior to sterilisation.

Analytical methods

Analysis of substrates and products was performed as previously described (87). Gases were analysed by GC-TCD using a molsieve 5A column (H_2, CO) or a Poraplot Q column (CO_2). Both columns were obtained from Chrompack (Middelburg, The Netherlands). Sulfate was analysed by HPLC using an Ionpac AS9-SC column and ED 40 electrochemical detector (column and detector by Dionex, Sunnyvale, USA)(181). Organic acids were analysed by HPLC using a Polyspher OA HY column (300-6.5mm, Merck, Darmstadt, Germany) and RI SE-61 refractive index detector (Shodex, Tokyo Japan)(181). Sulfide was analysed according to the colorimetric method described by Trüper and Schlegel (217). Optical density of culture samples were measured at 660 nm (OD660) on a U1500 spectrophotometer (Hitachi Instruments, San Jose, US). Total amounts of CO, CO_2, and H_2S were expressed as mmol L^{-1} medium, representing total amounts of these gases distributed over aqueous and gaseous phase and their dissociated states, taking into account the effect of temperature, activity and pH.

Nuclear Magnetic Resonance

Incorporation of ^{13}C in formate and acetate was analysed by proton nuclear magnetic resonance (^1H-NMR) in samples obtained from *A. fulgidus* cultures with initial $^{13}CO/^{12}CO_2$ (80:20) gas phase. Gas phases with 99.4 % ^{13}CO (Campro, Veenendaal, The Netherlands) were prepared as described above. The amount of yeast extract added to the medium was reduced to 0.05 g L^{-1}. Concentrations of acetate and formate were analysed by HPLC. ^1H-NMR spectra determined relative abundance of isotopomers of acetate ($^{12}C_2$-$^{12}C_1$, $^{13}C_2$-$^{12}C_1$, $^{12}C_2$-$^{13}C_1$, $^{13}C_2$-$^{13}C_1$) and formate (^{13}C, ^{12}C). Cultures were prepared as described and placed at 80 °C without shaking for 24 h to obtain an active culture with minimal CO conversion. Subsequently, cultures were placed at 80 °C and 200 rpm to stimulate gas/liquid mass transfer. Culture samples were withdrawn 6, 10, 14, 25 and 240 h. after placement at 200

rpm and centrifuged for 10' at 16 x g. To 450 µl culture supernatant 50 µl D_2O was added before ^{1}H-NMR spectra were recorded at 500.13 MHz on a Bruker AMX-500 NMR spectrometer. Each spectrum was a superposition of 2000 FID's accumulated in 16k data points; a 60° pulse was used and the inter pulse time was 1 s.

Results and Discussion

A. fulgidus is a strict anaerobic hyperthermophilic archaeon that oxidises lactate completely to CO_2 with sulfate as electron acceptor (200,201). Chemolithoautotrophically *A. fulgidus* VC-16 reduces thiosulfate with H_2, but not sulfate (249). For complete oxidation of lactate and autotrophic carbon fixation *A. fulgidus* depends on the activity of ACS/CODH (43,143,200,231). Besides the bifunctional archaeal type CODH, a monofunctional CODH similar to *cooS* of *R. rubrum* and *C. hydrogenoformans* is present in the genome of *A. fulgidus* (79,112). The presence of *cooS* and *cooF* in the genome of *A. fulgidus* indicates the possibility of growth with CO as a substrate by this organism, as proposed previously (112). However, thus far no experimental evidence was provided for this hypothesis.

CO inhibits growth of many strict anaerobic micro-organisms (47). Especially sulfate reducing bacteria seem to be sensitive towards CO, though this strongly depends on the species or even strain. Some species do not grow with CO levels (P_{CO}) above 2 kPa while others tolerate a P_{CO} up to 20 kPa. Only *Desulfotomaculum carboxydovorans* was reported to grow with CO levels up to 200 kPa without noticeable inhibition. Under these conditions it converts CO to $H_2 + CO_2$ instead of reducing sulfate (157). *Desulfotomaculum kuznetsovi* and *Desulfotomaculum thermobenzoicum* subsp. *thermosyntrophycum* grow with CO up to 70 kPa but revert to acetogenesis instead of sulfate reduction (156). Methanogenic archaea have been described to grow at low levels of CO, though growth is often poor (45). Recently, adaptation of *Methanosarcina acetivorans* C2A to CO was achieved by multiple transfers with increasing CO levels (171). When we inoculated *A. fulgidus* in serum vials with lactate (30 mM), sulfate (15 mM) and a 170 kPa CO:CO_2 (80:20) gas phase, growth started only after 14 days. Thereafter, the strain could be subcultured on CO with a lag phase comparable to lactate/sulfate grown cultures (data not shown). CO adapted and unadapted *A. fulgidus* were compared in an experiment where inoculations were made in serum vials with lactate (30 mM) and sulfate (15 mM) and a P_{CO} of 0, 5, 10, 20, 40, 80 and 136 kPa. In addition, serum vials with a P_{CO} of 80 kPa but without lactate were inoculated. Culture turbidity was measured daily and concentrations

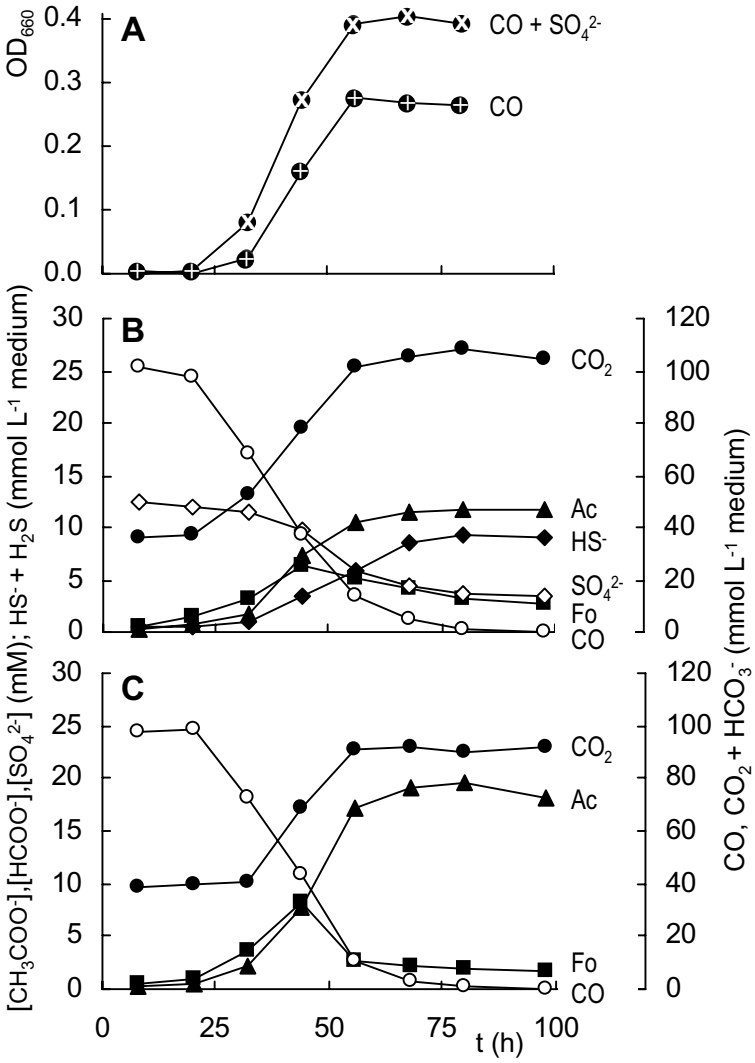

Figure 1:

Change in culture OD (A) and formation of acetate (Ac), formate (Fo), CO_2 and sulfide during growth of *A. fulgidus* VC-16 with CO in the presence (B) and absence (C) of sulfate. Data for CO_2 include calculated amounts of HCO_3^- and data for HS^- include calculated amounts of H_2S.

of substrates and products were analysed at the start and end of the experiment. CO levels up to 40 kPa did not or only slightly affect the lag phase in cultures inoculated with unadapted cells. CO levels of 40 and 80 kPa resulted in a noticeable lag phase increase of 2 to 13 days. Interestingly, cultures with a P_{CO} of 80 kPa without lactate showed a 4 day shorter lag phase than similar cultures with lactate.

Growth of *A. fulgidus* with CO and sulfate (15 mM) was studied in more detail. CO adapted cells were used to inoculate bottles with 200 mL medium and a 385 mL CO/CO_2 headspace. Bottles were

shaken (130 rpm) to improve mass transfer. Gas phase and liquid samples were taken at 12 h intervals and used for H_2, CO, CO_2, sulfide, and VFA analysis. After 140 h of cultivation cultures were allowed to cool down to room temperature upon which the final pH was measured (pH 6.3). Results are plotted in Figure 1A and 1B. CO (102 mmol L^{-1} medium) and sulfate (9.1 mM) were consumed by the cultures and this led to formation of CO_2 (68 mmol L^{-1} medium) and sulfide (8.6 mM). Organic acid analysis revealed the intermediate formation of formate (6.4 mM maximum) and the accumulation of acetate (11 mM). H_2 and methane were not detected. The OD_{660} of the cultures increased to 0.41 indicating growth (Fig.1A). The formation of acetate indicates that *A. fulgidus* can grow homoacetogenically with CO possibly even in the absence of sulfate. Therefore, a similar growth experiment was performed simultaneously with medium where $MgSO_4$ was replaced by $MgCl_2$ (Fig.1A and 1C). In these cultures CO (98 mmol L^{-1} medium) was consumed completely with formation of acetate (18 mM) and CO_2 (53 mmol L^{-1} medium). Intermediate formate (8.2 mM) was formed. The formation of acetate and CO_2 led to a final pH of 5.2. Cultures without sulfate obtained lower optical densities (0.27 vs. 0.41 at 660 nm). Besides acetate and formate small amounts (<0.5 mM) of propionate and butyrate were detected by HPLC. In ^{1}H-NMR experiments described below, no formate was detected in late culture samples while HPLC indicated presence of approximately 2 mM residual formate. This is remarkable since ^{1}H-NMR is a more sensitive detection technique for formate than HPLC. Closer inspection revealed that an unknown compound with similar retention time to formate in HPLC at 30 and 60 °C was formed.

A possible path for carbon flow in the CO metabolism of *A. fulgidus* to formate and acetate is drawn in Figure 2. Published results for *Methanosarcina barkeri* served as a basis, though some steps

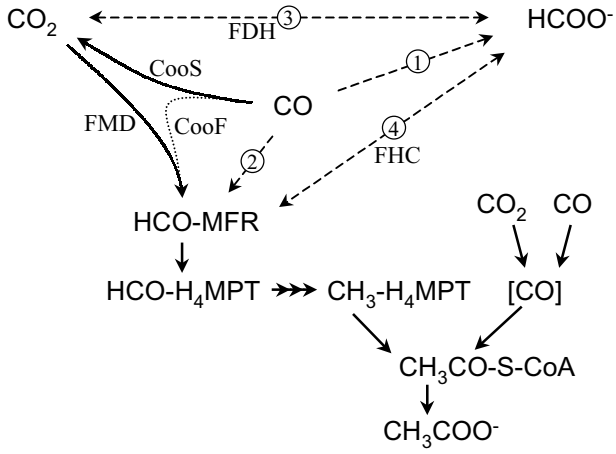

Figure 2:

Proposed carbon flow to acetate in the resultant metabolic pathway with the proposed function of CooS and CooF and hypothetical pathway for formation of formate during growth of *A. fulgidus* on CO. CO oxidation by CooS is coupled via electron transfer by CooF (dotted line) to reduction of CO_2 by FMD which yields formyl-MFR. Dashed and numbered lines represent hypothetical steps to formate as explained in the text. FDH = formate dehydrogenase, FMD = formyl-methanofuran dehydrogenase, FHC = formyl transferase/hydrolase complex.

remain hypothetical. *M. barkeri* reduces CO_2 to formyl-methanofuran (formyl-MFR) by a formylmethanofuran dehydrogenase (FMD). With H_2 as electron donor this reduction is energy driven through Ech (85). Coupling of H_2 oxidation and pmf by Ech bridges the gap between the electrode potentials of the H_2/H^+ (E°' -414 mV) and CO_2/formyl-MFR (E°' -530 mV) redox couples and makes the reduction of CO_2 possible (16,85). *M. barkeri* Ech catalyses the reversible reduction of ferredoxin with H_2 (137). CO_2 reduction to formyl-MFR in Δ*ech* mutants was restored if CO or pyruvate were added (203). Electron transfer from pyruvate and CO oxidation to protons, likely proceeds through ferredoxin (203). A ferredoxin-like protein, CooF, is functionally associated with the monofunctional CODH CooS in *R. rubrum* and *C. hydrogenoformans* and mediates electron transfer to a H_2-evolving hydrogenase (59,106,189,210), similar to *M. barkeri* Ech (85). The lack of H_2 formation by *A. fulgidus* from CO corresponds with the absence of an *ech* in its genome. Instead of a role in H_2 formation a possible physiological function of CooS and CooF in *A. fulgidus* is the oxidation of CO and transfer of electrons to FMD. Presence of these components and the components necessary for transfer of the formyl moiety to tetrahydromethanopterin and further steps of the reductive acetyl-CoA pathway were demonstrated before or have been annotated in the genome of *A. fulgidus* (43,112,144). Summarized, it is proposed that CooS oxidises CO to CO_2 and CooF transfers the released electrons to FMD which reduces CO_2 to formyl-MFR.

