Hydrogen metabolism and syntrophic interactions of *Sulfurospirillum* spp. in anaerobic co-cultures

Dissertation

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für meine Eltern

Contents

Glossary

[Ade](N)Cba	Adeninyl-(nor)cobamide
Ado	5'-Deoxyadenosyl-
AK	Acetate kinase
AP-P	(*R*)-1-Aminopropan-2-ol *O*-2-phosphate
ATP	Adenosintriphosphate
BN-PAGE	Blue native polyacrylamide gel electrophoresis
BTEX	Aromatic hydrocarbons (benzene, toluene, ethylbenzene, xylene)
Bza	Benzimidazole
Cba	Cobamide
CSIA	Compound-specific isotope analysis
*c*DCE	*cis*-1,2-Dichloroethene
DMB	5,6-Dimethylbenzimidazole
[DMB](N)Cba	5,6-Dimethylbenimidazolyl-(nor)cobamide
DMN	2,3-Dimethyl-1,4-napthoquinone
EA-P	Ethanolamine *O*-phosphate
Ech	Energy-converting hydrogenase
EPS	Extracellular polymeric substances
FdhF	Formate dehydrogenase H
[FeS]	Iron-sulfur cluster
FHL	Formate hydrogen lyase
FISH	Fluorescence *in situ* hybridization
HQNO	2-n-Heptyl-4-hydroxychinolin-N-oxid
Hup	Cytoplasmic uptake hydrogenase
Hyc	*E. coli* Hydrogenase 3
HydB	Catalytic subunit MBH
Hyf	Hyf-like hydrogenase/*E. coli* Hydrogenase 4
HyfG	Catalytic subunit Hyf-like hydrogenase
HyfR	Formate-dependent transcriptional activator (Hyf)
(i)LDH	(quinone-independent) Lactate dehydrogenase
LutABC	Lactate utilization proteins in *Bacillus subtilis*
MBH/Hyd	Membrane-bound hydrogenase

5-MeBza	5-Methylbenzimidazole
[5-MeBza]Cba	5-Methylbenzimidazolyl-cobamide
OHRB	Organohalide-respiring bacteria
5-OMeBza	5-Methoxybenzimidazole
[5-OMeBza]Cba	5-Methoxybenzimidazolyl-cobamide
PAH	Polycyclic aromatic hydrocarbons
PCE	Tetrachlorethene
PCB	Polychlorinated biphenyls
PCDD/PCDF	Polychlorinated dibenzo-*p*-dioxins/dibenzofuranes
PceA	Tetrachloroethene reductive dehalogenase
PCNB	Pentachloronitrobenzene
PFOR	Pyruvate:ferredoxin oxidoreductase
PFL	Pyruvate formate lyase
PoxB	Quinol-dependent pyruvate dehydrogenase
PTA	phosphotransacetylase
RDase/Rdh	Reductive dehalogenase
ROS	Reactive oxygen species
(r)TCA	(reductive) Tricarboxylic acid cycle
TCE	Trichloroethene
TCP	Trichlorphenol
VC	Vinyl chloride

Summary

The massive global release of halogenated hydrocarbons (organohalides) over the last century caused severe detrimental effects on human and environmental health by contaminating many different ecosystems. Research on the biological degradation of organohalides revealed the discovery of several bacteria able to dehalogenate and thus detoxify these pollutants by utilizing them as terminal electron acceptors in organohalide respiration.

The organohalide-respiring ε-proteobacterium *Sulfurospirillum multivorans* was extensively studied for its tetrachloroethene (PCE) reductive dehalogenase PceA and has become a model organism for organohalide respiration. However, the hydrogen metabolism which might be involved in the respiratory chain and possible syntrophic interactions of this organism with bacteria in its natural habitat has never been characterized and are the focus of this study. Four [NiFe] hydrogenases have been identified in the genome, of which one membrane-bound H_2-oxidizing (MBH) and one H_2-producing (Hyf) enzyme were found to be expressed. Biochemical characterization of a MBH-enrichment implicate the enzyme as possible electron donating system of the organohalide respiratory chain. The other hydrogenase was transcribed during pyruvate fermentation and screening of different *Sulfurospirillum* spp. revealed a fermentative hydrogen production of these bacteria for the first time. Fermentation balance experiments and comparative proteomics were performed to elucidate the metabolic pathways of pyruvate fermentation and a remarkable upregulation of the Hyf hydrogenase and other pyruvate-metabolizing enzymes was observed.

Co-cultivation of *S. multivorans* with another organohalide-respiring bacterium, *Dehalococcoides mccartyi*, introduced the organism as a hydrogen-producing syntrophic partner and uncovered a new ecological role. An interspecies hydrogen and cobamide transfer enabled a fast and complete dechlorination of the prominent groundwater pollutant PCE to ethene which is of high interest for bioremediation processes.

Zusammenfassung

Die massive weltweite Freisetzung von halogenierten Kohlenwasserstoffen im letzten Jahrhunderthat gefährdende Auswirkungen auf Mensch und Umwelt, hervorgerufen durch die Kontamination vieler verschiedener Ökosysteme. Untersuchungen zum biologischen Abbau dieser Schadstoffe führten zur Entdeckung von Bakterien die in der Lage sind Diese Verbindungen zu entgiften und als terminale Elektronenakzeptoren in der Organohalid-Atmung zu nutzen.

Das organohalid-respirierende ε-Proteobakterium *Sulfurospirillum multivorans* wurde hinsichtlich seiner Tetrachlorethen(PCE)-reduktiven Dehalogenase intensiv untersucht. Der an der Atmungskette beteiligte Wasserstoffmetabolismus und die ökologischen Interaktionen des Organismus (z.B. Syntrophie) wurden dagegen nie charakterisiert, und waren daher Fokus dieser Arbeit. Im Genom wurden vier [NiFe] Hydrogenasen detektiert, von denen eine membrangebundene H_2-oxidierende (MBH) und eine H_2-produzierende (Hyf) Hydrogenase transkribiert wurden. Die biochemische Charakterisierung einer MBH-Anreicherung ergab eine mögliche Beteiligung der MBH an der Organohalid-Atmungskette. Die andere Hydrogenase ist vermutlich in der Pyruvatfermentation involviert und Wachstumsexperimente zeigten erstmals eine fermentative Wasserstoffproduktion von verschiedenen *Sulfurospirillum* Arten. Mit Hilfe von Fermentationsbilanzen und vergleichenden Proteomstudien wurden die Stoffwechselwege dieser Fermentation aufgeklärt und es konnte eine Hochregulation der Hyf-Hydrogenase und anderer assoziierter Enzyme gezeigt werden.

Die Ko-Kultivierung von *S. multivorans* mit einem anderen organohalid-atmenden Bakterium, *Dehalococcoides mccartyi*, stellte den Organismus erstmals als wasserstoffproduzierenden syntrophen Partner vor und wies der Gattung eine neue ökologische Rolle zu. Der interspezies Wasserstoff- und Corinoidtransfer zwischen beiden führte zu einer schnellen und vollständigen Dechlorierung der Grundwasserkontaminante PCE, welche für Bioremediationsprozesse von großem Interesse ist.

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1 Introduction

1.1 Syntrophic relationships among prokaryotes

The interspecies electron transfer within syntrophic relationships in microbial communities is an intermediary key step in the anaerobic degradation of complex organic matter. In this special type of symbiotic cooperation, microorganisms exhibiting various metabolic lifestyles fulfill the degradation of a certain compound that cannot be carried out alone by one of the involved prokaryotes (McInerney *et al.*, 2009). For example, the complete degradation of complex organic compounds such as cellulose, polysaccharides, lipids and proteins to methane and carbon dioxide requires the interaction of at least four metabolically different groups of bacteria (Fig. 1). Primary fermenters break down the compounds to smaller molecules like pyruvate, lactate, propionate, butyrate and ethanol or directly to substrates for methanogenesis as hydrogen, formate, acetate, and carbon dioxide. The intermediate products such as pyruvate and lactate are further degraded by secondary fermenters into substrates which can be utilized by methanogens and iron- and sulfate-reducers (McInerney *et al.*, 2008; Schink, 2002; Schink, 1991; Schink and Stams, 2013; Stams, 1994). The basis of these various degradation processes is the exchange of metabolites between the community members including an extracellular transport of electrons which can be achieved by chemical compounds itself such as hydrogen, formate and acetate or organic shuttles like humic acids, quinones and riboflavins. Hydrogen plays a key role in this interspecies hydrogen transfer, which is often described as the 'heart of syntrophy' (McInerney *et al.*, 2011). Many syntrophic relationships in methanogenic, sulfate or iron reducing and denitrifying environments are described with hydrogen as electron carrier (Morris *et al.*, 2013). However, the interspecies electron transfer is not exclusively achieved by a hydrogen transfer. A co-culture of *Syntrophobacter fumaroxidans* and *Methanospirillum hungatei* was shown to prefer an interspecies formate transfer rather than

a hydrogen transfer, and similarly, acetate serves as the electron carrier in methanogenic associations (de Bok *et al.*, 2002; Platen *et al.*, 1994; Platen and Schink, 1987). Depending on the metabolic lifestyles of the organisms, syntrophic relations can be grouped into facultative or obligate associations. The latter associations depend directly on the hydrogen-consuming partner maintaining a low hydrogen partial pressure which favours endergonic substrate oxidation. Electrons gained from oxidation are bound either to NADH or ferredoxin and used for proton reduction. Formation of hydrogen from NADH is only feasible at low hydrogen levels since the redoxpotental of this reaction is unfavourable and would hamper growth at high hydrogen levels. Instead, ferredoxins of the *Allochromatium vinosum* type are more favourable electron carriers due to their low redox potential (Moulis *et al.*, 1996). In contrast, facultative syntrophs are still able to oxidize NADH at high hydrogen concentrations and bypass hydrogen production by reducing internal metabolites. This was reported for *Ruminococcus flavefaciens* and *Selemomonas ruminatium*, which ferment glucose at high hydrogen levels additionally to succinate and lactate (Chen and Wolin, 1977; Latham and Wolin, 1977).

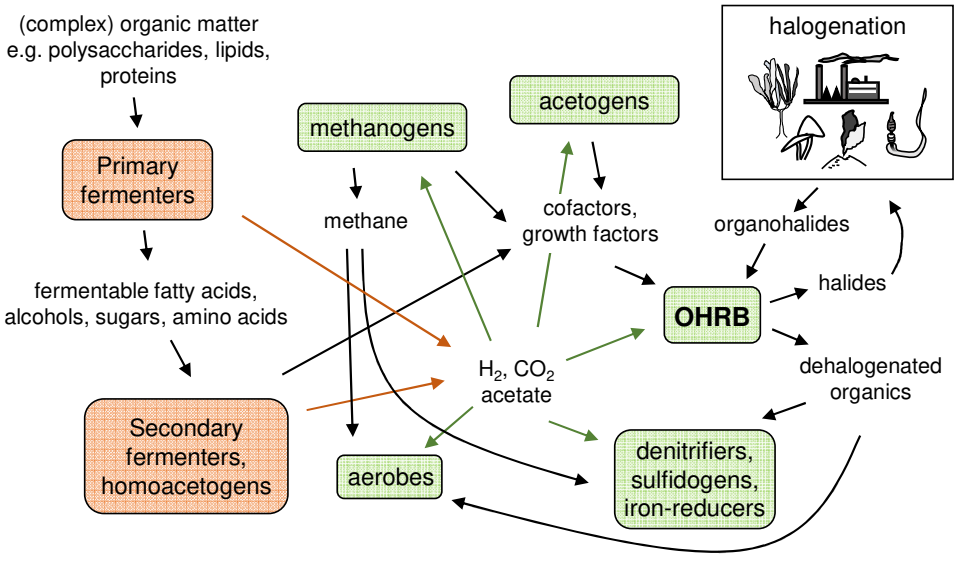

Figure 1: Schematic overview of catabolic food webs and syntrophic interactions in microbial communities containing organohalide-respiring bacteria (OHRB). Key physiological groups are framed in boxes and the exchanges of metabolites between these groups are indicated by arrows. Color code; orange: H_2-producer, green: H_2-consumer.

In syntrophic associations, these organisms benefit from a higher ATP yield as shown for *Ruminococcus albus* which higlights the importance of removing hydrogen when NADH oxidation is restricted to proton reduction. (Stams and Plugge, 2009).

Syntrophy is often at the thermodynamic limit of life since Gibbs free energy changes are at the minimum energy gain required for ATP synthesis (Schink and Stams, 2006). The generation of ATP under the conditions in a living cell including the heat loss requires +70 kJ mol^{-1} (Schink, 1990). Peter Mitchell postulated in 1966 a vectorial transport of three protons necessary for ATP formation (Mitchell, 2011). Therefore, the minimum Gibbs free energy needed is about -20 kJ mol^{-1} which is equivalent to one third ATP (Schink, 1990; Schink and Thauer, 1988). The stoichiometry of proton translocation and ATP synthesis is not restricted to 3:1 and has been reported in the recent years as 4:1 or even 5:1 which lowers the minimum energy needed to translocate one proton across the cytoplasmic membrane to -15 - -10 kJ mol^{-1} (Seelert *et al.*, 2000; Stock *et al.*, 1999).

Syntrophic interactions are widespread in nature and can be found in various habitats including methanogenic and sulfidogenic environments, and even hyperthermophilic habitats. A unique syntrophic association between *Nanoarchaeum equitans* and *Ignicoccus hospitalis,* identified in hot submarine vents, led to the discovery of a new archaeal phylum. Additionally, the relationship demonstrates another common feature of syntrophic interactions, namely, the formation of cell aggregates and the reduction of intermicrobial distances. A tight physiological association and physical contact between both organisms was reported. At the attachment sites, the periplasmic space of *I. hospitalis* cells was narrow, resulting in a close contact between the outer membrane and cytoplasmic membrane of *N. equitans* cells (Huber *et al.*, 2002; Jahn *et al.*, 2008). The advantages of cell-to-cell contact were previously reported by Schink and Thauer in 1988 (Schink and Thauer, 1988). According Fick's law, the diffusion rates of metabolites like hydrogen or formate are increased at low distances, which is of high importance for growth kinetics and substrate consumption rates. Cell aggregation is an optimal interaction for an enhanced

interspecies hydrogen and metabolite transfer. Despite the hyperthermophilic association, densely packed granules were also obtained in high-rate methanogenic bioreactors for efficient waste water treatment (Hulshoff Pol *et al.*, 2004; Lettinga *et al.*, 1980).

A more detailed understanding of these metabolic and physiological cooperations in the future might enable the isolation and cultivation of currently uncultured microorganisms based on a syntrophic co-culture approach.

1.2 Catabolic food webs and syntrophy in OHRB communities

Chlorinated hydrocarbons were widely used for different industrial processes as solvents (e.g. tetrachloroethene (PCE, trichloroethene (TCE), chloroform), polymers (e.g. polyvinylchloride) and pesticides including herbicides, fungicides and insecticides (e.g. Bromoxynil, Pentachloronitrobenzene [PCNB], Trichlorphenol [TCP]) and were released for many years in high quantities into the environment despite their toxicity (Mohn and Tiedje, 1992). In addition to this massive anthropogenic release, halogenated organic compounds are produced and released naturally from abiotic sources including geological processes like wildfires, rock disruptions, and volcanoes and biotically by lignin-degrading fungi, marine sponges, algae and lichens (Abrahamsson *et al.*, 1995; Gribble, 2012; Harper and Hamilton, 2003; Jordan *et al.*, 2000; Swarts *et al.*, 1998). The ongoing pollution, high environmental persistence and bioaccumulation of carcinogenic organohalides in food chains can have severe detrimental effects on human health (Henschler, 1994). Many biotechnological and geotechnological processes have been developed and already applied for the remediation of contaminated environments. Due to its ecological compatibility, bioremediation processes based on organohalide-respiring bacteria (OHRB) are promising approaches and the application of these consortia into contaminated soil was recently started. These bacteria, especially *Dehalococcoides mccartyi,* use a wide range of organohalides as electron acceptor and enables the degradation of a wide range of chlorinated organic compounds such as PCE, polychlorinated biphenyls (PCB), dioxins

(PCDD) and furans (PCDF) (Bunge and Lechner, 2009). Organohalide-respiring communities are characterized by a heterogenic structure and multitude of potential food webs among their members. Besides OHRB, fermenting bacteria, methanogens, homoacetogens, sulfidogens, denitrifiers and aerobes shape the community structure (Fig. 1). Also, OHRB populations show a heterogeneous composition and form distinctive groups: facultative organohalide respirers found in the Firmicutes (*Desulfitobacterium* spp.) and δ- and ε-Proteobacteria (*Desulfomonile* spp. and *Sulfurospirillum* spp.); and obligate organohalide respirers also found in the Firmicutes (*Dehalobacter* spp.) and Chloroflexi (*Dehalococcoides* spp. and *Dehalogenimonas* spp.). The facultative OHRB exhibit a versatile metabolism and utilize numerous electron acceptors, whereas obligate OHRB are restricted to organohalides as electron acceptors and hydrogen as electron donor (Maphosa *et al.*, 2010). Fermenting bacteria degrade more complex organic matter to organic acids (e.g. pyruvic, lactic, butyric, formic), alcohols, CO_2 and H_2 which in turn can be used by obligate OHRB as electron donors and corbon sources. Different studies showed the presence of fermenting bacteria like *Desulfovibrio* spp., different Bacteroidetes and Clostridiales, homoacetogens and acetoclastic methanogens beeing responsible for hydrogen and acetate production (Diekert and Wohlfarth, 1994; Grostern *et al.*, 2009; Grostern and Edwards, 2006a; Heimann *et al.*, 2006; Lee *et al.*, 2011; Lovley and Ferry, 1985). For example, the sulfidogen *Desulfovibrio fructosovorans* fermented fructose to hydrogen which was subsequently used as electron donor for dechlorination of PCE to *cis*-1,2-dichloroethene (*c*DCE) by the co-cultivated *Desulfitobacterium hafniense* strain TCE1 suggesting a hydrogen transfer (Fig. 2A) (Drzyzga and Gottschal, 2002). Another mixed culture containing *Syntrophomonas wolfei* and *Dehalococcoides mccartyi* strain 195 revealed an efficient and complete dechlorination of PCE to ethene (Fig. 2B) (Mao *et al.*, 2015). In a 3-chlorobenzoate degrading tri-culture, the methanogen *Methanospirillum* sp. consumed hydrogen and stimulated in turn benzoate fermentation of BZ-2 which was produced by *Desulfomonile tiedjei* (Fig. 2C).

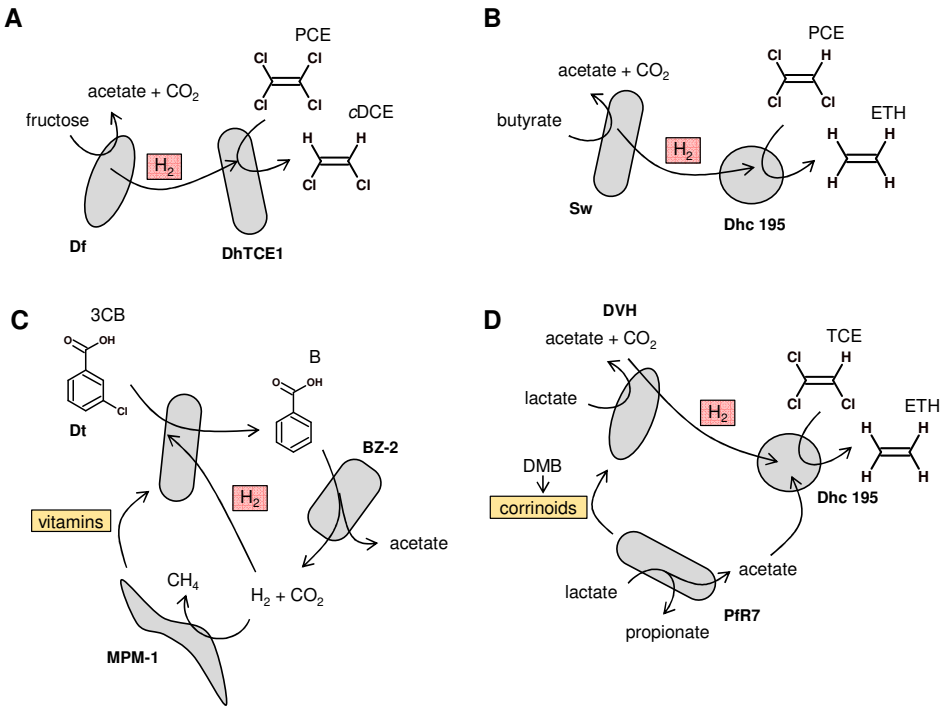

Figure 2: Syntrophic interactions between dechlorinating and non-dechlorinating bacteria. (A) Syntrophic lactate fermentation by *Desulfovibrio fructosivorans* (Df) and PCE-to-*c*DCE dechlorinator *Desulfitobacterium hafniense* strain TCE1 (DhTCE1) (Drzyzga and Gottschal, 2002). (B) Complete dechlorination of PCE to ethene by *Syntrophomonas wolfei* (Sw) and *Dehalococcoides mccartyi* strain 195 (Dhc 195) (Mao *et al.*, 2015). (C) Dechlorination of 3-chlorobenzoate (3CB) to benzoate (B) and exchange of growth factors/vitamins in a tri-culture of the benzoate-fermenting bacterium BZ-2, *Desulfomonile tiedjei* (DT) and *Methanospirillum* sp. (MPM-1) (Mohn and Tiedje, 1992; Tiedje and Stevens, 1988). (D) Interspecies hydrogen and corrinoid transfer in a TCE-to-ethene dechlorinating tri-culture of *Desulfovibrio vulgaris* Hildenborough (DVH), *Pelosinus fermentans* R7 (PfR7) and *D. mccartyi* strain 195 (Dhc 195) (Men *et al.*, 2014). Interspecies hydrogen transfer is indicated by a red box and interspecies corrinoid by a yellow box. PCE - tetrachloroethene, TCE - trichloroethene, *c*DCE - *cis*-1,2-dichloroethene.

Methanospirillum sp. was also shown to produce growth stimulating vitamins used by *D. tiedjei* (Mohn and Tiedje, 1992; Tiedje and Stevens, 1988). Besides hydrogen, also acetate and formate are additional energy sources in syntrophic communities and can also be used as the sole electron donor for reductive dechlorination.

The presence of only a few electron donors and low concentrations of these, and the occurance of multiple alternative electron acceptors such as nitrate, sulfate and iron, in sometimes high concentrations, lead to competition between methanogens, acetogenes, sulfate and nitrate reducers with dechlorinating bacteria for electron donors (Fennell and Gossett, 1998; Smatlak *et al.*, 1996). At exceeding electron donor concentrations, various electron-accepting processes will simultaneously occur, while at limiting conditions,

organisms possessing the highest hydrogen affinities and higher energy-yielding metabolism will outcompete the less effective organisms. However, dechlorinating bacteria are reported to exhibit extremely low hydrogen affinities below the thresholds for methanogenesis, acidogenesis and sulfate reduction. Therefore, obligate OHRB are well-adapted competitors for hydrogen among other community members and will thrive-in anaerobic environments as long as chlorinated electron acceptors are available (Löffler *et al.*, 1999; Lovley and Goodwin, 1988; Smatlak *et al.*, 1996; Yang and McCarty, 1998).

In addition to the transfer of energy sources and metabolites, transport and supply of growth factors (e.g. vitamins) in syntrophic relationships has been reported. The most important growth factors in OHRB communities are corrinoids, since organohalide respirers use corrinoids (e.g. vitamin B_{12}) as co-factor for the key enzyme in OHR, the reductive dehalogenase. Several obligate OHRB, e.g. D. mccartyi, are dependent on corrinoids due to missing genes for *de novo* corrinoid biosynthesis. Different co-culture experiments revealed an interspecies cobamide transfer between the corrinoid producing *Acetobacterium*, *Sporomusa*, *Geobacter* or *Methanosarcina* and different *D. mccartyi* strains (He *et al.*, 2007; Yan *et al.*, 2013; Yan *et al.*, 2012). For example, an exchange of corrinoids between *Methanospirillum*sp. and *Desulfomonile tiedjei* was shown (Fig. 2C) (Mohn and Tiedje, 1992; Tiedje and Stevens, 1988).

The syntrophic associations among OHRB and other members of microbial communities play a crucial role in supporting the detoxification and bioremediation of contaminated groundwater and soils. Additionally, these associations enable highly effective cooperations producing rapid and complete dechlorination of organohalides. This finding shows signifciant potential for environmental applications.

1.3 Hydrogenases and their roles in syntrophic microbial communities

Key enzymes involved in microbial hydrogen metabolism and thus in interspecies hydrogen transfer are hyrogenases catalyzing the reversible conversion of molecular hydrogen: $H_2 \leftrightarrow 2H^+ + 2e^-$. In presence of a terminal electron acceptor, organisms oxidize hydrogen and provide reducing equivalents for respiratory processes which can be coupled to energy conservation in respiratory chains. In case of a low potential electron donor and a missing electron acceptor, hydrogen is produced by proton reduction and enables dispose of excess reductants (Vignais and Billoud, 2007; Vignais *et al.*, 2001). Based on the metal content of their active sites containing either a Ni and Fe atom or one or two Fe atoms, they can be classified into three phylogenetic distinct groups: [NiFe], [FeFe] and [Fe] hydrogenases, whereas [Fe] enzymes are restricted to methanogens. The [FeFe] hydrogenases are primarily found in H_2-producing organisms including fermenting bacteria, and sulfate-reducers and [NiFe] hydrogenases are mainly present in H_2-utilizing organisms. The [NiFe] are the most divers class and were therefore further classified into four groups based on phylogeny, function, cellular localization, and composition (Schwartz *et al.*, 2013). Briefly, periplasmic faced membrane-bound hydrogenases catalyzing respiratory hydrogen oxidation linked to quinone reduction are classified into group 1. Group 2 resembles sensory hydrogenases and cytoplasmic cyanobacterial uptake hydrogenases playing a role in nitrogen fixation. Group 3 represents multimeric enzymes interacting with cofactors (e.g. F_{420}, NAD) in the cytoplasm of which some function bidirectional. Hydrogenases which evolve hydrogen and generate a proton gradient during proton reduction and thus conserve energy are arranged in group 4.

Fermenters like *Clostridium pasteurianum*, *Syntrophomonas wolfei* or *Thermotoga maritima* often harbor [FeFe] hydrogenases and produce hydrogen during sugar fermentation. Hydrogen production in *Pyrococcus furiosus*, *E. coli* and Sporomusa ovata is achieved by a membrane-bound [NiFe] hydrogenase (Adams, 1990; Breznak, 2006; Dubini *et al.*, 2002; McTernan *et al.*, 2015; Schut and Adams, 2009; Sieber *et al.*, 2010).

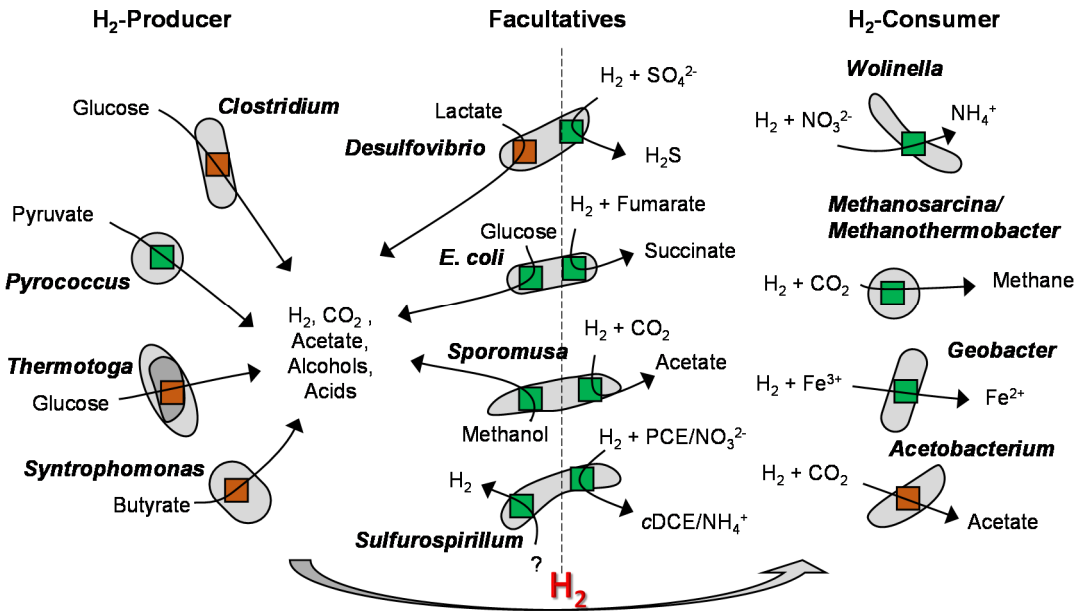

Figure 3: Schematic overview of hydrogenases and their roles in key microbial groups. Representatives include fermenting bacteria, acetogens, methanogens, iron-/sulfate-/nitrate-reducer and organohalide-respiring bacteria. [NiFe] hydrogenases are indicated by a green square and [FeFe] hydrogenases by a brown square. The direction of the interspecies hydrogen transfer is indicated by a grey arrow from H_2-producer to H_2-consumer. Reactions are stoichiometrically not balanced.

[FeFe] enzymes are reported not to function bidirectional and show a catalytic bias towards hydrogen oxidation *in vitro*, the [FeFe] hydrogenases of *C. pasteurianum* preferentially oxidize hydrogen (Therien *et al.*, 2017). Hydrogen is the electron donor and energy source for physiological divers organisms. [NiFe] hydrogenases catalyze hydrogen uptake for nitrate reduction in *Wolinella succinogenes* and *Geobacter sulfurreducens*, or CO_2 reduction in *Methanosarcina barkeri* and *Methanothermobacter thermoautotrophicus* (Coppi, 2005; Meuer *et al.*, 2002; Tanner and Paster, 1992). Bacteria harboring H_2-oxidizing and H_2-producing hydrogenases can switch between both functions enabling a versatile metabolism. For example, *Desulfovibrio vulgaris* Hildenborough produces hydrogen during lactate fermentation via a [FeFe] hydrogenase and oxidizes hydrogen in the presence of sulfate (Fauque *et al.*, 1988). *Sulfurospirillum multivorans* was shown to utilize hydrogen with tetrachloroethene or nitrate as electron acceptor and is therefore considered as a hydrogen consumer (Scholz-Muramatsu *et al.*, 1995). However, a total of four [NiFe] hydrogenases suggests a more complex hydrogen metabolism.

1.4 Organohalide respiration, [NiFe] hydrogenases and ecology of *Sulfurospirillum multivorans*

The gram-negative Epsilonproteobacterium *Sulfurospirillum multivorans* was isolated from dechlorinating and methanogenic enrichment cultures obtained from the activated sludge of a wastewater treatment plant in Stuttgart (Germany). It utilizes pyruvate as an electron donor and the highly persistent groundwater pollutant tetrachloroethene (PCE) as an electron acceptor (Scholz-Muramatsu *et al.*, 1995). Isolation attempts were based on the ability of *S. multivorans* to use PCE as an electron acceptor in an anaerobic respiration process. In this, the reductive dechlorination of PCE to *c*DCE is coupled to energy conservation by electron transport phosphorylation, and ATP synthesis is driven by a proton motive force. The key enzyme for the dechlorination of PCE is the tetrachloroethene reductive dehalogenase (Rdh) PceA, which has a periplasmic orientation, and is anchored to the cytoplasmic membrane via a small hydrophobic protein PceB (John *et al.*, 2006; Neumann *et al.*, 1996; Neumann *et al.*, 1998). PceA harbors two [4Fe4S] clusters and a corrinoid cofactor, and both are essential for the intramolecular electron transport. The corrinoid is located at the active site and directly involved in the reaction mechanism (Bommer *et al.*, 2014; Kunze *et al.*, 2017). The corrinoid cofactor is a norpseudo-B_{12} with adenine as lower ligand and an ethanolamine-phosphate moiety in the nucleotide loop. Production and utilization of that special type of corrinoid is only known for *S. multivorans* so far, but occurs most likely also in other PCE-dechlorinating *Sulfurospirillum* spp. (Fig. 4 A, B) (Butler *et al.*, 2006; Buttet *et al.*, 2018; Kräutler *et al.*, 2003). In general, complete *de novo* corrinoid biosynthesis involves more than 25 enzymes and was shown until now exclusively in prokaryotes. The last step in the cobamide biosynthesis is the incorporation of the lower ligand via a nucleotide loop assembly pathway which is variable among different microorganisms and can be either a purinyl, benzimidazolyl or a phenolyl moiety (Chan *et al.*, 2014; Claas *et al.*, 2010; Hazra *et al.*, 2013). In addition to endogenously synthesized bases, exogenously provided lower ligand bases can also be incorporated into the

cobamide (Cheong *et al.*, 2001; Renz, 1999; Stupperich *et al.*, 1988). This 'guided biosynthesis' and the ability to exchange the lower ligand enables higher flexibility towards the occurence and availability of these bases in nature. Furthermore, it leads to a functionalization of corrinoids in *D. mccartyi* which will be discussed extensively in the next chapter. *S. multivorans* was shown to exchange adenine by 5,6-dimethylbenimidazole (DMB) when amended (Keller *et al.*, 2014; Reinhold *et al.*, 2012). All genes essential for the *de novo* 'anaerobic pathway' of corrinoid biosynthesis cluster together with *pceA* and *pceB* and accessory genes referred to as the organohalide respiration gene region (Appendix Fig. 2, Appendix Tab. 1) (Goris *et al.*, 2014; Gruber *et al.*, 2011).