The formation of formate by *A. fulgidus* is not explained by the above proposed pathway. Formation of formate was also observed for *Methanosarcina acetivorans* C2A grown on CO, in addition to formation of methane and acetate, but remained unexplained (171). Higher CO levels led to more acetate, more formate and less methane. Rother and Metcalf (171) discussed several possibilities for a physiological role and for the mechanism of formate formation. Steps indicated by dashed lines in Figure 2 represent hypothesized paths of formate formation. CO and/or CO_2 are the possible precursors of formate, either by direct conversion or via formyl-MFR by hydrolysis of this compound. The biocatalytic hydration of CO to formate was speculated upon, but supportive biochemical results are lacking (Fig.2, step 1)(171,210). Similarly, it can be speculated that formyl-MFR is formed directly from CO and MFR (Fig.2, step 2). To improve insights on formate formation and the nature of its precursor, ^{1}H-NMR spectroscopy of successive samples of medium supernatant of *A. fulgidus* cultures incubated with an initial gas phase of $^{13}CO/^{12}CO_2$ (80:20) was performed (Fig.3). The data show the relative 13-C labelling of the four isotopomers of acetate and of formate (Table 1). Although obtained

data are not fully conclusive, the dominant conversions are clear. Oxidation of ^{13}CO to $^{13}CO_2$ by *A. fulgidus* will result in an increase in label in the HCO_3^-/CO_2 pool, while the relative amount of label in CO will remain unchanged or show a decrease if $^{12}CO_2$ is converted into CO. The relative increase of label in the HCO_3^-/CO_2 pool will affect labelling of formate and acetate accordingly when CO_2 is incorporated. Trends in the labelling data over time indicate that CO_2 is the precursor of formate. The concentration of unlabelled formate was highest in the first sample analysed (t = 6 h), and decreased in later samples, while the concentration of labelled formate increased between 6 h and 10 h samples. After 10 h the total formate decreased to undetectable levels at 25 h. The relative increase in label at the methyl group (C_2) of acetate indicates that CO_2 is its precursor in similar way as described for formate. The relatively high labelled fraction of formate (43 %) compared to C_2 label in acetate (30 %) at 6 h and in later samples is caused by uptake of formate. This indicates that formation and uptake of formate are simultaneous processes, rather than sequential. Labelling of C_1-acetate (68 %-72 %) clearly exceeds labelling of formate and of C_2-acetate and indicates that CO is a precursor for C_1-acetate. However, CO_2 as precursor cannot be excluded.

The results obtained by ^{1}H-NMR spectroscopy of *A. fulgidus* culture samples grown with ^{13}CO indicate that CO_2 is the precursor of formate. It is not possible to distinguish whether formate is

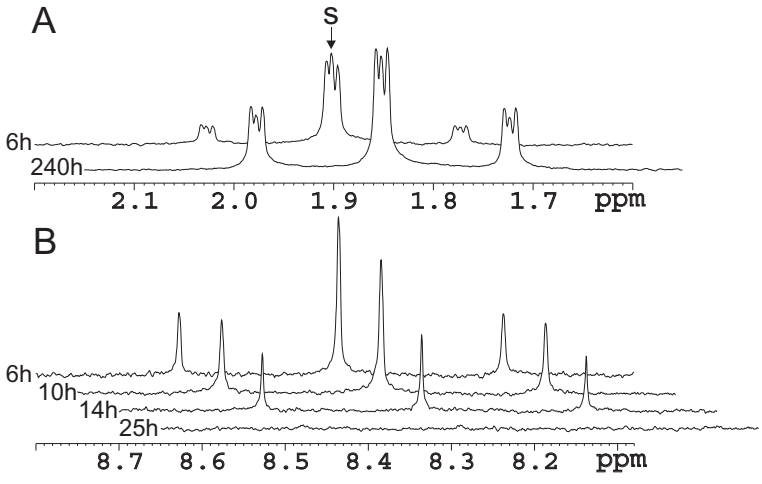

Figure 3:

^{1}H-NMR spectra of acetate (A) and formate (B) present in samples obtained from *A. fulgidus* cultures grown with ^{13}CO as substrate. Respective concentrations of acetate and formate are listed in Table 1. Singlet acetate, marked by S, served as reference and was set at 1.9 ppm in accordance with its known relative position towards tetramethylsilan. X-axes correspond to the topmost spectra; each successive spectrum is plotted offset by 0.05 ppm.

Table 1: Relative and absolute ^{13}C-labelling of formate and acetate isotopomers over time in ^{13}CO fed *A. fulgidus* cultures.

t(h)[1]	CO left (%)	Acetate (mM)	Formate (mM)	isotopomers acetate (%)						formate (%)	
				$^{13}C_1$-$^{13}C_2$	$^{12}C_1$-$^{13}C_2$	$^{13}C_1$-$^{12}C_2$	$^{12}C_1$-$^{12}C_2$	$^{13}C_2$-Ac	$^{13}C_1$-Ac	^{13}C-Fo	^{12}C-Fo
6	55	4.8	10	22	8	46	24	30	68	43	57
10	31	7.1	11	28	9	43	20	37	71	51	49
14	14	11	7.0	33	11	39	16	44	72	61	39
25	1.8	14	2.6	36	13	36	15	49	72	nd[2]	nd
240	na[3]	na	na	37	13	36	14	50	73	nd	nd

[1] number of hours after cultures were placed at 200 rpm; [2] nd = not detected; [3] na = not analysed

formed directly by reduction of CO_2 or via hydrolysis of HCO-MPT. If the mechanism responsible for formate formation in *A. fulgidus* and *M. acetivorans* is similar, then it is unlikely that CO_2 is directly reduced to formate (Fig.3, step 3). Formate production by *M. acetivorans* is irreversible (171). For *M. acetivorans* C2A no formate dehydrogenase (FDH) was annotated in the genome and no FDH activity was observed in cell extracts (171). FDH was also not annotated in the genome of *A. fulgidus* VC-16, but growth with formate as electron donor and sulfate or thiosulfate as electron acceptor was demonstrated (249). Additionally, a blast search with *Pyrococcus abyssi* formate dehydrogenase gene (PAB1389, PAB1390) indicated that open reading frames AF1202 and AF1203 in the *A. fulgidus* genome are putative formate dehydrogenase genes. Formation of formate from formyl-MFR (Fig.3, step 4) or formyl-tetrahydromethanopterin (formyl-H_4MPT) is plausible. From *Methylobacterium extorquens* an enzyme complex, Fhc, was isolated that hydrolyses formyl-MFR and thus forms formate (162). It was recently shown that a mutant of *M. barkeri* strain Fusaro that lacked the operon encoding for N^5-methyl-tetrahydrosarcinapterin:coenzyme-M methyltransferase, coupled the reduction of methanol to methane to the oxidation of acetate to formate and CO_2 (237). With ^{13}C-NMR spectroscopy it was demonstrated that this mutant forms formate exclusively from the C_2 of acetate (237). Although the obtained results for *A. fulgidus* do not exclude the direct formation of formate from CO or CO_2, the formation of formate from formyl-MFR is the most likely. Then possibly energy is conserved in the conversion of formyl-MFR or formyl-H_4MPT to formate, since a $\Delta G°'$ of -16 kJ mol^{-1} was estimated for the conversion of formyl-MFR to formate and MFR (130). The reaction with formyl-H_4MPT is slightly more endergonic $\Delta G°'$ (-9 kJ mol^{-1})(130). Possibly formate is converted by *A. fulgidus* through oxidation of formate to CO_2 by FDH. Oxidation of formate could be coupled to the reductive steps of acetate formation or to the reduction of sulfate or

another electron acceptor. The latter raises the possibility that formate instead of CO is the direct electron donor for sulfate reduction in the *A. fulgidus* cultures with CO and sulfate.

Here we demonstrated that CO is an excellent growth substrate for *A. fulgidus*. Sulfate is reduced with CO as electron donor, instead of the proposed hydrogenogenic metabolism (112). Additionally, *A. fulgidus* grows homoacetogenically with CO in the absence of sulfate. Therefore, *A. fulgidus* is not strictly dependent on sulfate or thiosulfate reduction for growth. Intermediate formate accumulates in the presence and in the absence of sulfate. Formate might therefore serve as the direct electron donor for sulfate reduction rather than CO. Interestingly, sulfate reduction to sulfide is not inhibited by CO. This, in contrast to several sulfate reducing bacteria (47,156). Based on the presented results a pathway for acetogenic CO oxidation by *A. fulgidus* was proposed. Our results indicate the existence of an unidentified enzyme complex in *A. fulgidus* that hydrolyses formyl-MFR or formyl-H_4MPT to formate.

The observation that *A. fulgidus* does not reduce sulfate with H_2 (249), but does so with CO creates the opportunity for its application in synthesis-gas biotechnology. Synthesis gas is a mixture of mainly CO and H_2 produced by gasification of natural gas or other hydrocarbons and is a cheap H_2 source (202). Alternatives to current technology are desired to remove CO in synthesis gas below levels of 10 ppm. One solution is provided by catalysts that selectively oxidise CO and not H_2 (196). *A. fulgidus* is essentially a CO selective catalyst and thus holds the potential for a biotechnological process to produce a high grade H_2 gas from synthesis gas. (186,226)

The capacity of *A. fulgidus* to grow with CO could have relevance for a yet unaddressed research field. The novel CO sensor CooA (169) or any member of the CRP/FNR family of regulators appear to be absent in the genome of *A. fulgidus* (26). CooA independent anti-inflammatory and anti-apoptotic responses of human cells to CO indicate the existence of yet unknown CO signal transduction pathways (170). *A. fulgidus* may proof to be a suitable model organism for identification of ancestry CO signal transduction other than through CooA.

Acknowledgements

The authors are grateful to Dr. Pieter de Waard for assistance in graphical output of NMR data, and to Dr. Jeanine Geelhoed for useful comments regarding the manuscript.

Summary and concluding remarks

Technological Perspective

Microbial CO metabolism was studied in detail with the ultimate aim to assess the feasibility of a biotechnological process that could replace the existing water gas shift technology in the production of a fuel cell grade hydrogen gas from synthesis gas. It is expected that a biotechnological process is less sensitive to impurities present in synthesis gas and can reach lower CO thresholds, and thus might be more cost effective than conventional catalysts. Low CO thresholds are especially required for polymer electrolyte membrane fuel cells. These fuel cells have a broad application potential in a future hydrogen economy. The bulk of hydrogen produced today is derived from natural gas in a steam reforming process that forms CO besides H_2. CO is removed by its conversion to H_2 in the water gas shift reaction. However, due to thermodynamic limitations of the process operation temperature, the required CO thresholds are not obtained (<10 ppm). CO conversion at more ambient temperatures is in that respect advantageous for biological CO conversion to obtain a fuel cell grade H_2.

It was demonstrated in Chapter 2 that batch cultures of *Carboxydothermus hydrogenoformans* converted CO to levels below 2 ppm while accumulating H_2. In these cultures it was necessary to remove CO_2 from the gas phase. Without removal of CO_2, CO thresholds approached 100 ppm. CO limits that are generally communicated for PEM-FC, indicate 10 ppm as allowable threshold for CO in H_2 gas. The potential of a biotechnological process is thus supported, however, the time needed to reach these thresholds was considerable and needs attention. Biomass activity and gas/liquid mass transfer are possible rate limiting factors in the biological water gas shift reaction. As demonstrated in Chapter 3, higher conversion rates are possible in batch culture than achieved with the cultivation technique used in Chapter 2. In fact, it appears that only in the early stage of batch cultivation the biomass is limiting CO conversion rates. After some time biomass has grown to sufficient density and gas/mass transfer becomes limiting. Even in the most turbulent shaking regime allowed by the incubator, gas/liquid mass transfer remained rate limiting. Success of a tentative biotechnological process therefore likely depends on gas/liquid mass transfer rates of specific reactor types and associated restrictions dictated by process economics.

Physiological Perspective

In recent years it has become clear that CO is used as a substrate by a diverse group of strict anaerobic micro-organisms. While it previously was believed that CO predominantly inhibited the

growth of many anaerobes, albeit at higher partial pressures than aerobes. Currently research has indicated that CO is a versatile substrate which is effectively used in many microbial metabolisms. The advances that were made especially show that many electron acceptors can be reduced by a wide variety of micro organisms with CO as electron donor. The advances could be made due to the fact that CO is generally neglected as a substrate in physiological studies of novel anaerobic isolates. Genome sequencing projects also demonstrate that the enzymes, CO dehydrogenases, involved in the CO metabolism are present in already well known organisms that have never been tested with CO. An example of this is *Archaeoglobus fulgidus*, which was tested for its ability to grow with CO in Chapter 5. While it was speculated that *A. fulgidus* could oxidise CO coupled to the reduction of protons to H_2, it grew acetogenically with CO instead. In fact, *A. fulgidus* is the first true homo-acetogenic archaeon known. Remarkable of its metabolism was the intermediate accumulation of formate. It was proposed that *A. fulgidus* forms acetate via the acetyl-CoA pathway. In this pathway, CO_2 is reduced to form the methyl group of acetate. However, formate is not an intermediate in the expected pathway in which CO_2 is reduced to form formyl-methanofuran and subsequently formyl-tertahydromethanopterin. Besides the formation of acetate, *A. fulgidus* can reduce sulfate to sulfide with CO as electron donor. It distinguishes itself from bacterial sulfate reducers by its tolerance to CO. While most known sulfate-reducing bacteria are inhibited by elevated levels of CO, *A. fulgidus* was not noticeably inhibited in the presence of 136 kPa CO. Since *A. fulgidus* is not capable of growth with H_2 and CO_2 or sulfate as electron acceptors, this organism could be employed to selectively oxidise CO in gas mixtures containing H_2 and remove trace amounts of CO to levels below 10 ppm.

Selective oxidation of CO is not possible with *C. hydrogenoformans*. Although *C. hydrogenoformans* can reduce various electron acceptors with CO, it also does so with H_2 (Chapter 4). *C. hydrogenoformans* is a true CO specialist. It is able to grow hydrogenogenically with CO, to reduce various other electron acceptors with CO, and to use CO as sole source of energy and carbon. *C. hydrogenoformans* contains five CO dehydrogenase genes. Besides the identified activities in H_2 formation, in NADPH generation and in autotrophic carbon fixation for three of these CO dehydrogenase, two CO dehydrogenases are still without function (Chapter 5). Conditions that may lead to expression of these CO dehydrogenases are proposed. In nitrate amended cultures a CO dehydrogenase was present, that was not present in cells grown with other substrates. Wu *et al.* (2005) already indicated that a CO dehydrogenase of *C. hydrogenoformans* might be involved in oxidative stress response and be expressed under micro aerophilic conditions. Clearly the microbial physiology regarding CO is still incomplete and is more diverse than thought up to now. Further studies are needed in this respect.

References

1. **Abrini, J., H. Naveau, and E. J. Nyns.** 1994. *Clostridium autoethanogenum*, *sp. nov.*, an anaerobic bacterium that produces ethanol from carbon monoxide. Archives of Microbiology **161:**345-351.
2. **Afting, C., A. Hochheimer, and R. K. Thauer.** 1998. Function of H_2-forming methylenetetrahydromethanopterin dehydrogenase from *Methanobacterium thermoautotrophicum* in coenzyme F_{420} reduction with H_2. Archives of Microbiology **169:**206-210.
3. **Afting, C., E. Kremmer, C. Brucker, A. Hochheimer, and R. K. Thauer.** 2000. Regulation of the synthesis of H_2-forming methylenetetrahydromethanopterin dehydrogenase (Hmd) and of HmdII and HmdIII in *Methanothermobacter marburgensis*. Archives of Microbiology **174:**225-232.
4. **Amend, J. P., and E. L. Shock.** 2001. Energetics of overall metabolic reactions of thermophilic and hyperthermophilic Archaea and Bacteria. FEMS Microbiology Reviews **25:**175-243.
5. **Anonymous.** 1997. Kyoto, Japan., 1-10 December.
6. **Appleby, J.** 1995. Fuel cells, p. 1408-1437. *In* A. Bisio and S. Boots (ed.), Encyclopedia of energy, technology and the environment, vol. 2. John Wiley and Sons, New York.
7. **Ariga, O., M. Kimura, M. Taya, and T. Kobayashi.** 1986. Kinetic evaluation and characterization of ceramic honeycomb-monolith bioreactor. Journal of Fermentation Technology **64:**327-334.
8. **Armor, J. N.** 1999. The multiple roles for catalysis in the production of H_2. Applied Catalysis A **176:**159-176.
9. **Atimtay, A. T., S. Emri, T. Bagci, and A. U. Demir.** 2000. Urban CO exposure and its health effects on traffic policemen in Ankara. Environmental Research **82:**222-230.
10. **Bae, J. H., and P. L. McCarty.** 1993. Variation of carbon-monoxide production during methane fermentation of glucose. Water Environment Research **65:**890-898.
11. **Bagramyan, K., and A. Trchounian.** 2003. Structural and functional features of formate hydrogen lyase, an enzyme of mixed-acid fermentation from *Escherichia coli*. Biochemistry-Moscow **68:**1159-1170.
12. **Baker, B. J., and J. F. Banfield.** 2003. Microbial communities in acid mine drainage. FEMS Microbiology Ecology **44:**139-152.
13. **Bartish, C. M., and G. M. Drissel.** 1978. Carbon monoxide, p. 772-793. *In* Kirk-Othmer (ed.), Encyclopedia of chemical technology, 3 ed. John Wiley & Sons, New York.
14. **Belgiorno, V., G. De Feo, C. Della Rocca, and R. M. A. Napoli.** 2003. Energy from gasification of solid wastes. Waste Management **23:**1-15.
15. **Berkessel, A., and R. K. Thauer.** 1995. On the mechanism of catalysis by a metal-free hydrogenase from methanogenic archaea - enzymatic transformation of H_2 without a metal and its analogy to the chemistry of alkanes in superacidic solution. Angewandte Chemie **34:**2247-2250.
16. **Bertram, P. A., and R. K. Thauer.** 1994. Thermodynamics of the formylmethanofuran dehydrogenase reaction in *Methanobacterium thermoautotrophicum*. European Journal of Biochemistry **226:**811-818.
17. **Bonch Osmolovskaya, E. A., M. L. Miroshnichenko, A. I. Slobodkin, T. G. Sokolova, G. A. Karpov, N. A. Kostrikina, D. G. Zavarzina, M. I. Prokof'eva, I. I. Rusanov, and N. V. Pimenov.** 1999. Biodiversity of anaerobic lithotrophic prokaryotes in terrestrial hot springs of kamchatka. Mikrobiologiya **68:**343-351.
18. **Bond, P. L., S. P. Smriga, and J. F. Banfield.** 2000. Phylogeny of microorganisms populating a thick, subaerial, predominantly lithotrophic biofilm at an extreme acid mine drainage site. Applied and Environmental Microbiology **66:**3842-3849.
19. **Boonstra, J., R. v. Lier, G. Janssen, H. Dijkman, and C. J. N. Buisman.** 1999. Biological treatment of acid mine drainage, p. 559-567. *In* R. Amils and A. Ballester (ed.), Biohydrometallurgy and the environment; Toward the mining of the 21st century, vol. 9B. Elsevier, Amsterdam.
20. **Boshoff, G., J. Duncan, and P. D. Rose.** 2004. Tannery effluent as a carbon source for biological sulphate reduction. Water Research **38:**2651-2658.

21. **Boshoff, G., J. Duncan, and P. D. Rose.** 2004. The use of micro-algal biomass as a carbon source for biological sulphate reducing systems. Water Research **38:**2659-2666.

22. **Bott, M. H., B. Eikmanns, and R. K. Thauer.** 1985. Defective formation and or utilization of carbon-monoxide in H_2 CO_2 fermenting methanogens dependent on acetate as carbon source. Archives of Microbiology **143:**266-269.

23. **Bradford, M. M.** 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.

24. **Bredwell, M. D., P. Srivastava, and R. M. Worden.** 1999. Reactor design issues for synthesis-gas fermentations. Biotechnology Progress **15:**834-844.

25. **Brimblecombe, P., C. Hammer, H. Rohde, A. Ryaboshapko, and C. F. Boutron.** 1989. Human influence on the sulfur cycle. *In* P. Brimblecombe and A. Y. Lein (ed.), Evolution of the global biogeochemical sulfur cycle, vol. Scope 39. John Wiley and Sons, New York.

26. **Brinkman, A.** 2002. Regulation of transcription in hyperthermophilic archaea. Ph.D. thesis. Wageningen University, Wageningen.

27. **Buisman, C. J. N., B. G. Geraats, P. IJspeert, and G. Lettinga.** 1990. Optimization of sulfur production in a biotechnological sulfide-removing reactor. Biotechnology and Bioengineering **35:**50-56.

28. **Bullister, J. L., N. L. Guinasso, and D. R. Schink.** 1982. Dissolved hydrogen, carbon monoxide, and methane at the cepex site. Journal of Geophysical Research Oceans and Atmospheres **87:**2022-2034.

29. **Buurman, G., S. Shima, and R. K. Thauer.** 2000. The metal-free hydrogenase from methanogenic archaea: evidence for a bound cofactor. FEBS Letters **485:**200-204.

30. **Chang, I. S., P. K. Shin, and B. H. Kim.** 2000. Biological treatment of acid mine drainage under sulphate-reducing conditions with solid waste materials as substrate. Water Research **34:**1269-1277.

31. **Charlson, R. J., S. E. Schwartz, J. M. Hales, R. D. Cess, J. A. Coakley, J. E. Hansen, and D. J. Hofmann.** 1992. Climate forcing by anthropogenic aerosols. Science **255:**423-430.

32. **Chen, C. C., C. Y. Lin, and M. C. Lin.** 2002. Acid-base enrichment enhances anaerobic hydrogen production process. Applied Microbiology and Biotechnology **58:**224-228.

33. **Chidthaisong, A., and R. Conrad.** 2000. Specificity of chloroform, 2-bromoethanesulfonate and fluoroacetate to inhibit methanogenesis and other anaerobic processes in anoxic rice field soil. Soil Biology & Biochemistry **32:**977-988.

34. **Colby, J., E. Williams, and A. P. F. Turner.** 1985. Applications of CO-utilizing microorganisms. Trends in Biotechnology **3:**12-17.

35. **Colleran, E., S. Finnegan, and P. Lens.** 1995. Anaerobic treatment of sulfate-containing waste streams. Antonie van Leeuwenhoek **67:**29-46.

36. **Collins, M. D., P. A. Lawson, A. Willems, J. J. Cordoba, J. Fernandezgarayzabal, P. Garcia, J. Cai, H. Hippe, and J. A. E. Farrow.** 1994. The phylogeny of the genus *Clostridium* - proposal of 5 new genera and 11 new species combinations. International Journal of Systematic Bacteriology **44:**812-826.

37. **Conrad, R., and W. Seiler.** 1980. Photo-oxidative production and microbial consumption of carbon monoxide in seawater. FEMS Microbiology Letters **9:**61-64.

38. **Conrad, R., and W. Seiler.** 1985. Characteristics of abiological carbon monoxide formation from soil organic-matter, humic acids, and phenolic-compounds. Environmental Science and Technology **19:**1165-1169.

39. **Conrad, R., W. Seiler, G. Bunse, and H. Giehl.** 1982. Carbon monoxide in sea-water (Atlantic-ocean). Journal of Geophysical Research Oceans and Atmospheres **87:**8839-8852.

40. **Conrad, R., and R. K. Thauer.** 1983. Carbon monoxide production by *Methanobacterium thermoautotrophicum*. FEMS Microbiology Letters **20:**229-232.

41. **Cortright, R. D., M. Sanchez-Castillo, and J. A. Dumesic.** 2002. Conversion of biomass to 1,2-propanediol by selective catalytic hydrogenation of lactic acid over silica-supported copper. Applied Catalysis B **39:**353-359.

42. **Cox, H. H. J., and M. A. Deshusses.** 2001. Biotrickling filters, p. 99-131. *In* C. Kennes and M. C. Veiga (ed.), Bioreactors for waste gas treatment. Kluwer Academic Publishers, Dordrecht.

43. **Dai, Y. R., D. W. Reed, J. H. Millstein, P. L. Hartzell, D. A. Grahame, and E. DeMoll.** 1998. Acetyl-CoA decarbonylase/synthase complex from *Archaeoglobus fulgidus*. Archives of Microbiology **169:**525-529.

44. **Daniel, S. L., T. Hsu, S. I. Dean, and H. L. Drake.** 1990. Characterization of the hydrogen- and carbon monoxide-dependent chemolithotrophic potentials of the acetogens *Clostridium thermoaceticum* and *Acetogenium kivui*. Journal of Bacteriology **172:**4464-4471.

45. **Daniels, L., G. Fuchs, R. K. Thauer, and J. G. Zeikus.** 1977. Carbon monoxide oxidation by methanogenic bacteria. Journal of Bacteriology **132:**118-126.

46. **Dashekvicz, M. P., and R. L. Uffen.** 1979. Identification of a carbon monoxide metabolizing bacterium as a strain of *Rhodopseudomonas gelatinosa* (Molisch) Vanniel. International Journal of Systematic Bacteriology **29:**145-148.

47. **Davidova, M. N., N. B. Tarasova, F. K. Mukhitova, and I. U. Karpilova.** 1994. Carbon monoxide in metabolism of anaerobic bacteria. Canadian Journal of Microbiology **40:**417-425.

48. **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.

49. **De Heyder, B., A. Overmeire, H. Van Langenhove, and W. Verstraete.** 1994. Ethene removal from a synthetic waste gas using a dry biobed. Biotechnology and Bioengineering **44:**642-648.

50. **de Smul, A.** 1998. A combined biotechnological and physico-chemical process for the desulfurization of waste waters. University of Ghent, Ghent, Belgium.

51. **Dellepiane, D., B. Bosio, and E. Arato.** 2003. Clean energy from sugarcane waste: feasibility study of an innovative application of bagasse and barbojo. Journal of Power Sources **122:**47-56.

52. **Diekert, G.** 1990. CO_2 reduction to acetate in anaerobic bacteria. FEMS Microbiology Letters **87:**391-395.