The reductive dehalogenase PceA is the electron-accepting system in the organohalide respiration chain and receives the electrons from menaquinone. The involvement of menaquinone was shown in inhibition experiments with the semiquinone analogon 2-n-heptyl-hydroxychinolin-n-oxide (HQNO), which led to a complete inhibition of PCE dechlorination (Krauter, 2006; Scholz-Muramatsu *et al.*, 1995). When utilizing pyruvate as electron donor, the electron donating system of the respiration chain might be either a pyruvate:ferredoxin oxidoreductase (PFOR) or a quinol-dependent pyruvate dehydrogenase (PoxB) as both enzymes were found in the proteome of cells grown with pyruvate (Goris *et al.*, 2015; Goris *et al.*, 2014). In the case of hydrogen or formate as electron donor, presumably a membrane-bound and periplamically orientated hydrogenase or formate dehydrogenase provide the electrons (Miller *et al.*, 1996; Schmitz and Diekert, 2004; Schmitz and Diekert, 2003).

The genomes of *Sulfurospirillum* spp. harbor genes encoding different sets of [NiFe] hydrogenases which can be grouped either into hydrogen oxidizing or hydrogen producing hydrogenases. The marine species *S. carboxydovorans* and *S. arcachonense* show less variation, while *S. cavolei* exhibits an additional [FeFe] hydrogenase gene cluster unique to that organism (Tab. 1). In *S. multivorans*, four [NiFe] hydrogenases were found by genome sequencing and annotation which is discussed in depth in chapter 2.1 (Goris *et al.*, 2014).

Briefly, a membrane-bound hydrogenase (MBH) presumbed catalyzes the oxidation of H_2 and transfers the electrons via a membrane-integral cytochrome *b* (HydC) to the menaquinone pool of a respiratory chain as shown for the close related MBH of *Wolinella succinogenes* (Dross *et al.*, 1992; Gross *et al.*, 2004). The second enzyme, HupSL, is closely related to the uptake hydrogenase 3 of *Aquifex aeolicus* and might be also involved in the reductive tricarboxylic acid (TCA) cycle by delivering low potential electrons or recycling hydrogen generated during nitrogen fixation mediated by a nitrogenase (Guiral *et al.*, 2005; Ju *et al.*, 2007). The third and fourth enzymes show similarities to energy-converting membrane-bound hydrogen-producing [NiFe] hydrogenases (group 4). One (EchEDFC) is related to the CO-induced membrane-bound hydrogenase of *Carboxydothermus hydrogenoformans* which receives electrons from CO oxidation catalyzed by a CO dehydrogenase and reduces protons to H_2 (Soboh *et al.*, 2002). The fourth hydrogenase, Hyf, is related to Hyd3 (Hyc) and Hyd 4 (Hyf) of *E. coli* and to other epsilonpoteobacterial group 4 hydrogenases found in *Arcobacter nitrofigilis* and *Campylobacter consicus* (Appendix Fig. 1A, B).

Table 1: Habitats, metabolic features and hydrogenase gene clusters of selected *Sulfurospirillum* isolates.

***Sulfurospirillum* spp.**	**Habitat**	**Metabolism**	**Hydrogenases**				
			[NiFe]				[FeFe]
			H_2 oxidation		H_2 production		
			MBH	Hup	Ech	Hyf	
S. multivorans	PCE-contaminated groundwater	Versatile, organohalide respiration	+	+	+	+	-
S. halorespirans			+	+	+	+	-
S. cavolei	Petroleum-contaminated groundwater	Versatile	+	+	+	+	+
S. deleyianum	Pond sediment		+	-	+	+	-
S. arsenophilum	Arsenic-contaminated sediments		+	+	+	+	-
S. carboxydovorans	Marine sediments		+	-	-	+	-
S. arcachonense		Sulfur reducer	+	-	-	+	-

The Hyc is a central component of the formate hydrogenlyase (FHL) complex and was shown to be involved in the mixed acid fermentation (Fig. 6A) (Doberenz *et al.*, 2014; Knappe and Sawers, 1990; Sargent, 2016; Sawers, 1994). A second FHL complex (FHL-2) in *E. coli* might be formed by the Hyf which is supported by the presence of genes coding for accessory proteins needed for formate oxidation (Fig. 6B, Appendix Fig. 1A) (Bagramyan and Trchounian, 2003; Pinske and Sawers, 2016; Sargent, 2016). The physiological role of the Hyf complex of *S. multivorans* remains unclear, since a PFL is not encoded in the genome. Instead of forming a FHL complex, the Hyf could dispose reducing equivalents it was reported for the group 4 hydrogenase of *Pyrococcus furiosus* (McTernan *et al.*, 2015; Schut *et al.*, 2013).

Several studies revealed the presence of *Sulfurospirillum* spp. up to high abundancies (one-third of the total bacterial population) in *D.mccartyi*-containing microbial communities and stated dechlorination and hydrogen utilization as the ecological role within these consortia (Dugat-Bony *et al.*, 2012; Rossetti *et al.*, 2008; van der Zaan *et al.*, 2009; Yohda *et al.*, 2015). Furthermore, the association of *Sulfurospirillum* and *Dehalococcoides* was already described in an enrichment culture obtained from PCE-contaminated groundwater (SL2) in which both were the predominant bacteria fulfilling a concerted PCE-to-ethene dechlorination (Maillard *et al.*, 2011). In addition to the utilization of chlorinated hydrocarbons as electron acceptors, *S. multivorans* is able to utilize various other electron acceptors including fumarate, nitrate, sulfur, thiosulfate, arsenate, selenate or 5% oxygen (Goris and Diekert, 2016; Goris *et al.*, 2014; Luijten *et al.*, 2004; Scholz-Muramatsu *et al.*, 1995). This versatile metabolism enables *S. multivorans* and also other *Sulfurospirillum* spp. to participate in more diverse ecological cycles than the halogen cycle. The organisms are globally distributed and have been identified in marine and river sediments, hyperthermophilic deep-sea hydrothermal vents, groundwater aquifers, soil and even arsenate- and selenate-contaminated environments (Tab. 1) (Ahmann *et al.*, 1994; Finster *et al.*, 1997; Jensen and Finster, 2005; Luijten *et al.*, 2003; Maillard *et al.*, 2011; Stolz *et al.*,

1999). Epsilonproteobacteria are ubiquitous and play significant roles in the global carbon, sulphur and nitrogen cycles (Campbell *et al.*, 2006; Corre *et al.*, 2001; Gevertz *et al.*, 2000; Nakagawa *et al.*, 2005). In such roles, they are known to utilize hydrogen exclusively as electron donors in addition to sulphur and formate (Campbell *et al.*, 2006). Cultivation experiments, molecular methods (e.g. FISH) and genome sequencing in combination with phylogenetical 16S rRNA gene analysis gave first insights into the phylogenetic diversity and ecophysiology of this phylum. However, the physiological properties and metabolic flexibilities raise additional questions about their interaction with other microorganisms in microbial communities, and further studies are needed to improve our understanding of the symbiotic and especially syntrophic relationships of these organisms.

1.5 The obligate organohalide respiring *Dehalococcoides mccartyi*

The genus *Dehalococcoides*, within the phylum Chloroflexi, comprises the only known organisms so far to completely dechlorinate the carcinogenic PCE to the benign ethane and are classified as the same species, *Dehalococcoides mccartyi* (Löffler *et al.*, 2013b). In contrast, other OHRB are only capable of dechlorinating PCE to *c*DCE (Fig. 4) (Futagami *et al.*, 2008; Lorenz and Löffler, 2016). In the past 15 years, several other *Dehalococcoides* strains have followed the original isolation of strain 195 exhibiting different dechlorination characteristics (He *et al.*, 2005; Maymó-Gatell *et al.*, 2001). Additionally, *Dehalococcoides*-containing enrichments and mixed cultures were shown to completely dechlorinate PCE to ethene (Fig. 4). Strain BTF08 was enriched with PCE up to >98% from the heavily polluted megasite in the Bitterfeld region (Germany). The residual population was identified by 16S rRNA gene clone libraries to be closely related to *Sulfurospirillum* spp. (99%) (Cichocka *et al.*, 2010; Pöritz *et al.*, 2013). Despite the different dehalogenation spectrum, all *Dehalococcoides* strains described so far are restricted to organohalide respiration and exclusively utilize hydrogen as an electron donor (Maymó-Gatell *et al.*, 1997).

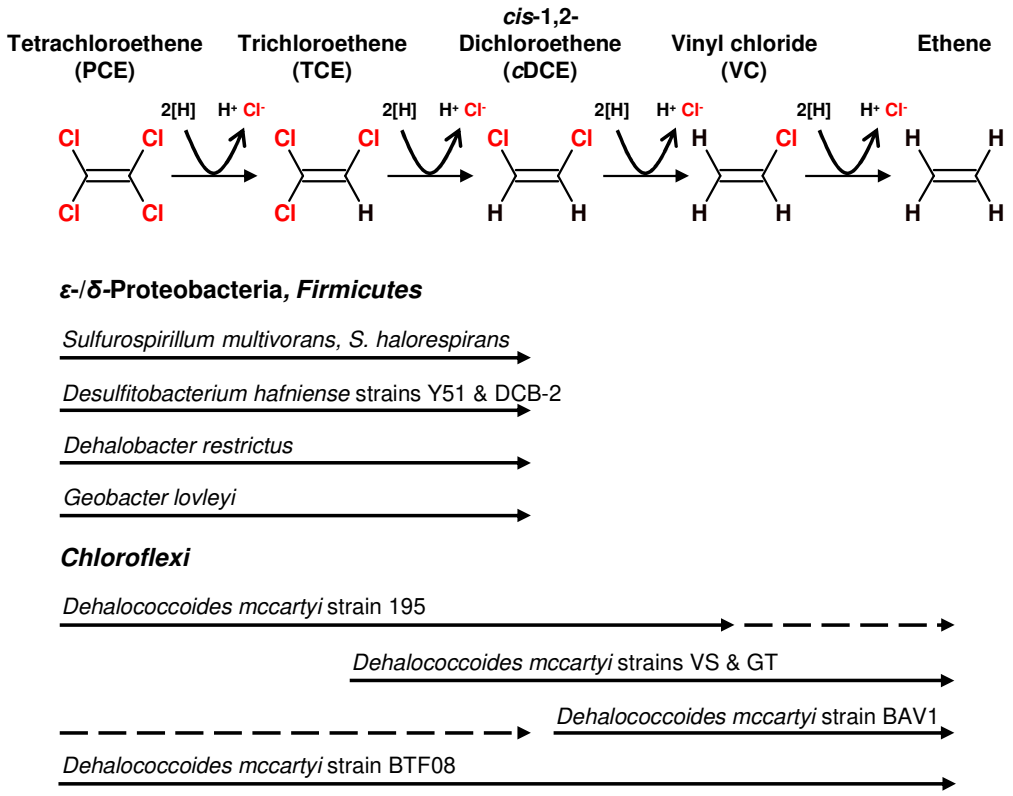

Figure 4: Comparison of PCE dechlorination capabilities among different bacterial genera. Solid arrows indicate a coupling of dechlorination to growth and energy conservation. Dashed arrows represent a cometabolic degradation.

Another common feature of all strains is an unusual cell morphology and electron microscopic analysis exhibiting small, disc-shaped cells (0.4 – 0.5 µm in diameter and 0.1 to 0.2 µm thickness) with irregular biconcave emarginations on both flat sides (Löffler *et al.*, 2013b). The cell surface sometimes exhibits filamentous attachments which might play a role in cell aggregation and could enable a cell-to-cell contact (Lendvay *et al.*, 2003). On thin-section electron micrographs, no peptidoglycan layer were visible and the cell wall ultrastructure resembled the S-layer structure of archaeal cell walls (Albers and Meyer, 2011). The lack of peptidoglycan enables a resistance to antibiotics and also permits growth in the presence of vancomycin and ampicillin which is used to enrich *Dehalococcoides mccartyi* (Löffler *et al.*, 2013b; Maymó-Gatell *et al.*, 1997).

The high degree of specialization and restriction to organohalide respiration is also reflected in the highly streamlined *Dehalococcoides* genomes, containing 5 to 38 different *rdhA* genes (McMurdie *et al.*, 2009; Seshadri *et al.*, 2005; Türkowsky *et al.*, 2018). Elucidation of

the RDase substrate spectra remains due to the low biomass production a challenging aim and needs more biochemical studies. Up to date, only a few RDases with distinct functions assigned have been identified by micro array, transcriptional studies and analysis of proteins loaded onto native PAGEs (Krajmalnik-Brown *et al.*, 2004; Kublik *et al.*, 2016; Magnuson *et al.*, 2000; Parthasarathy *et al.*, 2015). Even the prediction of the substrate range from the amino acid sequence cannot be applied, since orthologs with up to 99% sequence identity show no transcription on the desired substrates compared to their counterparts (Türkowsky *et al.*, 2018). Despite their dependence on corrinoids as cofactors for RDases, the *de novo* vitamin B_{12} biosynthesis is incomplete and the corrin ring cannot be synthesized in *D. mccartyi*. Although, all genes necessary for corrinoid salvaging and remodeling are clustered with a duplicate in strain 195 and a single gene cluster in BTF08 (Appendix Tab. 1, Appendix Fig. 2) (Seshadri *et al.*, 2005). Salvaging of exogenous corrinoids includes the uptake by a corrinoid specific ABC transporter (BtuFCD) and their modification (Fig. 5). Only three types of B_{12} are functional for *D. mccartyi* strain 195 and can be directly used for incorporation into the RDases: cobalamin (with DMB as lower ligand), 5-methylbenzimidazolyl-cobamide ([5-MeBza]Cba) and 5-methoxybenzimidazolyl-cobamide ([5-OMeBza]Cba) (Fig. 5 A,B) (Escalante-Semerena, 2007; McMurdie *et al.*, 2009; Yi *et al.*, 2012). Non-functional B_{12} types are modified and remodeled by CbiBZ and CobCDSTU. The original lower ligands are replaced by 5,6-dimethylbenzimidazol (DMB), 5-methylbenzimidazol (5-MeBza) or 5-methoxybenzimidazol (5-OMeBza) catalyzed by the cobinamide amidohydrolase CbiZ, which was shown to remove the nucleotide loop and lower ligand of the non-functional adeninylcobamide in *Rhodobacter sphaeroides* (Fig. 5C) (Gray and Escalante-Semerena, 2009a). Even the functional [5-MeBza]Cba was remodeled into cobalamin in the presence of DMB suggesting a high preference for DMB as lower ligand (Yi *et al.*, 2012).

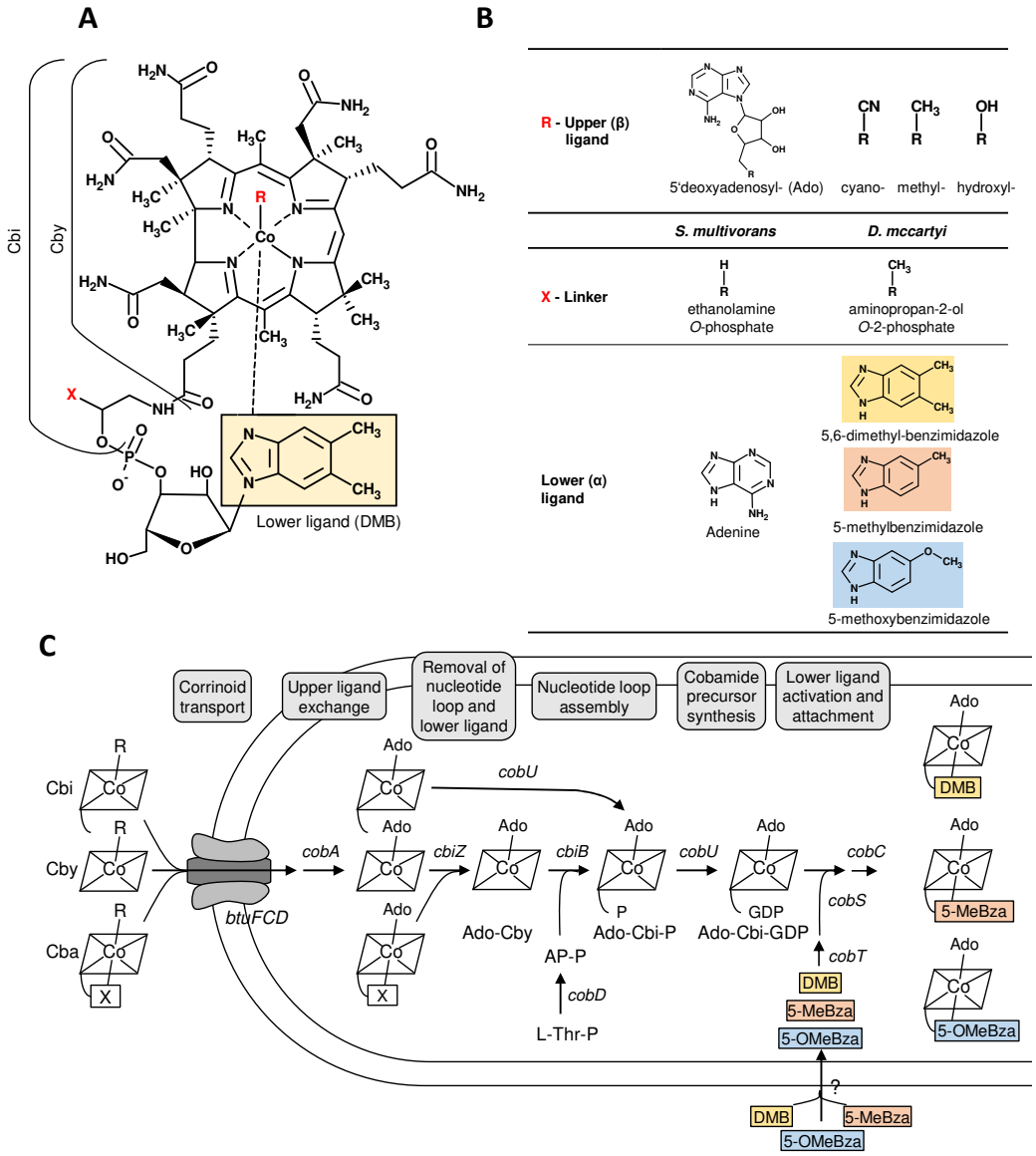

Figure 5: Salvaging and remodeling pathways of different corrinoids in *D. mccartyi*. (A) Complete cobamide (Cba) is shown with 5,6-dimethylbenzimidazole (DMB) as lower ligand (in box) and structures of cobinamide (Cbi) and cobyric acid (Cby) are indicated by brackets. (B) Possible upper ligands of corrinoids and linker found in *S. multivorans* and *D. mccartyi*. Depiction of lower ligands functional in corrinoids of RDases in *D. mccartyi* (framed in coloured boxes) and adenine, the lower ligand also found in norpseudo-B_{12} of *S. multivorans*. (C) Salvaging and remodeling of corrinoids in *D. mccartyi*. Complete corrinoids or derivatives are imported by BtuFCD and salvaging occurs by cbiZ- and cbiB-dependent pathway or the cobU-dependent pathway. CobA fuses deoxyadenosine (Ado) as upper ligand to the cobalt. Remodeled cobamides (without lower ligand and nucleotide loop) receives the aminopropanol nucleotide loop and an imported and activated lower ligand. DMB - 5,6-dimethylbenzimidazol, 5-MeBza - 5-methylbenzimidazol, 5-OMeBza - 5-methoxybenzimidazole.

Due to their nutritional requirements, relying on hydrogen as electron donor, acetate as carbon source and corrinoids as cofactors of RDases, *D. mccartyi* strains are highly dependent on their surrounding microbial community providing all of these substrates and

growth factors (Fig. 1 & 2). Several studies revealed the interspecies hydrogen transfer and the exchange of other metabolites and growth factors between *D. mccartyi* strains and fermenting bacteria. In these studies, the interspecies hydrogen transfer and the exchange of other metabolites and growth factors were observed. The association with potential fermenting bacteria, such as *Desulfovibrio desulfuricans*, *Desulfovibrio vulgaris* Hildenborough, *Acetobacterium woodii* or *Syntrophomonas wolfei* was shown to be highly beneficial for *D. mccartyi,* and co-cultivation with one of these bacteria enabled faster dechlorination, higher growth rates and biomass production compared to pure cultures (He *et al.*, 2007; Mao *et al.*, 2015; Men *et al.*, 2014). In addition to metabolite exchanges, different types of corrinoids are provided by the microbial community. For example, *Desulfovibrio*, *Acetobacterium*, *Clostridium*, *Sporomusa* and methanogenic archaea often found in *Dehalococcoides*-containing communities are reported to produce corrinoids with different lower ligand bases (Guimarães *et al.*, 1994; Stupperich *et al.*, 1988; Stupperich and Kräutler, 1988; Yan *et al.*, 2012). An interspecies cobamide transfer was demonstrated in a co-culture of *D. mccartyi* strains BAV1 and FL2 with *Geobacter lovleyi,* the methanogenic *Methanosarcina barkeri* strain Fusaro and the acetogen *Sporomusa ovata* (Yan *et al.*, 2013; Yan *et al.*, 2012).

The high adaptation to a large variety of organohalides as electron acceptors and the unique characteristic to completely dechlorinate PCE to ethene, makes *D. mccartyi* a promising candidate for *in situ* bioremediation technologies. The introduction of *Dehalococcoides*-containing mixed cultures into contaminated groundwater and soil is currently being performed as the subject of several on-going research projects. Long-term field studies have already demonstrated a successful and promising outcome of this special type of bioaugmentation (McCarty, 2010; Stroo *et al.*, 2013).

1.6 Aims of the study

Besides the putative involvement of the membrane-bound hydrogenase in hydrogen oxidation, little was known about the hydrogen metabolism of *Sulfurospirillum multivorans*, which is a model organism for organohalide respiration. Especially the detection of four [NiFe] hydrogenase gene clusters in its genome suggest a more complex hydrogen metabolism than restricted to organohalide respiration. The presence of a potential hydrogen-evolving hydrogenase, also in other *Sulfurospirillum* spp., might allow hydrogen evolution under certain growth conditions. However, experimental evidence for these functions is pending.

First, biochemical characterization as well as transcriptional analysis should reveal the hydrogen metabolism and the physiological roles of the four hydrogenases of *S. multivorans*. An enrichment and proteinbiochemical analysis of the uptake hydrogenase could provide insight into the subunit composition of these enzymes and activity assays with quinone analogous might suggest an interaction with the organohalide respiratory chain. Hydrogen production mediated by one or both of the putative hydrogen-evolving enzymes should be elucidated in fermentative cultivation experiments and in comparative proteomic studies

Furthermore, the presumed hydrogen production could represent a novel ecological role of *Sulfurospirillum* spp, since the organisms are hitherto exclusively recognized as hydrogen consumers. A co-culture approach with hydrogen-consuming bacteria would proof the ability of *Sulfurospirillum* spp to act as a hydrogen-producing syntrophic partner in microbial food webs. Finally, possible syntrophic relations could be characterized when the syntrophic interactions within OHRB communities could be unraveld when co-cultivating the organism with another organohalide-respiring bacterium, *Dehalococcoides mccartyi*. An interspecies hydrogen transfer between both based on an obligate relationship might enable a complete dechlorination of PCEat higher rates compared to the pure cultures.

2 Manuscripts

2.1 The NiFe Hydrogenases of the Tetrachloroethene-Respiring Epsilonbacterium *Sulfurospirillum multivorans*: Biochemical Studies and Transcription analysis

Kruse S, Goris T, Wolf M, Wei X, Diekert G (2017) *Frontiers in Microbiology* 8:444

2.2 Hydrogen production by *Sulfurospirillum* spp. enables syntrophic interactions of Epsilonproteobacteria

Kruse S, Goris T, Adrian L, Westermann M, Diekert G (2018)
under revision in *Nature Communications*

2.3 Syntrophy between *Dehalococcoides* and *Sulfurospirillum* leads to rapid and complete dechlorination of tetrachloroethene

Kruse S, Türkowsky D, Matturro B, Franke S, Birkigt J, Nijenhuis I, Westermann M, Rossetti S, Jehmlich N, Adrian L, Diekert G, Goris T
in preparation for submission in *ISME Journal*

2.1 The NiFe Hydrogenases of the Tetrachloroethene-Respiring Epsilonproteobacterium *Sulfurospirillum multivorans*: Biochemical Studies and Transcription analysis

Kruse S*, Goris T*, Wolf M, Wei X, Diekert G (2017) *Frontiers in Microbiology* 8:444

*These authors contributed equally to this work.

Genome sequencing of the organohalid-respiring epsilonproteobacterium *Sulfurospirillum multivorans* revealed genes coding for four different NiFe hydrogenases of unknown physiological function. The involvement of these enzymes in the hydrogen metabolism and their regulation in this organism was investigated for the first time. Quantitative real-time PCR analysis of hydrogenase transcript levels showed a transcription of only two hydrogenases: a putative uptake hydrogenase and a putative hydrogen producing hydrogenase. Enzyme activity assays and protein biochemical analysis of an enrichment of the uptake hydrogenase suggested a periplasmic membrane-bound enzyme being the electron donating system for the organohalide respiratory chain.

My own contributions to this publication covers about: 45%.

All quantitative real-time PCR experiments, enzyme assays, enrichments and biochemical analysis presented in this publication were performed by myself. Tobias Goris and Gabriele Diekert initiated and supervised the study. Design of the experiments, analysis of data and drafting of the manuscript were done by Tobias Goris and Stefan Kruse. Initial biochemical experiments and initial transcription analysis were done by Xi Wei and Maria Wolf.

Supplementary information is given in appendix, pp. v - ix

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ORIGINAL RESEARCH
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The NiFe Hydrogenases of the Tetrachloroethene-Respiring Epsilonproteobacterium *Sulfurospirillum multivorans*: Biochemical Studies and Transcription Analysis

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The organohalide-respiring Epsilonproteobacterium *Sulfurospirillum multivorans* is able to grow with hydrogen as electron donor and with tetrachloroethene (PCE) as electron acceptor; PCE is reductively dechlorinated to *cis*-1,2-dichloroethene. Recently, a genomic survey revealed the presence of four gene clusters encoding NiFe hydrogenases in its genome, one of which is presumably periplasmic and membrane-bound (MBH), whereas the remaining three are cytoplasmic. To explore the role and regulation of the four hydrogenases, quantitative real-time PCR and biochemical studies were performed with *S. multivorans* cells grown under different growth conditions. The large subunit genes of the MBH and of a cytoplasmic group 4 hydrogenase, which is assumed to be membrane-associated, show high transcript levels under nearly all growth conditions tested, pointing toward a constitutive expression in *S. multivorans*. The gene transcripts encoding the large subunits of the other two hydrogenases were either not detected at all or only present at very low amounts. The presence of MBH under all growth conditions tested, even with oxygen as electron acceptor under microoxic conditions, indicates that MBH gene transcription is not regulated in contrast to other facultative hydrogen-oxidizing bacteria. The MBH showed quinone-reactivity and a characteristic UV/VIS spectrum implying a cytochrome *b* as membrane-integral subunit. Cell extracts of *S. multivorans* were subjected to native polyacrylamide gel electrophoresis (PAGE) and hydrogen oxidizing activity was tested by native staining. Only one band was detected at about 270 kDa in the particulate fraction of the extracts, indicating that there is only one hydrogen-oxidizing enzyme present in *S. multivorans*. An enrichment of this enzyme and SDS PAGE revealed a subunit composition corresponding to that of the MBH. From these findings we conclude that the MBH is the electron-donating enzyme system in the PCE respiratory chain. The roles for the other three hydrogenases remain unproven. The group 4 hydrogenase might be involved in hydrogen production upon fermentative growth.

Keywords: organohalide respiration, hydrogenase, real-time PCR, regulation of gene expression, hydrogen, anaerobic respiration

INTRODUCTION

Molecular hydrogen (H_2) is one of the primary electron donors for many anaerobic respiratory processes mediated by prokaryotes. Organohalide respiration, in which energy conservation is coupled to the reductive dehalogenation of halogenated organic compounds, is no exception in this regard. Obligate organohalide-respiring bacteria such as *Dehalococcoides mccartyi* and *Dehalobacter restrictus* rely solely on H_2 as electron donor (Holliger et al., 1998; Löffler et al., 2013), while most versatile organohalide-respiring organisms, e.g., *Desulfitobacterium* spp. or *Sulfurospirillum* spp., use H_2 as one of many electron donors (Scholz-Muramatsu et al., 1995; Luijten et al., 2003; Villemur et al., 2006; Goris and Diekert, 2016). One feature, which is common to many genomes of organohalide-respiring bacteria, is the presence of multiple hydrogenase-encoding gene clusters (Kube et al., 2005; Seshadri et al., 2005; Nonaka et al., 2006; Kruse et al., 2013, 2015; Goris et al., 2014). Besides H_2 oxidation in respiratory processes, which is usually mediated by a membrane-bound NiFe hydrogenase, several different cellular metabolic processes are thought to recruit the other hydrogenase enzymes for either H_2 oxidation or H^+ reduction. The roles of these hydrogenases are unknown and under debate (Seshadri et al., 2005; Rupakula et al., 2013; Goris et al., 2014; Mansfeldt et al., 2014; Kruse et al., 2015). For example, cytoplasmic hydrogenases with an $NAD(P)^+$ binding motif might be responsible for generating reducing equivalents for biosynthetic pathways or for balancing the cellular redox state. Group 4 Ech-type hydrogenases, which often harbor several large membrane-integral subunits, might also be involved in energy-conserving processes, as reported for methanogens (Hedderich and Forzi, 2005; Welte et al., 2010).