53. **Diekert, G. B., and R. K. Thauer.** 1978. Carbon-monoxide oxidation by *Clostridium thermoaceticum* and *Clostridium formicoaceticum*. Journal of Bacteriology **136:**597-606.

54. **Dobbek, H., V. Svetlitchnyi, L. Gremer, R. Huber, and O. Meyer.** 2001. Crystal structure of a carbon monoxide dehydrogenase reveals a [Ni-4Fe-5S] cluster. Science **293:**1281-5.

55. **Drake, H. L., and S. L. Daniel.** 2004. Physiology of the thermophilic acetogen *Moorella thermoacetica*. Research in Microbiology **155:**422-436.

56. **Du Preez, L. A., and J. P. Maree.** 1994. Pilot-scale biological sulfate and nitrate removal utilizing producer gas as energy-source. Water Science and Technology **30:**275-285.

57. **Du Preez, L. A., J. P. Odendaal, J. P. Maree, and M. Ponsonby.** 1992. Biological removal of sulfate from industrial effluents using producer gas as energy-source. Environmental Technology **13:**875-882.

58. **Ebrahimi, S., C. Picioreanu, J. B. Xavier, R. Kleerebezem, M. Kreutzer, F. Kapteijn, J. A. Moulijn, and M. C. M. v. Loosdrecht.** 2005. Biofilm growth pattern in honeycomb monolith packings: Effect of shear rate and substrate transport limitations. Catalysis Today **105:**448-454.

59. **Ensign, S. A., and P. W. Ludden.** 1991. Characterization of the carbon monoxide oxidation-hydrogen evolution system of *Rhodospirillum rubrum*: Role of a 22-kDa iron-sulfur protein in mediating electron transfer between carbon monoxide dehydrogenase and hydrogenase. Journal of Biological Chemistry **266:**18395-18403.

60. **Ergas, S. J.** 2001. Membrane bioreactors, p. 163-177. *In* C. Kennes and M. C. Veiga (ed.), Bioreactors for waste gas treatment. Kluwer Academic Publishers, Dordrecht.

61. **Faaij, A., J. van Doorn, T. Curvers, L. Waldheim, E. Olsson, A. van Wijk, and C. Daey Ouwens.** 1997. Characteristics and availability of biomass waste and residues in the Netherlands for gasification. Biomass and Bioenergy **12:**225-240.

62. **Faaij, A., R. van Ree, L. Waldheim, E. Olsson, A. Oudhuis, A. van Wijk, C. Daey Ouwens, and W. Turkenburg.** 1997. Gasification of biomass wastes and residues for electricity production. Biomass and Bioenergy **12:**387-407.

63. **Fain, D. E.** 2000. Mixed gas separation technology using inorganic membranes. Membrane Technology**:**9-13.

64. **Fardeau, M. L., M. B. Salinas, S. L'Haridon, C. Jeanthon, F. Verhe, J. L. Cayol, B. K. C. Patel, J. L. Garcia, and B. Ollivier.** 2004. Isolation from oil reservoirs of novel thermophilic anaerobes phylogenetically related to *Thermoanaerobacter subterraneus*: reassignment of *T. subterraneus*, *Thermoanaerobacter yonseiensis*, *Thermoanaerobacter tengcongensis* and *Carboxydibrachium pacificum* to *Caldanaerobacter subterraneus gen. nov., sp. nov., comb. nov.* as four novel subspecies. International Journal of Systematic and Evolutionary Microbiology **54:**467-474.

65. **Faur Ghenciu, A.** 2002. Review of fuel processing catalysts for hydrogen production in PEM fuel cell systems. Current Opinion in Solid State and Materials Science **6:**389-399.

66. **Fauville, A., B. Mayer, R. Frommichen, K. Friese, and J. Veizer.** 2004. Chemical and isotopic evidence for accelerated bacterial sulphate reduction in acid mining lakes after addition of organic carbon: laboratory batch experiments. Chemical Geology **204:**325-344.

67. **Ferguson, J. F., and J. M. H. Pietari.** 2000. Anaerobic transformations and bioremediation of chlorinated solvents. Environmental Pollution **107:**209-215.

68. **Ferry, J. G.** 1995. CO dehydrogenase. Annual Review of Microbiology **49:**305-33.

69. **Fischer, F., W. Zillig, K. O. Stetter, and G. Schreiber.** 1983. Chemolithoautotrophic metabolism of anaerobic extremely thermophilic archaebacteria. Nature **301:**511-513.

70. **Fischer, K.** 2000. Biofilters, p. 321-332. *In* J. Klein and J. Winter (ed.), Biotechnology Vol 11C Environmental processes III; Solid waste and waste gas treatment, preparation of drinking water. Wiley-VCH, Weinheim.

71. **Fox, J. D., Y. He, D. Shelver, G. P. Roberts, and P. W. Ludden.** 1996. Characterization of the region encoding the CO-induced hydrogenase of *Rhodospirillum rubrum*. Journal of Bacteriology **178:**6200-6208.

72. **Fox, J. D., R. L. Kerby, G. P. Roberts, and P. W. Ludden.** 1996. Characterization of the CO-induced, CO-tolerant hydrogenase from *Rhodospirillum rubrum* and the gene encoding the large subunit of the enzyme. Journal of Bacteriology **178:**1515-24.

73. **Frostl, J., C. Seifritz, and H. Drake.** 1996. Effect of nitrate on the autotrophic metabolism of the acetogens *Clostridium thermoautotrophicum* and *Clostridium thermoaceticum*. Journal of Bacteriology **178:**4597-4603.

74. **Fuchs, G.** 1989. Alternative pathways of autotrophic CO_2 fixation, p. 365–382. *In* H. G. Schlegel and B. Bowien (ed.), Autotrophic bacteria. Springer-Verlag, Berlin.

75. **Fuchs, G., U. Schnitke, and R. K. Thauer.** 1974. Carbon monoxide oxidation by growing cultures of *Clostridium pasteurianaum*. European Journal of Biochemistry **49:**111-115.

76. **Gale, J.** 2004. Geological storage of CO_2: What do we know, where are the gaps and what more needs to be done? Energy **29:**1329-1338.

77. **Gerhardt, M., V. Svetlichny, T. G. Sokolova, G. A. Zavarzin, and M. Ringpfeil.** 1991. Bacterial CO utilization with H_2 production by the strictly anaerobic lithoautotrophic thermophilic bacterium *Carboxydothermus hydrogenus* DSM 6008 isolated from a hot swamp. FEMS Microbiology Letters **83:**267-272.

78. **Gibert, O., J. de Pablo, J. L. Cortina, and C. Ayora.** 2004. Chemical characterisation of natural organic substrates for biological mitigation of acid mine drainage. Water Research **38:**4186-4196.

79. **Gonzalez, J. M., and F. T. Robb.** 2000. Genetic analysis of *Carboxydothermus hydrogenoformans* carbon monoxide dehydrogenase genes *cooF* and *cooS*. FEMS Microbiology Letters **191:**243-247.

80. **Goorissen, H. P., A. J. M. Stams, and T. A. Hansen.** 2004. Methanol utilization in defined mixed cultures of thermophilic anaerobes in the presence of sulfate. FEMS Microbiology Ecology **49:**489-494.

81. **Gosselink, J. W.** 2002. Pathways to a more sustainable production of energy: sustainable hydrogen - a research objective for Shell. International Journal of Hydrogen Energy **27:**1125-1129.

82. **Grethlein, A. J., R. M. Worden, M. K. Jain, and R. Datta.** 1991. Evidence for production of n-butanol from carbon-monoxide by Butyribacterium-methylotrophicum. Journal of Fermentation and Bioengineering **72:**58-60.

83. **Haug, A. T., R. E. White, J. W. Weidner, and W. Huang.** 2002. Development of a novel CO tolerant proton exchange membrane fuel cell anode. Journal of the Electrochemical Society **149:**862-867.

84. **Hedderich, R.** 2004. Energy-converting [NiFe] hydrogenases from archaea and extremophiles: Ancestors of complex I. Journal of Bioenergetics and Biomembranes **36:**65-75.

85. **Hedderich, R., L. Forzi, B. Soboh, and A. Stojanowic.** 2004. Energy-converting NiFe hydrogenases from archaea and bacteria: ancestors of complex P. Biochimica et Biophysica Acta **1658:**14-14.

86. **Heilig, H. G. H. J., E. G. Zoetendal, E. E. Vaughan, P. Marteau, A. D. L. Akkermans, and W. M. de Vos.** 2002. Molecular diversity of *Lactobacillus spp*. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. Applied and Environmental Microbiology **68:**114-123.

87. **Henstra, A. M., and A. J. M. Stams.** 2004. Novel physiological features of *Carboxydothermus hydrogenoformans* and *Thermoterrabacterium ferrireducens*. Applied and Environmental Microbiology **70:**7236-7240.

88. **Hickey, R. F., J. Vanderweilen, and M. S. Switzenbaum.** 1987. Production of trace levels of carbon-monoxide during methanogenesis on acetate and methanol. Biotechnology Letters **9:**63-66.

89. **Higgins, I. J., D. J. Best, and R. C. Hammond.** 1980. New findings in methane-utilizing bacteria highlight their importance in the biosphere and their commercial potential. Nature **286:**561-564.

90. **Hochgesand, G.** 1970. Rectisol and purisol. Industrial and Engineering Chemistry **62:**37.

91. **Hoehler, T. M., M. J. Alperin, D. B. Albert, and C. S. Martens.** 2001. Apparent minimum free energy requirements for methanogenic Archaea and sulfate-reducing bacteria in an anoxic marine sediment. FEMS Microbiology Ecology **38:**33-41.

92. **Huang, S., P. A. Lindahl, C. Wang, G. N. Bennett, F. B. Rudolph, and J. B. Hughes.** 2000. 2,4,6-Trinitrotoluene reduction by carbon monoxide dehydrogenase from *Clostridium thermoaceticum*. Applied and Environmental Microbiology **66:**1474-1478.

93. **Hugenholtz, J., and L. G. Ljungdahl.** 1990. Metabolism and energy generation in homoacetogenic *Clostridia*. FEMS Microbiology Letters **87:**383-389.

94. **Imhoff, J. F., H. G. Truper, and N. Pfennig.** 1984. Rearrangement of the species and genera of the phototrophic purple nonsulfur bacteria. International Journal of Systematic Bacteriology **34:**340-343.

95. **Inman, R. E., R. B. Ingersol, and E. A. Levy.** 1971. Soil - natural sink for carbon monoxide. Science **172:**1229.

96. **Jansen, K., R. K. Thauer, F. Widdel, and G. Fuchs.** 1984. Carbon assimilation pathways in sulfate reducing bacteria - formate, carbon-dioxide, carbon-monoxide, and acetate assimilation by *Desulfovibrio baarsii*. Archives of Microbiology **138:**257-262.

97. **Janssen, A. J. H., H. Dijkman, and G. Janssen.** 2000. Novel biological processes for the removal of H_2S and SO_2 from gas streams., p. 265-280. *In* P. N. L. Lens and L. W. Hulshoff Pol (ed.), Environmental technologies to treat sulfur pollutions; principles and engineering. IWA Publishing, London.

98. **Janssen, A. J. H., S. Meijer, J. Bontsema, and G. Lettinga.** 1998. Application of the redox potential for controlling a sulfide oxidizing bioreactor. Biotechnology and Bioengineering **60:**147-155.

99. **Janssen, A. J. H., R. Ruitenberg, and C. J. N. Buisman.** 2001. Industrial applications of new sulphur biotechnology. Water Science and Technology **44:**85-90.

100. **Jensen, A., and K. Finster.** 2005. Isolation and characterization of *Sulfurospirillum carboxydovorans sp. nov.*, a new microaerophilic carbon monoxide oxidizing epsilon Proteobacterium. Antonie van Leeuwenhoek **87:**339-353.

101. **Johnson, B.** 2000. Biological removal of sulfurous compounds from inorganic wastewaters., p. 175-205. *In* P. N. L. Lens and L. W. Hulshoff Pol (ed.), Environmental technologies to treat sulfur pollutions; principles and engineering. IWA Publishing, London.

102. **Johnson, B., and K. B. Hallberg.** 2004. Acid mine drainage remediation options: a review. The Science of the Total Environment **338:**3-14.

103. **Jung, G. Y., H. O. Jung, J. R. Kim, Y. Ahn, and S. Park.** 1999. Isolation and characterization of *Rhodopseudomonas palustris* P4 which utilizes CO with the production of H_2. Biotechnology Letters **21:**525-529.

104. **Jung, G. Y., J. R. Kim, H. O. Jung, J. Y. Park, and S. Park.** 1999. A new chemoheterotrophic bacterium catalyzing water-gas shift reaction. Biotechnology Letters **21:**869-873.

105. **Jung, G. Y., J. R. Kim, J. Y. Park, and S. Park.** 2002. Hydrogen production by a new chemoheterotrophic bacterium *Citrobacter sp*. Y19. International Journal of Hydrogen Energy**:**601-610.

106. **Kerby, R. L., S. S. Hong, S. A. Ensign, L. J. Coppoc, P. W. Ludden, G. P. Roberts, G. O. Tan, S. Ciurli, M. J. Scott, B. Hedman, R. H. Holm, Z. R. Korszun, P. J. Stephens, and K. O. Hodgson.** 1992. Genetic and physiological characterization of the *Rhodospirillum rubrum* carbon monoxide dehydrogenase system: On the structure of the nickel/iron/sulfur center of the carbon monoxide dehydrogenase from *Rhodospirillum rubrum*: an x-ray absorption spectroscopy study. Journal of Bacteriology **174:**5284-5294.