Sulfurospirillum multivorans is a versatile organohalide-respiring Epsilonproteobacterium that uses H_2 or other compounds such as formate or pyruvate as electron donors and chlorinated ethenes or, e.g., nitrate or fumarate as electron acceptor (Scholz-Muramatsu et al., 1995; Goris and Diekert, 2016). Recently, it has also been shown that the organism is able to utilize O_2 as terminal electron acceptor under microoxic conditions with about 5% O_2 in the gas phase (Goris et al., 2014). The genome of *S. multivorans* contains gene clusters encoding four different NiFe hydrogenases. One is predicted to be a periplasmic, membrane-bound H_2-oxidizing enzyme (membrane-bound hydrogenase, MBH, HydABC encoded by SMUL_1423-1425), which is very similar (50 to 76% subunit amino acid sequence identity) to the characterized uptake hydrogenase of *Wolinella succinogenes* (Dross et al., 1992). The small subunit of the MBH of both organisms, HydA, contains a TAT signal peptide which is cleaved off after maturation and transport of the MBH into the periplasm. HydC is a membrane-integral cytochrome *b* subunit which connects the hydrogenase to the quinone pool (Gross et al., 2004). The other three hydrogenases contain no signal peptide motif and are therefore considered to be cytoplasmic (Goris et al., 2014). One of these three enzymes (HupSL, encoded by SMUL_1421-1422) is related to cytoplasmic H_2-consuming hydrogenases and regulatory hydrogenases, the other two can be classified as group 4 hydrogenases, which are known to produce H_2 (Vignais and Billoud, 2007). The hydrogenase encoded by the *ech* gene cluster (structural proteins EchEDFC encoded by SMUL_1307-1310) bears similarities to the CO-induced hydrogenase of *Carboxydothermus hydrogenoformans* (Soboh et al., 2002), but the *S. multivorans* genome does not contain genes coding for a CO dehydrogenase. Hydrogenase membrane subunit genes were not found on the *ech* gene cluster, which is remarkable, since all group 4 hydrogenase gene clusters normally contain genes encoding membrane-integral subunits (Greening et al., 2016). The fourth hydrogenase comprises eight subunits (HyfABCEFGHI encoded by SMUL_2383-2390), of which four are predicted to be membrane-integral. This hydrogenase is similar to hydrogenase 4 (Hyf) of *Escherichia coli*. This enzyme found in several Enterobacteriaceae might interact with a cytoplasmic formate dehydrogenase (FdhF) to form a formate hydrogen lyase (FHL) complex (Trchounian et al., 2012; Sargent, 2016). At least two of the hydrogenases of *S. multivorans*, the MBH and the Hyf, are produced during tetrachloroethene (PCE) respiration with either pyruvate or formate as sole electron donor (Goris et al., 2015). The periplasmically oriented MBH is assumed to be the main H_2-oxidizing enzyme in *S. multivorans*, since H_2-oxidizing activity was detected in whole cells and the majority of this activity was found to be membrane-associated (Miller et al., 1996). However, these assays were performed with cells and extracts from *S. multivorans* grown with H_2/fumarate, while a study with H_2/PCE-grown cells was never carried out. Deduced from amino acid sequence similarity, HupSL was discussed to play a role in either the recycling of H_2 produced cytoplasmically (e.g., during N_2 fixation) or to deliver low-potential reducing equivalents for anabolic purposes comparable to *Aquifex aeolicus* hydrogenase III (Guiral et al., 2005). However, it could also be involved in H_2-dependent PCE respiration. The two group 4 hydrogenases might play a role in H_2 production from excess reducing equivalents during fermentative growth. The putative roles of the four hydrogenases in *S. multivorans* are depicted in **Figure 1**.

Usually hydrogenase expression underlies specific regulation depending on their physiological role and growth conditions (Kovács et al., 2005; Greening and Cook, 2014). For example, uptake hydrogenases gene transcription is upregulated when H_2 is available, NiFe hydrogenase gene expression is down-regulated when the cofactor nickel is absent, and O_2-sensitive hydrogenases are not expressed under oxic conditions. However, in both Epsilonproteobacteria and organohalide-respiring bacteria nearly nothing is known about hydrogenase gene regulation. In this study we wanted to (1) reveal the hydrogen uptake metabolism of *S. multivorans* during H_2-driven PCE respiration and characterize the uptake hydrogenase involved in this process and (2) investigate the transcript profiles of hydrogenase catalytic subunit genes during different growth conditions, which might give information about the regulation of the four hydrogenases and their physiological role in *S. multivorans*.

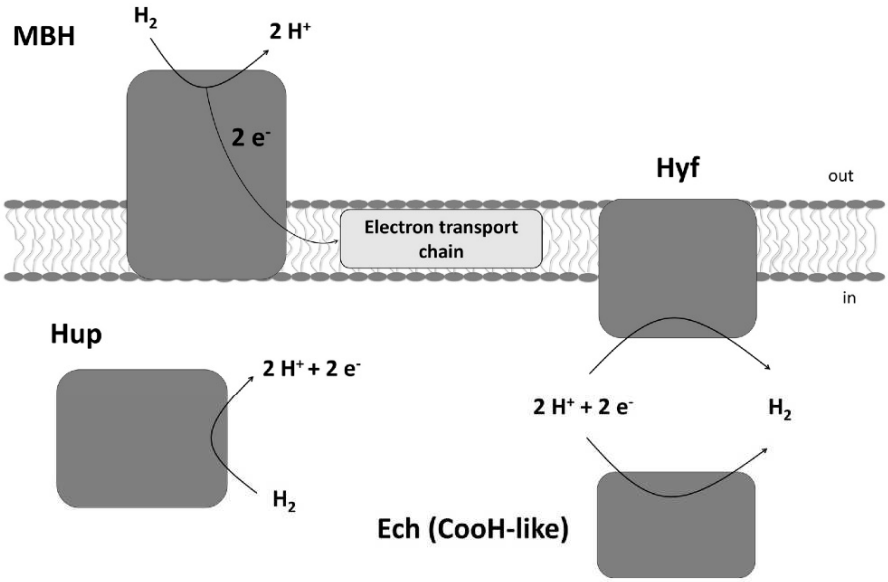

FIGURE 1 | Putative physiological roles of the four hydrogenases of *Sulfurospirillum multivorans* and their subcellular localization. MBH, membrane-bound hydrogenase HydABC; Hup, cytoplasmic uptake hydrogenase HupSL; Hyf, HyfABCEFGHI similar to *E. coli* hydrogenase 4; Ech, EchEDFC, similar to the CO-induced hydrogenase of *Carboxydothermus hydrogenoformans*, but in *S. multivorans* lacking any membrane subunit.

EXPERIMENTAL PROCEDURES

Cultivation of *S. multivorans*

Sulfurospirillum multivorans (DSMZ 12446) was grown anaerobically at 28°C in rubber stoppered glass bottles shaken at 150 rpm with an aqueous to gas phase ratio of 1:1. The medium was described previously containing cysteine as reductant but did not contain vitamin B_{12} (cyanocobalamin) here (Scholz-Muramatsu et al., 1995). Before autoclaving, the gas phase contained 150 kPa N_2. Pyruvate (40 mM) was used as electron donor and fumarate (40 mM), PCE, nitrate (10 mM), or 5% O_2 as electron acceptor. In the latter case, O_2 concentration in the gas phase was measured with the Microx 4 oxygen meter (PreSens Precision Sensing GmbH, Regensburg, Germany). The amount of 5% O_2 in the gas phase corresponded to approximately 0.5 mg/L medium in the liquid phase. Cells growing with pyruvate as electron donor were cultivated at least 50 transfers without H_2 in the gas phase to test for possible long-term regulation effects. PCE was added to the medium in a nominal concentration of 10 mM from a 0.5 M PCE hexadecane stock solution. When cells were cultivated with H_2 as electron donor, a gas phase of H_2 (150 kPa) was applied and acetate (5 mM) was added as carbon source. For experiments with N_2 as sole nitrogen source, ammonium chloride was omitted from the basal medium and N_2 (150 kPa) was used as gas phase. The following energy substrate combinations were used: pyruvate/fumarate, pyruvate/PCE, pyruvate/O_2, pyruvate alone, H_2/PCE, and H_2/nitrate. Growth was monitored photometrically at 578 nm.

Cell Harvesting and Samples Preparation

Sulfurospirillum multivorans cells were harvested oxically in the mid-exponential growth phase by centrifugation (12,000 × *g*, 10 min at 10°C). Resulting cell pellets were washed twice in 50 mM Tris-HCl (pH 7.5) and resuspended in two volumes (2 ml per g cells) of the same buffer with additional DNase I (AppliChem, Darmstadt, Germany) and protease inhibitor (one tablet for 10 ml buffer; cOmplete Mini, EDTA-free; Roche, Mannheim, Germany). Cell disruption was carried out semi-anoxically using a French Press cell (1500 psi), which was made anoxic in a glovebox (Coy Laboratory Products Inc., Grass Lake, MI, USA), while the disruption itself was performed outside the tent. The obtained crude extracts were used for biochemical experiments. Cells were fractionated by ultracentrifugation (36,000 × *g*, 45 min at 4°C), supernatants were designated soluble fractions (SF). The pellets were resuspended in two volumes (2 ml per g membrane pellet) 50 mM Tris-HCl (pH 7.5); a part of this suspension was used further as membrane fraction (MF), while the rest was mixed with 0.1% Triton X-100 and stirred at 4°C overnight. Solubilized membrane extract (ME) was obtained from the resulting supernatant after ultracentrifugation (36,000 × *g*, 45 min at 4°C).

Enrichment of the Membrane-Bound Hydrogenase

Enrichment was carried out at room temperature in an anoxic chamber with an atmosphere of 98% N_2 and 2% H_2 (Coy Laboratory Products Inc., Grass Lake, MI, USA) using an ÄKTA-FPLC system (GE Healthcare Europe GmbH, Freiburg, Germany). Cells were grown in 2 L Schott bottles (aqueous to gas phase ratio 1:1) with pyruvate and fumarate. Harvesting, disruption and fractionation was done as described in the previous chapter, except that 1% digitonin was used as detergent. The filtered solubilized ME was fractionated via an anion exchange column (Q-Sepharose HP column 10/10, GE Healthcare Europe GmbH, Freiburg, Germany). The Q-Sepharose column was pre-equilibrated with 50 mM Tris-HCl (pH 8.0), 0.5 mM DTT and 0.1% (v/v) Triton X-100. Subsequently, the column was washed with 200 ml of the same buffer. Elution of proteins was achieved with a linear salt gradient from 0 to 0.5 M KCl at a flow rate of 2 ml/min. Fractions containing hydrogenase activity eluted at approximately 0.2 M KCl. H_2-oxidizing activity was checked photometrically and by activity stained blue native (BN) polyacrylamide gel electrophoresis (PAGE), while purity was checked by SDS PAGE and silver-stained BN PAGE.

Blue Native Gel Activity Staining PAGE and SDS PAGE

Non-denaturing PAGE was performed with native gels from SERVA (SERVA*GEL* N, Heidelberg, Germany). The anaerobic running buffer system was composed of a cathode buffer (50 mM Tricine, 15 mM BisTris-HCl, 1 ml/L Serva Blue G; pH 7.0) and anode buffer (15 mM BisTris-HCl; pH 7.0). Samples were mixed with twofold loading dye (1 M 6-Aminocaproic acid; 100 mM BisTris-HCl pH 7.0; 100 mM NaCl; 20% glycerol; 0.1% Serva Blue G250). The protein marker used was a SERVA Native Marker Liquid Mix. The gels were run in a vertical polyacrylamide electrophoresis system (Minigel-Twin, Biometra GmbH, Göttingen, Germany) starting with 50 V for 10 min and subsequently 200 V for about 2 h. The gel electrophoresis was performed in an anoxic glovebox (Coy Laboratory Products Inc., Grass Lake, MI, USA). H_2-oxidizing activity was visualized as follows: The gel was transferred to an anoxic Schott bottle filled with 50 mM Tris-HCl (pH 8.0) buffer containing 1 mM benzyl viologen (BV) as primary electron acceptor and redox mediator and 1 mM triphenyl tetrazolium chloride (TTC) as terminal electron acceptor for permanent staining. The bottle was then flushed with pure H_2 for 30 min. The incubation was carried out in a 28°C water bath until first bands were visible and then stopped by removing the native gel from the bottle. Fractions obtained from enrichment procedure were subjected to SDS-PAGE, which was silver stained after the run.

Photometric Measurement of Hydrogenase Activity

H_2-oxidizing activity was measured spectrophotometrically in butyl rubber stoppered glass cuvettes filled with 1 ml H_2-saturated buffer (50 mM Tris-HCl, pH 8.0) made anoxic by flushing with H_2 for 5 min. Redox dyes used were 1 mM benzyl viologen (BV), 1 mM methyl viologen (MV), or 1 mM methylene blue (MB) at 30°C. The colorization caused by the reduction of BV or MV was recorded at 578 nm and decolorization of MB was monitored at 570 nm. Additionally, activity was measured with the menaquinone analogs 1,4-naphthoquinone and 2,3-dimethyl-1,4-naphthoquinone (DMN). Changes in absorption during reduction of these derivatives were recorded at 270 nm. The anoxic reaction buffer contained 0.2 mM DMN or 1,4-naphthoquinone in 50 mM glycylglycine (pH 8.0) and 0.5 mM DTT. The assay was performed in rubber-stoppered quartz cuvettes, flushed with H_2. Reduction of DMN was recorded using the absorbance difference at 270 and 290 nm. Determination of protein concentration was done according to the method of Bradford (1976). Activity values are given in nanokatal (oxidation of 1 nmol H_2 per second). Temperature and pH dependance was measured with enriched hydrogenase using a 50 mM Britton-Robinson buffer system (50 mM H_3BO_3, 50 mM H_3PO_4, 50 mM acetate) in a pH range from 5.5 to 10 and a temperature range between 10 and 50°C.

Isolation of RNA, Reverse Transcription, and Quantitative Real-Time PCR

Isolation of total RNA from *S. multivorans* was done using the RNeasy minikit (Qiagen, Hilden, Germany). Remaining genomic DNA was removed with DNase I (RNase free; Roche, Mannheim, Germany). For quality check of the RNA, agarose gel electrophoresis was performed. For all quantitative real-time PCR (qPCR) experiments, the RNA was isolated in the mid-exponential growth phase of three independently grown cultures. For cDNA synthesis, 1 μg of RNA was used as starting material in the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Schwerte, Germany). The reaction mixture contained 1 μg RNA, 2.5 μl reverse primer, 3.5 μl 5x reaction buffer, and 2 μl 10 mM dNTP mix. It was filled up to a final volume of 17.5 μl with PCR-grade water (Fermentas, St. Leon Rot, Germany). To 10.5 μl of the mix, 0.5 μl RevertAid Reverse transcriptase (RT; 200 U/μl) was added, the residual amount (mix without RT) was used as negative control. The mix was incubated for 1 h at 42°C in a thermo cycler (Mastercycler, Personal, Eppendorf, Hamburg, Germany) and the reaction was stopped at 72°C for 5 min. Transcript levels of the different genes were compared by qPCR with the Maxima SYBR green qPCR master mix (Fermentas, St. Leon Rot, Germany); the primer pairs used are listed in Supplementary Table 1. The assay was performed in triplicates in a CFX96 qPCR machine (Bio-Rad, Munich, Germany). The PCR reaction mixture included 2.5 μl cDNA, 0.5 μM of each primer, and 6 μl 2x Maxima SYBR green qPCR master mix and was filled up to a final volume of 12 μl with PCR-grade water. Two negative controls were used, one with water as qPCR template and a reverse transcriptase negative control for each tested gene. Melting curve analysis was performed to exclude the formation of primer dimers or unspecific byproducts. Before performing the qPCR experiments, primer efficiency was tested for all primer pairs used in this study to ensure accurate and comparable amplification (see Supplementary Data Sheet 2). Three different control genes for normalization were tested: 16S

rRNA, *recA*, *rpoB*. Only the first was useful as control gene under all growth conditions applied. Obtained data were thus normalized to 16S cDNA (diluted 1:10,000) and the calculation of the relative gene expression level was done according to the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008), except where stated otherwise. For statistical analysis, student's *t*-test was performed on the $^{\Delta CT}$-values; *p*-values lower than 0.05 were regarded as significant.

RESULTS

Transcript Levels of Hydrogenase Genes under Different Growth Conditions

To determine and compare the transcript levels of the hydrogenase genes under different growth conditions, *S. multivorans* was grown with the following substrate combinations: pyruvate plus fumarate (standard condition), PCE, 5% O_2, or without external electron acceptor, or H_2 plus PCE or nitrate. In addition, N_2-fixing conditions were achieved with N_2 as sole nitrogen source. Prior to the main qPCR experiments, three housekeeping genes were tested for their suitability as reference genes: 16S rRNA, *recA* and *rpoB*. The latter was not considered further due to unspecific products and primer specificities out of an acceptable range (more than 10% deviation from primer specificities for the other primer pairs). The *recA* gene was unsuitable, since the transcript levels varied too much among replicates and under the different growth conditions (see Supplementary Data Sheet 2). Therefore, hydrogenase qPCR data were normalized to the 16S rRNA gene when comparing transcript levels under different growth conditions. For hydrogenase qPCR, the following genes encoding the catalytic subunits of each hydrogenase gene cluster were chosen: *hydB* (SMUL_1424) of the MBH gene cluster, *hupL* (SMUL_1422) from the cytoplasmic uptake hydrogenase (*hupSL*), *echE* (SMUL_1307) from the CooH-like hydrogenase and *hyfG* (SMUL_2388) from the Hyf hydrogenase. Primers used in qPCR are listed in Supplementary Table 1.

Transcripts of only two hydrogenase genes, *hydB* and *hyfG* (large subunits of the MBH and of Hyf) were found at levels of 0.03 to 0.08 (after normalization to 16S rRNA transcript level) under nearly all tested growth conditions. This was even the case when pyruvate rather than H_2 was used as electron donor in cells cultivated for at least 50 transfers with pyruvate as sole energy source. A long-term transcriptional regulatory effect, as was seen for *pceA* (John et al., 2009), can therefore be excluded. Opposed to *hydB* and *hyfG*, *hupL* and *echE* transcripts were not detected under most conditions, except very low transcript levels under pyruvate/fumarate (below 0.001, see **Figure 2** and Supplementary Data Sheet 2). To establish the hydrogenases that are involved in H_2-dependent PCE respiration, *S. multivorans* was grown in the presence of 100% H_2 in the gas phase as electron donor and 10 mM PCE in a hexadecane phase as electron acceptor. Only an insignificant increase in *hydB* transcript level (about twofold, *p*-value 0.224) and a decrease of *hyfG* (about threefold, *p*-value 0.562) was observed (**Figure 2**). To compare these transcript levels to those of another anaerobic H_2-dependent respiration, cells were grown with nitrate as electron acceptor and H_2 as electron donor. Nitrate rather than fumarate was chosen as electron acceptor, since *S. multivorans* was shown to be capable of growth with fumarate alone by disproportionation (Scholz-Muramatsu et al., 1995). The *hydB* gene transcript level dropped slightly, but insignificantly compared to H_2/PCE (twofold, *p*-value 0.384), while *hyfG* was reduced significantly in H_2/nitrate cells (about 30-fold lower; *p*-value 0.022) (**Figure 2**).

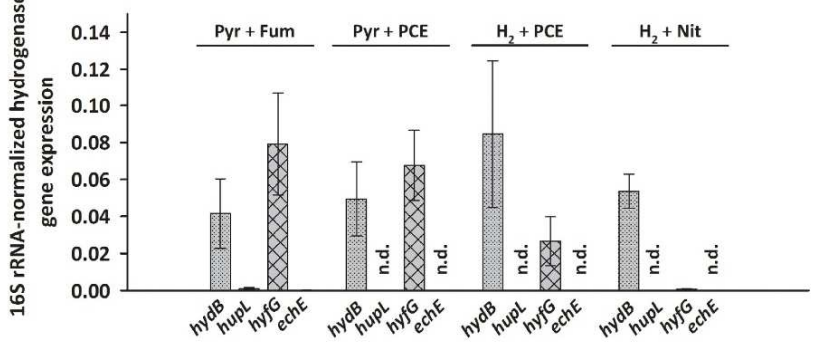

FIGURE 2 | Transcription pattern of hydrogenase catalytic subunit genes of *S. multivorans* grown under different growth conditions. Transcript levels of hydrogenase genes were normalized to those of the 16S rRNA gene. All data were obtained from three biological replicates and three technical replicates. When amplification was detected only in one biological replicate, the hydrogenase gene was designated as not detected (n.d.). The raw data of all values are shown in the Supplementary Data Sheet 2. *hydB*, membrane-bound hydrogenase (MBH); *hupL*, cytoplasmic uptake hydrogenase; *hyfG*, Hyf-hydrogenase; *echE*, Ech-like hydrogenase. Pyr, pyruvate; Fum, fumarate; Nit, nitrate; PCE, tetrachloroethene.

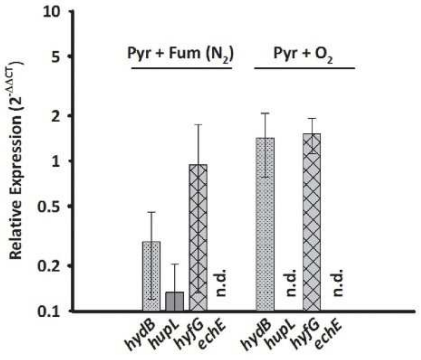

FIGURE 3 | Transcription pattern of hydrogenase catalytic subunit genes of *S. multivorans* with N_2 as sole N-source and 5% O_2 as electron acceptor. Transcript levels obtained from cells grown with N_2 as N-source and 5% O_2 were normalized to the transcript levels of the corresponding hydrogenase genes of pyruvate/fumarate grown cells. All data were obtained from three biological replicates and three technical replicates. When amplification was detected only in one biological replicate, the hydrogenase gene was designated as not detected (n.d.). The raw data of all values are shown in the Supplementary Data Sheet 2 and Figure 1 shows the data normalized to 16S rRNA gene. *hydB*, membrane-bound hydrogenase (MBH); *hupL*, cytoplasmic uptake hydrogenase; *hyfG*, Hyf-hydrogenase; *echE*, Ech-like hydrogenase. Pyr, pyruvate; Fum, fumarate; NH_4Cl, with ammonium chloride; N_2, N_2 as sole N-source.

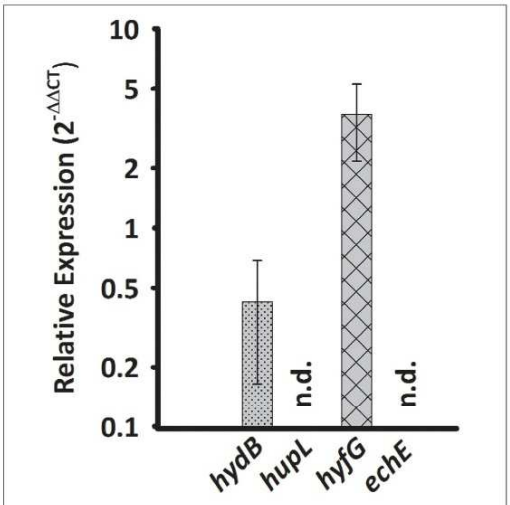

FIGURE 4 | Transcription pattern of hydrogenase catalytic subunit genes of *S. multivorans* grown fermentatively with pyruvate. Transcript levels obtained from cells grown with pyruvate in the absence of an electron acceptor were normalized to the transcript levels of the corresponding hydrogenase genes of pyruvate/fumarate grown cells. All data were obtained from three biological replicates and three technical replicates. When amplification was detected in less than two biological replicates, the hydrogenase gene was designated as not detected (n.d.). The raw data of all values are shown in the Supplementary Data Sheet 2 and Figure 1 shows the data normalized to 16S rRNA gene. *hydB*, membrane-bound hydrogenase (MBH); *hupL*, cytoplasmic uptake hydrogenase; *hyfG*, Hyf-hydrogenase; *echE*, Ech-like hydrogenase. Pyr, pyruvate; Fum, fumarate.

Since HupSL might be involved in recycling hydrogen produced during N_2 fixation, as was shown for similar enzymes in cyanobacteria (Tamagnini et al., 2007; Bothe et al., 2010) and *S. multivorans* was shown to fix N_2 (Ju et al., 2007; Goris et al., 2014), the transcript levels of *hupL* were investigated with N_2 as sole N-source. *S. multivorans* was grown under standard conditions (pyruvate/fumarate plus NH_4Cl as N-source) and with medium containing N_2 as sole N-source. The *hupL* transcript level did not increase with N_2 as sole N-source when compared to cells grown with NH_4^+. Instead, *hupL* and *hydB* levels decreased about 7.5- and 3-fold, albeit insignificantly with a *p*-value of 0.592 and 0.892, respectively (**Figure 3**).

Aerobic growth of *S. multivorans* with pyruvate and an O_2-concentration of about 5% in the gas phase was described recently (Goris et al., 2014). Since NiFe hydrogenases are generally inactivated by O_2 (Stiebritz and Reiher, 2012), it was of interest whether O_2 could possibly cause a down-regulation of hydrogenase gene expression as found for other bacteria (Kovács et al., 2005). Therefore, qPCR of cells grown in the presence of 5% O_2 in the gas phase with pyruvate as electron donor was performed to test if hydrogenase transcription occurs in the presence of oxygen. Cells were harvested at an O_2 concentration in the medium of at least 0.2 mg/L. Surprisingly, the transcript levels of *hydB* and *hyfG* were similar or insignificantly higher (about twofold for *hydB*, *p*-value of 0.553) under microoxic conditions compared to cells grown with fumarate as electron acceptor (**Figure 3**), while *hupL* and *echE* transcripts were not detected. The cells were, however, not able to grow with H_2 as electron donor and 5% O_2 as electron acceptor and acetate as carbon source.

Sulfurospirillum multivorans was described to grow fermentatively with pyruvate as the sole energy source (Scholz-Muramatsu et al., 1995). To investigate whether one of the putative H_2-producing hydrogenases shows higher transcript levels under this growth condition, we compared qPCR results obtained from pyruvate-grown cells with those from pyruvate/fumarate-grown cells. The *hydB* transcript level was lowered insignificantly (*p*-value 0.229) about threefold, while the *hyfG* level was increased by a factor of 3 with a *p*-value of 0.056 (**Figure 4**).

Biochemical Characterization of the Membrane-Bound Hydrogenase

To biochemically characterize the H_2-oxidizing enzyme of cells grown under PCE-respiring conditions, we measured the H_2-uptake activity of subcellular fractions from *S. multivorans* in different enzymatic assays. First, crude extracts of cells grown with pyruvate or H_2 as electron donor and PCE as

electron acceptor were tested spectrophotometrically for H_2-oxidizing activity with several different artificial redox mediators as electron acceptors. The activity with BV was by far the highest and about five times higher than the activities with MV and approximately tenfold of the activity with MB (**Figure 5A**). The other redox mediators tested, NAD^+, nitro blue tetrazolium chloride (NBT) or phenazine methosulfate (PMS), and negative controls without H_2 or without cell extracts showed no activity (Supplementary Table 2). In accordance with the quantitative PCR results, there was not much difference in the activity levels between pyruvate/PCE- and H_2/PCE-grown cells, with a specific H_2-oxidizing activity of 60 nkat/mg protein with BV as electron acceptor for pyruvate/PCE cells, 50 nkat/mg for pyruvate/fumarate cells and 70 nkat/mg for H_2/PCE cells (**Figure 5A**). Crude extracts obtained from cells grown with pyruvate as electron donor and 5% O_2 as electron acceptor showed H_2-dependent enzyme activity with BV (25 ± 2.3 nkat/mg). After subcellular fractionation, the highest specific activity (about 60 nkat/mg) was found in the MF while only around one tenth (7 and 5 nkat/mg for extracts obtained from pyruvate- and H_2-grown cells, respectively) was measured for the SF. The activities of crude extract, MF and solubilized MEs were about 50 to 70 nkat/mg (**Figure 5B**).

The different subcellular fractions were subjected to native PAGE followed by activity staining. This method allows to estimate the size and activity of catalytically active enzyme complexes and is often routinely used for the characterization of hydrogenases (Ballantine and Boxer, 1985; Pinske et al., 2012). For this purpose, *S. multivorans* cells were grown with either pyruvate or H_2 as electron donor and PCE as electron acceptor. Both, cell extracts and subcellular fractions (SF and ME solubilized with digitonin), were subjected to activity staining with BV as primary and TTC (triphenyl tetrazolium chloride) as secondary electron acceptor. After incubation of the gel with H_2 for 15–30 min at 28°C, a distinct band showing the typical reddish color of reduced TTC appeared at a size of about 270 kDa. This band was missing or only weakly visible in the SF (**Figure 6**). A prolonged incubation time up to 12 h did not lead to an additional band in the activity staining with membrane or SFs, suggesting the involvement of only one hydrogenase in H_2 oxidation, albeit a hydrogenase not performing H_2 oxidation under the given conditions might have been overlooked in this assay. The size of 270 kDa correlates to a dimeric form of the heterotrimeric *S. multivorans* MBH, which is predicted to be around 244 kDa (predicted sizes for the maturated MBH – large subunit HydB: 62 kDa, small subunit HydA: 34 kDa, and the membrane-integral cytochrome *b*, HydC: 26 kDa). This value, however, does not take into account the unknown contribution of the digitonin micelle size.

The presumable physiological electron acceptor for the MBH is menaquinone, as was shown for the MBH from *W. succinogenes* (Dross et al., 1992). To test the H_2-dependent reactivity of the MBH of *S. multivorans* with quinones, H_2 oxidation activity was measured spectrophotometrically with two different quinone analogs. Similar to the MBH of *W. succinogenes*, crude and MEs of *S. multivorans* showed activity with 2,3-dimethyl-1,4-naphtoquinone (DMN) (**Table 1**). Extracts from cells grown with H_2 showed approximately 10% higher activities than those from cells grown with pyruvate (**Table 1**). The hydrophilic quinone analog, 1,4-naphthoquinone, was neither reduced by the MBH of *S. multivorans* nor by that of *W. succinogenes* (Dross et al., 1992).

To investigate the involvement of a cytochrome *b*, spectroscopic changes in UV/VIS absorption of MFs were recorded before and after the addition of H_2. The MF showed an absorption maximum at 414 nm in the absence of H_2. After flushing the sample with H_2, the Soret band at 414 nm shifted to 427 nm and two peaks at 530 and 561 nm appeared (**Figure 7A**).

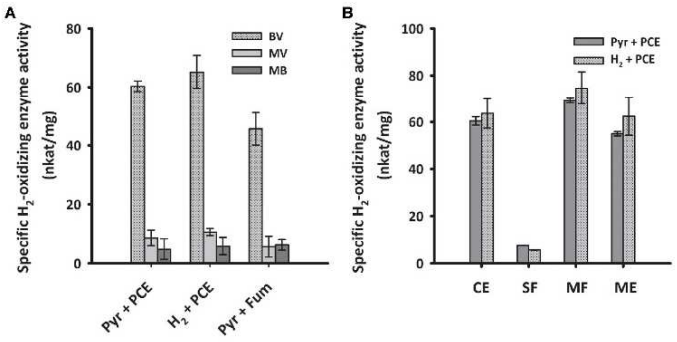

FIGURE 5 | H_2-oxidizing activities of crude extracts with different redox dyes (A) and enzyme activity of subcellular fractions of *S. multivorans* measured with benzyl viologen **(B)**. **(A)** Specific H_2-oxidizing enzyme activity (nkat/mg) of crude extracts from cells grown under different cultivation conditions. **(B)** Specific H_2-oxidizing enzyme activity (nkat/mg) of different cell fractions from pyruvate/PCE and H_2/PCE-grown cells. Data are calculated from three independent biological and technical replicates. Cells were fractionated according to the "Experimental Procedures" section and solubilization was done with 0.1% Triton X-100. For concentrations of redox dyes, see section "Experimental Procedures". BV, benzyl viologen; MV, methyl viologen; MB, methylene blue. Pyr, pyruvate; Fum, fumarate; CE, crude extract; SF, soluble fraction; MF, membrane fraction; ME, membrane extract.

FIGURE 6 | Analysis of the localization and oligomeric organization of the H_2-oxidizing enzyme of *S. multivorans*. Blue native activity stained gels of *S. multivorans* cell fractions are shown. Marker lane was cut off and silver stained separately. One microgram protein was applied to each lane. Cells were grown with pyruvate/PCE or H_2/PCE. MF, membrane fraction; SF, soluble fraction; ME, membrane extract; M, marker lane.

When calculating the difference spectra of H_2-reduced minus oxidized state, the spectrum showed α-, β-, and γ-absorption peaks at 561, 530, and 427 nm, respectively (**Figure 7B**). These spectral properties correspond to absorption spectra of *b*-type cytochromes (Yoon et al., 2008; Eguchi et al., 2012).

Enrichment of the Membrane-Bound Hydrogenase

Attempts to express the MBH of *S. multivorans* heterologously did not lead to the formation of an active enzyme, therefore, an enrichment from MEs was carried out (**Figure 8**). After solubilization of the MF with digitonin, 18% of hydrogenase enzyme activity from the MF could be recovered. An enrichment

TABLE 1 | H_2-oxidizing activities of different cell fractions with DMN as electron acceptor.

Growth substrates	Cell fraction	Specific hydrogenase activity (nkat/mg) H_2 –> DMN
Pyruvate + PCE	ME	41.9 ± 4.67
	MF	26.9 ± 3.14
H_2 + PCE	ME	54.4 ± 5.57
	MF	29.9 ± 6.38

DMN, 2,3-dimethyl-1,4-naphthoquinone; MF, membrane fraction; ME, membrane extract (solubilized membranes).

of 221-fold was obtained using anion exchange chromatography with a Q-Sepharose column (**Table 2**). After elution, the fractions with the highest activities (fractions 19–23) were applied to SDS-PAGE, native PAGE and BN activity staining (Supplementary Figure 2). In the latter, all fractions showed the remarkable red band at 270 kDa, which was most intense in fractions 20 and 21. Similar to the cell extracts, no second band was visible after prolonged incubation (up to 12 h) in the reaction mixture. Silver stained native PAGE showed the same 270 kDa band in all applied fractions with the most intense band in fraction 20 (**Figure 8A**). Other distinct bands in fraction 20 appeared around 50 and 130 kDa. After SDS-PAGE, all tested fractions showed a band at about 65 kDa, which corresponds to the size of HydB (62 kDa). This band was strongest in fractions 20 and 21. Additionally, a band at nearly 35 kDa was showing up in fractions 20 to 23, correlating to the theoretical size of the mature small subunit of the MBH (34 kDa). Several bands between 25 and 30 kDa were visible in all fractions, but none of them could be attributed clearly to HydC (26 kDa). One predominant band at 25 kDa observed in fractions 20 and 21 might represent a cytochrome *b*, especially since both fractions showed activity with DMN as electron acceptor (Supplementary Figure 2). Fraction 20 appeared to exhibit less additional bands than

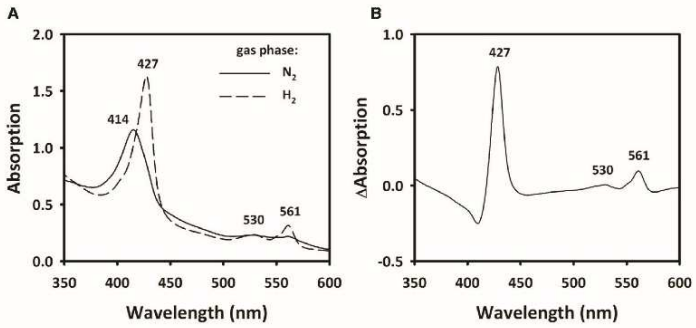

FIGURE 7 | Absorption spectra of membrane extract of *S. multivorans*. (A) The solid line represents the membrane fraction under N_2 as gas phase. Incubation of the sample for 10 min under H_2 leads to the spectrum represented by the dashed line **(B)** Difference spectra of the H_2-reduced minus N_2-incubated state. Numbers indicate wavelengths of characteristic absorption peaks of *b*-type cytochromes. Cells were grown with pyruvate/PCE.

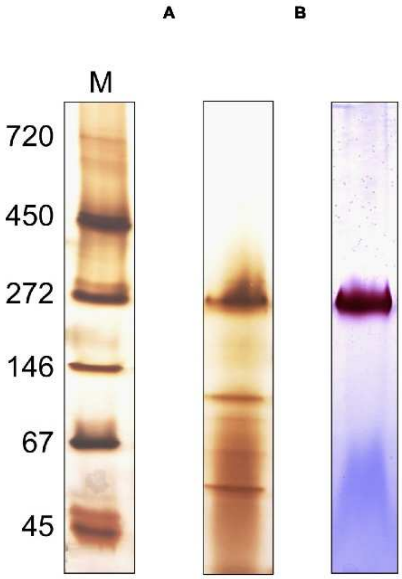

FIGURE 8 | Oligomeric analysis of the enriched MBH of *S. multivorans*. **(A)** Silver-stained Native PAGE and **(B)** activity stained BN-PAGE of the enriched MBH. Ten microgram of protein was applied on native PAGE and 1 μg for hydrogenase activity stain. M: marker lane. Cells were grown with pyruvate/fumarate.

fraction 21 after SDS-PAGE (Supplementary Figure 2), therefore, we chose this fraction for further purification. Hydrophobic interaction chromatography or gel filtration did not lead to further enrichment of active trimeric MBH, so that further characterization was performed with fraction 20. The presence of the membrane-integral cytochrome *b* (HydC) in this fraction was shown using UV/Vis spectroscopy. The same characteristic absorption spectra and difference spectrum were recorded as those obtained for the MF (Supplementary Figure 3). Compared to the membranes, the enriched MBH showed a higher absorption in the difference spectrum, suggesting a higher cytochrome *b* concentration. The optimal condition for H_2 oxidation by fraction 20 was at pH 8.0 and 40°C when BV was used as electron acceptor in the photometric assay.

DISCUSSION

To identify hydrogenases involved in the hydrogen-oxidation during PCE respiration in *S. multivorans*, we performed transcription analysis and biochemical investigations with cells grown in the presence of different electron donor/acceptor combinations. Of the previously identified four NiFe hydrogenases encoded in the genome of *S. multivorans*, a membrane-bound hydrogenase similar to the hydrogen-oxidizing MBH from *W. succinogenes* was suggested to play a role in hydrogen oxidation during PCE respiration. However, the only evidence for this assumption was based on amino acid sequence similarities to other hydrogen-oxidizing enzymes and cell extracts grown with H_2 and fumarate (Miller et al., 1996; Goris et al., 2014), which did not allow for an unambiguous conclusion of its role in PCE respiration. Since up to now no detailed information on the composition of organohalide respiratory chains is available, it is feasible that one of the other three hydrogenases might be involved. In the transcript level analysis presented here, only two of the four hydrogenase large subunit genes were found to be transcribed to a considerable amount. The first is *hydB*, encoding the large subunit of the MBH similar to that of *W. succinogenes*, while the second, *hyfG*, belongs to a group 4 hydrogenase (Vignais and Billoud, 2007) similar to hydrogenases 3 and 4 of *E. coli* (Hyc and Hyf). The *hydB* transcript level was found not to be significantly altered during any of the tested growth conditions. The presence of the MBH and Hyf with pyruvate or formate as electron donor was already seen in a previous proteomic study (Goris et al., 2015) (Supplementary Data Sheet 3). This points toward a constitutive expression of the MBH in *S. multivorans*. As this was tested in cells long-term cultivated without H_2, a regulatory effect as reported earlier for PCE respiration (John et al., 2009) can be also excluded.

Post-transcriptional regulation and maturation could also have an influence on the hydrogen-oxidizing activity and the responsible hydrogenase. The biochemical analyses with extracts obtained from cells grown under different conditions revealed comparable H_2-oxidizing activities regardless of the electron donor or acceptor used. Also, H_2 had no apparent influence on the maturation of the MBH, since the H_2-oxidizing activity in the ME did not change when cultures were grown with H_2. Furthermore, we conclude that only one hydrogenase plays a role in hydrogen oxidation under all growth conditions tested, since only one band was detected in BN activity staining regardless of the growth condition. However, the blue native activity staining was performed only with the primary electron acceptor BV. Therefore, the presence of another hydrogen-oxidizing enzyme

TABLE 2 | Enrichment of membrane-bound hydrogenase of *S. multivorans*.

Purification step	Activity (nkat/ml)	Protein (mg/ml)	Specific activity (nkat/mg)	Yield (%)	Purification (fold)
Membrane fraction	92	20	4.6	100	1
Digitonin solubilization	403.1	3.6	112	18	24.3
Q-Sepharose HP	1326	1.3	1020	6.5	221

Enzyme activity was measured with benzyl viologen. Cells were grown with pyruvate/fumarate.

requiring a different electron acceptor cannot be completely ruled out. Activity-stained BN PAGE of a hydrogen-oxidizing enzyme enriched from *S. multivorans* showed a band corresponding to the same size. SDS PAGE analysis of the subunit composition revealed subunits with molecular masses corresponding to the three subunits HydABC of the MBH. Spectroscopic analysis of the enriched enzyme revealed characteristic spectra of *b*-type cytochromes, indicating the presence of a cytochrome *b* in the enrichment. These results are in accordance with spectra obtained from membranes and from the purified MBH of *W. succinogenes*. In addition, the H_2-dependent reduction of the quinone analog DMN indicates that electrons derived from H_2 oxidation are transferred via the membrane-integral cytochrome *b* to the menaquinone pool (**Figure 9A**). Previous studies suggested the involvement of menaquinone and a quinol dehydrogenase in the PCE respiratory chain (Goris et al., 2014, 2015), which supports the assumption that the MBH-mediated hydrogen oxidation is coupled to PCE reduction via menaquinone.

The apparent molecular mass of the native enzyme points to a heterotrimeric MBH dimer. This is opposed to a putative trimeric composition of the heterotrimeric complex of *Cupriavidus necator* MBH (Frielingsdorf et al., 2011). Instead, the *S. multivorans* complex resembles the *E. coli* hydrogenase 1 crystallized as a heterotrimer (Volbeda et al., 2013), albeit the latter was reported to contain only one cytochrome for a hydrogenase dimer. A dimeric structure is not only found for *E. coli* hydrogenase 1, but also found in several other hydrogenase crystal structures, although these were solved without a membrane-integral subunit (Wulff et al., 2016).

The broad array of growth conditions used for real-time PCR experiments in this study revealed that under none of the conditions tested neither *hupL* nor *echE* were induced. In contrast, *hydB* was expressed under all growth conditions, even with O_2 as electron acceptor. The latter observation was surprising, since Epsilonproteobacteria including *S. multivorans* do not harbor oxygen-tolerant NiFe hydrogenases and most NiFe hydrogenases suffer from inactivation when exposed to O_2. Therefore, many facultative anaerobic bacteria employ mechanisms to down-regulate the expression of hydrogenase genes in the presence of O_2. For example, hydrogenase gene transcription is under control of regulators ArcAB and Fnr in other facultative anaerobic proteobacteria including *E. coli* (Richard et al., 1999; Kovács et al., 2005). None of these regulators is found in Epsilonproteobacteria, which might explain the lack of O_2-induced down-regulation of hydrogenase gene expression. Many free-living Epsilonproteobacteria are facultative microaerophilic and often found in the oxic–anoxic interface, where the O_2 concentration varies (Vetriani et al., 2003; Grote et al., 2012). Therefore, a constitutive expression of the hydrogen-uptake system and the lack of an O_2-sensitive regulation system may be beneficial to these organisms. Although all attempts to grow *S. multivorans* with H_2 and O_2 as energy substrates failed so far, it is feasible that the organism might use H_2 as additional electron donor under very low O_2 partial pressure as described elsewhere for other Epsilonproteobacteria (e.g., *Campylobacter jejuni* and *Helicobacter pylori*) (Laanbroek et al., 1977, 1978; Carlone and Lascelles, 1982; Maier et al., 2003). Since crude extracts from cells grown with O_2 still showed around half of the activity as anaerobically grown cells, this seems feasible for *S. multivorans*.

The presence of H_2 in the gas phase did not raise the transcript level of the MBH significantly. On the molecular level, this might be attributed to a missing H_2 sensor such as that found in some "Knallgas" bacteria (Kleihues et al., 2000; Friedrich et al., 2005). The absence of regulatory proteins with the exception of the nickel-dependent NikR (Goris et al., 2014) is in accordance with a constitutive expression of the MBH in *S. multivorans*. The constitutive expression of the MBH might also be an advantage in anoxic environments where nutrient composition and H_2 levels are often changing.

The role of the Hyf hydrogenase in *S. multivorans* remains enigmatic. The key enzyme in mixed acid fermentation in *E. coli* is the pyruvate formate lyase (PFL), generating formate, which is the substrate for the FHL complex. An ortholog of the PFL is not encoded in the genome of *S. multivorans*. While the amino acid sequences of *S. multivorans* Hyf subunits are similar (33 to 50% amino acid sequence identity) to those of hydrogenase 4 of *E. coli*, the *S. multivorans hyf* gene cluster does not contain any genes encoding formate-metabolism related proteins as found in the *hyf* cluster of *E. coli* (e.g., the formate channel *focB* or a formate-sensitive transcriptional regulator). A gene encoding a putative cytoplasmic formate dehydrogenase is found in the genome of *S. multivorans*, but the according protein sequence shows only 30% amino acid sequence identity to FdhF of the FHL complex of *E. coli*. Additionally, an ortholog of the *S. multivorans* Fdh is found in many epsilonproteobacterial genomes not encoding the Hyf hydrogenase. All this renders participation of Hyf in an FHL complex in *S. multivorans* an unlikely scenario. Instead, we assume that Hyf could serve to dispose of excess reducing equivalents, accepting electrons, e.g., from ferredoxin reduced by a pyruvate:ferredoxin oxidoreductase (PFOR), similar to the group 4 hydrogenase of *Pyrococcus furiosus* (McTernan et al., 2015). The ferredoxin and the PFOR were found to be present in the proteome in *S. multivorans* previously (Goris et al., 2015). We assume that *S. multivorans* produces H_2 especially under fermentative conditions (**Figure 9B**) or when the concentration of the electron acceptor may be limiting. This is probably the case for PCE, which, even in polluted environments, could be present at low concentrations due to its low solubility. In contrast, nitrate may be available at higher concentrations, which might explain the down-regulation exclusively with H_2/nitrate as substrates. The role and function of this hydrogenase is subject to further studies currently conducted in our laboratory.

The role of the cytoplasmic uptake hydrogenase HupSL remains unclear in *S. multivorans*. A TetR-like transcriptional repressor which is encoded directly upstream of the *hupSL* cluster (Goris et al., 2014) might repress the transcription of the enzyme under the tested growth conditions. The function of HupSL in recycling of H_2 as a byproduct of N_2 fixation can be excluded, since growth with N_2 as sole N-source did not lead to an elevated transcript level, which would have been expected in that case. A role in reducing a cytoplasmic low-potential

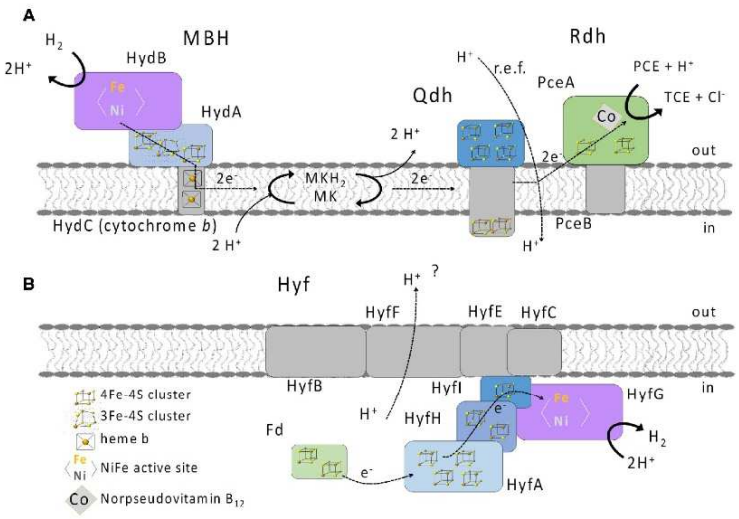

FIGURE 9 | Putative physiological roles of the MBH (A) and Hyf hydrogenase **(B)** in *S. multivorans*. Qdh, quinol dehydrogenase; PceAB, PCE reductive dehalogenase; MK, menaquinone; MKH2, reduced MK; Fd, ferredoxin; PCE, tetrachloroethene; TCE, trichloroethene; i, inside (cytoplasm); o, outside (periplasm).

electron donor such as reduced ferredoxin providing electrons for the reductive TCA cycle seems feasible, as was suggested for a similar hydrogenase in *A. aeolicus* (Guiral et al., 2005). The proteins for the rTCA cycle are encoded in the genome of *S. multivorans* (Goris et al., 2014), but autotrophic growth was never observed. Like for the *hupL* gene, also *echE* transcripts were only found in negligible amounts in this study. All Ech subunits bear high similarity to the CO-induced hydrogenase of *C. hydrogenoformans*, in which the hydrogenase genes are located adjacent to a CO dehydrogenase gene (Soboh et al., 2002). In the genome of *Sulfurospirillum* sp. SCADC, this structure is still conserved (Tan and Foght, 2014; Goris and Diekert, 2016) and *S. carboxydovorans* is reported to use CO as electron donor (Jensen and Finster, 2005). Hence, a loss of the CO dehydrogenase gene and non-functionality of the CO-induced like hydrogenase in *S. multivorans* is possible. However, it is also feasible that growth conditions, which induce transcription of the *hup* and *ech* genes, were not tested in this study. For example, pH variation or different levels of H_2 concentrations, which could have an effect on hydrogenase expression, was not included here.

CONCLUSION

In this study we showed that *S. multivorans* involves a membrane-bound NiFe hydrogenase similar to the MBH of *W. succinogenes* for H_2 oxidation in the PCE-respiratory chain. The MBH, which was enriched with a redox-active, membrane-integral cytochrome *b*, reacts most likely with the quinone pool and forms a native complex of around 270 kDa. The transcript studies presented here show that the MBH is not subject to a distinct H_2- or O_2-dependent regulation as reported for other organisms. Besides the MBH, only one more hydrogenase gene transcript (Hyf type) was found under the growth conditions applied in this study. This cytoplasmic, membrane-bound hydrogenase complex with similarities to Hyf from *E. coli* is most likely playing a role in proton reduction under electron acceptor-limiting conditions.

Taken together, this study allows a deeper insight into the H_2 metabolism of a model organohalide-respiring bacterium and a free-living Epsilonproteobacterium.

AUTHOR CONTRIBUTIONS

TG and GD initiated and supervised the study, SK and TG designed experiments, analyzed data and drafted the manuscript. SK performed the biochemical and real-time PCR experiments, MW performed initial real-time PCR experiments, including primer design and testing of optimal conditions, XW performed initial biochemical experiments. All authors curated, read, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00444/full#supplementary-material

REFERENCES

Ballantine, S. P., and Boxer, D. H. (1985). Nickel-containing hydrogenase isoenzymes from anaerobically grown *Escherichia coli* K-12. *J. Bacteriol.* 163, 454–459.

Bothe, H., Schmitz, O., Yates, M. G., and Newton, W. E. (2010). Nitrogen fixation and hydrogen metabolism in cyanobacteria. *Microbiol. Mol. Biol. Rev.* 74, 529–551. doi: 10.1128/MMBR.00033-10

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254. doi: 10.1016/0003-2697(76)90527-3

Carlone, G. M., and Lascelles, J. (1982). Aerobic and anaerobic respiratory systems in *Campylobacter fetus* subsp. *jejuni* grown in atmospheres containing hydrogen. *J. Bacteriol.* 152, 306–314.

Dross, F., Geisler, V., Lenger, R., Theis, F., Krafft, T., Fahrenholz, F., et al. (1992). The quinone-reactive Ni/Fe-hydrogenase of *Wolinella succinogenes*. *Eur. J. Biochem.* 206, 93–102. doi: 10.1111/j.1432-1033.1992.tb16905.x

Eguchi, S., Yoon, K. S., and Ogo, S. (2012). O2-stable membrane-bound [NiFe]hydrogenase from a newly isolated *Citrobacter* sp. S-77. *J. Biosci. Bioeng.* 114, 479–484. doi: 10.1016/j.jbiosc.2012.05.018

Friedrich, B., Buhrke, T., Burgdorf, T., and Lenz, O. (2005). A hydrogen-sensing multiprotein complex controls aerobic hydrogen metabolism in *Ralstonia eutropha*. *Biochem. Soc. Trans.* 33, 97–101. doi: 10.1042/BST0330097

Frielingsdorf, S., Schubert, T., Pohlmann, A., Lenz, O., and Friedrich, B. (2011). A trimeric supercomplex of the oxygen-tolerant membrane-bound [NiFe]-hydrogenase from *Ralstonia eutropha* H16. *Biochemistry* 50, 10836–10843. doi: 10.1021/bi201594m

Goris, T., and Diekert, G. (2016). "The genus *Sulfurospirillum*," in *Organohalide-Respiring Bacteria*, eds L. Adrian and F. Löffler (Berlin: Springer).

Goris, T., Schiffmann, C. L., Gadkari, J., Schubert, T., Seifert, J., Jehmlich, N., et al. (2015). Proteomics of the organohalide-respiring Epsilonproteobacterium *Sulfurospirillum multivorans* adapted to tetrachloroethene and other energy substrates. *Sci. Rep.* 5:13794. doi: 10.1038/srep13794

Goris, T., Schubert, T., Gadkari, J., Wubet, T., Tarkka, M., Buscot, F., et al. (2014). Insights into organohalide respiration and the versatile catabolism of *Sulfurospirillum multivorans* gained from comparative genomics and physiological studies. *Environ. Microbiol.* 16, 3562–3580. doi: 10.1111/1462-2920.12589

Greening, C., Biswas, A., Carere, C. R., Jackson, C. J., Taylor, M. C., Stott, M. B., et al. (2016). Genomic and metagenomic surveys of hydrogenase distribution indicate H2 is a widely utilised energy source for microbial growth and survival. *ISME J.* 10, 761–777. doi: 10.1038/ismej.2015.153

Greening, C., and Cook, G. M. (2014). Integration of hydrogenase expression and hydrogen sensing in bacterial cell physiology. *Curr. Opin. Microbiol.* 18, 30–38. doi: 10.1016/j.mib.2014.02.001

Gross, R., Pisa, R., Sänger, M., Lancaster, C. R., and Simon, J. (2004). Characterization of the menaquinone reduction site in the diheme cytochrome b membrane anchor of *Wolinella succinogenes* NiFe-hydrogenase. *J. Biol. Chem.* 279, 274–281. doi: 10.1074/jbc.M310610200

Grote, J., Schott, T., Bruckner, C. G., Glöckner, F. O., Jost, G., Teeling, H., et al. (2012). Genome and physiology of a model Epsilonproteobacterium responsible for sulfide detoxification in marine oxygen depletion zones. *Proc. Natl. Acad. Sci. U.S.A.* 109, 506–510. doi: 10.1073/pnas.1111262109

Guiral, M., Aubert, C., and Giudici-Orticoni, M. T. (2005). Hydrogen metabolism in the hyperthermophilic bacterium *Aquifex aeolicus*. *Biochem. Soc. Trans.* 33, 22–24. doi: 10.1042/BST0330022

Hedderich, R., and Forzi, L. (2005). Energy-converting [NiFe] hydrogenases: more than just H2 activation. *J. Mol. Microbiol. Biotechnol.* 10, 92–104. doi: 10.1159/000091557

Holliger, C., Hahn, D., Harmsen, H., Ludwig, W., Schumacher, W., Tindall, B., et al. (1998). *Dehalobacter restrictus* gen. nov. and sp. nov., a strictly anaerobic bacterium that reductively dechlorinates tetra- and trichloroethene in an anaerobic respiration. *Arch. Microbiol.* 169, 313–321. doi: 10.1007/s002030050577

Jensen, A., and Finster, K. (2005). Isolation and characterization of *Sulfurospirillum carboxydovorans* sp nov., a new microaerophilic carbon monoxide oxidizing epsilon Proteobacterium. *Antonie Van Leeuwenhoek* 87, 339–353. doi: 10.1007/s10482-004-6839-y

John, M., Rubick, R., Schmitz, R. P., Rakoczy, J., Schubert, T., and Diekert, G. (2009). Retentive memory of bacteria: long-term regulation of dehalorespiration in *Sulfurospirillum multivorans*. *J. Bacteriol.* 191, 1650–1655. doi: 10.1128/JB.00597-08

Ju, X., Zhao, L., and Sun, B. (2007). Nitrogen fixation by reductively dechlorinating bacteria. *Environ. Microbiol.* 9, 1078–1083. doi: 10.1111/j.1462-2920.2006.01199.x

Kleihues, L., Lenz, O., Bernhard, M., Buhrke, T., and Friedrich, B. (2000). The H2 sensor of *Ralstonia eutropha* is a member of the subclass of regulatory [NiFe] hydrogenases. *J. Bacteriol.* 182, 2716–2724. doi: 10.1128/JB.182.10.2716-2724.2000

Kovács, A. T., Rákhely, G., Balogh, J., Maróti, G., Fülöp, A., and Kovács, K. L. (2005). Anaerobic regulation of hydrogenase transcription in different bacteria. *Biochem. Soc. Trans.* 33, 36–38. doi: 10.1042/BST0330036

Kruse, T., Maillard, J., Goodwin, L., Woyke, T., Teshima, H., Bruce, D., et al. (2013). Complete genome sequence of *Dehalobacter restrictus* PER-K23(T.). *Stand. Genomic Sci.* 8, 375–388. doi: 10.4056/sigs.3787426

Kruse, T., van de Pas, B. A., Atteia, A., Krab, K., Hagen, W. R., Goodwin, L., et al. (2015). Genomic, proteomic, and biochemical analysis of the organohalide respiratory pathway in *Desulfitobacterium dehalogenans*. *J. Bacteriol.* 197, 893–904. doi: 10.1128/JB.02370-14

Kube, M., Beck, A., Zinder, S. H., Kuhl, H., Reinhardt, R., and Adrian, L. (2005). Genome sequence of the chlorinated compound-respiring bacterium *Dehalococcoides* species strain CBDB1. *Nat. Biotechnol.* 23, 1269–1273. doi: 10.1038/nbt1131

Laanbroek, H., Kingma, W., and Veldkamp, H. (1977). Isolation of an aspartate-fermenting, free-living *Campylobacter* species. *FEMS Microbiol. Lett.* 1, 99–102. doi: 10.1111/j.1574-6968.1977.tb00590.x

Laanbroek, H. J., Stal, L. H., and Veldkamp, H. (1978). Utilization of hydrogen and formate by *Campylobacter* spec. under aerobic and anaerobic conditions. *Arch. Microbiol.* 119, 99–102. doi: 10.1007/BF00407935

Löffler, F. E., Yan, J., Ritalahti, K. M., Adrian, L., Edwards, E. A., Konstantinidis, K. T., et al. (2013). *Dehalococcoides mccartyi* gen. nov., sp. nov., obligately organohalide-respiring anaerobic bacteria relevant to halogen cycling and bioremediation, belong to a novel bacterial class, *Dehalococcoidia* classis nov., order *Dehalococcoidales* ord. nov. and family *Dehalococcoidaceae* fam. nov., within the phylum *Chloroflexi*. *Int. J. Syst. Evol. Microbiol.* 63, 625–635. doi: 10.1099/ijs.0.034926-0

Luijten, M. L., de Weert, J., Smidt, H., Boschker, H. T., de Vos, W. M., Schraa, G., et al. (2003). Description of *Sulfurospirillum halorespirans* sp. nov., an anaerobic, tetrachloroethene-respiring bacterium, and transfer of *Dehalospirillum multivorans* to the genus *Sulfurospirillum* as *Sulfurospirillum multivorans* comb. nov. *Int. J. Syst. Evol. Microbiol.* 53, 787–793. doi: 10.1099/ijs.0.02417-0

Maier, R. J., Olson, J., and Olczak, A. (2003). Hydrogen-oxidizing capabilities of *Helicobacter hepaticus* and in vivo availability of the substrate. *J. Bacteriol.* 185, 2680–2682. doi: 10.1128/JB.185.8.2680-2682.2003

Mansfeldt, C. B., Rowe, A. R., Heavner, G. L., Zinder, S. H., and Richardson, R. E. (2014). Meta-analyses of *Dehalococcoides mccartyi* strain 195 transcriptomic profiles identify a respiration rate-related gene expression

transition point and interoperon recruitment of a key oxidoreductase subunit. *Appl. Environ. Microbiol.* 80, 6062–6072. doi: 10.1128/AEM.02130-14

McTernan, P. M., Chandrayan, S. K., Wu, C. H., Vaccaro, B. J., Lancaster, W. A., and Adams, M. W. (2015). Engineering the respiratory membrane-bound hydrogenase of the hyperthermophilic archaeon *Pyrococcus furiosus* and characterization of the catalytically active cytoplasmic subcomplex. *Protein Eng. Des. Sel.* 28, 1–8. doi: 10.1093/protein/gzu051

Miller, E., Wohlfarth, G., and Diekert, G. (1996). *Studies on tetrachloroethene respiration in Dehalospirillum multivorans. Arch. Microbiol.* 166, 379–387. doi: 10.1007/BF01682983

Nonaka, H., Keresztes, G., Shinoda, Y., Ikenaga, Y., Abe, M., Naito, K., et al. (2006). Complete genome sequence of the dehalorespiring bacterium *Desulfitobacterium hafniense* Y51 and comparison with *Dehalococcoides ethenogenes* 195. *J. Bacteriol.* 188, 2262–2274. doi: 10.1128/JB.188.6.2262-2274.2006

Pinske, C., Jaroschinsky, M., Sargent, F., and Sawers, G. (2012). Zymographic differentiation of [NiFe]-hydrogenases 1, 2 and 3 of *Escherichia coli* K-12. *BMC Microbiol.* 12:134. doi: 10.1186/1471-2180-12-134

Richard, D. J., Sawers, G., Sargent, F., McWalter, L., and Boxer, D. H. (1999). Transcriptional regulation in response to oxygen and nitrate of the operons encoding the [NiFe] hydrogenases 1 and 2 of *Escherichia coli*. *Microbiology* 145(Pt 10), 2903–2912. doi: 10.1099/00221287-145-10-2903

Rupakula, A., Kruse, T., Boeren, S., Holliger, C., Smidt, H., and Maillard, J. (2013). The restricted metabolism of the obligate organohalide respiring bacterium *Dehalobacter restrictus*: lessons from tiered functional genomics. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 368, 20120325. doi: 10.1098/rstb.2012.0325

Sargent, F. (2016). The model [NiFe]-hydrogenases of *Escherichia coli*. *Adv. Microb. Physiol.* 68, 433–507. doi: 10.1016/bs.ampbs.2016.02.008

Schmittgen, T. D., and Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc.* 3, 1101–1108. doi: 10.1038/nprot.2008.73

Scholz-Muramatsu, H., Neumann, A., Messmer, M., Moore, E., and Dieker, G. (1995). Isolation and characterization of *Dehalospirillum multivorans* gen. nov., sp. nov., a tetrachloroethene-utilizing, strictly anaerobic bacterium. *Arch. Microbiol.* 163, 48–56. doi: 10.1007/BF00262203

Seshadri, R., Adrian, L., Fouts, D. E., Eisen, J. A., Phillippy, A. M., Methe, B. A., et al. (2005). Genome sequence of the PCE-dechlorinating bacterium *Dehalococcoides ethenogenes*. *Science* 307, 105–108. doi: 10.1126/science.1102226

Soboh, B., Linder, D., and Hedderich, R. (2002). Purification and catalytic properties of a CO-oxidizing:H2-evolving enzyme complex from *Carboxydothermus hydrogenoformans*. *Eur. J. Biochem.* 269, 5712–5721. doi: 10.1046/j.1432-1033.2002.03282.x

Stiebritz, M., and Reiher, M. (2012). Hydrogenases and oxygen. *Chem. Sci.* 3, 1739–1751. doi: 10.1039/c2sc01112c

Tamagnini, P., Leitão, E., Oliveira, P., Ferreira, D., Pinto, F., Harris, D. J., et al. (2007). Cyanobacterial hydrogenases: diversity, regulation and applications. *FEMS Microbiol. Rev.* 31, 692–720. doi: 10.1111/j.1574-6976.2007.00085.x

Tan, B., and Foght, J. (2014). Draft genome sequences of *Campylobacterales (Epsilonproteobacteria)* obtained from methanogenic oil sands tailings pond metagenomes. *Genome Announc.* 2:e1034-14. doi: 10.1128/genomeA.01034-14

Trchounian, K., Poladyan, A., Vassilian, A., and Trchounian, A. (2012). Multiple and reversible hydrogenases for hydrogen production by *Escherichia coli*: dependence on fermentation substrate, pH and the F(0)F(1)-ATPase. *Crit. Rev. Biochem. Mol. Biol.* 47, 236–249. doi: 10.3109/10409238.2012.655375

Vetriani, C., Tran, H. V., and Kerkhof, L. J. (2003). Fingerprinting microbial assemblages from the oxic/anoxic chemocline of the Black Sea. *Appl. Environ. Microbiol.* 69, 6481–6488. doi: 10.1128/AEM.69.11.6481-6488.2003

Vignais, P. M., and Billoud, B. (2007). Occurrence, classification, and biological function of hydrogenases: an overview. *Chem. Rev.* 107, 4206–4272. doi: 10.1021/cr050196r

Villemur, R., Lanthier, M., Beaudet, R., and Lepine, F. (2006). The *Desulfitobacterium* genus. *FEMS Microbiol. Rev.* 30, 706–733. doi: 10.1111/j.1574-6976.2006.00029.x

Volbeda, A., Darnault, C., Parkin, A., Sargent, F., Armstrong, F. A., and Fontecilla-Camps, J. C. (2013). Crystal structure of the O(2)-tolerant membrane-bound hydrogenase 1 from *Escherichia coli* in complex with its cognate cytochrome b. *Structure* 21, 184–190. doi: 10.1016/j.str.2012.11.010

Welte, C., Kratzer, C., and Deppenmeier, U. (2010). Involvement of Ech hydrogenase in energy conservation of *Methanosarcina mazei*. *FEBS J.* 277, 3396–3403. doi: 10.1111/j.1742-4658.2010.07744.x

Wulff, P., Thomas, C., Sargent, F., and Armstrong, F. A. (2016). How the oxygen tolerance of a [NiFe]-hydrogenase depends on quaternary structure. *J. Biol. Inorg. Chem.* 21, 121–134. doi: 10.1007/s00775-015-1327-6

Yoon, K. S., Tsukada, N., Sakai, Y., Ishii, M., Igarashi, Y., and Nishihara, H. (2008). Isolation and characterization of a new facultatively autotrophic hydrogen-oxidizing Betaproteobacterium, *Hydrogenophaga* sp. AH-24. *FEMS Microbiol. Lett.* 278, 94–100. doi: 10.1111/j.1574-6968.2007.00983.x

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2.2 Hydrogen production by *Sulfurospirillum* spp. enables syntrophic interactions of Epsilonproteobacteria

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Based on the previous study investigating the hydrogen metabolism of *Sulfurospirillum multivorans*, the hydrogen production capacity and metabolism of several *Sulfurospirillum* spp. and an involvement of the putative hydrogen producing hydrogenase was elucidated. Cultivation experiments on a fermentative growth condition revealed a hydrogen production of Epsilonproteobacteria for the first time which were described and characterized as hydrogen consumers exclusively. Enzyme assays, comparative proteomics and genomics showed two different pyruvate fermentation pathways among the tested *Sulfurospirillum* spp. and a multisubunit, membrane-bound hydrogenase was identified being responsible for the hydrogen production. Furthermore, the hydrogen production capacity of *S. multivorans* allocated a new ecological role as a syntrophic partner in microbial communities and an interspecies hydrogen transfer with a hydrogen consuming methanogen in a co-culture experiment was observed.