107. **Kerby, R. L., P. W. Ludden, and G. P. Roberts.** 1995. Carbon monoxide-dependent growth of *Rhodospirillum rubrum*. Journal of Bacteriology **177:**2241-2244.

108. **Kerkhoven, R., F. H. J. van Enckevort, J. Boekhorst, D. Molenaar, and R. J. Siezen.** 2004. Visualization for genomics: the Microbial Genome Viewer. Bioinformatics **20:**1812-1814.

109. **Kim, B., R. Grote, D. Lee, G. Antranikian, and Y. Pyun.** 2001. *Thermoanaerobacter yonseiensis* sp. nov., a novel extremely thermophilic, xylose-utilizing bacterium that grows at up to 85 degrees C. International Journal of Systematic and Evolutionary Microbiology **51:**1539-1548.

110. **King, G. M.** 1999. Attributes of atmospheric carbon monoxide oxidation by Maine forest soils. Applied and Environmental Microbiology **65:**5257-5264.

111. **Klemps, R., H. Cypionka, F. Widdel, and N. Pfennig.** 1985. Growth with hydrogen, and further physiological-characteristics of *Desulfotomaculum* species. Archives of Microbiology **143:**203-208.

112. **Klenk, H. P., R. A. Clayton, J. F. Tomb, O. White, K. E. Nelson, K. A. Ketchum, R. J. Dodson, M. Gwinn, E. K. Hickey, J. D. Peterson, D. L. Richardson, A. R. Kerlavage, D. E. Graham, N. C. Kyrpides, R. D. Fleischmann, J. Quackenbush, N. H. Lee, G. G. Sutton, S. Gill, E. F. Kirkness, B. A. Dougherty, K. McKenney, M. D. Adams, B. Loftus, S. Peterson, C. I. Reich, L. K. McNeil, J. H. Badger, A. Glodek, L. X. Zhou, R. Overbeek, J. D. Gocayne, J. F. Weidman, L. McDonald, T. Utterback, M. D. Cotton, T. Spriggs, P. Artiach, B. P. Kaine, S. M. Sykes, P. W. Sadow, K. P. Dandrea, C. Bowman, C. Fujii, S. A. Garland, T. M. Mason, G. J. Olsen, C. M. Fraser, H. O. Smith, C. R. Woese, and J. C. Venter.** 1997. The complete genome sequence of the hyperthermophilic, sulphate-reducing archaeon *Archaeoglobus fulgidus*. Nature **390:**364-370.

113. **Kluyver, A. J., and C. Schnellen.** 1947. On the fermentation of carbon monoxide by pure cultures of methane bacteria. Archives of Biochemistry **14:**57-70.

114. **Koros, W. J., and R. Mahajan.** 2001. Pushing the limits on possibilities for large scale gas separation: which strategies? Journal of Membrane Science **181:**141-141.

115. **Kozianowski, G., F. Canganella, F. A. Rainey, H. Hippe, and G. Antranikian.** 1997. Purification and characterization of thermostable pectate-lyases from a newly isolated thermophilic bacterium, *Thermoanaerobacter italicus* sp. nov. Extremophiles **1:**171-182.

116. **Krumholz, L. R., and M. P. Bryant.** 1985. *Clostridium pfennigii sp. nov.* uses methoxyl groups of monobenzenoids and produces butyrate. International Journal of Systematic Bacteriology **35:**454-456.

117. **Kuenen, J. G.** 1975. Colorless sulfur bacteria and their role in sulfur cycle. Plant and Soil **43:**49-76.

118. **Kurt, M., I. J. Dunn, and J. R. Bourne.** 1987. Biological denitrification of drinking-water using autotrophic organisms with H_2 in a fluidized-bed biofilm reactor. Biotechnology and Bioengineering **29:**493-501.

119. **Lapidus, A. L., S. Y. Grobovenko, F. K. Mukhitova, and S. V. Kiyashko.** 1989. Enzyme-catalyzed synthesis of hydrocarbons and oxygen-containing-compounds from CO and H_2. Journal of Molecular Catalysis **56:**260-265.

120. **Ledjeff-Hey, K., J. Roes, and R. Wolters.** 2000. CO_2-scrubbing and methanation as purification system for PEFC. Journal of Power Sources **86:**556-561.

121. **Levy, P. F., G. W. Barnard, D. V. Garciamartinez, J. E. Sanderson, and D. L. Wise.** 1981. Organic-acid production from CO_2-H_2 and CO-H_2 by mixed-culture anaerobes. Biotechnology and Bioengineering **23:**2293-2306.

122. **Lide, D. R.** 2001. CRC handbook of chemistry and physics, 82 ed. CRC Press, Boca Raton.

123. **Lindahl, P. A.** 2002. The Ni-containing carbon monoxide dehydrogenase family: Light at the end of the tunnel? Biochemistry **41:**2097-2105.

124. **Lindahl, P. A., and B. Chang.** 2001. The evolution of acetyl-CoA synthase. Origins of Life and Evolution of the Biosphere **31:**403-434.

125. **Lorowitz, W. H., and M. P. Bryant.** 1984. *Peptostreptococcus productus* strain that grows rapidly with CO as the energy source. Applied and Environmental Microbiology **47:**961-964.

126. **Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, A. Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jacob, W. Förster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. König, T. Liss, R. Lüßbmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K.-H. Schleifer.** 2004. ARB: A software environment for sequence data. Nucleic Acids Research **32:**1363-1371.

127. **Lupton, F. S., R. Conrad, and J. G. Zeikus.** 1984. CO metabolism of *Desulfovibrio vulgaris* strain Madison - physiological-function in the absence or presence of exogeneous substrates. FEMS Microbiology Letters **23:**263-268.

128. **Lynd, L., R. Kerby, and J. G. Zeikus.** 1982. Carbon monoxide metabolism of the methylotrophic acidogen *Butyribacterium methylotrophicum*. Journal of Bacteriology **149:**255-63.

129. **Lyon, E. J., S. Shima, G. Buurman, S. Chowdhuri, A. Batschauer, K. Steinbach, and R. K. Thauer.** 2004. UV-A/blue-light inactivation of the 'metal-free' hydrogenase (Hmd) from methanogenic archaea - The enzyme contains functional iron after all. European Journal of Biochemistry **271:**195-204.

130. **Maden, B. E.** 2000. Tetrahydrofolate and tetrahydromethanopterin compared: functionally distinct in C_1 metabolism. Biochemical Journal **350:**609-629.

131. **Maness, P. C., and P. F. Weaver.** 2002. Hydrogen production from a carbon-monoxide oxidation pathway in *Rubrivivax gelatinosus*. International Journal of Hydrogen Energy **27:**1407-1411.

132. **Markowitz, V. M., F. Korzeniewski, K. Palaniappan, E. Szeto, G. Werner, A. Padki, X. Zhao, I. Dubchak, P. Hugenholtz, I. Anderson, A. Lykidis, K. Mavromatis, N. Ivanova, and N. C. Kyrpides.** 2006. The integrated microbial genomes (IMG) system. Nucleic Acids Research **34:**D344-348.

133. **Maschio, G., A. Lucchesi, and G. Stoppato.** 1994. Production of syngas from biomass. Bioresource Technology **48:**119-126.

134. **Matsumura, Y.** 2002. Evaluation of supercritical water gasification and biomethanation for wet biomass utilization in Japan. Energy Conversion and Management **43:**1301-1310.

135. **Mazumder, T. K., N. Nishio, and S. Nagai.** 1985. Carbon monoxide conversion to formate by *Methanosarcina barkeri*. Biotechnology Letters **7:**377-382.

136. **Merida, W., P. C. Maness, R. C. Brown, and D. B. Levin.** 2004. Enhanced hydrogen production from indirectly heated, gasified biomass, and removal of carbon gas emissions using a novel biological gas reformer. International Journal of Hydrogen Energy **29:**283-290.

137. **Meuer, J., S. Bartoschek, J. Koch, A. Kunkel, and R. Hedderich.** 1999. Purification and catalytic properties of ECH hydrogenase from *Methanosarcina barkeri*. European Journal of Biochemistry **265:**325-335.

138. **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. Proceedings of the National Academy of Sciences of the United States of America **99:**5632-5637.

139. **Meyer, O., K. Frunzke, D. Gadkari, S. Jacobitz, I. Hugendieck, and M. Kraut.** 1990. Utilization of carbon monoxide by aerobes: recent advances. FEMS Microbiology Letters **87:**253-260.

140. **Meyer, O., and H. G. Schlegel.** 1983. Biology of aerobic carbon-monoxide oxidizing bacteria. Annual Review of Microbiology **37:**277-310.

141. **Midilli, A., M. Dogru, G. Akay, and C. R. Howarth.** 2002. Hydrogen production from sewage sludge via a fixed bed gasifier product gas. International Journal of Hydrogen Energy **27:**1035-1041.

142. **Min, H., and S. H. Zinder.** 1990. Isolation and characterization of a thermophilic sulfate-reducing bacterium *Desulfotomaculum thermoacetoxidans sp. nov.* Archives of Microbiology **153:**399-404.

143. **Möller-Zinkhan, D.** 1990. Anaerobic lactate oxidation to 3 CO2 by *Archaeoglobus fulgidus* via the carbon monoxide dehydrogenase pathway: demonstration of the acetyl-CoA carbon-carbon cleavage reaction in cell extracts. Archives of Microbiology **153:**215-218.

144. **Möller-Zinkhan, D., G. Borner, and R. K. Thauer.** 1989. Function of methanofuran, tetrahydromethanopterin, and coenzyme-F420 in *Archaeoglobus fulgidus*. Archives of Microbiology **152:**362-368.

145. **Mörsdorf, G., K. Frunzke, D. Gadkari, and O. Meyer.** 1992. Microbial growth on carbon monoxide. Biodegradation **3:**61-82.

146. **Nagpal, S., S. Chuichulcherm, L. Peeva, and A. Livingston.** 2000. Microbial sulfate reduction in a liquid-solid fluidized bed reactor. Biotechnology and Bioengineering **70:**370-380.

147. **Nazina, T. N., A. E. Ivanova, L. P. Kanchaveli, and E. P. Rozanova.** 1988. A new spore-forming thermophilic methylotrophic sulfate-reducing bacterium, *Desulfotomaculum kuznetsovii sp. nov.* Microbiology **57:**659-663.

148. **Newsome, D. S.** 1980. Water-Gas Shift Reaction. Catalysis reviews: science and engineering **21:**275-281.

149. **Nübel, U., B. Engelen, A. Felske, J. Snaidr, A. Wieshuber, R. Amann, W. Ludwig, and H. Backhaus.** 1996. Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. Journal of Bacteriology **178:**5636-5643.

150. **O'Brien, J. M., R. H. Wolkin, T. T. Moench, J. B. Morgan, J. G. Zeikus, F. S. Lupton, and R. Conrad.** 1984. Association of hydrogen metabolism with unitrophic or mixotrophic growth of *Methanosarcina barkeri* on carbon monoxide. Journal of Bacteriology **158:**373-5.

151. **Oh, S. E., S. van Ginkel, and B. E. Logan.** 2003. The relative effectiveness of pH control and heat treatment for enhancing biohydrogen gas production. Environmental Science and Technology **37:**5186-5190.

152. **Oremland, R. S., and D. G. Capone.** 1988. Use of specific inhibitors in biogeochemistry and microbial ecology. Advances in Microbial Ecology **10:**285-383.

153. **Otsuka, K., Y. Shigeta, and S. Takenaka.** 2002. Production of hydrogen from gasoline range alkanes with reduced CO_2 emission. International Journal of Hydrogen Energy **27:**11-18.

154. **Oude Elferink, S. J. W. H., A. Visser, L. W. Hulshoff Pol, and A. J. M. Stams.** 1994. Sulfate reduction in methanogenic bioreactors. FEMS Microbiology Reviews **15:**119-136.

155. **Papageorgopoulos, D. C., M. Keijzer, and F. A. de Bruijn.** 2002. The inclusion of Mo, Nb and Ta in Pt and PtRu carbon supported electrocatalysts in the quest for improved CO tolerant PEMFC anodes. Electrochimica Acta **48:**197-204.

156. **Parshina, S. N., S. Kijlstra, A. M. Henstra, J. Sipma, C. M. Plugge, and A. J. M. Stams.** 2005. Carbon monoxide conversion by thermophilic sulfate-reducing bacteria in pure culture and in co-culture with *Carboxydothermus hydrogenoformans*. Applied Microbiology and Biotechnology **68:**390-396.

157. **Parshina, S. N., J. Sipma, Y. Nakashimada, A. M. Henstra, H. Smidt, A. M. Lysenko, P. N. L. Lens, G. Lettinga, and A. J. M. Stams.** 2005. *Desulfotomaculum carboxydivorans* sp. nov., a novel sulfate-reducing bacterium capable of growth at 100 % CO. International Journal of Systematic and Evolutionary Microbiology **55:**2159-2165.