My own contributions to this publication covers about: 50%.

All growth experiments and enzyme assays were conducted by myself. The mass spectrometric measurements were done in cooperation with Lorenz Adrian (Centre for Environmental Research, Leipzig). Electron microscopy was done in cooperation with Martin Westermann (University Hospital Jena). Tobias Goris and Gabriele Diekert initiated and supervised the study. Design of the experiments, analysis of data and drafting of the manuscript were done by Stefan Kruse and Tobias Goris.

Supplementary information is given in appendix, pp. x - xxix

Hydrogen production by *Sulfurospirillum* spp. enables syntrophic interactions of Epsilonproteobacteria

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Abstract

Hydrogen-producing bacteria are of environmental and biotechnological importance in anoxic environments, since hydrogen is an important electron donor for prokaryotes and of interest as an alternative energy source. Epsilonproteobacteria, inhabiting ecologically, clinically or biotechnologically relevant environments, are currently considered to be hydrogen-oxidizing bacteria exclusively. Here, we report hydrogen production upon pyruvate fermentation for a genus of free-living Epsilonproteobacteria, *Sulfurospirillum* spp., which inhabit e.g. sediments, wastewater plants, bioelectrodes, oil reservoirs, contaminated areas, and marine habitats. The amount of hydrogen production was largely different in two subgroups of *Sulfurospirillum* spp., represented by *S. cavolei* and *S. multivorans*. The former is shown to be the more potent hydrogen producer and excretes acetate as sole organic acid, while the latter exhibited a more flexible fermentation, producing additionally lactate and succinate. The observed hydrogen production could be assigned to a group 4 hydrogenase similar to hydrogenase 4 (Hyf) in *E. coli*. We propose that *Sulfurospirillum* spp. produce molecular hydrogen with electrons derived from pyruvate oxidation by pyruvate:ferredoxin oxidoreductase and reduced ferredoxin. This hypothesis is supported by comparative proteome data, in which both PFOR and ferredoxin as well as hydrogenase 4 are up-regulated in fermentatively cultivated cells. A co-culture experiment with *S. multivorans* and *Methanococcus voltae* cultivated with lactate as sole substrate shows a syntrophic interaction between both organisms, since the former cannot grow fermentatively on lactate alone and the latter relies on hydrogen as electron donor. This opens up new perspectives on microbial communities, since Epsilonproteobacteria could play a yet unrecognized role as hydrogen producers in anoxic microbial communities.

Introduction

Hydrogen gas (H_2), an important energy substrate for many bacteria and archaea, plays a crucial role in the anaerobic food web, e.g. in syntrophic interactions. It is produced by fermenting bacteria as a result of the disposal of excess reducing equivalents. Other prokaryotes may use it as an electron donor for e.g. sulfate respiration or methanogenesis. In syntrophic interactions, the H_2-producing bacterium is dependent on the H_2 uptake by its syntrophic partner, which sustains a low H_2 partial pressure and thus enables H_2 production, which would otherwise thermodynamically be unfavorable[1-3]. For example, butyrate, propionate or acetate-oxidizing anaerobic bacteria that form H_2 as fermentation product are dependent on H_2-oxidizing microorganisms such as methanogenic archaea[4-6]. It was shown that the interspecies H_2 transfer becomes more efficient when syntrophs and methanogens are in close physical contact[7,8]. The syntrophic degradation of propionate by a co-culture of *Pelotomaculum thermopropionicum* and *Methanothermobacter thermoautotrophicus* as well as butyrate degradation coupled to organohalide respiration by *Syntrophomonas wolfei* and *Dehalococcoides mccartyi* 195 resulted in aggregate formation and cell-to-cell contact of the involved organisms[9,10]. In addition to the importance of H_2 in microbial food webs, H_2 is considered to be an

alternative energy source and biohydrogen production by microorganisms is discussed as one way to generate environmentally compatible fuels[11].

Epsilonproteobacteria are hitherto considered to be H_2-consuming organisms exclusively and H_2-oxidizing enzymes of only a few Epsilonproteobacteria are characterized so far, e.g. the membrane-bound uptake hydrogenases of *Helicobacter pylori* and *Wolinella succinogenes*[12,13]. H_2 production has never been shown to be performed by any Epsilonproteobacterium so far, although in recent years several Epsilonproteobacteria, especially marine, deep vent-inhabiting species, were reported to encode putative H_2-evolving hydrogenases in their genomes[14-22]. *Sulfurospirillum* spp. are free-living, metabolically versatile Epsilonproteobacteria, many of which are known for their ability to respire toxic or environmentally harmful compounds such as arsenate, selenate or organohalides (e.g. tetrachloroethene - PCE)[23,24]. The anaerobic respiration with PCE, leading to the formation of *cis*-1,2-dichloroethene (*c*DCE), was studied in detail in *S. multivorans* (formerly known as *Dehalospirillum multivorans*)[26,27]. Several *Sulfurospirillum* spp. were found in contaminated sediments, wastewater plants, marine environments or on biocathodes[19,23,27,28]. The role of *Sulfurospirillum* in such environments is unclear.

In previous studies, four gene clusters, each encoding a [NiFe] hydrogenase, were found in the genome of *S. multivorans*[20] and most other *Sulfurospirillum* spp.[23]. Two of these appear to be H_2-producing, the other two are potential H_2-uptake enzymes as deduced from sequence similarity to known hydrogenases. Of these four hydrogenases, one of each type, H_2-oxidizing and H_2-producing, were previously detected in *S. multivorans*[26,29]. The periplasmically oriented H_2-oxidizing enzyme is very similar to the characterized *W. succinogenes* and *H. pylori* membrane-bound hydrogenase (MBH). It comprises three subunits, the large subunit, harboring the NiFe active site, a small subunit for electron transfer with three FeS clusters, and a membrane-integral cytochrome *b*. The putative H_2-producing, cytoplasmically oriented enzyme (Hyf) is a large, complex enzyme with eight subunits, four of them presumably membrane-integral. Regarding amino acid sequence and subunit architecture, this hydrogenase is similar to hydrogenase 4 of *E. coli*, part of a putative second formate hydrogen lyase (FHL)[44]. However, in *S. multivorans*, Hyf is unlikely to form an FHL complex since the corresponding gene cluster does not encode any formate-specific proteins as is the case for the FHL complexes in *E. coli* (Supplementary Figure 1).

Here, we show that several *Sulfurospirillum* spp. produce H_2 upon pyruvate fermentation. *S. cavolei* was observed to produce more H_2 than other *Sulfurospirillum* spp., which is caused by a different fermentation metabolism. To unravel the metabolism and the hydrogenase equipment of both organisms, label-free comparative proteomics was carried out. A co-culture experiment of *S. multivorans* with the methanogenic archaeon *Methanococcus voltae* revealed an interspecies H_2 transfer between both organisms suggesting a hitherto undiscovered contribution of *Sulfurospirillum* spp. and other Epsilonproteobacteria to the microbial anaerobic food web as H_2 producers.

Methods

Cultivation of bacteria

S. multivorans (DSMZ 12446) was cultivated under anaerobic conditions at 28°C in a defined mineral medium[30] without vitamin B_{12} (cyanocobalamin). Pyruvate (40 mM) was used as electron donor and fumarate (40 mM) as electron acceptor. For fermentation experiments, all cultivations were performed with pyruvate (40 mM) or lactate (40 mM) as sole energy source in the absence of an electron acceptor and without yeast extract. Bacteria were grown in serum bottles with a ratio of aqueous to gas phase of 1:1. If not stated otherwise, the gas phase was N_2 (150 kPa). For the cultivation with 100% H_2 in the gas phase, nitrogen was completely removed after autoclaving by flushing with H_2 and an overpressure of 50 kPa was applied. Fermentation balance experiments were performed at 28°C in 1 L Schott bottles placed in a Fermentation apparatus to allow for the expansion of the gases during the cultivation and to determine the stoichiometry of dissolved and gaseous fermentation products (Supplementary Figure 2). For CO_2 quantification, the gas phase of the Schott bottle was connected via a tube to a washing flask filled with 200 mL 4 M KOH to bind produced CO_2 as carbonate. Downstream, the gas phase of the washing flask was further connected to a water-filled measuring cylinder placed up-side down in a water bath. The amount of H_2 was determined volumetrically viathe displaced volume of water in the measuring cylinder that correlates with the amount of H_2 produced. The concentration was calculated using the ideal gas equation. The adaptation experiment included a transfer in the next sub-cultivation step every 48 h with 10% inoculum. *Clostridium pasteurianum* W5 was cultivated in anoxic media composed of 1 L basal medium (autoclaved) supplemented with the following anoxic solutions: 100 mL phosphate buffer (142 g L^{-1} K_2HPO_4, 15 g L^{-1} KH_2PO_4) and 5 mL iron solution (10 g L^{-1} $FeSO_4 \cdot 7\ H_2O$). The basal medium contained per L 142 mg NaCl, 1.42 g NH_4Cl, 284 mg $MgSO_4 \cdot 7\ H_2O$, 14.2 mg $Na_2MoO_4 \cdot 2\ H_2O$, 28.4 mg D(+) biotin and 1.42 mg 4-aminobenzoate. Cells were grown in rubber-stoppered serum bottles with a ratio of aqueous to gas phase of 1:4. Pyruvate (40 mM) and glucose (20 mM) were used as substrates. *Desulfitobacterium hafniense* DCB-2 was cultivated in medium described previously[31]. The medium composition of the co-culture of *S. multivorans* and *Methanococcus voltae* DSMZ 1537 was identical to that described by Whitman *et al.*[32], except that 5 g L^{-1} NaCl were added. Electron donor was 15 mM lactate. *C. pasteurianum* W5, *D. hafniense* DCB-2 and *E. coli* JM109 were taken from the strain collection of our laboratory and *M. voltae* was obtained from the German Collection of Microorganism (DSMZ, Braunschweig, Germany).

Cell harvesting and preparation of cell suspensions and subcellular fractions

S. multivorans, S. cavolei and *C. pasteurianum* W5 cells were harvested in the mid-exponential growth phase in an anoxic glove box (COY, 134 Laboratory, Grass Lake, Michigan, USA) by centrifugation (12,000 x g, 10 min at 10°C). For the preparation of cell suspensions, the obtained cell pellets were washed twice in anoxic 100 mM MOPS-KOH-buffer (pH 7.0) and resuspended in two volumes (2 mL per g cells) of the same buffer. Subcellular fractionation was done by washing the cell pellet twice in 50 mM Tris-HCl (pH 8.0) and resuspension (2 mL per g cells) in the same buffer

containing DNaseI (AppliChem, Darmstadt, Germany) and protease inhibitor (one tablet for 10 mL buffer; complete Mini, EDTA-free; Roche, Mannheim, Germany). The resuspended cells were disrupted using a beadmill (10 min at 25 Hz; MixerMill MM400, Retsch GmbH, Haan, Germany) with an equal volume of glass beads (0.25–0.5 mm diameter, Carl Roth GmbH, Karlsruhe, Germany). The crude extracts were separated from the glass beads by centrifugation (14,000 x g, 2 min) under anaerobic conditions and ultracentrifuged (36,000 x g, 45 min at 4°C). The obtained supernatants were considered as soluble fractions (SF). The pellets were washed twice with 50 mMTris-HCl (pH 8.0) including protease inhibitor (one tablet for 10 mL buffer; cOmplete Mini, EDTA-free; Roche, Mannheim, Germany) and resuspended in the same buffer. The suspension was stated as membrane fraction (MF).

Measurement of hydrogenase activity

H_2 oxidizing activity was measured in H_2-saturated buffer (50 mMTris-HCl, pH 8.0) with 1 mM benzyl viologen (BV) or methyl viologen (MV) at 30°C as artificial electron acceptors. The reduction of the redox dyes was followed at 578 nm using a Cary 100 spectrophotometer (Agilent Technologies, Waldbronn, Germany). H_2-evolving activities of cell extracts were determined gas chromatographically with 1 mM MV as electron donor: MV was reduced with 20 mM sodium dithionite in an anoxic buffer system (50 mMTris-HCl, pH 8.0). Protein concentration was determined according to the method of Bradford[33]. Hydrogenase enzyme activities are given in nanokatal units (1 nmol H_2 evolved per second).

Analytical methods

Liquid samples were taken anaerobically, filtered with 0.2 µm-syringe filters (MiniSart RC4, Sartorius, Göttingen, Germany) and acidified with concentrated H_2SO_4 (2.5 µL mL^{-1} sample volume). Organic acids were separated by HPLC at 50°C on an AMINEX HPX-87H column (7.8 x 300 mm,BioRad, Munich, Germany) with a cation H guard pre-column using 5 mM H_2SO_4 as mobile phase at a flow rate of 0.7 mL min^{-1}. The injection volume was 20 µL per sample. All acids (e.g. pyruvate, acetate, lactate, succinate and fumarate) were monitored by their absorption at 210 nm. Retention times were compared to known standards and concentrations were calculated using calibration curves. H_2 was measured gas chromatographically with 99.999% argon as the carrier gas using a thermal conductivity detector (AutoSystem, Perkin Elmer, Berlin, Germany). Samples for gas analysis were taken from the gas phase with gas-tight syringes (Hamilton, Bonaduz, Switzerland). Concentrations were calculated using calibration curves. CO_2 formed during the cultivation was determined gravimetrically. To 15 mL of the solution of the CO_2 trap 7.5 mL NH_4Cl (1 M) and 15 mL $BaCl_2$ (1 M) were added and the pH was adjusted to 9 with concentrated HCl (37%). After stirring for 2 h at room temperature, the precipitated barium carbonate was filtered with filter circles and dried over night at 80°C.

Isolation of RNA, reverse transcription (RT) and polymerase chain reaction (PCR)

Total RNA from three independent *S. cavolei* cultures was isolated from cells in the mid-exponential growth phase using the RNeasy minikit (Qiagen, Hilden, Germany). Residual genomic DNA (gDNA) in the RNA samples was removed with DNase I (RNase free; Roche, Mannheim, Germany). RNA quality was checked by visual inspection after agarose gel electrophoresis using distinct rRNA bands as control. Synthesis of cDNA was done with 1 µg RNA as starting material in the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Schwerte, Germany). The RT-PCE mixture contained 1 µg RNA, 2.5 µL reverse primer, 2 µL 10 mM dNTP mix and 3.5 µL 5x reaction buffer and PCR-grade water (Fermentas, St. Leon Rot, Germany) was added to a final volume of 17.5 µL. ReverseAid Reverse transcriptase (RT, 0.5 µL, 200 U mL^{-1}) was added to 10.5 µL of the mixture (positive control), residual 7 µl of the reaction mixture without RT were used as negative control. Reaction mixtures were incubated for 1 h at 42°C, the reaction was stopped for 5 min at 72°C. PCR was performed with each 1 µL of positive and negative reactions, 2.5 µL forward and reverse primer, 1 µL 10 mM dNTP mix, 2.5 µL 10x reaction buffer, 14.5 µL PCR-grade water (Fermentas, St. Leon Rot, Germany) and 1 µL *Taq* polymerase (0.1 U $µL^{-1}$, Thermo Scientific, Schwerte, Germany) in a thermocycler (Mastercycler, Personal, Eppendorf, Hamburg) with the following program: 95°C for 5 min, 30 cycles of 95°C 1 min, 52°C for 30 s, 72°C for 1 min and final elongation at 72°C for 10 min. *S. halorespirans* gDNA was isolated using the innuPREP Bacteria DNA kit (Analytik Jena AG, Jena, Germany) according to the manufactorer's instructions. Quality of the extracted DNA was confirmed by gel electrophoresis. The PCR reaction mixture contained 1 µg DNA, 2.5 µL forward and reverse primer, 1.5 µL 10 mM dNTP mix, 5 µL HF reaction buffer and 0.5 µL Phusion DNA polymerase (2 U $µL^{-1}$, Thermo Scientific, Schwerte, Germany). The mix was filled up to 25 µl with PCR-grade water (Fermentas, St. Leon Rot, Germany). The PCR program included following steps: 96°C for 5 min, 30 cycles of 96°C 1 min, 60°C for 30 s, 72°C for 30 s and final elongation at 72°C for 10 min in a thermo cycler (Mastercycler, Personal, Eppendorf, Hamburg). Used primer pairs are listed in Supplementary Table 4.

Field emission-scanning electron microscopy (FE-SEM)

Field emissionscanning electron microscopy (FE-SEM) was performed with co-cultures of *S. multivorans* and *Methanococcus voltae*. After incubation of 3 mL culture in 2.5% glutaraldehyde for 15 min, the cells were pre-fixed for 2 h on poly-L-lysin coated cover slides (12 mm, Fisher Scientific, Schwerte, Germany). Washing of cover slides was done using 0.1 M sodium cacodylate (pH 7.2) (>98% purity, Sigma Aldrich, Steinheim, Germany) for three times. Subsequently, cells were post-fixed with 1% osmium tetroxide in the same cacodylate buffer and dehydrated with different ethanol concentrations. Critical point drying was done in a Leica EM CPD200 Automated Critical Point Dryer (Leica, Wetzlar, Germany) and the samples were coated with 6 nm platinum in a BAL-TEC MED 020 Sputter Coating System (BAL-TEC, Balzers, Liechtenstein). They were visualized at different magnifications using a Zeiss-LEO 1530 Gemini field emission scanning electron microscope (Carl Zeiss, Oberkochen, Germany).

Sample preparation, mass spectrometry and proteome data analysis

Protein concentration of extracted proteins was determined using a Bradford reagent (Bio-Rad, Munich, Germany) with bovine serum albumin as standard. For protein identifications 20 µg of crude extracts were first cleaned from cations and cell debris by running shortly into an SDS gel. For this, the gel was run at 13 mA until the proteins entered the separating gel at a depth of about 3-5 mm. Then the protein band was cut out, reduced, alkylated and proteolytically digested with trypsin (Promega, Madison, WI, USA) and subsequently desalted with C18 ZipTips as described[34].

Mass spectrometry was performed using an Orbitrap Fusion (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a TriVersa NanoMate (Advion, Ltd., Harlow, UK). 5 µL of the peptide solution were separated with a Dionex Ultimate 3000 nano-LC system (Dionex/Thermo Fisher Scientific, Idstein, Germany) using a 15 cm analytical column (Acclaim PepMap RSLC, 2 µm C18 particles, Thermo Scientific) at 35°C.. Liquid chromatography was done with a constant flow of 300 nL min^{-1} with a mixture of solvent A (0.1% formic acid) and B (80% acetonitrile, 0.08% formic acid) in a linear 90 min gradient of 4% to 55% solvent B.

MS1 scans were taken with a cycle time of 3 s in the Orbitrap mass analyzer between 350 and 2,000 *m/z* at a resolution of 120,000, automatic gain control (AGC) target 4×10^5, maximum injection time 50 ms. Data-dependent acquisition (DDA) was employed selecting for highly intense ions ($>5\times10^4$) and charge state between +2 and +7 with a precursor ion isolation windows of 1.6 *m/z*. Fragmentation was done via higher energy dissociation (HCD) at 30% energy, and also measured in the Orbitrap analyzer at a resolution of 120,000 with an AGC target of 5×10^4 and a maximum injection time of 120 ms. Fragmentation events were done within the 3 s of cycle time until the next MS1 scan was done excluding the same mass (±10 ppm) for further precursor selection for 45 s.

Mass spectrometric data were analyzed with Proteome Discoverer 1.4 (Thermo Scientific) against the NCBI *S. multivorans* database (CP007201.1) with the search engines SequestHT and MS Amanda. Oxidation of methionine was set as dynamic, carbamidomethylation of cysteine as static modification; two missed cleavages were accepted, mass tolerance of MS1 and MS2 measurements were set to 5 ppm and 0.05 Da, respectively. A percolator false discovery rate (FDR) threshold of <0.01 was set for peptide identification. Label-free quantification of proteins was done with the area of the three most abundant peptides of each protein. The values were logarithmized (log10) and normalized (see Supplementary Dataset 1) and a two-tailed T-test was applied. Significance values (p-values) of <0.05 were considered to indicate statistical significance. Only proteins identified in at least two of the three replicates were quantified, otherwise, proteins were considered to be identified but were not quantified

Results

1. Adaptation of *Sulfurospirillum multivorans* to pyruvate-fermenting conditions

In previous studies, *S. multivorans* and other *Sulfurospirillum* spp. were shown to grow fermentatively on pyruvate[23,30,35]. Only few data on growth behavior are available in the literature, but *S. multivorans* was reported to exhibit poor growth on pyruvate as sole energy source compared to respiratory growth with pyruvate and fumarate or tetrachloroethene (PCE) as electron acceptor[30]. However, we observed an adaptation of *S. multivorans* to fermentative growth on pyruvate. After about twenty transfers with 10% inoculum each, a growth rate of 0.09 h^{-1} was determined (growth rate on pyruvate/fumarate, 0.19 h^{-1}, Figure 1). During the adaptation to pyruvate fermentation, the growth rate increased on average by 0.02 h^{-1} with each transfer (Supplementary Figure 3). In addition, the lag phase duration decreased from initially 40h to 5h. After 18 transfers, no further significant increase of the growth rate was observed. This adaptation process was also observed for *S. cavolei*, *S. delyianum* and *S. arsenophilum*. For *S. barnesii* and *S. halorespirans*, no growth on pyruvate alone was detected, even after several subcultivation steps.

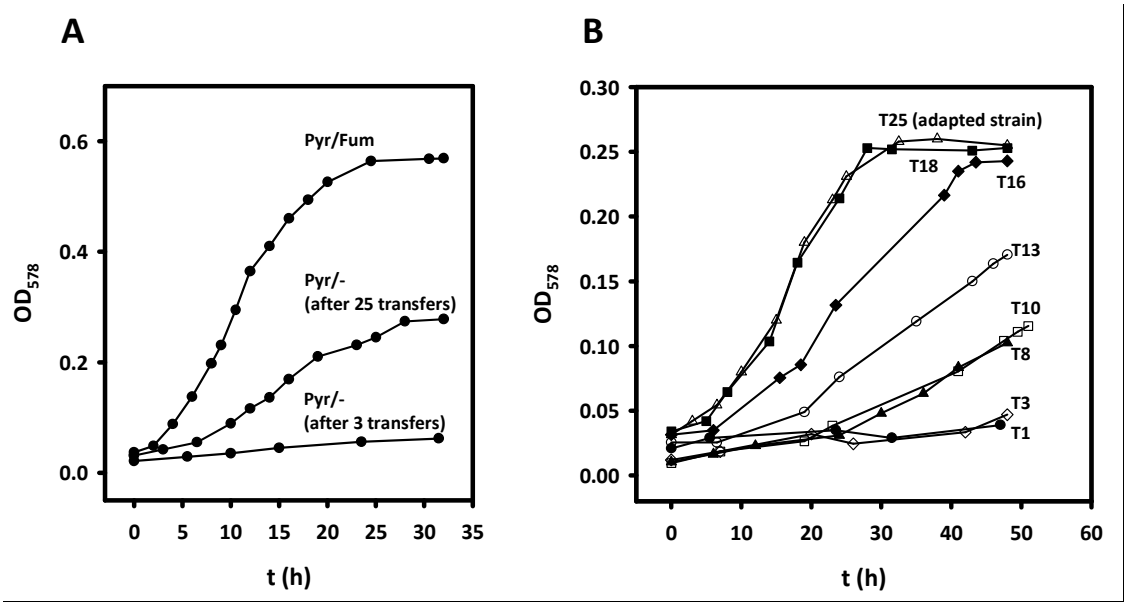

Figure 1: Adaptation of *S. multivorans* to pyruvate-fermenting conditions. A) Growth curves with pyruvate as sole growth substrate after three and twenty-five transfers; A culture with pyruvate/fumarate after three transfers is shown for comparison. B) Growth during continuous transfer on pyruvate without electron acceptor. Each transfer (10% inoculum) was done after 48 hours cultivation. Data were obtained from at least two independent biological replicates and are representatives. T - number of transfer step, Pyr - pyruvate, Fum - fumarate, OD_{578} - optical density at 578 nm.

2. Fermentative growth and H_2 production by *Sulfurospirillum* spp.

To get deeper insight into the fermentation pathways and H_2 production capabilities of *Sulfurospirillum* spp., several species were cultivated with pyruvate as sole substrate. Six species were tested for pyruvate fermentation, of which *S. barnesii* and *S. halorespirans* were not able to grow even after cultivation for several months. *S. cavolei*, *S. deleyianum* and *S. arsenophilum* were able to grow on pyruvate alone and showed the same adaptation behavior as *S. multivorans*, albeit they grew at slower rates (0.03 h^{-1}, 0.06 h^{-1}, and 0.004 h^{-1}, respectively). H_2 production was measured for all fermentatively growing *Sulfurospirillum* spp., but the produced amount differed, depending on the species. *S. cavolei* produced the highest amount of H_2 followed by *S. arsenophilum*. *S. deleyianum* and *S. multivorans* produced about 100 µmol H_2 per 100 mL culture. *D. hafniense* DCB-2, a known pyruvate-fermenting organohalide-respiring bacterium grows similar to *Sulfurospirillum* spp. (Figure 2A) but produced only minor amounts of H_2 (20 µmol) (Figure 2b). Fermentative growth on lactate was not observed for any of the organisms including *D. hafniense* DCB-2 even after cultivation for several months.

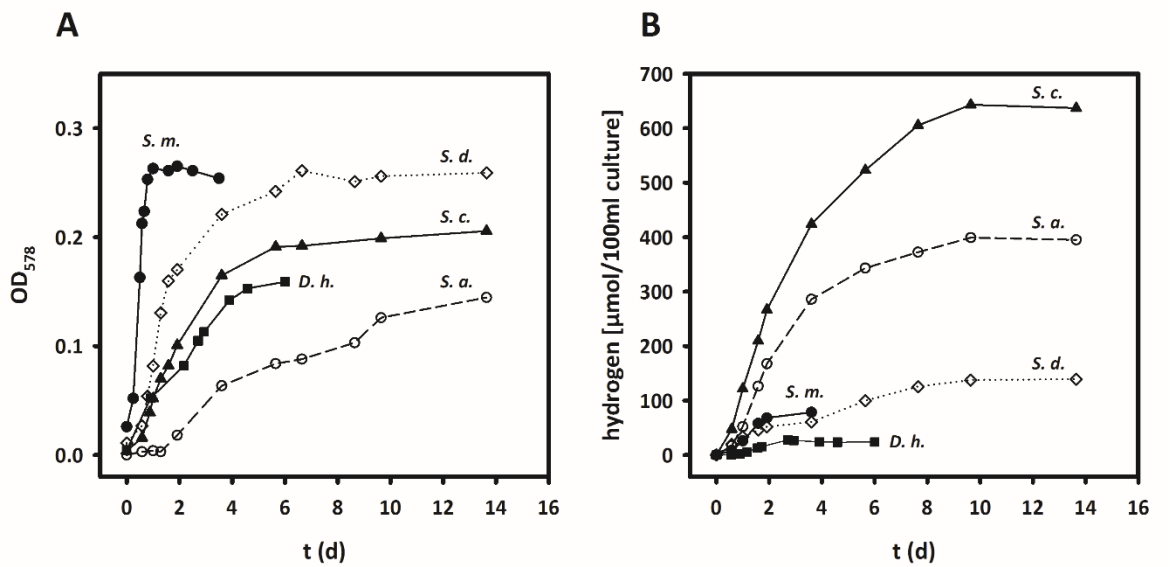

Figure 2: Growth (A) and H_2 production (B) by *Sulfurospirillum* spp. and *D. hafniense* strain DCB-2 during fermentative growth on 40 mM pyruvate after adaptation. The graph is a representative of three independent replicates. *S.m.* - *S. multivorans*, *S.d.* - *S. deleyianum*, *S.c.* - *S. cavolei*, *S.a.* - *S. arsenophilum*, *D.h.* - *D. hafniense* DCB-2.

3. Fermentative metabolism of *S. multivorans* and *S. cavolei*

To unravel the fermentative metabolism of two *Sulfurospirillum* spp. showing different H_2 production patterns during growth on pyruvate, *S. multivorans* and *S. cavolei* were cultivated in a fermentation apparatus in which the gas phase of the Schott bottle was connected to CO_2 and H_2 traps (see Supplementary Figure 2) to avoid increasing gas partial pressures and hence a possible product inhibition on H_2 production or growth (see also next chapter). Enhanced H_2 evolution was measured when compared to the serum bottle experiment, with up to hundred times more H_2 produced, while the growth was slower than in the previous setup (Figure 3A). After consumption of 40 mM pyruvate, 27 mM acetate, 10 mM lactate, 3 mM succinate,10 mM H_2 and 28 mM CO_2 were measured as fermentation products of *S. multivorans* (Figure 3A). *S. cavolei* showed slower growth than *S. multivorans* and a much higher amount of H_2 evolved. During growth, which took 8 to 10 days, pyruvate (40 mM) was used up completely and 38 mM acetate, 36 mM H_2 and 38 mM CO_2 were the only products detected (Figure 3B). No other organic acids or alcohols were dected for both. *S. deleyianum* showed similar fermentation products as observed with *S. multivorans* (Supplementary Figure 4). The stoichiometry of the fermentation was verified by calculating the carbon recovery and an oxidation/reduction balance (Supplementary Table 1, Eqns (I) and (II)). In *S. multivorans*, the amount of reducing equivalents generated from pyruvate oxidation was calculated to be 54 [H], which fits to the amount of used reducing equivalents for the production of molecular hydrogen, lactate and succinate (52 [H], Supplementary Table 1). In *S. cavolei*, pyruvate oxidation leads to the generation of 76 [H], which were almost exclusively (72 [H]) used for proton reduction to H_2. In addition, the carbon recovery is in agreement with the theoretical values and is 102.5% for *S. multivorans* and 95% for *S. cavolei*. The anabolic assimilation of the carbon source is minor with approximately 2.5 mM for *S. multivorans* and 2 mM for *S. cavolei* as calculated from OD and dry weight.