158. **Perry, R. H., D. W. Green, and J. O. Maloney.** 1997. Perry's chemical engineers' handbook, 7 ed. McGraw-Hill, New York.

159. **Philip, L., and M. A. Deshusses.** 2003. Sulfur dioxide treatment from flue gases using a biotrickling filter - Bioreactor system. Environmental Science and Technology **37:**1978-1982.

160. **Plaggemeier, T., and O. Lämmerzahl.** 2000. Treatment of waste gas pollutants in trickling filters., p. 333-344. *In* J. Klein and J. Winter (ed.), Biotechnology Vol 11C Environmental processes III; Solid waste and waste gas treatment, preparation of drinking water. Wiley-VCH, Weinheim.

161. **Plugge, C. M., M. Balk, and A. J. M. Stams.** 2002. *Desulfotomaculum thermobenzoicum subsp. thermosyntrophicum subsp. nov.*, a thermophilic, syntrophic, propionate-oxidizing, spore-forming bacterium. International Journal of Systematic and Evolutionary Microbiology **52:**391-399.

162. **Pomper, B. K., O. Saurel, A. Milon, and J. A. Vorholt.** 2002. Generation of formate by the formyltransferase/hydrolase complex (Fhc) from *Methylobacterium extorquens* AM1. FEBS Letters **523:**133-137.

163. **Pruschek, R., G. Oeljeklaus, G. Haupt, G. Zimmerman, D. Jansen, and J. S. Ribberink.** 1997. Energy Convers. Mgmt. **38:**s153-s158.

164. **Ragsdale, S. W.** 2004. Life with carbon monoxide. Critical Reviews in Biochemistry and Molecular Biology **39:**165-195.

165. **Rajagopalan, S., R. P. Datar, and R. S. Lewis.** 2002. Formation of ethanol from carbon monoxide via a new microbial catalyst. Biomass and Bioenergy **23:**487-493.

166. **Reiser, M.** 2000. Membrane processes and alternative techniques, p. 345-353. *In* J. Klein and J. Winter (ed.), Biotechnology Vol 11C Environmental processes III; Solid waste and waste gas treatment, preparation of drinking water. Wiley-VCH, Weinheim.

167. **Richterich, R.** 1965. Klinische chemie: Theorie und praxis, 1 ed. Karger, Basel.

168. **Riveros, H. G., J. L. Arriaga, J. Tejeda, A. Julian-Sanchez, and H. Riveros-Rosas.** 1998. Ozone and its precursors in the atmosphere of Mexico City. Journal of the Air & Waste Management Association **48:**866-871.

169. **Roberts, G. P., R. L. Kerby, H. Youn, and M. Conrad.** 2005. CooA, a paradigm for gas sensing regulatory proteins. Journal of Inorganic Biochemistry **99:**280-292.

170. **Roberts, G. P., H. Youn, and R. L. Kerby.** 2004. CO-sensing mechanisms. Microbiology and Molecular Biology Reviews **68:**453-473.

171. **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. Proceedings of the National Academy of Sciences of the United States of America **101:**16929-16934.

172. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution **4:**406-425.

173. **Sandrock, G.** 1999. A panoramic overview of hydrogen storage alloys from a gas reaction point of view. Journal of Alloys and Compounds **295:**877-888.

174. **Sapra, R., K. Bagramyan, and M. W. W. Adams.** 2003. A simple energy-conserving system: Proton reduction coupled to proton translocation. Proceedings of the National Academy of Sciences of the United States of America **100:**7545-7550.

175. **Savage, M. D., Z. G. Wu, S. L. Daniel, L. L. Lundie, and H. L. Drake.** 1987. Carbon monoxide-dependent chemolithotrophic growth of *Clostridium thermoautotrophicum*. Applied and Environmental Microbiology **53:**1902-6.

176. **Schauder, R., A. Preuss, M. Jetten, and G. Fuchs.** 1989. Oxidation and reductive acetyl coenzyme A/carbon monoxide dehydrogenase pathway in *Desulfobacterium autotrophicum*: 2. Demonstration of the enzymes of the pathway and comparison of carbon monoxide dehydrogenase. Archives of Microbiology **151:**84-89.

177. **Schink, B.** 1997. Energetics of syntrophic cooperation in methanogenic degradation. Microbiology and Molecular Biology Reviews **61:**262-280.

178. **Schippert, E., and H. Chmiel.** 2000. Bioscrubbers, p. 305-320. *In* J. Klein and J. Winter (ed.), Biotechnology Vol 11C Environmental processes III; Solid waste and waste gas treatment, preparation of drinking water. Wiley-VCH, Weinheim, Germany.

179. **Schobert, H. H., and C. Song.** 2002. Chemicals and materials from coal in the 21st century. Fuel **81:**15-32.

180. **Scholten, J. C. M., R. Conrad, and A. J. M. Stams.** 2000. Effect of 2-bromo-ethane sulfonate, molybdate and chloroform on acetate consumption by methanogenic and sulfate-reducing populations in freshwater sediment. FEMS Microbiology Ecology **32:**35-42.

181. **Scholten, J. C. M., 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.

182. **Sharak Genthner, B. R., and M. P. Bryant.** 1982. Growth of *Eubacterium limosum* with carbon monoxide as the energy source. Applied and Environmental Microbiology **43:**70-74.

183. **Sharak Genthner, B. R., and M. P. Bryant.** 1987. Additional characteristics of one-carbon-compound utilization by *Eubacterium limosum* and *Acetobacterium woodii*. Applied and Environmental Microbiology **53:**471-476.

184. **Shen, G. J., J. S. Shieh, A. J. Grethlein, M. K. Jain, and J. G. Zeikus.** 1999. Biochemical basis for carbon monoxide tolerance and butanol production by *Butyribacterium methylotrophicum*. Applied Microbiology and Biotechnology **51:**827-832.

185. **Shima, S., E. J. Lyon, M. S. Sordel-Klippert, M. Kauss, J. Kahnt, R. K. Thauer, K. Steinbach, X. L. Xie, L. Verdier, and C. Griesinger.** 2004. The cofactor of the iron-sulfur cluster free hydrogenase Hmd: Structure of the light-inactivation product. Angewandte Chemie **43:**2547-2551.

186. **Sipma, J., P. N. L. Lens, A. J. M. Stams, and G. Lettinga.** 2003. Carbon monoxide conversion by anaerobic bioreactor sludges. FEMS Microbiology Ecology **44:**271-277.

187. **Sipma, S., A. M. Henstra, S. N. Parshina, P. N. L. Lens, G. Lettinga, and A.J.M. Stams.** 2006. Microbial CO conversions with applications in synthesis gas purification and biodesulfurization. Critical Reviews in Biotechnology **26:** in press.

188. **Slobodkin, A., A. L. Reysenbach, N. Strutz, M. Dreier, and J. Wiegel.** 1997. *Thermoterrabacterium ferrireducens* gen. nov., sp. nov., a thermophilic anaerobic dissimilatory Fe(III)-reducing bacterium from a continental hot spring. International Journal of Systematic Bacteriology **47:**541-547.

189. **Soboh, B., D. Linder, and R. Hedderich.** 2002. Purification and catalytic properties of a CO-oxidizing: H_2-evolving enzyme complex from *Carboxydothermus hydrogenoformans*. European Journal of Biochemistry **269:**5712-5721.

190. **Soboh, B., D. Linder, and R. Hedderich.** 2004. A multisubunit membrane-bound [NiFe] hydrogenase and an NADH-dependent Fe-only hydrogenase in the fermenting bacterium *Thermoanaerobacter tengcongensis*. Microbiology **150:**2451-2463.

191. **Sokolova, T. G., J. M. Gonzalez, N. A. Kostrikina, N. A. Chernyh, T. V. Slepova, E. A. Bonch-Osmolovskaya, 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. International Journal of Systematic and Evolutionary Microbiology **54:**2353-2359.

192. **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. International Journal of Systematic and Evolutionary Microbiology **51:**141-149.

193. **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.

194. **Sokolova, T. G., N. A. Kostrikina, N. A. Chernyh, T. V. Kolganova, T. P. Tourova, and E. A. Bonch-Osmolovskaya.** 2005. *Thermincola carboxydiphila* gen. nov., sp. nov., a novel anaerobic, carboxydotrophic, hydrogenogenic bacterium from a hot spring of the Kae Baikal area. International Journal of Systematic and Evolutionary Microbiology **55:**2069-2073.

195. **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. International Journal of Systematic and Evolutionary Microbiology **52:**1961-1967.

196. **Song, C.** 2002. Fuel processing for low-temperature and high-temperature fuel cells: Challenges, and opportunities for sustainable development in the 21st century. Catalysis Today **77:**17-49.

197. **Stackebrandt, E., C. Sproer, F. A. Rainey, J. Burghardt, O. Pauker, and H. Hippe.** 1997. Phylogenetic analysis of the genus *Desulfotomaculum*: Evidence for the misclassification of *Desulfotomaculum guttoideum* and description of *Desulfotomaculum orientis* as *Desulfosporosinus orientis gen. nov., comb. nov*. International Journal of Systematic Bacteriology **47:**1134-1139.

198. **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. Applied and Environmental Microbiology **59:**1114-1119.

199. **Steele, B. C. H., and A. Heinzel.** 2001. Materials for fuel-cell technologies. Nature **414:**345-352.

200. **Stetter, K. O.** 1988. *Archaeoglobus fulgidus gen. nov., sp. nov.* a new taxon of extremely thermophilic archaebacteria. Systematic and Applied Microbiology **10:**172-173.

201. **Stetter, K. O., G. Lauerer, M. Thomm, and A. Neuner.** 1987. Isolation of Extremely Thermophilic Sulfate Reducers - Evidence for a Novel Branch of Archaebacteria. Science **236:**822-824.

202. **Stiegel, G. J., and R. C. Maxwell.** 2001. Gasification technologies: the path to clean, affordable energy in the 21st century. Fuel Processing Technology **71:**79-97.

203. **Stojanowic, A., and R. Hedderich.** 2004. CO_2 reduction to the level of formylmethanofuran in *Methanosarcina barkeri* is non-energy driven when CO is the electron donor. FEMS Microbiology Letters **235:**163-167.

204. **Sun, J., J. Desjardins, J. Buglass, and K. Liu.** 2005. Noble metal water gas shift catalysis: Kinetics study and reactor design. International Journal of Hydrogen Energy **30:**1259-1264.

205. **Sutton, D., B. Kelleher, and J. R. H. Ross.** 2001. Review of literature on catalysts for biomass gasification. Fuel Processing Technology **73:**155-173.

206. **Svetlichny, V. A., T. G. Sokolova, M. Gerhardt, N. A. Kostrikina, and G. A. Zavarzin.** 1991. Anaerobic extremely thermophilic carboxydotrophic bacteria in hydrotherms of Kuril Islands. Microbial Ecology **21:**1-10.

207. **Svetlichny, V. A., T. G. Sokolova, M. Gerhardt, M. Ringpfeil, N. A. Kostrikina, and G. A. Zavarzin.** 1991. *Carboxydothermus hydrogenoformans*, new genus new species, a carbon monoxide utilizing thermophilic anaerobic bacterium from hydrothermal environments of Kunashir Island. Systematic and Applied Microbiology **14:**254-260.

208. **Svetlichnyi, 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.

209. **Svetlitchnyi, V., H. Dobbek, W. Meyer-Klaucke, T. Meins, B. Thiele, P. Romer, R. Huber, and O. Meyer.** 2004. A functional Ni-Ni-[4Fe-4S] cluster in the monomeric acetyl-CoA synthase from *Carboxydothermus hydrogenoformans*. Proceedings of the National Academy of Sciences of the United States of America **101:**446-451.

210. **Svetlitchnyi, V., C. Peschel, G. Acker, and O. Meyer.** 2001. Two membrane-associated NiFeS-carbon monoxide dehydrogenases from the anaerobic carbon-monoxide-utilizing eubacterium *Carboxydothermus hydrogenoformans*. Journal of Bacteriology **183:**5134-5144.

211. **Symonds, R. B., W. I. Rose, G. J. S. Bluth, and T. M. Gerlach.** 1994. Volcanic gas studies - methods, results, and applications, p. 1-66. *In* M. R. Carroll and J. R. Holloway (ed.), Volatiles in Magmas, vol. 30.

212. **Szekeres, S., I. Kiss, T. T. Bejerano, and M. I. M. Soares.** 2001. Hydrogen-dependent denitrification in a two-reactor bio-electrochemical system. Water Research **35:**715-719.

213. **Szewzyk, R., and N. Pfennig.** 1990. Competition for ethanol between sulfate-reducing and fermenting bacteria. Archives of Microbiology **153:**470-477.

214. **Tanner, R. S., L. M. Miller, and D. Yang.** 1993. *Clostridium ljungdahlii sp. nov.*, an acetogenic species in clostridial ribosomal-RNA homology group-I. International Journal of Systematic Bacteriology **43:**232-236.

215. **Thauer, R. K., K. Jungermann, and K. Decker.** 1977. Energy-conservation in chemotropic anaerobic bacteria. Bacteriological Reviews **41:**100-180.

216. **Tiedje, J. M.** 1988. Ecology of denitrification and dissimilatory nitrate reduction to ammonium, p. 179-244. *In* A. J. B. Zehnder (ed.), Biology of anaerobic microorganisms. John Wiley & Sons, New York.