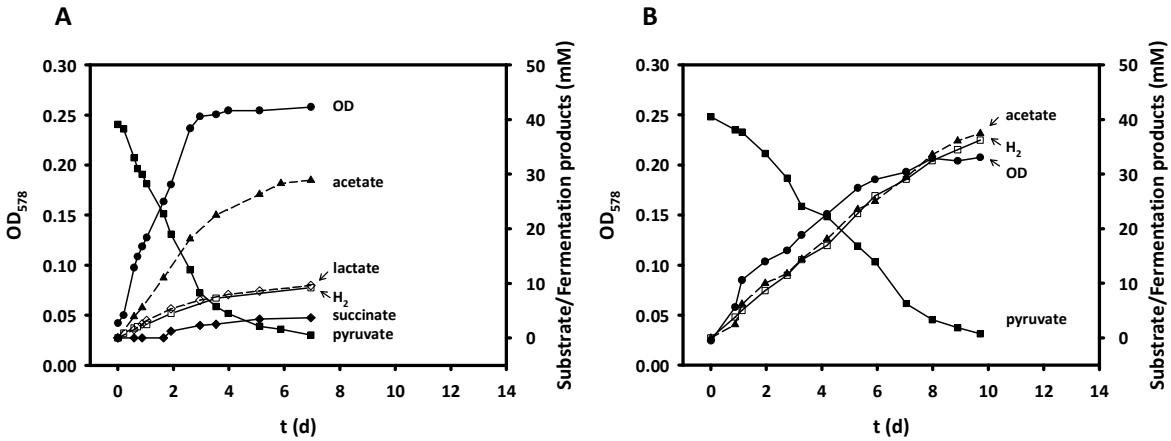

Figure 3: Fermentation balance of *S. multivorans* (A) and *S. cavolei* (B) during fermentative growth on 40 mM pyruvate measured in the fermentation apparatus.

Eqn. (I) 1 Pyruvate → 0.7 Acetate + 0.25 Lactate + 0.075 Succinate + 0.25 H_2 + 0.7 CO_2

Eqn. (II)1 Pyruvate → 0.95 Acetate + 0.9 H_2 + 0.95 CO_2

4. Product inhibition by H_2 on fermentative growth in *S. cavolei* and *S. multivorans*

The different amount of H_2 produced in the growth experiments in serum bottles and the fermentation apparatus imply a product inhibition of H_2 on H_2 production. To investigate the effect of H_2 in the gas phase on the fermentative growth of *S. multivorans* and *S. cavolei*, both organisms were cultivated in serum bottles with a gas phase of 100% H_2 or 100% nitrogen (Figure 4). With nitrogen as gas phase, *S. multivorans* and *S. cavolei* showed similar growth and production rates of organic acids as observed in the fermentation apparatus. A strong negative effect on growth was observed with 100% H_2 in the gas phase. *S. multivorans* was still able to ferment pyruvate but showed an inhibited growth and a lower cell density compared to the culture without H_2 in gas phase, while *S. cavolei* was almost completely inhibited (Figure 4A). The restricted growth is also reflected by a lower pyruvate consumption rate (Figure 4B). In addition, the formation of fermentation products shifted from acetate production to lactate and succinate formation in *S. multivorans* (Figure 4C-E). *S. cavolei* produced neither lactate nor succinate and only minor amounts of acetate.

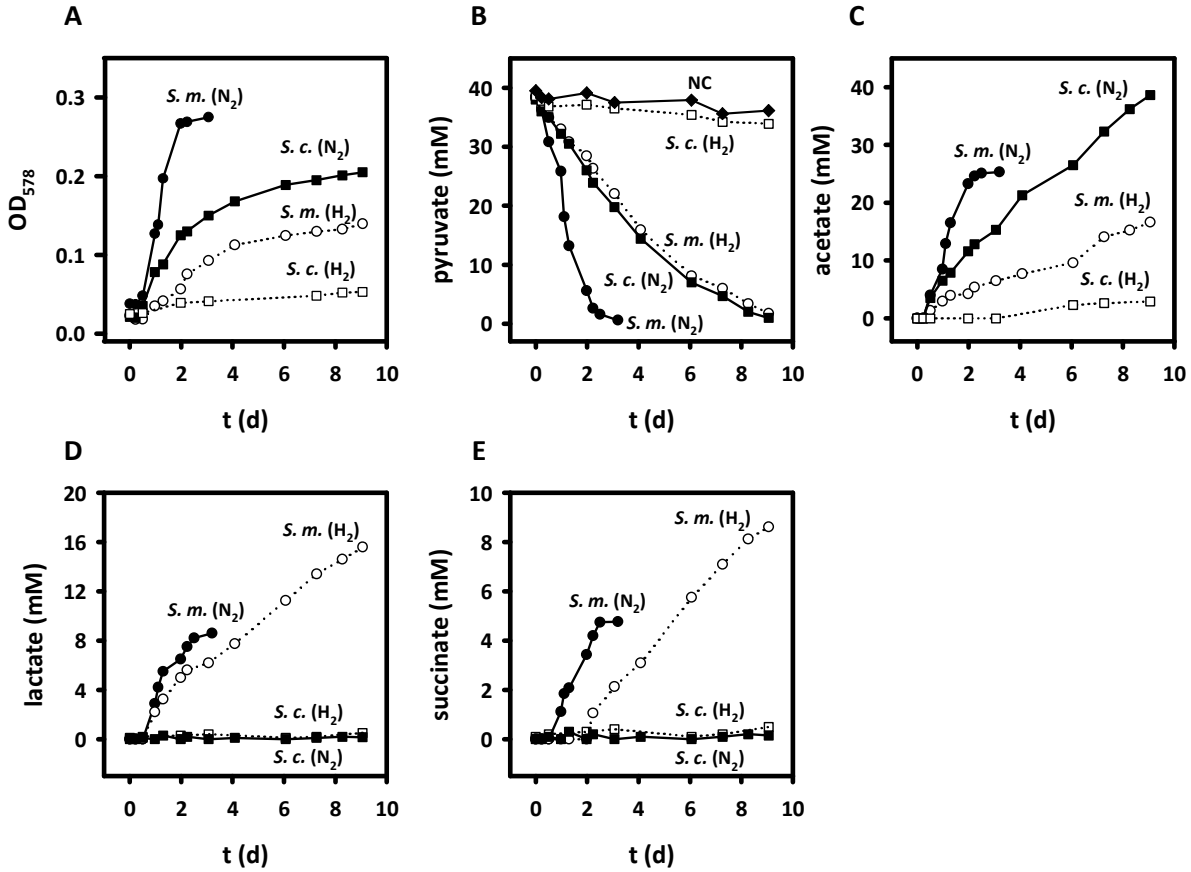

Figure 4: Growth and formation of fermentation products during cultivation under 100% nitrogen (N_2) and 100% H_2 atmosphere with pyruvate as sole energy source. Growth curve (A), pyruvate consumption (B) and acetate (C), lactate (D) and succinate (E) production are shown. Organic acids were measured via HPLC. Each cultivation was conducted in three biological replicates. S.m. - *S. multivorans*, S.c. - *S. cavolei*, N_2 - nitrogen, H_2 - hydrogen, NC - negative control (cell-free medium).

5. Hydrogenase activities by cell suspensions of *Sulfurospirillum* spp.

The H_2 production and oxidation capability of cell suspensions of *S. multivorans* and *S. cavolei* was analyzed to obtain further evidence about the hydrogenase involved in the production and oxidation reaction. Transcriptional and proteomic studies revealed the presence of two [NiFe] hydrogenases in *S. multivorans*[29]: a hydrogen-oxidizing periplasmic membrane-bound hydrogenase (MBH) and a putative H_2-producing cytoplasmic membrane-bound hydrogenase (Hyf). These two hydrogenases might be distinguished by their different subcellular localization and thus their accessibility to redox mediators like viologens in hydrogenase acitivity assays. Photometrically measured H_2-oxidizing activity was detected in whole cell suspensions as well as in membrane and soluble fractions (Table 1). In contrast, H_2-producing activity, as monitored by GC, was only measured with membrane fractions but not in whole cell suspensions of *S. multivorans* and *S. cavolei* with approximately 1.5-fold higher activity in *S. cavolei* (Table 1). The membrane fractions of *S. multivorans* and *S. cavolei* cells grown on pyruvate as sole energy source were about 2-fold more active in H_2-production than those of cells cultivated under respiratory growth conditions with pyruvate plus fumarate, while the latter exhibited slightly more H_2 oxidation activity. *Clostridium pasteurianum* W5, which is known to harbor a cytoplasmic soluble H_2-producing hydrogenase, exhibited hydrogenase activity only in the soluble fractions and showed no H_2 producing activity in cell suspensions with methyl viologen as electron donor (Supplementary Table 2), thus serving as a control for the hydrogenase localization experiment.

Table 1: Hydrogen-producing and oxidizing activities of cell suspensions and cellular fractions of *S. multivorans* and *S. cavolei* cultivated with pyruvate or with pyruvate/fumarate. Data are derived from three independent biological replicates and show means ± standard deviation.

Cellular fraction	Hydrogenase activity (nkat mg^{-1})					
	S. multivorans			*S. cavolei*		
	MV → H_2	H_2 → BV	H_2 → MV	MV → H_2	H_2 → BV	H_2 → MV
Cell suspensions						
Pyr	< 0.01	4.1 ± 0.5	0.7 ± 0.3	< 0.01	5.5 ± 0.6	1.4 ± 0.3
Membrane fractions						
Pyr	12.3 ± 2.4	23.5 ± 2.1	n.d.	20.6 ± 3.7	10.1 ± 0.5	n.d.
Pyr + Fum	5.7 ± 1.5	36.6 ± 3.3	n.d.	10.1 ± 2.4	13.5 ± 1.6	n.d.
Soluble fractions						
Pyr	< 0.01	n.d.	n.d.	< 0.01	n.d.	n.d.
Pyr + Fum	< 0.01	2.3 ± 0.3	n.d.	< 0.01	1.9 ± 0.3	n.d.

MV → H_2 indicates H_2 formation activity, H_2 → BV/MV indicates H_2 oxidation. MV - methyl viologen, BV - benzyl viologen. Pyr - pyruvate , Fum - fumarate, n.d. - not determined

6. Comparative genomics and proteomics

To unravel the cause of the different fermentative metabolisms of the two *Sulfurospirillum* sp., a comparative genomic analysis was done with the RAST sequence comparison tool[36]. Additionally, proteomes of *S. cavolei* NRBC109482 and *S. multivorans* cultivated under fermenting and respiring conditions with fumarate as electron acceptor were analyzed. Bidirectional blast hits with more than 50% amino acid sequence identity were considered as orthologs, proteins putatively fulfilling the same functions in both organisms. The genomes were overall similar, with 2057 of 2768 of the encoded proteins in *S. cavolei* being orthologs. Only few of the non-orthologous proteins in *S. cavolei* could be considered to play a role in the fermentation based on their annotation and putative involvement in one of the pathways connected to fermentative catabolism. Among the proteins encoded in the *S. cavolei* genome (annotated RefSeq WGS accession number NZ_AP014724), which do not have an ortholog in *S. multivorans*, we found a cluster encoding an [FeFe] hydrogenase known to contribute to fermentative H_2 production in many bacteria, e.g. Clostridia (Supplementary Figure 5). A nearly identical gene cluster is found in the other two genomes of *S. cavolei* strains UCH003 and MES, the latter of which was assembled from a metagenome[19]. The large [FeFe] hydrogenase catalytic subunit gene, *hydA*, is disrupted by a stop codon resulting from a nucleotide insertion only in *S. cavolei* strain NRBC109482. The mutation was confirmed by PCR and Sanger sequencing. RT PCR analysis suggested that *hydA* was transcribed under pyruvate-fermenting growth conditions (Supplementary Figure 6). However, the [FeFe] hydrogenase was not identified in the proteome of *S. cavolei*.

Of the proteins related to pyruvate metabolism, a pyruvate,water dikinase (phosphoenolpyruvate [PEP] synthetase) is encoded in the genome of *S. multivorans* (encoded by SMUL_1602), but not in *S. cavolei*. This enzyme is responsible for the ATP-dependent synthesis of phosphoenolpyruvate from pyruvate in gluconeogenesis (Supplementary Figure 7). The PEP synthetase was found in 6.3-fold higher abundance (p-value 0.02) in the proteome of fermentatively cultivated *S. multivorans* cells (Supplementary Table 3). In *S. cavolei*, PEP might be formed from pyruvate via oxaloacetate by two reactions catalyzed by pyruvate carboxylase and PEP carboxykinase. These two enzymes are encoded in one gene cluster (SCA02S_RS02520 and SCA02S_RS02525, respectively, Supplementary Figure 8). In *S. multivorans* these proteins (SMUL_0789 and SMUL_0791) cluster with a gene encoding a subunit similar to the membrane subunit of a putative Na^+-translocating oxaloacetate decarboxylase (SMUL_0790), of which an ortholog is not encoded in *S. cavolei* (Supplementary Figure 8). Both pyruvate carboxylase/oxaloacetate decarboxylase and PEP carboxykinase were found in the proteomes of both organisms in slightly higher amounts in cells grown with pyruvate only (Supplementary Dataset 2). Similar to *S. cavolei*, also *S. arsenophilum*, producing larger amounts of H_2 than *S. multivorans* (Figure 2), lacks the putative oxaloacetate decarboxylase subunit gene.

The Hyf hydrogenase was found in high abundancies especially in the proteome of *S. multivorans* cultivated with pyruvate alone. Here, four out of eight of the structural subunits were found in the 10% of the most abundant proteins, while none were found in the top 10% under respiratory conditions. In *S. cavolei*, the hydrogenase-4 subunits were not as abundant as in *S. multivorans* with only two out of six quantified subunits in the top 20% (Supplementary Dataset 2). In both organisms, a significantly higher amount of Hyf subunits was quantified under fermentative growth conditions (*S. multivorans:* 4- to 27-fold for the structural subunits HyfA-HyfI, all p-values are <0.001, *S. cavolei*: 2- to 5-fold for HyfA-HyfI, all p-values are <0.05; Figure 5, Supplementary Table 3, Supplementary Dataset 2). Interestingly, the Hyf gene cluster is disrupted at one site in *S. halorespirans*, which cannot grow on pyruvate alone. Genome sequencing[37] revealed a transposase insertion at *hyfB* which might result in a non-functional gene *S. halorespirans*. The transposon insertion was confirmed by PCR using *hyfB*-specific primers flanking the transposase (Supplementary Figure 9). The membrane-bound subunits HyfE and HyfF were found in fermenting cells of *S. multivorans* exclusively. Sequence comparison of the Hyf hydrogenase of *S. multivorans* shows similarities to the proton-pumping complex I of *Thermus thermophilus* (Supplementary Figure 10). An analysis regarding the potential proton pumping capabilities of the *S. multivorans* Hyf deduced from conserved amino acids which are responsible for proton pumping in complex I of *T. thermophilus* and a comparison to the *E. coli* FHL is given in the Supplementary information (Supplementary Note 1, Supplementary Table 6 and Supplementary Figures 11 - 13).

A search for the *hyf* gene cluster in genomes of Epsilonproteobacteria shows that it is ubiquitous in, but not limited to, *Sulfurospirillum* spp (Supplementary Table 5). Four out of 15 *Sulfurospirillum* sp. genomes harbor a second *hyf* gene cluster co-located with genes encoding a formate transporter and a formate dehydrogenase (Supplementary Figure 1). In *Arcobacter* spp. and the marine species *Caminibacter mediatlanticus* and *Lebetimonas* spp., only the latter gene cluster encoding a putative FHL complex is found. In several *Campylobacter* spp. including *C. concisus*, a *hyf* gene cluster with a formate transporter gene was identified (Supplementary Figure 1), while a second group of *Campylobacter* (including *C. fetus*) does not encode any formate-related proteins (Supplementary Table 5).

A pyruvate:ferredoxin oxidoreductase (PFOR) and a ferredoxin (Fd) showed also a higher abundance in both *Sulfurospirillum* sp. under fermenting conditions (*S. multivorans*: PFOR 2-fold, Fd 6-fold, *S. cavolei*: PFOR 4-fold, Fd 2-fold, all p-values are <0.01; Figure 5, Supplementary Table 3). A second pyruvate-oxidizing enzyme, a quinone-dependent pyruvate dehydrogenase encoded exclusively in the genome of *S. multivorans*, was significantly lower abundant during pyruvate fermentation (7-fold, p-value 0.02). The enzymes responsible for ATP generation via substrate-level phosphorylation, phosphotransacetylase and acetate kinase, are slightly higher abundant during pyruvate fermentation in both *Sulfurospirillum* sp. (approximately 2-fold for both enzymes in *S. multivorans,* p-values are <0.01 and approximately 3-fold in *S. cavolei,* p-values are <0.001; Figure 7, Supplementary Table 3). The malic enzyme is higher abundant during fermentation in *S. multivorans* (3.7-fold, p-value <0.001, Supplementary Table 3) and not quantified in any proteome

of *S. cavolei* (Supplementary Table 3). The subunits of the membrane-bound hydrogenase (MBH) were quantified in either unsignificantly lower amounts (HydAB, approximately 2-fold, p-values 0.40 and 0.07) or slightly higher amounts (HydC, approximately 2-fold, p-value 0.01) under fermenting conditions for *S. multivorans*. In contrast, HydABC were found in significantly lower amounts in *S. cavolei* when grown fermentatively. Of the cytoplasmic H_2-producing hydrogenase (Ech-like), only one subunit (present in the lower 50% abundant proteins) was quantified in *S. multivorans* grown with pyruvate alone. In *S. cavolei*, five of six Ech-like hydrogenase subunits were quantified in cells cultivated with pyruvate alone and two of six subunits in pyruvate/fumarate-cultivated cells, all of the them in the lowest third abundant proteins. No subunit of the cytoplasmic uptake hydrogenase (HupSL) was found in any of the proteomes.

A putative lactate dehydrogenase (SMUL_0438, SCA02S_RS08360) with 35% amino acid sequence identity to a characterized lactate-producing lactate dehydrogenase from *Selenomonas ruminantium*[38] was not detected in any proteome. This is in accordance to the lack of pyridine dinucleotide-dependent lactate-oxidizing or pyruvate-reducing activities in cell extracts of *S. multivorans* (data not shown, methods described in the Supplement). Several candidates for pyridine dinucleotide-independent lactate dehydrogenases (iLDH) are encoded in the genome of *S. multivorans*. Since *S. deleyianum* shows also lactate production during pyruvate fermentation, only genes present as orthologs in both genomes were considered to be responsible for lactate production in *Sulfurospirillum* spp. Functionally characterized iLDHs are flavin and FeS-cluster-containing oxidoreductases[40] or enzymes related to malate:quinone oxidoreductase[40]. Only two candidates of the former class were identified in the genome, encoded by SMUL_1449 and SMUL_2229. Of these, only the latter gene product was detected in the proteome, however, not in altered amounts under fermentative conditions when compared to respiratory cultivation.

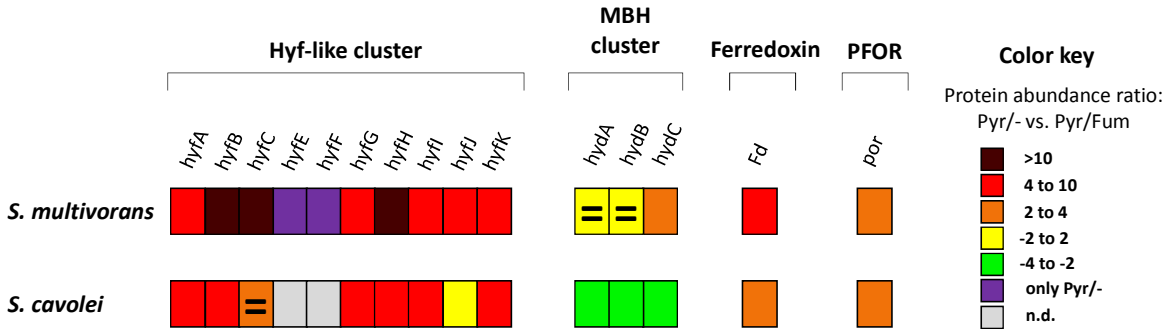

Figure 5: Comparative proteomics of proteins possibly involved in pyruvate fermentation of *S. multivorans* and *S. cavolei*. Comparison of cells grown with pyruvate alone was done with cells grown with pyruvate/fumarate. For quantified proteins the protein intensity ratio is given as colored squares. Non-significantly altered proteins levels are marked with an equal sign (p-values >0.05). Proteins exclusively found in pyruvate fermenting cells are colored purple. All data were obtained from 3 independent biological replicates. Hyf-like - Hyf hydrogenase (SMUL_2383-2392; SCA02S_RS01920-RS01965), MBH - membrane-bound hydrogenase (SMUL_1423-1425; SCA02S_RS01350-RS01360), Fd - ferredoxin (SMUL_0303; SCA025_RS12260), PFOR - pyruvate:ferredoxin-oxidoreductase (SMUL_2630; SCA02S_RS04525). Pyr - pyruvate, Fum - fumarate.

7. *Sulfurospirillum multivorans* as a syntrophic partner for *Methanococcus voltae*

To unravel the potential role of *S. multivorans* in a syntrophic partnership as H_2 producer, a co-culture with *Methanococcus voltae* was prepared. *M. voltae* is a methanogenic archaeon dependent on either H_2 or formate as electron donor and CO_2 as electron acceptor[32]. To investigate the syntrophic interaction of the two organisms, the co-culture was cultivated with lactate, which could not serve as a fermentation substrate for pure *S. multivorans* cultures. A syntrophic, hydrogen-consuming partner keeping H_2 concentration at a low level in co-cultures might render lactate fermentation by *S. multivorans* thermodynamically feasible in a co-culture. In the corresponding co-culture, 15 mM lactate was consumed in approximately two weeks while methane was formed, indicating lactate fermentation by *S. multivorans* and H_2 transfer to *M. voltae* as syntrophic partner (Figure 6 A,B). Electron microscopic analyses of the co-culture revealed cell aggregates with sizes between 50 and 600 µm (Figure 6C, Supplementary Figure 14). These aggregates showed a compact network of the rod-shaped *S. multivorans* and coccoidal *M. voltae* with net-forming flagellum-like structures surrounding the organisms. The cells in the aggregates were embedded in extracellular polymeric substances (EPS)-like structures, which might aid cell-to-cell contact.

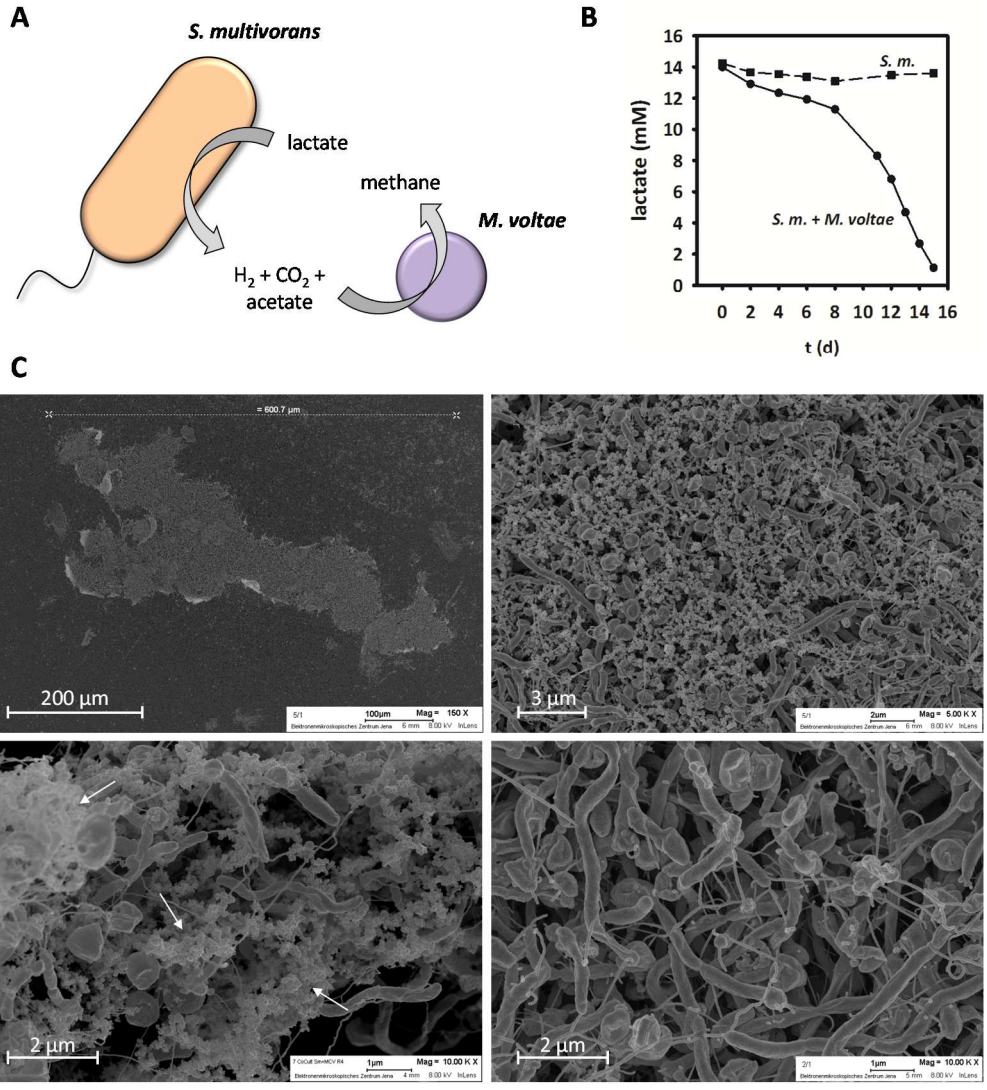

Figure 6: Syntrophic co-culture of *S. multivorans* and *Methanococcus voltae*. (A) scheme of syntrophic interactions and exchange of metabolites and (B) lactate concentration in *S. multivorans* pure culture and co-culture of *S. multivorans* and *M. voltae*. (C) Electron microscopic images of aggregates, Magnifications, 150 x (whole aggregate, upper row left), 5,000 x (upper right), 10,000 x (lower images). Sections of the lower images were obtained from different areas of the aggregate. White arrows indicate EPS-like structures. Cultivation experiments included three biological replicates in which similar aggregates were formed. S.m. - *S. multivorans*.

Discussion

In this study, production of H_2 was observed for several *Sulfurospirillum* species during pyruvate fermentation, which is the first evidence of H_2 production for Epsilonproteobacteria, which hitherto were generally regarded as H_2 oxidizers[14,35,41,42]. Specifically, we report H_2 production for *S. multivorans, S. cavolei, S. arsenophilum* and *S. deleyianum* during fermentative growth on pyruvate. Sequential subcultivation on pyruvate alone revealed a continuous adaptation of *Sulfurospirillum* spp. to a fermentative metabolism. The mechanisms behind this long-term adaptation process in *Sulfurospirillum* spp. remain unresolved for now and might include genomic rearrangements and/or population dynamics, but also a long-term regulatory effect similar to the one observed for *S. multivorans* after continuous transfer without PCE as electron acceptor[43] might play a role. The basis for the latter effect is also unknown to date.

Two different fermentation balances were observed for the different *Sulfurospirillum* spp. tested. While *S. cavolei* showed the highest H_2 production rate and produced, besides hydrogen, acetate and CO_2, *S. deleyianum* and *S. multivorans*, displaying lower H_2 production, additionally produced succinate and lactate. Pyruvate is most likely oxidized to acetate by the pyruvate:ferredoxin oxidoreductase, which showed an upregulation in the proteome of fermentatively cultivated compared to fumarate-respiring cells in both, *S. multivorans* and *S. cavolei*. In contrast, the quinone-dependent pyruvate dehydrogenase (PoxB) which could transfer electrons generated upon pyruvate fermentation to menaquinone, is downregulated in fermenting cells and therefore most likely does not contribute significantly to pyruvate oxidation under this condition. A pyruvate formate lyase is not encoded in any *Sulfurospirillum* spp., which, in addition to the low protein abundance of a cytoplasmic formate dehydrogenase in *S. multivorans* and *S. cavolei*, argues against the role of the Hyf in a formate hydrogen lyase complex as opposed to the suggested function for Hyf in *E. coli*[44]. The generated acetyl-CoA is used to generate acetate and one mol ATP per mol pyruvate via substrate-level phosphorylation.

Electrons generated upon pyruvate oxidation are most likely transferred in both organisms to a ferredoxin of the *Allochromatium vinosum*-type, which is known for the very negative redox potentials of its two [4Fe4S] clusters[45]. The proteome data and biochemical experiments presented in our study strongly suggest that the Hyf (hydrogenase 4) of *Sulfurospirillum* spp. accepts electrons from the reduced ferredoxin to reduce two protons to hydrogen. Hyf is significantly upregulated, whereas the other hydrogenases are either detected only in low amounts in the proteome data or are unaltered or downregulated under fermentative cultivation. Furthermore, reduced methyl viologen served as electron donor for H_2 production only with crude extract and not with intact cells, suggesting a

cytoplasmic localization of the hydrogen-producing hydrogenase since methyl viologen should not have access to the cytoplasm. A cytoplasmic localization was also suggested previously for Hyf of *S. multivorans* based on the lack of a signal peptide in any of the corresponding subunit amino acid sequences [20,29]. The involvement of a Hyf in H_2 production via pyruvate oxidation was also observed for a group 4 hydrogenase from *Pyrococcus furiosus* [46] and a genetically modified *E. coli* strain[47]. The structure and subunit composition of several group 4 hydrogenases suggested their involvement in the generation of a proton motive force, thereby contributing to ATP formation[48,49]. A thorough alignment analysis of the subunits of *Sulfurospirillum* spp. Hyf indicated that most of the important residues in the membrane helices are conserved, thus making a role in energy conservation of this hydrogenase a possible scenario. The difference in the amount of H_2 produced with *S. cavolei* producing more H_2 than *S. multivorans* can be explained by two different fermentation metabolism types. Opposed to *S. cavolei*, reducing equivalents can be channelled into the production of lactate and succinate by *S. multivorans* (as was also observed for *S. deleyianum*) upon pyruvate fermentation. Succinate might be produced from fumarate (fumarate reductase) via malate (fumarase), which could be formed from pyruvate via reductive decarboxylation to malate by the malic enzyme. This enzyme, which often functions in the reverse direction e. g. in C_4 plants, is upregulated in *S. multivorans* under fermentative conditions. This finding supports the involvement of the malic enzyme in conversion of pyruvate to malate. The malic enzyme was not detected in the proteomes of *S. cavolei*, which might at least partially explain the different fermentation balances.

The origin of lactate in *S. multivorans* is not clear. An NAD+-dependent lactate dehydrogenase was not detected in any of the proteomes and no $NAD(P)^+$-dependent lactate production could be measured. Most likely, the lactate dehydrogenase is misannotated in the genome of *S. multivorans*, as reported for a related protein of *Campylobacter jejuni*[50]. A possible source of lactate could be the reduction of pyruvate via an unknown, NAD^+-independent lactate dehydrogenase (iLDH). Some of these are characterized to be functional in the direction of lactate oxidation[51,52] and could act in the reverse direction to produce lactate in *Sulfurospirillum* spp., possibly with reduced ferredoxin as electron donor. Several candidates of iLDHs are encoded in the genome of *S. multivorans*, but only one of them shows a slight upregulation on pyruvate alone. A homolog of the corresponding gene cluster is not encoded in the lactate-producing *S. deleyianum*, making it an unlikely candidate for lactate production. A glycolate oxidase was shown to be responsible for lactate oxidation in *Pseudomonas putida*[39,53] and a homolog is encoded in both lactate-producing *Sulfurospirillum* spp. This protein, however, is not upregulated upon pyruvate fermentation and further studies are needed to identify the lactate-producing enzyme in *S. multivorans*.