217. **Trüper, H. G., and H. G. Schlegel.** 1964. Sulfur metabolism in *Thiorhodaceae*. I. Quantitative measurements on growing cells of *Chromatium okenii*. Antonie van Leeuwenhoek **30:**225-238.

218. **Tsukamoto, T. K., H. A. Killion, and G. C. Miller.** 2004. Column experiments for microbiological treatment of acid mine drainage: low-temperature, low-pH and matrix investigations. Water Research **38:**1405-1418.

219. **Uffen, R. L.** 1976. Anaerobic growth of a *Rhodopseudomonas* species in the dark with carbon monoxide as sole carbon and energy substrate. Proceedings of the National Academy of Sciences of the United States of America **73:**3298-302.

220. **Uffen, R. L.** 1981. Metabolism of carbon monoxide. Enzyme and Microbial Technology **3:**197-206.

221. **Vallero, M. V. G., J. Sipma, A. Annachhatre, P. N. L. Lens, and L. W. Hulshoff Pol.** 2002. Biotechnological treatment of sulfur-containing wastewaters, p. 233-268. *In* M. Fingerman and R. Nagabhushanam (ed.), Recent advances in marine biotechnology, vol. 8. Science Publishers Inc., Enfield, USA.

222. **van der Drift, A., J. van Doorn, and J. W. Vermeulen.** 2001. Ten residual biomass fuels for circulating fluidized-bed gasification. Biomass and Bioenergy **20:**45-56.

223. **Van Groenestijn, J. W.** 2001. Bioscrubbers, p. 133-162. *In* C. Kennes and M. C. Veiga (ed.), Bioreactors for waste gas treatment. Kluwer Academic Publishers, Dordrecht, The Netherlands.

224. **van Houten, R. T., L. W. Hulshoff Pol, and G. Lettinga.** 1994. Biological sulfate reduction using gas-lift reactors fed with hydrogen and carbon dioxide as energy and carbon source. Biotechnology and Bioengineering **44:**586-594.

225. **van Houten, R. T., and G. Lettinga.** 1996. Biological sulphate reduction with synthesis gas: microbiology and technology, p. 793-799. *In* R. H. Wijffels, R. M. Buitelaar, C. Bucke, and J. Tramper (ed.), Progress in biotechnology, vol. 11. Elsevier, Amsterdam.

226. **van Houten, R. T., H. van der Spoel, A. C. van Aelst, L. W. Hulshoff Pol, and G. Lettinga.** 1996. Biological sulfate reduction using synthesis gas as energy and carbon source. Biotechnology and Bioengineering **50:**136-144.

227. **van Houten, R. T., S. Y. Yun, and G. Lettinga.** 1997. Thermophilic sulphate and sulphite reduction in lab-scale gas-lift reactors using H_2 and CO_2 as energy and carbon source. Biotechnology and Bioengineering **55:**807-814.

228. **Vega, J. L., E. C. Clausen, and J. L. Gaddy.** 1990. Design of bioreactors for coal synthesis gas fermentations. Resources Conservation and Recycling **3:**149-160.

229. **Vignais, P. M., B. Billoud, and J. Meyer.** 2001. Classification and phylogeny of hydrogenases. FEMS Microbiology Reviews **25:**455-501.

230. **Visser, A., Y. Gao, and G. Lettinga.** 1993. Effects of short-term temperature increases on the mesophilic anaerobic breakdown of sulfate containing synthetic waste-water. Water Research **27:**541-550.

231. **Vorholt, J., J. Kunow, K. O. Stetter, and R. K. Thauer.** 1995. Enzymes and coenzymes of the Carbon-monoxide dehydrogenase pathway for autotrophic CO_2 fixation in *Archaeoglobus lithotrophicus* and the lack of Carbon-monoxide dehydrogenase in the heterotrophic *A. profundus*. Archives of Microbiology **163:**112-118.

232. **Wasserfallen, A., J. Nolling, P. Pfister, J. Reeve, and E. C. 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.* International Journal of Systematic and Evolutionary Microbiology **50:**43-53.

233. **Weijma, J., E. A. A. Bots, G. Tandlinger, A. J. M. Stams, L. W. Hulshoff Pol, and G. Lettinga.** 2002. Optimisation of sulphate reduction in a methanol-fed thermophilic bioreactor. Water Research **36:**1825-1833.

234. **Weijma, J., T. M. Chi, L. W. H. Pol, A. J. M. Stams, and G. Lettinga.** 2003. The effect of sulphate on methanol conversion in mesophilic upflow anaerobic sludge bed reactors. Process Biochemistry **38:**1259-1266.

235. **Weijma, J., A. J. M. Stams, L. W. H. Pol, and G. Lettinga.** 2000. Thermophilic sulfate reduction and methanogenesis with methanol in a high rate anaerobic reactor. Biotechnology and Bioengineering **67:**354-363.

236. **Weinberg, M. V., F. E. Jenney Jr, X. Cui, and M. W. W. Adams.** 2004. Rubrerythrin from the hyperthermophilic archaeon *Pyrococcus furiosus* is a rubredoxin-dependent, iron-containing peroxidase. Journal of Bacteriology **186:**7888.

237. **Welander, P. V., and W. W. Metcalf.** 2005. Loss of the mtr operon in *Methanosarcina* blocks growth on methanol, but not methanogenesis, and reveals an unknown methanogenic pathway. Proceedings of the National Academy of Sciences of the United States of America **102:**10664-10669.

238. **Werther, J., and T. Ogada.** 1999. Sewage sludge combustion. Progress in Energy and Combustion Science **25:**55-116.

239. **White, H., H. Lebertz, I. Thanos, and H. Simon.** 1987. *Clostridium thermoaceticum* forms methanol from carbon-monoxide in the presence of viologen dyes. FEMS Microbiology Letters **43:**173-176.

240. **Widdel, F., and T. A. Hansen.** 1991. The dissimilatory sulfate- and sulfur-reducing bacteria, p. 583-624. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The prokaryotes, 2 ed. Springer Verlag, New York.

241. **Willems, A., M. Gillis, and J. De Ley.** 1991. Transfer of *Rhodocyclus gelatinosus* to *Rubrivivax gelatinosus* new genus, new combination and phylogenetic relationships with *Leptothrix sphaerotilus-natans, Pseudomonas saccharophila* and *Alcaligenes latus*. International Journal of Systematic Bacteriology **41:**65-73.

242. **Wolfe, M. T., J. Heo, J. S. Garavelli, and P. W. Ludden.** 2002. Hydroxylamine Reductase Activity of the Hybrid Cluster Protein from Escherichia coli. Journal of Bacteriology **184:**5898-5902.

243. **Wolfrum, E. J., and A. S. Watt.** 2002. Bioreactor design studies for a hydrogen-producing bacterium. Applied Biochemistry and Biotechnology **98:**611-625.

244. **Wood, H. G., and L. G. Ljungdahl.** 1991. Autotrophic character of the acetogenic bacteria, p. 201-250. *In* J. M. Shively and L. L. Barton (ed.), Variations in autotrophic life. Academic Press, London.

245. **Wu, M., Q. Ren, A. S. Durkin, S. C. Daugherty, L. M. Brinkac, R. J. Dodson, R. Madupu, S. A. Sullivan, J. F. Kolonay, W. C. Nelson, L. J. Tallon, K. M. Jones, L. E. Ulrich, J. M. Gonzalez, I. B. Zhulin, F. T. Robb, and J. A. Eisen.** 2005. Life in hot carbon monoxide: The complete genome sequence of *Carboxydothermus hydrogenoformans* Z-2901. PLoS Genetics **1:**e65.

246. **Yang, H. C., and H. L. Drake.** 1990. Differential-effects of sodium on hydrogen-dependent and glucose-dependent growth of the acetogenic bacterium *Acetogenium kivui*. Applied and Environmental Microbiology **56:**81-86.

247. **Young, L., and C. C. P. Pian.** 2003. High-temperature, air-blown gasification of dairy-farm wastes for energy production. Energy **28:**655-672.

248. **Zeikus, J. G.** 1983. Metabolism of one-carbon compounds by chemotropic anaerobes. Advances in Microbial Physiology **24:**215-299.

249. **Zellner, G., E. Stackebrandt, H. Kneifel, P. Messner, U. B. Sleytr, E. C. Demacario, H. P. Zabel, K. O. Stetter, and J. Winter.** 1989. Isolation and characterization of a thermophilic, sulfate reducing archaebacterium, *Archaeoglobus fulgidus* strain-Z. Systematic and Applied Microbiology **11:**151-160.

250. **Zirngibl, C., W. van Dongen, B. Schworer, R. von Bunau, M. Richter, A. Klein, and R. K. Thauer.** 1992. H_2-forming methylenetetrahydromethanopterin dehydrogenase, a novel type of hydrogenase without iron-sulfur clusters in methanogenic archaea. European Journal of Biochemistry **208:**511-520.

251. **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. Applied and Environmental Microbiology **64:**3854-3859.

252. **Zuo, Y., and R. D. Jones.** 1995. Formation of carbon monoxide by photolysis of dissolved marine organic material and its significance in the carbon cycling of the oceans. Naturwissenschaften **82:**472-474.

Samenvatting

Er wordt veel verwacht van waterstof, de brandstof van de toekomst volgens velen. Voordat het zover is zullen echter een flinke technologische sprong moeten worden gemaakt. Om van alle voordelen die waterstof als brandstof biedt gebruik moet op het gebied van productie, opslag en gebruik in brandstofcellen nieuwe technologie moeten worden ontwikkeld. Een belangrijke duwende kracht achter deze ontwikkelingen zal van economische aard zijn. Waterstof technologie kan alleen het gebruik van de huidige fossiele brandstoffen en verbrandingsmotoren vervangen als het economische voordeel groot genoeg is. Een klein aspect, een mogelijk voordelige biologische stap in de chemisch technologische productie van waterstof uit fossiele brandstoffen, is onderwerp van dit proefschrift.

Het grootste deel van de wereldwijd gevormde waterstof wordt geproduceerd uit methaan (aardgas). Door aanwezigheid van CO is dit waterstofgas echter niet geschikt voor gebruik brandstofcellen van het PEM type (polymere electrolyt membraan). Een type dat vooral zal worden gebruikt in kleine apparaten en voertuigen. Waterstof dat wordt geproduceerd door electrolyse van water is wel geschikt voor PEM brandstofcellen, maar ook vele malen duurder. Door CO te verwijderen uit het waterstofgas dat uit aardgas wordt geproduceerd, kan dit geschikt worden gemaakt voor gebruik in PEM brandstofcellen. Daarvoor moet minder dan 10 ppm (0,001 %) CO overblijven om economisch rendabel te zijn volgens brandstofcellen bouwers. De chemische technologie is nog op zoek naar katalysatoren die dat kunnen.

Waterstofgas wordt geproduceerd uit methaan bij hoge temperatuur (ca. 800 ^{o}C) in aanwezigheid van stoom en een katalysator (steam reforming) volgens reactie 1. Dit geeft een gasmengsel, genaamd synthese gas, van voornamelijk koolstofmonoxide en waterstof. De CO kan met stoom verder worden omgezet in koolstof dioxide en meer waterstof met een andere katalysator volgens de ‘water-gas shift reaction’, reactie 2. In een industrieel proces wordt de reactie bij een hoge temperatuur (ca. 400 ^{o}C) uitgevoerd om het grootste deel van de CO om te zetten, gevolgd door een lage temperatuur stap (ca. 200 ^{o}C) om een zo laag mogelijke CO concentratie te krijgen. Deze CO concentratie ligt rond de 1000 ppm, 100x hoger dan gewenst. Een lagere CO spanning kan met reactie 2 alleen worden gehaald bij een lagere temperatuur, vanwege thermodynamische limieten. De chemische technologie richt zich op de zoektocht naar catalysatoren die voldoende actief zijn bij lagere temperaturen en richt zich daarbij op duurdere metalen. Een andere oplossing word gezocht in de verwijdering van CO uit het waterstofgas mengsel door selectieve oxydatie met zuurstof (reactie 3), waarbij de thermodynamische limieten niet een beperkende rol spelen. Hierbij is van belang dat niet de waterstof wordt geoxideerd (reactie 4). Catalysatoren die bij lage temperatuur actief zijn, zijn alom aanwezig in de natuur.

(1) $CH_4 + H_2O \rightarrow CO + 3\ H_2$

(2) $CO + H_2O \rightarrow CO_2 + H_2$

(3) $2\ CO + O_2 \rightarrow 2\ CO_2$

(4) $2\ H_2 + O_2 \rightarrow 2\ H_2O$

(5) $4\ CO + 2\ H_2O \rightarrow 2\ CO_2 + CH_3COOH$

De water-gas shift reactie (reactie 2) wordt ook uitgevoerd door micro-organismen die daaruit voldoende energie halen om te kunnen groeien. Door de relatief lage temperatuur (30 °C – 80 °C) waarbij groei plaatsvind bestaat de mogelijkheid dat de lage CO waarden wel gehaald kunnen worden. Bovendien bestaat de verwachting dat micro-organismen minder gevoelig zijn voor verontreinigingen dan chemisch technologische catalysatoren. Deze verontreinigingen, voornamelijk sulfide, zijn al aanwezig in het gebruikte aardgas. Een hogere tolerantie tegen verontreinigingen betekent ook dat goedkopere grondstoffen (kolen) gebruikt kunnen voor de productie van synthese gas, in plaats van aardgas.