The different disposal of excess reducing equivalents during fermentation enables *S. multivorans* to grow with pyruvate even with 100% H_2 in the gas phase, whereas the growth of *S. cavolei* was nearly completely abolished under these conditions. This correlates with a shift towards a higher production of lactate and succinate and a lower acetate and H_2 production of *S. multivorans* under these conditions. H_2 production via Hyf is obviously subject to product inhibition and *S. multivorans* is able to circumvent this by using alternative cytoplasmic electron sinks upon fermentation. The inability of *Sulfurospirillum* spp. to use lactate as sole substrate in pure cultures is most probably due to the

thermodynamically unfavorable lactate oxidation to pyruvate upon H_2 production. However, a syntrophic partnership of *S. multivorans* with a hydrogen-consumer, *Methanococcus voltae*, enabled lactate utilization by *S. multivorans* and led to the formation of large cell aggregates of the two organisms presumably via the formation of EPS.

These findings confirm our suggested role of *Sulfurospirillum* spp. as H_2 producers in anaerobic food webs. Additionally, this role as a potential H_2 producer is most likely not limited to this genus. In a genome mining approach, *hyf* gene clusters were found among several genera of Epsilonproteobacteria inhabiting a wide range of habitats. Some *Campylobacter* spp. known to be opportunistic or foodborne pathogens encode the same Hyf as *Sulfurospirillum* spp., while *hyf* gene clusters containing either a formate channel gene in different *Campylobacter* spp. or additionally a cytoplasmic formate dehydrogenase in other phyla might indicate the formation of a formate hydrogen lyase complex. Since a PFL is missing in these bacteria, it might be presumed that extracellular formate might aid growth in these bacteria as reported for *Thermococcus* spp.[54]. Some *Sulfurospirillum* spp. even encode for both, a FHL-independent Hyf and one presumably forming an FHL complex, pointing towards separate regulation and roles of both hydrogenases and thus for even more physiological diversity in this genus.

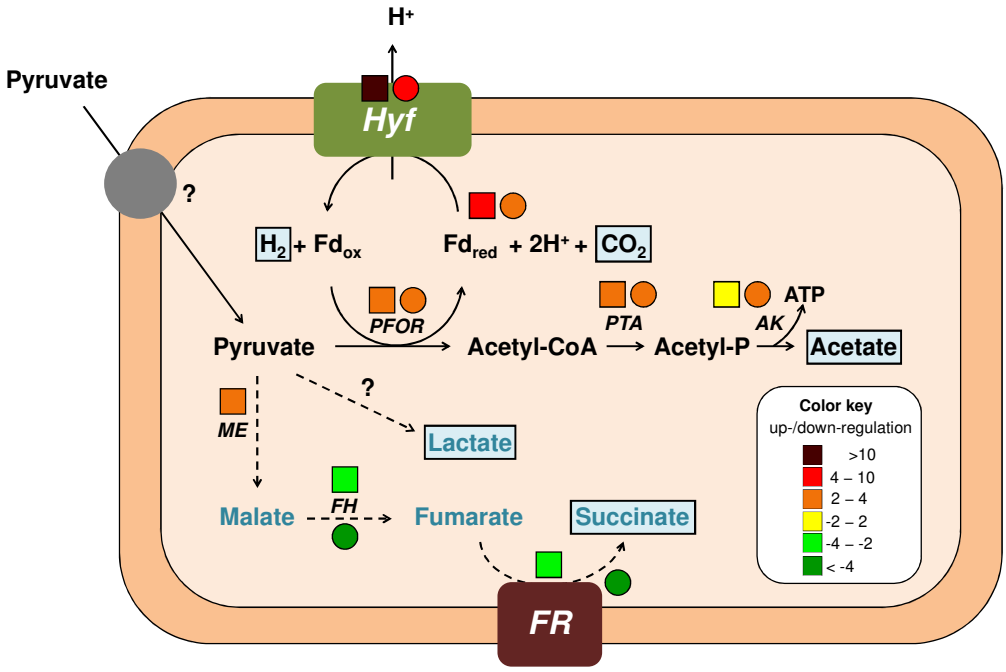

Figure 7: Tentative scheme of pyruvate fermentation metabolism in *S. multivorans* and *S. cavolei*. Reactions represented by solid arrows belong to the core pyruvate metabolism and are catalyzed by both organisms. Reactions with dashed arrows are solely catalyzed by *S. multivorans,* fumarate hydratase and fumarate reductase are also present in *S. cavolei*. Hyf might pump protons via its membrane-integral subunits (Supplementary Note 1, Supplementary Table 6 and Supplementary Figures 10 - 13) which could lead to additional ATP production via a chemiosmotoQuestion marks indicate enzymes not identified. Fermentation products are highlighted in light blue boxes. Protein abundance ratios (pyruvate alone versus pyruvate/fumarate) are indicated by colored squares (*S. multivorans*) and circles (*S. cavolei*) at the protein abbreviations. Color code of the ratios is given in the box at the lower right. Hyf - Hyf-like hydrogenase (SMUL_2383-2392; SCA02S_RS01920-RS01965), PFOR - pyruvate:ferredoxin oxidoreductase (SMUL_2630; SCA02S_RS04525), PTA - phosphotransacetylase (SMUL_1483; SCA02S_RS00245), AK - acetate kinase (SMUL_1484; SCA02S_RS00240), ME - malic enzyme (SMUL_3158; corresponding enzyme in *S. cavolei* is not present), FH - fumarate hydratase (SMUL_1459, SMUL_1679-1680; SCA02S_RS00615-RS00620), FR - fumarate reductase (SMUL_0550-0552; SCA02S_RS07735-RS07740).

Conclusion

Taken together, our results show that several Epsilonproteobacteria have to be considered as H_2 producers and serve as syntrophic partners in e.g. the presence of lactate, which is a widely distributed organic electron donor in natural habitats. H_2 production in *Sulfurospirillum* spp. under the tested conditions relies on Hyf, a multisubunit, membrane-bound and cytoplasmically oriented group 4 NiFe hydrogenase similar to the one used in a second *E. coli* formate hydrogen lyase complex and probably functioning as a proton pump. Adaptation to fermentative conditions seems to be common in *S. multivorans* and related strains, although the underlying mechanism of this process is still unclear. Two seperate clades of *Sulfurospirillum* spp. have different fermentation pathways, the *S. cavolei* clade producing more H_2 and exclusively one organic acid, namely acetate, in comparison to *S. multivorans*, which additionally produces lactate and succinate. All these findings imply an even higher versatility for Epsilonproteobacteria than previously thought and a new ecological role for *Sulfurospirillum* spp., which inhabit a large range of environmentally or biotechnologically important habitats such as wastewater plants, oil reservoirs, bioelectrodes, contaminated sediments or marine areas.

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Author Contributions

SK performed the wetlab work, SK and TG planned experiments, TG initiated the study, TG and GD supervised the study, LA performed mass-spectrometric analysis, SK, TG and GD analyzed and discussed data, MW was responsible for electron microscopy, SK and TG drafted the manuscript, all authors revised, read and approved this manuscript.

References

1. McInerney, M. et al. Physiology, ecology, phylogeny, and genomics of microorganisms capable of syntrophic metabolism. *Ann N Y Acad Sci.* 1125, 58-72 (2008).
2. Sieber, J.R., McInerney, M.J., & Gunsalus, R.P. Genomic Insights into Syntrophy: The Paradigm for Anaerobic Metabolic Cooperation. *Ann Rev Microbiol* 66, 429-452 (2012).
3. Schink B. Energetics of syntrophic cooperation in methanogenic degradation. *Microbiol Mol Biol Rev* 61*,* 262–280. (1997).
4. Schink, B. & Stams, A. Prokaryotic Communities and Ecophysiology. The Prokaryotes (eds. E. Rosenberg, E. DeLong , S. Lory, E. Stackebrandt & F. Thompson) 471-493 (Springer, Berlin Heidelberg; 2013).
5. Stams, A.J. & Plugge, C.M. Electron transfer in syntrophic communities of anaerobic bacteria and archaea. *Nat Rev Microbiol* 7, 568-577 (2009).
6. Morris, B.E., Henneberger, R., Huber, H. & Moissl-Eichinger, C. Microbial syntrophy: Interaction for the common good. *FEMS Microbiol Rev* 37, 384-406 (2013).
7. Stams, A.J. Metabolic interactions between anaerobic bacteria in methanogenic environments. *Antonie Van Leeuwenhoek* 66, 271-294 (1994).
8. de Bok, F.A., Plugge, C.M. & Stams, A.J. Interspecies electron transfer in methanogenic propionate degrading consortia. *Water Res* 38, 1368-1375 (2004).
9. Ishii, S., Kosaka, T., Hori, K., Hotta, Y. & Watanabe, K. Coaggregation facilitates interspecies hydrogen transfer between Pelotomaculum thermopropionicum and Methanothermobacter thermautotrophicus. *Appl Environ Microbiol* 71, 7838-7845 (2005).
10. Mao, X., Stenuit, B., Polasko, A. & Alvarez-Cohen, L. Efficient metabolic exchange and electron transfer within a syntrophic trichloroethene-degrading coculture of *Dehalococcoides mccartyi* 195 and *Syntrophomonas wolfei*. *Appl Environ Microbiol* 81, 2015–2024 (2015).
11. Lee, H.S., Vermaas, W.F. & Rittmann, B.E. Biological hydrogen production: prospects and challenges. *Trends Biotechnol* 28, 262-271 (2010).
12. Dross, F. et al. The quinone-reactive Ni/Fe-hydrogenase of *Wolinella succinogenes*. *Eur J Biochem* 206, 93-102 (1992).
13. Maier, R.J. et al. Hydrogen uptake hydrogenase in *Helicobacter pylori*. *FEMS Microbiol Lett* 141, 71-76 (1996).
14. Meyer, J.L. & Huber, J.A. Strain-level genomic variation in natural populations of *Lebetimonas* from an erupting deep-sea volcano. *ISME J* 8, 867-880 (2014).
15. Handley, K.M. et al. The complete genome sequence for putative H_2- and S-oxidizer Candidatus *Sulfuricurvum* sp., assembled de novo from an aquifer-derived metagenome. *Environ Microbiol* 16, 3443-3462 (2014).
16. Nakagawa, S. et al. Deep-sea vent epsilon-proteobacterial genomes provide insights into emergence of pathogens. *Proc Natl Acad Sci U S A* 104, 12146-12150 (2007).
17. Roalkvam, I. et al. Physiological and genomic characterization of *Arcobacter anaerophilus* IR-1 reveals new metabolic features in Epsilonproteobacteria. *Front Microbiol* 6, 987 (2015).
18. Campbell, B.J. et al. Adaptations to submarine hydrothermal environments exemplified by the genome of *Nautilia profundicola*. *PLoS Genet* 5, e1000362 (2009).
19. Ross, D.E., Marshall, C.W., May, H.D. & Norman, R.S. Comparative Genomic Analysis of *Sulfurospirillum cavolei* MES Reconstructed from the Metagenome of an Electrosynthetic Microbiome. *PLoS One* 11, e0151214 (2016).
20. Goris, T. et al. Insights into organohalide respiration and the versatile catabolism of *Sulfurospirillum multivorans* gained from comparative genomics and physiological studies. *Environ Microbiol* 16, 3562-3580 (2014).
21. Zhang, Y. & Sievert, S.M. Pan-genome analyses identify lineage- and niche-specific markers of evolution and adaptation in Epsilonproteobacteria. *Front Microbiol* 5, 110 (2014).
22. Han, Y. & Perner, M. The globally widespread genus *Sulfurimonas*: versatile energy metabolisms and adaptations to redox clines. *Front Microbiol* 6, 989 (2015).
23. Goris, T. & Diekert, G. The Genus *Sulfurospirillum*. Organohalide-respiring Bacteria. (eds. L. Adrian & F. Löffler) (Springer, Berlin Heidelberg; 2016).
24. Luijten, M. et al. Anaerobic reduction and oxidation of quinone moieties and the reduction of oxidized metals by halorespiring and related organisms. *FEMS Microbiol Ecol* 49, 145-150 (2004).
25. Neumann, A., Scholz-Muramatsu, H. & Diekert, G. Tetrachloroethene metabolism of *Dehalospirillum multivorans*. *Arch Microbiol* 162, 295-301 (1994).

26. Goris, T. et al. Proteomics of the organohalide-respiring Epsilonproteobacterium *Sulfurospirillum multivorans* adapted to tetrachloroethene and other energy substrates. *Sci Rep* 5, 13794 (2015).
27. Page, A. et al. Microbial diversity associated with a *Paralvinella sulfincola* tube and the adjacent substratum on an active deep-sea vent chimney. *Geobiology* 2, 225-238 (2004).
28. Jensen, A. & Finster, K. Isolation and characterization of *Sulfurospirillum carboxydovorans* sp nov., a new microaerophilic carbon monoxide oxidizing epsilon Proteobacterium. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* 87, 339-353 (2005).
29. Kruse, S., Goris, T., Wolf, M., Wei, X. & Diekert, G. The NiFe Hydrogenases of the Tetrachloroethene-Respiring Epsilonproteobacterium *Sulfurospirillum multivorans*: Biochemical Studies and Transcription Analysis. *Front Microbiol* 8, 444 (2017).
30. Scholz-Muramatsu, H., Neumann, A., Messmer, M., Moore, E. & Diekert, G. Isolation and characterization of *Dehalospirillum multivorans* gen. nov., sp. nov., a tetrachloroethene-utilizing, strictly anaerobic bacterium. *Arch Microbiol* 163, 48-56 (1995).
31. Neumann, A. et al. Phenyl methyl ethers: novel electron donors for respiratory growth of *Desulfitobacterium hafniense* and *Desulfitobacterium* sp strain PCE-S. *Arch Microbiol* 181, 245-249 (2004).
32. Whitman, W.B., Ankwanda, E. & Wolfe, R.S. Nutrition and carbon metabolism of *Methanococcus voltae*. *J Bacteriol* 149, 852-863 (1982).
33. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72, 248-254 (1976).
34. Kublik, A. et al. Identification of a multi-protein reductive dehalogenase complex in *Dehalococcoides mccartyi* strain CBDB1 suggests a protein-dependent respiratory electron transport chain obviating quinone involvement. *Environ Microbiol* 18, 3044-3056 (2016).
35. Kodama, Y., Ha, L. & Watanabe, K. *Sulfurospirillum cavolei* sp nov, a facultatively anaerobic sulfur-reducing bacterium isolated from an underground crude oil storage cavity. *International Journal of Systematic and Evolutionary Microbiology* 57, 827-831 (2007).
36. Aziz, R.K. et al. The RAST Server: rapid annotations using subsystems technology. *BMC Genomics* 9, 75 (2008).
37. Goris, T. et al. The complete genome of the tetrachloroethene-respiring Epsilonproteobacterium *Sulfurospirillum halorespirans*. *J Biotechnol* 255, 33-36 (2017).
38. Evans, J.D. & Martin, S.A. Cloning of the L-lactate dehydrogenase gene from the ruminal bacterium *Selenomonas ruminantium* HD4. *Curr Microbiol* 44, 155-160 (2002).
39. Jiang, T. et al. A Bacterial Multidomain NAD-Independent d-Lactate Dehydrogenase Utilizes Flavin Adenine Dinucleotide and Fe-S Clusters as Cofactors and Quinone as an Electron Acceptor for d-Lactate Oxidization. *J Bacteriol* 199 (2017).
40. Fuller, J.R. et al. Identification of a lactate-quinone oxidoreductase in *Staphylococcus aureus* that is essential for virulence. *Front Cell Infect Microbiol* 1, 19 (2011).
41. Campbell, B.J., Engel, A.S., Porter, M.L. & Takai, K. The versatile epsilon-proteobacteria: key players in sulphidic habitats. *Nat Rev Microbiol* 4, 458-468 (2006).
42. Miroshnichenko, M. et al. *Caminibacter profundus* sp nov., a novel thermophile of Nautiliales ord. nov within the class 'Epsilonproteobacteria', isolated from a deep-sea hydrothermal vent. *International Journal of Systematic and Evolutionary Microbiology* 54, 41-45 (2004).
43. John, M. et al. Retentive memory of bacteria: Long-term regulation of dehalorespiration in *Sulfurospirillum multivorans*. *J Bacteriol* 191, 1650-1655 (2009).
44. Mirzoyan, S., Romero-Pareja, P., Coello, M., Trchounian, A. & Trchounian, K. Evidence for hydrogenase-4 catalyzed biohydrogen production in *Escherichia coli*. *International Journal of Hydrogen Energy* 42, 21697-21703 (2017).
45. Kyritsis, P., Hatzfeld, O.M., Link, T.A. & Moulis, J.M. The two [4Fe-4S] clusters in *Chromatium vinosum* ferredoxin have largely different reduction potentials. Structural origin and functional consequences. *J Biol Chem* 273, 15404-15411 (1998).
46. McTernan, P.M. et al. Engineering the respiratory membrane-bound hydrogenase of the hyperthermophilic archaeon *Pyrococcus furiosus* and characterization of the catalytically active cytoplasmic subcomplex. *Protein Eng Des Sel* 28, 1-8 (2015).
47. Lamont, C.M. et al. Expanding the substrates for a bacterial hydrogenlyase reaction. *Microbiology* 163, 649-653 (2017).
48. Hedderich, R. Energy-converting [NiFe] hydrogenases from archaea and extremophiles: Ancestors of complex I. *J Bioenerg Biomembr* 36, 65-75 (2004).

49. Marreiros, B.C., Batista, A.P., Duarte, A.M. & Pereira, M.M. A missing link between complex I and group 4 membrane-bound [NiFe] hydrogenases. *Biochim Biophys Acta* 1827, 198-209 (2013).
50. Thomas, M.T. et al. Two respiratory enzyme systems in *Campylobacter jejuni* NCTC 11168 contribute to growth on L-lactate. *Environ Microbiol* 13, 48-61 (2011).
51. Pinchuk, G.E. et al. Genomic reconstruction of *Shewanella oneidensis* MR-1 metabolism reveals a previously uncharacterized machinery for lactate utilization. *Proc Natl Acad Sci U S A* 106, 2874-2879 (2009).
52. Chai, Y., Kolter, R. & Losick, R. A widely conserved gene cluster required for lactate utilization in *Bacillus subtilis* and its involvement in biofilm formation. *J Bacteriol* 191, 2423-2430 (2009).
53. Zhang, Y. et al. Coexistence of two d-lactate-utilizing systems in *Pseudomonas putida* KT2440. *Environ Microbiol Rep* 8, 699-207(2016).
54. Kim, Y.J. et al. Formate-driven growth coupled with H(2) production. *Nature* 467, 352-355 (2010).

2.3 Syntrophy between *Dehalococcoides* and *Sulfurospirillum* leads to rapid and complete dechlorination of tetrachloroethene

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The ability to produce hydrogen shed new light on the ecological role of *S. multivorans* and *Sulfurospirillum* spp. as syntrophic partners. To get more insights into possible syntrophic interactions with hydrogen consuming microorganisms in organohalide-respiring microbial communities, the organism was co-cultivated with another an obligate dechlorinator *Dehalococcoides mccartyi,* which are often found in these consortia and restricted to hydrogen as energy source and electron donor. An interspecies hydrogen transfer between both organisms enabled a complete dechlorination of tetrachloroethene to ethene at much higher rates compared to the single cultures. Additionally, an interspecies cobamide transfer was observed and *D. mccartyi* was shown to salvage and remodel a corrinoid produced by *S. multivorans*. The syntrophic relation between both led to the formation of cell aggregates. Electron microscopic analysis and FISH staining of these flocs revealed cells embedded in extracellular polymeric substances and in close physical contact which might aid in an enhanced exchange of metabolites. The established co-culture is a potential candidate for bioremediation and bioaugmentation processes by applying it to organohalide contaminated sites.

My own contributions to this publication covers about: 65%.

All growth experiments and analytic determinations of metabolites were conducted by myself. Following experiments were done in cooperation: Isotope fractionation with Steffi Franke and Ivonne Nijenhuis, vitamin B_{12} analysis with Jan Birkigt, Proteomic analysis with Dominique Türkowsky and Nico Jehmlich (all at Centre for Environmental Research, Leipzig). FISH analysis with Bruna Matturro and Simona Rossetti (CNR-IRSA, Rome, Italy), Electron microscopy with Martin Westermann (University Hospital Jena). Tobias Goris and Stefan Kruse initiated the study and Tobias Goris and Gabriele Diekert supervised the study. Stefan Kruse drafted the manuscript and Tobias Goris and Gabriele Diekert revised the draft.

Supplementary information is given in appendix, xxx - xxvii

Syntrophy between *Dehalococcoides* and *Sulfurospirillum* leads to rapid and complete dechlorination of tetrachloroethene

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Abstract

Sulfurospirillum multivorans was recently introduced as a syntrophic partner for the methanogen *Methanococcus voltae* by producing hydrogen during fermentative growth. Although, its ecological role in dechlorinating microbial communities remains elusive. First insights into syntrophic interactions of *S. multivorans* with other organohalide-respiring bacteria were gained in a co-culture with *Dehalococcoides mccartyi* strain BTF08 and 195 with tetrachloroethene (PCE) as electron acceptor. Lactate was used as electron donor and enabled an obligate syntrophic relation between both, since *S. multivorans* showed no growth on lactate alone and relies on *D. mccartyi* maintaining low hydrogen partial pressures. PCE was rapidly dechlorinated to *cis*-1,2-dichloroethene (*cis*-DCE) by *S. multivorans* as confirmed by carbon stable isotope analysis (CSIA) and subsequent interspecies hydrogen transfer led to complete conversion to ethene. Co-cultures exhibited 3- fold higher dechlorination rates (Sm/Dhc BTF08: 4.1 ± 0.2 µmol day^{-1}; Sm/Dhc 195: 4.8 ± 0.2 µmol day^{-1}) compared to the pure cultures and increased after refeeding of PCE (11.8 µmol day^{-1}). Additionally, dechlorination acitivity was restored in co-cultures amended with 5,6-dimethylbenzimidazole (DMB) indicating an interspecies cobamide transfer. MS analysis confirmed the remodeling of the nonfunctional norpseudovitamin-B_{12} produced by *S. multivorans* into cyanocobalamin supporting *D. mccartyi* growth. Formed cell aggregates were analyzed using field-emission scanning electron microscopy and FISH staining showed an equal distribution of both organisms within the aggregates. On electron micrographs, cells were surrounded by flagellum-like filaments and embedded in extracellular polymeric substances (EPS)-like structures. In contrast to the single culture, *D. mccartyi* showed an unusual cell morphology in the co-culture which is supported by the cell division protein FtsZ found to be downregulated in the proteome. This study provides the first in-depth analysis of the syntrophic interactions between two organohalide respiring bacteria, *S. multivorans* and *D. mccartyi*.

Introduction

Bacterial communities are characterized by various microbial interactions based on an exchange of metabolic products. Hydrogen, for example, is an important energy carrier in syntrophic communities, in which the gas is produced by fermenting bacteria and taken up by a hydrogen-consuming partner. This interspecies hydrogen transfer allows otherwise thermodynamically unfavorable reactions to proceed by shifting the reaction out of equilibrium. Therefore, the involved bacteria are physiologically dependent on each other (Morris *et al.*, 2013; Schink and Stams, 2013; Stams and Plugge, 2009). The prominent and potential carcinogenic groundwater pollutant tetrachloroethene (PCE) is often only completely dechlorinated to ethene in communities involving

interspecies hydrogen transfer (Henschler, 1994). The key step in this process is the reductive dehalogenation of the organohalide, e.g. PCE, hexachlorobenzene or polychlorinated biphenyls, which is used as terminal electron acceptors and coupled to energy conservation via electron transport phosphorylation. This anaerobic respiration is termed organohalide respiration (OHR) (Leys *et al.*, 2013). Key player in detoxification and bioremediation of PCE contaminated environments is the exclusively hydrogen-oxidizing *Dehalococcoides mccartyi*, Several strains of this species are currently the only known bacteria capable of complete dechlorination of PCE to non-toxic ethene (Maymó-Gatell *et al.*, 1999; Maymó-Gatell *et al.*, 1997; Maymó-Gatell *et al.*, 2001) However, these bacteria are characterized by low growth yields when cultivated in pure cultures, resulting in low dechlorination rates (Löffler *et al.*, 2013b). Additionally, the organisms are restricted to specific nutrient and vitamin requirements in their habitats (Zinder, 2016). Besides being restricted to hydrogen as electron donor, *D. mccartyi* uses only acetate as carbon source and organohalides as electron acceptors (Seshadri *et al.*, 2005). Additionally, these bacteria are not able to synthesize corrinoids *de novo* (Löffler *et al.*, 2013b; Schipp *et al.*, 2013). Corrinoids are cofactors of the key enzymes for organohalide respiration, reductive dehalogenases (RDases). While proteins for complete corrinoid biosynthesis are not encoded in the genomes of *D. mccartyi*, different studies revealed the bacterium's ability to salvage and remodel corrinoids (Kube *et al.*, 2005; McMurdie *et al.*, 2009). The corresponding pathways enable *D. mccartyi* to scavenge incomplete corrinoids and exchange the lower ligand to render the corrinoid functional leading to better growth and dechlorination (Men *et al.*, 2014; Seshadri *et al.*, 2005). The functionality of the corrinoid and thus the reductive dehalogenase is directly dependent on the type of the lower base in *D. mccartyi*. Only three types of corrinoids were shown to be functional in *D. mccartyi* strain 195: 5,6-dimethylbenzimidazolyl-cobamide ([DMB]-Cba), 5-methylbenzimidazolylcobamide ([5-MeBza]Cba) and 5-methoxybenzimidazolylcobamide ([5-OMeBza]Cba). Nonfunctional corrinoids e.g. 5-hydroxybenzimidazolyl-cobamide ([5-OHBza]Cba) or 7-adeninyl-cobamide ([Ade]Cba) can be converted into functional ones by replacement of the lower ligand when 5,6-dimethylbenzimidazole (DMB) is present (He *et al.*, 2007; Men *et al.*, 2014; Yi *et al.*, 2012). Different studies on dechlorinating communities containing *D. mccartyi* in association with fermenting, acetogenic and methanogenic bacteria revealed higher dechlorination and growth rates than those of pure cultures (DiStefano *et al.*, 1992; He *et al.*, 2003a; He *et al.*, 2003b; Maymó-Gatell *et al.*, 1997). It is assumed that cross-feeding and a constant supply of growth factors such as corrinoids and biotin enhance growth (Richardson, 2016). In the syntrophic interactions of this communities, non-dechlorinating fermenting bacteria provide hydrogen, acetate and CO_2 from e.g. lactate or butyrate fermentation and are dependent on hydrogen consumers which keep the hydrogen partial pressure low (Cheng *et al.*, 2010; Mao *et al.*, 2015; Men *et al.*, 2012; Richardson *et al.*, 2002). For example, co-culture experiments revealed an interspecies hydrogen transfer between *Desulfovibrio desulfuricans* fermenting lactate and *D. mccartyi* (He *et al.*, 2007). Additionally, acetogens like *Acetobacterium woodii, Sporomusa ovata* or *Desulfovibrio* and methanogenic archaea like *Methanococcus voltae* are able to produce a broad range of different types of corrinoid cofactors (Duhamel and Edwards, 2007; Guimarães *et al.*, 1994; Stupperich *et al.*, 1988; Stupperich and Kräutler, 1988). An

interspecies cobamide transfer was also shown between *Geobacter lovleyi* and *D. mccartyi* strain BAV1 and FL2 and *Methanosarcina barkeri* strain Fusaro and *D. mccartyi* strain BAV1, GT and FL2 when DMB was present (Yan *et al.*, 2013; Yan *et al.*, 2012). All these studies showed more robust growth of *D. mccartyi* in co-cultures, resulting in higher dechlorination rates and cell yields. However, one single organism providing hydrogen, acetate and corrinoid to *D. mccartyi* in a co-culture was not found so far. Such a syntrophic partner could aid in optimization of bioremediation and bioaugmentation using *D. mccartyi*-containing cultures (Major *et al.*, 2002). Recently, the PCE to *cis*-DCE-respiring Epsilonproteobacterium *Sulfurospirillum multivorans*, capable of *de novo* corrinoid production of norpseudo B_{12} (Kräutler *et al.*, 2003; Neumann *et al.*, 1994), was shown to produce hydrogen and acetate under fermentative growth conditions and might therefore be of interest for co-cultivation with *D. mccartyi*.

In this study, we report the syntrophic relation between *D. mccartyi* strains BTF08 in co-culture with *S. multivorans*. It was of interest, whether *S.multivorans* is able to supply *D. mccartyi* with the aforementioned supplements. These co-cultures showed an enhanced PCE to ethene dechlorination rate compared to pure cultures. Additionally, Interspecies cobamide transfer was detected. Electron microscopic and FISH analysis of co-cultures showed formation and association of both organisms in aggregates. The co-culture could be a potential candidate for bioremediation of PCE-contaminated sites and to study microbial interaction between different OHRB.

Materials and Methods

Growth conditions of pure cultures

D. mccartyi pure cultures BTF08 and 195 (received from Steffi Franke, UFZ Leipzig) were cultivated in 200 ml serum bottles containing 100 ml bicarbonate-buffered mineral salt medium with 5mM acetate and 148 nM vitamin B_{12} (cyanocobalamin, ca. 200 µg/L), reduced by Na_2S (Maymó-Gatell *et al.*, 1999). Anoxic atmosphere was established by 30 cycles gasing and degassing with nitrogen and final atmosphere of N_2:CO_2 (25:75 v/v). The composition of the basal medium was described previously. After autoclaving, hydrogen [150 kPa] was applied. PCE (>99% purity, Sigma Aldrich, Steinheim, Germany) and *cis*-DCE (97% purity, Sigma Aldrich, Steinheim, Germany) served as electron donors and were added with a microliter syringe (Hamilton, Bonaduz, Switzerland) to a final concentration of 0.35 mM (aqueous-phase concentration). Re-feeding of the cultures was done with the same dose of PCE or *cis*-DCE. After maximally three re-feeding steps, cultures were transferred [10% (v/v)] into fresh medium. To evaluate the effect of different types and concentrations of B_{12} on dechlorination activities, Dhc pure cultures received 54 nM or 54 pM norpseudo-B_{12} ([Ade]Cba) and 5-OMeBza-B_{12} ([5-OMeBza]Cba). Norpseudo-B_{12} was extracted according Keller *et al.* (Keller *et al.*, 2014) from 6L of *S. multivorans* grown anoxically with 40 mM pyruvate and 10 mM PCE as described elsewhere (Kruse *et al.*, 2017a). 5-OMeBza-B_{12} was obtained from 6L of *Desulfitobacterium hafniense* DCB2 grown anoxically with 40 mM pyruvate and 10 mM ClOHPA (3-chloro-4-hydroxy-

phenylacetate) with the addition of 25 µM 5-OMeBza (unpublished). Pure cultures of *S. multivorans* (DSMZ 12446) were maintained in the same mineral salt medium without the addition of acetate and hydrogen. All cultivation experiments were performed statically at 28°C in the dark and in biological triplicates.