Van *Carboxydothermus hydrogenoformans* is in hoofdstuk 2 bestudeerd welke uiteindelijke CO concentratie gehaald kan worden en de mogelijkheid om deze concentratie verder te verlagen. In cultures die gestart waren met een 100% CO gas fase bleef 117 ppm CO over. Door gevormd CO_2 weg te vangen konden CO concentraties berijkt worden van minder dan 2 ppm, de detectie limiet van de gebruikte apparatuur. Of deze concentraties werden berijkt door de omzetting volgens reactie 2, of door de omzetting van CO naar acetaat (reactie 5) is af te vragen. Als de omzetting volledig volgens reactie 2 plaatsvind betekent dit dat het microbieel metabolisme zeer dicht het thermodynamisch evenwicht kan naderen, waarbij vrijwel geen vrije energie beschikbaar is. Dit terwijl verondersteld wordt dat er een minimale hoeveelheid energie nodig is om een levende cel in stand te houden. Wanneer uitgegaan wordt van de vorming van acetaat kan aan de veronderstelling van een minimale energie quantum voldaan worden.

De omzetting van CO volgens reactie 2 naar CO_2 en H_2 door *C. hydrogenoformans* waarbij zeer lage CO concentraties werden berijkt, vereisten ook veel tijd om die concentraties te berijken. De vraag die gesteld kan worden is of de omzetting wel voldoende snel is om interessant te zijn voor een biotechnologisch proces. In hoofdstuk 3 is uitgegaan van mogelijke limitatie van de omzettings snelheid door activiteit van het micro-organisme en de mogelijke limitatie door massa overdracht van het gas CO naar de vloeistof fase en de afgifte van de producten, CO_2 en H_2, aan de gasfase. In een eenvoudig experiment, waarbij *C. hydrogenoformans* cultures werden geincubeerd bij verschillende schud regimes, om massa overdracht te beinvloeden, werd gekeken naar de snelheid waarbij de gasdruk van de cultures toenam. De druk geeft een indicatie van de omzetting doordat 1

gas molecule wordt opgenomen (CO) terwijl er 2 gevormd worden (CO_2 en H_2). Slechts bij zeer lage cel concentraties, zoals vlak na aanenting van het kweek medium, bleek de omzetting gelimiteerd door de biomassa. Een sterke reductie in de tijd nodig voor de omzetting van alle CO werd gevonden bij het meest wilde schud regime. Uit een zeer voorzichtige schatting bleek tenminste een 8 maal kortere tijd nodig te zijn voor een volledige omzetting van CO dan in de experimenten zoals uitgevoerd in hoofdstuk 2. Bij de meest optimistische schatting 30 maal. Het succes van een biotechnologisch proces voor de omzetting van CO volgens de watergas shift reactie lijkt dus af te hangen van technologische mogelijkheden voor een optimale gas/vloeistof massa transport.

Carboxydothermus hydrogenoformans is niet het enige micro-organisme dat met goed met koolstofmonoxide als substraat kan groeien, hoewel dat vooral duidelijk is geworden over de afgelopen jaren. Voorheen werd koolstofmonoxide bij hogere concentraties vooral gezien als remmer van groei. Koolstofmonoxide wordt daarom maar spaarzaam getoets als substraat. Recent zijn vele nieuwe microbiele soorten beschreven die met koolstofmonoxide als substraat groeien, maar ook al eerder geisoleerde soorten blijken in staat koolstofmonoxide te gebruiken. Hoofdstuk 4 beschrijft bijvoorbeeld de groei van *Thermoterrabacterium ferrireducens* met koolstofmonoxide. Deze verschilt van die van *C. hydrogenoformans* omdat *T. ferrireducens* niet in staat is H_2 te vormen met CO. In plaats daarvan koppelt *T. ferrireducens* de oxidatie van CO tot CO_2 aan de reductie van verschillende electronen acceptoren. Terwijl voorheen werd gedacht dat *C. hydrogenoformans* alleen kon groeien op de omzetting van CO naar H_2 volgens reactie 2, bleek het over vergelijkbare metabole capaciteiten te beschikken als *T. ferrireducens*.

De uitgebreide mogelijkheden voor de kweek van *C. hydrogenoformans* bood de mogelijkheid om een interessante waarneming te toetsen. Uit genoom onderzoek aan *C. hydrogenoformans* bleek dat het over vijf koolstofmonoxide dehydrogenases beschikte. Koolstofmonoxide dehydrogenase (CODH) katalyseert de oxidatie van CO naar CO_2 of de omgekeerde versie daarvan, de reductie van CO_2 naar CO. Hoewel het mogelijk is dat alle vijf CODH dezelfde fysiologische functie hebben, is een specifieke rol voor elk waarschijnlijker. In het geval dat de vijf verschillende CODH een eigen rol hebben en het waarschijnlijke geval dat ze alleen gemaakt worden door de cel (expressie) wanneer ze nodig zijn, maakt het mogelijk een functie toe te wijzen door cellen te kweken onder selectieve omstandigeheden. In hoofdstuk 5 is hier een aanzet toe gegeven door cellen te kweken in de aan en afwezigheid van CO en met organische en anorganische verbindingen als energie substraat. Verschillen in de expressie van de verschillende CODH werd waargenomen, hoewel de gebruikte techniek onvoldoende bleek om de verschillen te duiden. Wel heeft de studie suggesties opgeleverd voor kweek condities en rol van enzym complexen, mogelijk van belang voor vervolg onderzoek. Nieuwe genoom en proteoom technieken voor de analyse van expressie nivo's lijken beter geschikt voor het bestuderen van de fysiologische functie van de verschillende CODHs.

Niet alleen *Thermoterrabacterium ferrireducens* maar ook de archaeon *Archaeoglobus fulgidus* bleek goed met CO overweg te kunnen door acetaat en formiaat te maken (hoofdstuk 6). Zeer opmerkelijk omdat deze activiteit van slechts één andere archaeon, *Methanosarcina acetivorans*, bekend is. Met CO als electronen donor kan *Archaeoglobus fulgidus* ook sulfaat reduceren. Dat *A. fulgidus* met CO kan groeien betekent dat het in staat is om autotroof koolstof te fixeren. Het is zeer waarschijnlijk dat *A. fulgidus* hiervoor de acetyl-CoA route gebruikt en CO_2 reduceert tot een methanofuran gebonden formyl, dat vervolgens word overgedragen op tetrahydromethanopterin. In archaea wordt deze formyl groep verder gereduceerd in de acetyl-CoA pathway om als methyl groep van de CoA gebonden acetyl te eindigen. Omdat *A. fulgidus* een archaeon is, is de vorming van formiaat opmerkelijk. Dit in tegenstelling to bacterien waar de reductie van CO_2 via formiaat verloopt, naar een tetrahydrofolaat gebonden formyl. Met NMR werd aangetoond dat de fomiaat afkomstig is van CO_2 en niet CO. Vorming van de formiaat door reductie van CO_2 is mogelijk, maar minder waarschijnlijk dan de vorming uit formyl methanofuran, of formyl tetrahydromethanopterin, als met *Methanosarcina acetivorans* wordt vergeleken. Dit organisme mist het enzym formiaat dehydrogenase dat de directe omzetting van CO_2 naar formiaat catalyseerd. Als formiaat uit de gebonden formyl groep wordt gevormd, is het niet onwaarschijnlijk dat bij die stap metabole energie wordt vast gelegd. Een dergelijke reactie is echter nog niet eerder beschreven voor archaea in de literatuur.

Naast de nieuwe waarnemingen die met *Archaeoglobus fulgidus* zijn gedaan bestaat er voor dit organisme ook de mogelijkheid het toe te passen in een biotechnologisch proces om waterstof gas met zeer weinig CO te produceren. In plaats van de omzetting naar H_2 zoals uitgevoerd door *Carboxydothermus hydrogenoformans*, kan *A. fulgidus* in theorie als selectieve catalysator CO verwijderen door de oxidatie naar CO_2 te koppelen aan de reductie van sulfaat of de vorming van acetaat. Dit doordat *A. fulgidus* niet in staat is te groeien met waterstof als substraat.

De studies met *Carboxydothermus hydrogenoformans* en *Archaeoglobus fulgidus* geven aan dat de natuur interessante mogelijkheden biedt voor de productie van waterstof, maar ook andere chemicalien, uit synthese gas. Verdere studie moet aangeven of de voordelen van mogelijke biotechnologische toepassing voldoende opwegen tegen de nadelen om bestaande chemische technologie te kunnen vervangen. Ook binnen de microbiele fysiologie is nog voortgang te boeken op het gebied van koolmonoxide. Genoom analyses laten zien dat niet alleen *Carboxydothermus hydrogenoformans* over meerdere koolstofmonoxide dehydrogenases beschikt, maar ook organismen waarvan het minder wordt verwacht zoals bijvoorbeeld *Desulfitobacterium hafniense.* Verdere ontwikkeling van de inzichten rond de rol van koolstofmonoxide in strict anaerobe micro-organismen zal duiden op een voorname rol van CO in de vroege evolutie van het leven.

Curriculum Vitae

Anne Meint Henstra werd geboren op 27 juli 1976 te Tijnje (de Tynje). In 1994 behaalde hij zijn VWO-diploma aan de RSG te Heerenveen. In datzelfde jaar startte hij met de studie Biologie aan de Rijksuniversiteit Groningen, met als specialisatie Moleculaire Biologie. Als onderdeel van deze specialisatie verichtte hij een afstudeeronderzoek in de groep Microbiële Fysiologie van Prof. dr. L. Dijkhuizen aan de Rijksuniversiteit Groningen. Hier deed hij onderzoek onder begeleiding van dr. H. Kloosterman aan het nudix eiwit Mut dat in de actinomyceet *Amycolatopsis methanolica* betrokken is bij een verhoogde spontane mutatie frequentie van dit organisme. Een tweede afstudeeronderzoek werd uitgevoerd bij de groep Maag, Darm en Leverziekten op het Academisch Ziekenhuis in Groningen bij Dr. M. Müller onder leiding van dr. O. Mol. Onderwerp van dit onderzoek was de isolatie van het gen voor de humane galzout export pomp, een ABC-transporter. In augustus 1999 behaalde hij het doctoraal diploma. Na een korte zoektocht naar een interessant onderwerp starte hij in 2000 een promotie onderzoek bij het Laboratorium voor Microbiologie van de Wageningen Universiteit in de groep Microbiele Fysiologie onder begeleiding van Prof. dr. Fons Stams. Het project 'Microbial conversion of CO to H_2: a biological alternative for the water-gas shift reaction' werd gefinancierd door de stichting technologische wetenschappen (STW, WBC.5280). De resultaten van het onderzoek staan beschreven in dit proefschrift.

List of publications

Henstra, A.M. and A.J.M. Stams. 2004. Novel physiological features of *Carboxydothermus hydrogenoformans* and *Thermoterrabacterium ferrireducens*. Applied and Environmental Microbiology 70:7236-7240.

Sipma, J., A.M. Henstra, S.N. Parshina, P.N.L. Lens, G. Lettinga and A.J.M. Stams. 2006 Microbial CO conversions with application in synthesis gas purification and biodesulfurization. Critical Reviews in Biotechnology 26:541-547.

Henstra, A.M., C. Dijkema and A.J.M. Stams. submitted. *Archaeoglobus fulgidus* couples CO oxidation to sulfate reduction and acetogenesis with intermeate formate accumulation.

Henstra, A.M. and A.J.M. Stams. submitted. Ultra deep shift catalysis by *Carboxydothermus hydrogenoformans*.

Parshina S.N., S. Kijlstra, A.M. Henstra, J. Sipma, C.M. Plugge and A.J.M. Stams. 2005. Carbon monoxide conversion by thermophilic sulfate-reducing bacteria in pure culture and in co-culture with *Carboxydothermus hydrogenoformans*. Applied Microbiology and Biotechnology 68:390-396

Parshina S.N., J. Sipma, Y. Nakashimada, A.M. Henstra, H. Smidt, A.M. Lysenko, P.N.L. Lens, G. Lettinga and A.J.M. Stams. 2005. *Desulfotomaculum carboxidivorans* sp. nov., a novel sulfate-reducing bacterium capable of growth at 100% CO. International Journal of Systematic and Evolutionary Microbiology 55:2159-2165.

Jiang, B., P.L. Paulo, A.M. Henstra, W. van Doesburg, A.J.M. Stams. submitted. Novel one-carbon metabolism of *Moorella hydrogenoformans* sp. nov., isolated from a methanogenic bioreactor.

Dit onderzoek is gesteund door de Technologiestichting STW, een onderdeel van de Nederlandse Organisatie voor Wetenschappelijk onderzoek (NWO), onder project nummer STW-WBC5280. Mede mogelijk gemaakt door Paques B.V. (Balk) en Shell Global Solutions (Amsterdam).

This research was supported by the Technology Foundation STW, aplied science division of the Netherlands Organisation for Scientific Research (NOW), with project number STW-WBC5280. Supported by Paques B.V. (Balk, Netherlands) and Shell Global Solutions (Amsterdam, Netherlands).

ONTWERP OMSLAG
TAEKE CASTELEIN GRONINGEN
TEL: 050-5773038