Growth of co-cultures of *S. multivorans* and *Dehalococcoides*

Co-cultures of *S. multivorans* and *D. mccartyi* strain BTF08 or 195 were maintained in the same mineral salt medium as the pure cultures without the addition of acetate and hydrogen. The medium contained 25 mM lactate as the electron donor for *S. multivorans* and 0.35 mM PCE (aqueous-phase concentration) as the electron acceptor. Re-feeding of lactate and PCE was done in the same concentration as the initial dose. The B_{12}-dependance of the co-cultures was tested with 148 nM vitamin B_{12} serving as the positive control and without vitamin B_{12} additions. Additionally, cultures without vitamin B_{12} received 1 µM DMB (>99% purity, Sigma Aldrich, Steinheim, Germany). For isotope fractionation experiment, *S. multivorans* and Sm/Dhc BTF08 co-culture were cultivated in 50 ml serum bottles with 25 ml medium. Different replicate bottles were inoculated at the same time and stopped at different time points during the dehalogenation process by addition of 3 ml 2 M Na_2SO_4 (pH 1.0).

qPCR analysis of cell growth

DNA was extracted from 1 ml co-culture taken from different time points during the cultivation experiment using the NucleoSpin Tissue DNA extraction kit (Macherey-Nagel, Düren, Germany). Extraction procedure was according manufacturer's instructions. Quantitative PCR (qPCR) was applied to enumerate *Sulfurospirillum* and *Dehalococcoides* 16S rRNA gene copies. qPCR reaction mixture contained 1 µl of gDNA or standard, 6.25 µl 1x KAPA SYBR Fast master mix (Sigma Aldrich, Steinheim, Germany) and 0.208 µM forward and reverse primer. Primer used were Dhc_sp_16S_fw (5'-GTATCGACCCTCTCTGTGCCG-3') and Dhc_sp_16S_rev (5'-GCAAGTTCCTGACTTAACAGGTCGT-3') for *Dehalococcoides* sp. and Smul_16S_fw (5'-AGRGCTAGTTTACTAGAACTTAGAG-3') and Smul_16S_rev (5'-CAGTCTGATTAGAGTGCTCAG-3') for *S. multivorans*. The conditions of the PCR program were as followed: 95°C for 2 min (initial denaturation) followed by 40 cycles of 55°C (*S. multivorans* primer) or 60°C (*D. mccartyi* primer) for 20s (annealing), 72°C for 30s (elongation) and 95°C for 10s (denaturation). Each PCR included a melting curve for verification of specific target DNA amplification. Standard curves were done from extracted gDNA from different cell number preparations of *S. multivorans* and *D. mccartyi* strains BTF08 and 195. For this, each dilution step contained the same cell number of both organisms from which genomic DNA was isolated. The obtained C_T values were compared with the standard curve to determine the different cell numbers. All samples were conducted in three biological replicates with two corresponding technical replicates and three technical replicates were done for the calibration curve.

Analytical methods

Ethene and chlorinated ethenes were quantified gas chromatographically with a flame ionization detector (Clarus 500, Perkin Elmer, Rodgau, Germany) and a CP-PoraBOND Q FUSED SILICA 25 m x 0.32 mm column (Agilent Technologies, Böblingen, Germany). A headspace sample from 1 ml culture as well as from 1 ml gas phase was taken using a gas-tight syringe (Hamilton, Bonaduz, Switzerland) was analyzed. Separation of chlorinated ethenes was as followed: 4 min at 60 to 280°C in 10°C/min steps. The injector temperature was fixed at 250°C and detector temperature at 300°C. Standard curves of ethene and each chlorinated ethene were performed for peak area quantification and retention times were compared to known standards. Hydrogen was measured using a thermal conductivity detector (AutoSystem, Perkin Elmer, Rodgau, Germany). Organic acids were analyzed by HPLC and separated on an AMINEX HPX-87H column (7.8x300mm; BioRad, Munich, Germany).

Compound Specific Stable Isotope Analysis

Determination of the carbon isotope composition of the chlorinated ethenes in pure culture of *S. multivorans* and in co-culture of *S. multivorans* and *D. mccartyi* strain BTF08 was done using gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS, Thermo GC Trace 1320 combined with Thermo-Finnigan MAT 253 IRMS, Bremen, Germany) according to Schmidt *et al.* (Schmidt *et al.*, 2014). All samples were analyzed in technical triplicates. 2 ml liquid phase were taken from the respective sample and transferred to a He-flushed 10 ml crimped vial. Therefore, 1 ml headspace was taken via an autosampler (Thermo TriPlus RSH Autosampler) and injected in a gas chromatograph with a split ratio of 1:5. Chromatographic separation of chloroethenes was done using a DB-MTBE column (60 m x 0.32 mm x 1.8 µm, J&W Scientific, Waldbronn, Germany) according to following temperature program: 40°C for 5 min, increase to 250°C by 20°C min^{-1} and hold for 5 min utilizing helium as carrier gas at a flow rate of 2.0 ml min^{-1} (Injector at 250°C).

The carbon isotope composition is given in the δ-notation (‰) relative to the Vienna Pee Dee Belemnite standard (Coplen *et al.*, 2006). Carbon isotope fractionation was calculated using the Rayleigh equation (eq 1) where R_0 and R_t represents the isotope values and C_0 and C_t the concentrations at time 0 and t (Elsner, 2010; Elsner *et al.*, 2008).

$$ln\frac{Rt}{R0} = \varepsilon C * ln\frac{Ct}{C0} \qquad (1)$$

$$\varepsilon = (\alpha C - 1) \qquad (2)$$

The carbon isotope enrichment factor (ε_C) is defined in equation 2 and was determined with the carbon isotope fractionation factor αC which relates changes in the concentration of the isotopes to changes in their isotope composition. The 95% confidence interval was defined as the slope of the linear regression of the Rayleigh equation. Standard deviations were obtained from at least triplicate measurements (< 0.5 ‰).

B_{12} extraction and MS analysis

The B_{12} content of the whole serum bottle including supernatant and cells was analyzed. Therefore, the culture volume was reduced to 20 ml using a vacuum concentrator and subsequently 0.1 M potassium cyanide was added. After boiling of the samples for 20 min, cell debris was removed by centrifugation (10 min, 6700 x g, 8°C). The supernatant was applied onto a C-18 column (CHROMABOND C-18 ec, Macherey-Nagel, Düren, Germany) equilibrated with 5 mL 100% (v/v) methanol and 5 mL ultra pure water (UPW). Washing of the column was done with 5 mL UPW twice and B12-types were eluted with 5 mL 100% (v/v) methanol. The eluate was completely dryed in a vacuum dryer.

Scanning Electron Microscopy and fluorescence in situ hybridization

Field emission-scanning electron microscopy (FE-SEM) was performed with co-cultures of *S. multivorans* and *D. mccartyi* strain BTF08 and 195. Cells of 5 ml culture were incubated for 15 min with 2.5% glutaraldehyde and pre-fixed for 2 h on Poly-L-Lysin coated coverslides (12 mm, Fisher Scientific, Schwerte, Germany). Coverslides were washed three times with 0.1 M sodium cacodylate (pH 7.2) (>98% purity, Sigma Aldrich, Steinheim, Germany) and post-fixed for 1 h with 1% osmium tetroxyde in the same cacodylate buffer. After fixation, samples were dehydrated using different ethanol concentrations. Critical point drying was done in a Leica EM CPD200 Automated Critical Point Dryer (Leica, Wetzlar, Germany), followed by coating with 6 nm platinum in a BAL-TEC MED 020 Sputter Coating System (BAL-TEC, Balzers, Liechtenstein). Imaging of the samples was done with a Zeiss-LEO 1530 Gemini field emission scanning electron microscope (Carl Zeiss, Oberkochen, Germany) at different magnifications. Fluorescence in situ hybridization (FISH) was performed as described previously (Fazi *et al.*, 2008; Matturro *et al.*, 2013). In brief, samples were fixed with 10% (v/v) formaldehyde for 2 h at 4°C and the addition of one volume 96% (v/v) ethanol. Cells were filtered on polycarbonate membrane filters (47 mm diameter, 0.2 µm pore size, Nucleopore) and washed with Milli-Q water. FISH detection of *D. mccartyi* strain BTF08 and 195 was done with Cy3-labeled DHC1259t and DHC1259c probes and *S. multivorans* detection with FITC-labeled probes SULF F220ab (Rossetti *et al.*, 2008). Finally, filters were fixed on a microscope slide and stained with DAPI (4,6-diamidino-2-phenylindole). Imaging of un-aggregated cells was done with an epifluorescence microscope (Olympus, BX51) combined with an Olympus XM10 camera. Images were analyzed via Cell-F software. Aggregates were visualized using a confocal laser scanning microscopy (CSLM, Olympus FV1000).

SDS-PAGE and proteolytic digestion

Protein concentration was determined after protein extraction using the Bio-Rad Bradford reagent (Bio-Rad, Munich, Germany) and bovine serum albumin as protein standard. Crude extracts were loaded onto a SDS-PAGE. In total, 20 µg of protein lysates were applied. The gel was run until the samples entered the seperating gel. Afterwards, a 3-5mm protein band from each sample was cutted out and prepared for proteolytic cleavage using trypsin (Promega, Madison, WI, USA). Peptide lysates were extracted and desalted using C18 ZipTips (Merck Millipore, Darmstadt, Germany).

Mass spectrometry and proteome data analysis

Separation of tryptic peptides was performed using an Ultimate 3000 nanoRSLC instrument (Thermo Scientific, Germering, Germany) coupled to an Orbitrap Fusion mass spectrometer (Thermo Scientific, San Jose, CA, USA). A sample volume of 1 µL was loaded onto a trapping columnwith 300 µm inner diameter, packed with 5 µm C18 particles (µ-precolumn, Thermo Scientific) and separatedvia a 15cm analytical column (Acclaim PepMap RSLC, 2µm C18 particles,Thermo Scientific).The column oven temperature was constantly set to 35°C. During LC run, a constant flow of 300 nL/min (solvent A: 0.1% formic acid) was applied for a linear gradient of 4% to 55% solvent B (80% acetonitrile, 0.08% formic acid) in 90 min. Full MS scans were measured in the Orbitrap mass analyzer within the mass range of 400-1,700 *m/z* at 60,000 resolution using an automatic gain control (AGC) target of $4x10^5$ and maximum fill time of 50 ms. The MS instrument measured in data-dependent acquisition (DDA) mode using the highest intense ion and positive ion charge state ≥2 and ≤7 were selected for MS/MS. An MS/MS isolation window for MS ions in the quadrupole was set to 1.6 *m/z*. MS/MS scan events were repeated within 3 s of cycle time (Top Speed) using the higher energydissociationmode (HCD) at normalized collision induced energy of 35%, activation time of120 ms, and minimum of ion signal threshold for MS/MS of $5x10^4$counts. The exclusion time to reject masses from repetitive MS/MS fragmentation was set to 30 s.

LC-MS/MS data wereanalyzed using Proteome Discoverer (v1.4.1.14, Thermo Scientific). MS/MS spectra were searched against the *S. multivorans* database containing 3,191 different protein-coding sequence entries (downloaded February 17th 2014 from NCBI Genbank accession number CP007201) using the SEQUEST HT and MS Amanda algorithms with the following settings: trypsin as cleavage enzyme, oxidation on methionine as dynamic and carbamidomethylation on cysteine as static modification, up to two missed cleavages, MSmass tolerance set to 10 ppm and MS/MSmass tolerance to 0.05 Da, respectively. Only peptides with a false discovery rate (FDR)<0.01 were considered as identified (Supplement information Table S1). Quantification of proteins was performed using the average of TOP3 peptidearea. After log10 transformation, the protein values were mean normalized and bioinformatic analysis was applied by ANOVA and T-test statistics (GraphPad).

Results

Growth and dechlorination in *Sulfurospirillum-Dehalococcoides* co-cultures

To ensure optimal cultivation conditions for both organisms in the co-culture, *S. multivorans* was tested for its ability to grow with lactate and PCE in a medium previously optimized for *D. mccarty* and showed the same growth in this medium when compared to the original medium. With lactate as electron donor and PCE as electron acceptor, co-cultures of *S. multivorans* and *D. mccartyi* BTF08 dechlorinated 32.8 ± 2.6 µmol PCE completely to stoichiometric amounts of ethene within 8 days (4.1 ± 0.2 µmol/bottle/day, Figure 1A, Table 1). The culture was re-fed with 35 µmol PCE at day 8 and day 12. A complete dechlorination of PCE occurred in three days (Figure 2A, table 1). After re-feeding, the dechlorination rate increased 2.8-fold to 11.6 µmol/bottle/day. PCE was dechlorinated to *cis*-DCE already within one day and can be assigned to high dechlorination activities of *S. multivorans*. This was confirmed by elucidating the stable carbon isotope fractionation of PCE by growing cells of the co-culture compared to the pure culture of *S. multivorans.* No significant differences in the fractionation of PCE were found. The isotope signature of the co-culture slightly changed 0.7δ units, from about -30.2 to -29.5‰ (Supplementary Figure 1A). A similar fractionation was measured in the pure culture of *S. multivorans* (1.6δ units, from about -29.2 to -27.6‰). Additionally, the enrichment factor was calculated using the Rayleigh equation and the isotope slopes of both cultures were in the same range (Sm/BTF08 co-culture: ε_C = -0.4 ± 0.3 ‰; *S. multivorans* pure culture: ε_C = -2.0 ± 0.4 ‰, Supplementary Figure 1B). The fast dechlorination of PCE to *cis*-DCE was also reflected in a fast increase of the cell number from 1.6 ± 0.4 x 10^8 to 6.9 ± 0.2 x 10^8 cells/ml as analysed by qPCR (Figure 1C). The cell number of *D. mccartyi* BTF08 increased from 6.1 ± 0.6 x 10^7 to 1.5 ± 0.2 x 10^8 cells/ml. Both organism show a correlation between dechlorination and growth. The ratio between *S. multivorans* and *D. mccartyi* strain BTF08 changed from initially 2.6:1 to 4.6:1 after 15 days and two re-feeding steps. After 12 days, the initial lactate (2.4 ± 0.1 mmol/bottle) was completely consumed after the second dose of PCE was completely dechlorinated to ethene and was subsequently re-fed. Acetate production occurred continuously during the whole dechlorination process (up to 0.8 ± 0.01 mmol/bottle, Figure 1E) and no hydrogen was measured in the gas phase (not shown). The PCE to ethene dechlorination rate of the Sm/Dhc BTF08 co-culture is 4.5-fold faster compared to the *D. mccartyi* strain BTF08 culture. In the latter, PCE was completely reduced to ethene a rate of 0.9 ± 0.03 µmol/bottle/day which increased after re-feeding to 1.4 µmol/bottle/day (Table 1, Supplementary Figure 2A).

Similar growth characteristics and dechlorination behavior was observed in the Sm/Dhc 195 co-culture cultivated under the same conditions, except that VC was the major dechlorination product. A first dose of PCE (34.2 ± 1.2 µmol) was dechlorinated within 7 days in stoichiometric amounts to VC (4.8 ± 0.1 µmol/bottle/day, Figure 1B, D and F). The increase of cell number is slightly lower for *S. multivorans* (1.9 ± 0.3 x 10^8 to 6.1 ± 0.3 x 10^8) and slightly higher for *D. mccartyi* 195 (3.8 ± 1.1 x $10^{7)}$ to 1.5 ± 0.02 x 10^8 in this co-culture (Figure 1D). A low amount of ethene was produced starting at day 10 after the second dose of PCE was dechlorinated to VC, reaching 10.4 µmol/bottle after day 15. The Sm/Dhc 195 co-culture reduced PCE to VC 3.5-fold faster compared to the *D. mccartyi*

strain 195 pure culture (1.5 ± 0.04 µmol/bottle/day) increasing after a re-feeding of PCE to 3.3 µmol/bottle/day in the pure culture (Supplementary Figure 2B).

Table 1: Dechlorination rates of different co-culture set-ups *of S. multivorans* and *D. mccartyi* strains BTF08 or 195. n.d. - a 2nd or 3rd re-feeding step was not done.

		Dechlorination rate (µmol day^{-1} bottle^{-1})		
Organism(s)	**Electron donor**	1st dose PCE	2nd dose PCE (1st re-feeding)	3rd dose PCE (2nd re-feeding)
With amendment of vitamin B_{12}				
Dhc strain BTF08	H_2	0.9 ± 0.03	1.4	n.d.
Dhc strain 195	H_2	1.5 ± 0.04	3.3	n.d.
Sm + Dhc strain BTF08	Lactate	4.1 ± 0.2	11.6	11.6
Sm + Dhc strain 195	Lactate	4.8 ± 0.1	11.6	11.6
Without amendment of vitamin B_{12}				
Sm + Dhc strain BTF08	Lactate	∞	n.d.	n.d.
Sm + Dhc strain 195	Lactate	0.9 ± 0.004	3.6	3.6
Without amendment of vitamin B_{12} + DMB				
Sm + Dhc strain BTF08	Lactate	3.6 ± 0.2	8.0	8.0
Sm + Dhc strain 195	Lactate	0.8 ± 0.05	4.3	4.3

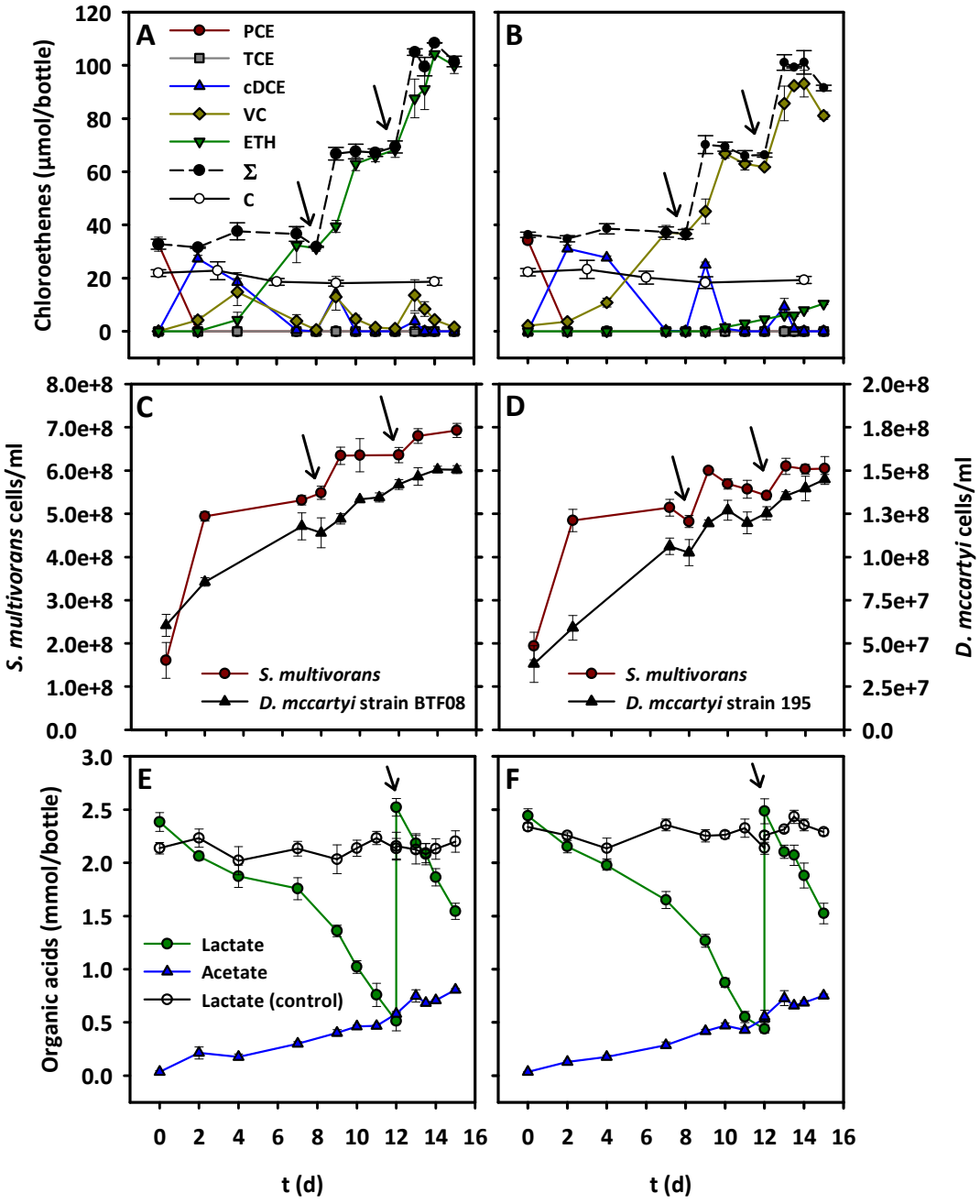

Figure 1: PCE dechlorination, growth and metabolite analysis of *S. multivorans* and *D. mccartyi* strains BTF08 or 195 co-cultures. (A) PCE dechlorination of Sm/Dhc BTF08 and (B) Sm/Dhc 195. (C) Growth curve of Sm/Dhc BTF08 and (D) Sm/Dhc 195. (E) Lactate consumption and acetate production of Sm/Dhc BTF08 and (F) Sm/Dhc 195. Arrows indicate the time points of re-feeding the culture with PCE or lactate. Please note the secondary y-axes for *D. mccartyi* cell numbers in C and D. Negative controls were run with autoclaved cells (abiotic controls). Standard deviation of three independent biological replicates are represented by error bars. C - abiotic negative control.

2. Corrinoid transfer in co-cultures and the effect of the lower ligand

D. mccartyi strains rely on externally provided corrinoids produced by the microbial community. Therefore, it was of interest if *S. multivorans* is able to provide functional corrinoids for *D. mccartyi* strains BTF08 and 195 when co-cultivating both organisms. In Sm/Dhc BTF08 co-cultures without the amendment of vitamin B_{12}, a stoichiometric dechlorination of PCE to *cis*-DCE was obtained (~30 µmol/bottle/day) (Figure 2A). No further dechlorination of *cis*-DCE to VC or ethene was measured, indicating that *S. multivorans* was responsible for the dechlorination.

The non-dechlorinating Sm/Dhc BTF08 co-culture was supplemented with 1 µM 5,6-dimethylbenzimidazole (DMB) resulting in a restored dechlorination (Figure 2C). *cis*-DCE produced by *S. multivorans* is completely reduced to stoichiometric amounts of ethene in 10 days (3.6 ± 0.2 µmol/bottle/day) which is similar to the corresponding co-culture with amended vitamin B_{12} (4.1 ± 0.2 µmol/bottle/day). The dechlorination rate increased after a re-feeding with PCE to 8 µmol/bottle/day. In contrast to the Sm/Dhc BTF08 co-culture, the Sm/Dhc 195 co-culture without vitamin B_{12} amendment (-B_{12}) dechlorinated PCE to VC, although at low rates before the re-feeding of PCE at day 35 (0.9 ± 0.004 µmol/bottle/day, Figure 2D). After 35 days, *cis*-DCE was dechlorinated to VC faster, at a 4-fold higher dechlorination rate (3.6 µmol/bottle/day) (Supplementary Figure 3).

D. mccartyi BTF08 and 195 pure cultures without the addition of vitamin B_{12} showed no dechlorination activity over more than 100 days (Figure 2B, E). The Sm/Dhc 195 co-culture showed no significant increase in dechlorination when adding 1 µM DMB (Figure 2F). Produced *cis*-DCE is reduced to VC in 35 days (0.8 ± 0.05 µmol/bottle/day) which is similar to the co-culture without DMB supplementation. Additionally, PCE was faster reduced to VC after a second and third dose of PCE (4.28 µmol/bottle/day). Mass spectrometric analysis revealed the detection of [Ade]NCba produced by *S. multivorans* and additionally [DMB]NCba and [DMB]Cba (Supplementary Figure 4).

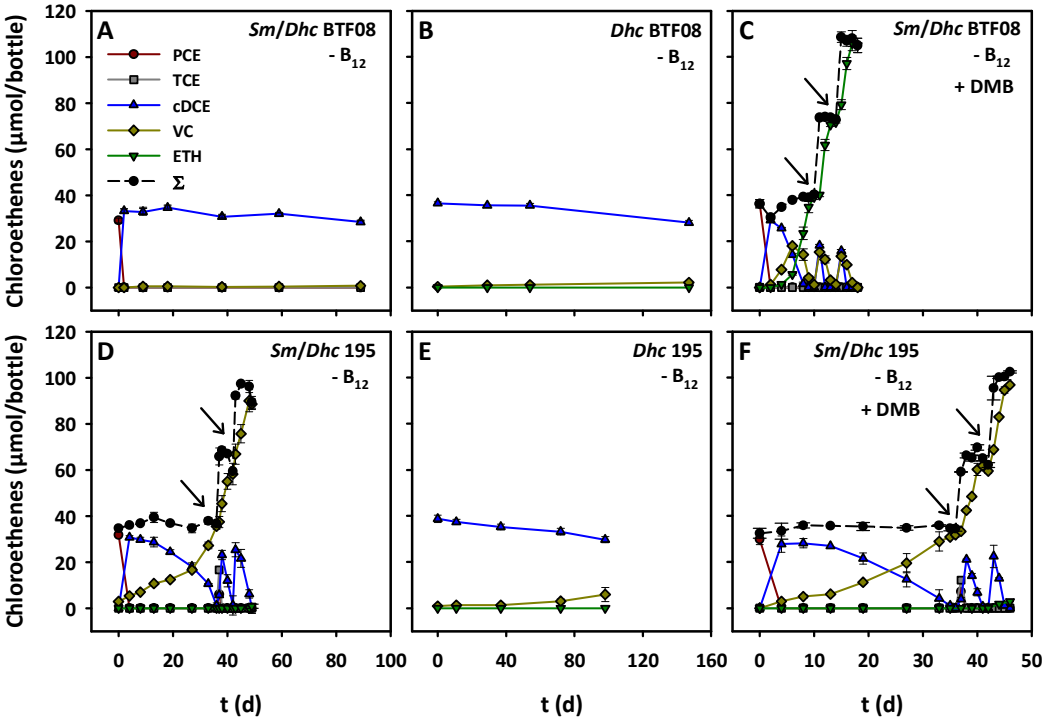

Figure 2: Dechlorination activity of *D. mccartyi* strains BTF08 and 195 in co-culture with *S. multivorans* without addition of vitamin B_{12}. (A) Sm/Dhc BTF08 with PCE as the electron acceptor. (B) Strain BTF08 with *cis*-DCE as electron acceptor (negative control). (C) Sm/Dhc BTF08 with PCE as electron acceptor and amendment of 1 µM DMB. (D) Sm/Dhc 195 with PCE as the electron acceptor. (E) Strain 195 with *cis*-DCE as electron acceptor (negative control). (F) Sm/Dhc 195 with PCE as electron acceptor and amendment of 1 µM DMB. Please not the different time scales. All growth experiments were conducted in independent biological triplicates. Arrows indicate re-feeding of PCE. ∑ = mass balance; sum of PCE, TCE, *cis*-DCE, VC and ethene.

3. Electron microscopy and FISH analysis of formed cell aggregates

During cultivation and approximately 25 to 30 transfers on lactate and PCE, the co-cultures showed a formation of large spherical aggregates already visible to the naked eye and reached a size of 1 to 2 mm in diameter (Figure 3A). Field emission-scanning electron microscopy was applied (FE-SEM) to get detailed insights into the composition and putative microbial interactions in these aggregates. After preparation for SEM, the aggregates showed sizes ranging from 30 to maximally 200 µm (Figure 3B and C, Supplementary Figure 5). Electron micrographs of both co-cultures showed a compact network of *S. multivorans* and *D. mccartyi* cells coiled around by net-forming filament-like structures. Additionally, the cells are embedded in an extracellular matrix (extracellular polymeric substance (EPS)-like structure) which might enable cell-to-cell contact (Figure 3D). FISH (fluorescence in situ hybridization) with specific oligonucleotide probes targeting 16S rRNA was performed to distinguish between *S. multivorans* and the *D. mccartyi* strains in the aggregates. 3-dimensional imaging showed a spatial organization and an equal distribution of both species within the aggregates (Figure 3E, Supplementary Figure 6). Additionally, the high resolution of the confocal laser scanning microscopy enabled visualization of single cell structures and revealed the same morphologies as in the electron micrographs. The sizes of the aggregates ranged from 50 to 100 µm in diameter. At magnifications of around 10.000x and 20.000x, *S. multivorans* showed a helical rod

shaped cell structure with a single flagellum and a length of 2 to 5 µm and a diameter of around 0.5 µm as described previously (Scholz-Muramatsu *et al.*, 1995) (Supplementary Figure 2). However, *D. mccartyi* showed an untypical cell morphology when co-cultivating with *S. multivorans*. Microscopic analysis of the pure culture revealed a disc-shaped irregular coccus of 0.5 µm in diameter, whereas the co-culture showed 0.5 µm large barrel-like cells with a flattened cell pole at one side and a ring-shaped septum (Figure 4) (Löffler *et al.*, 2013b). Although, FISH analysis of un-aggregated cells in the co-culture identified both organisms and clarified the presence of *D. mccartyi* (Supplementary Figure 7).

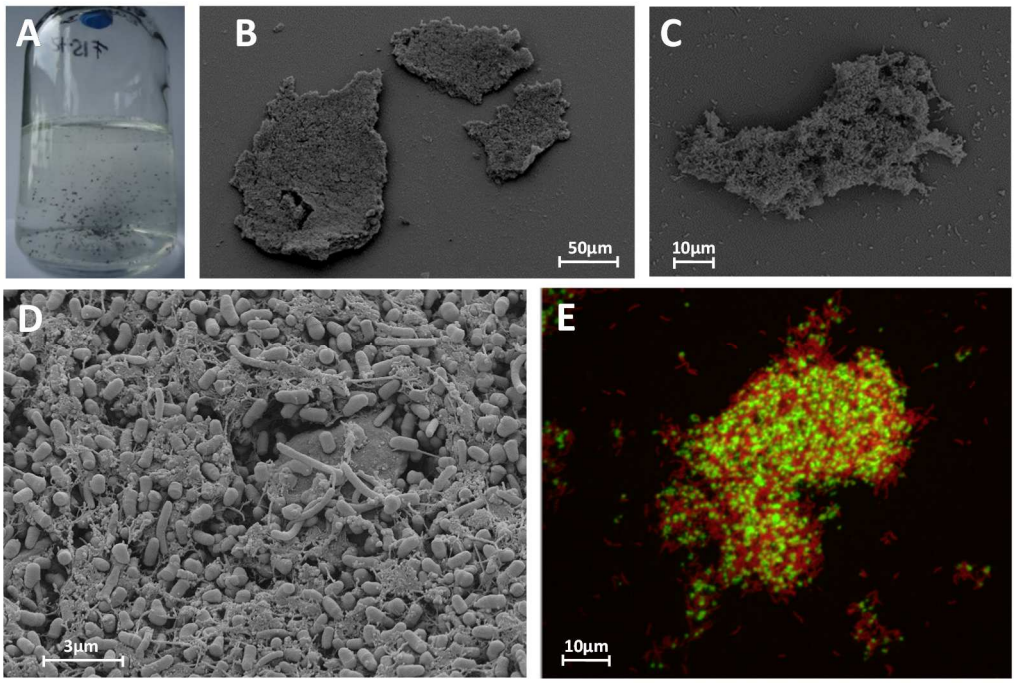

Figure 3: Microscopic analysis of formed cell aggregates in co-cultures of *S. multivorans* and *D. mccartyi* strain 195 and BTF08. (A) Serum bottle of a Sm/Dhc BTF08 co-culture. (B-D) Scanning electron micrographs of an aggregate of Sm/Dhc 195 (B) and Sm/Dhc BTF08 (C,D). (E) Confocal laser scanning image of FISH stained aggregates of Sm/Dhc BTF08. red: *S. multivorans*, green: *D. mccartyi*.

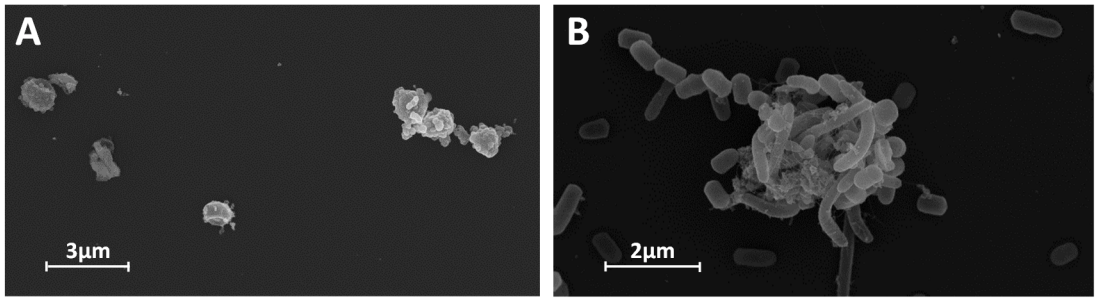

Figure 4: Different cell morphologies of *D. mccartyi* strain BTF08 cells in pure culture (A) and co-culture with *S. multivorans* (B).

Proteomics of pure and co-cultures

To get a picture of the physiological response of *S. multivorans* and *D. mccartyi* BTF08 to the dechlorination of PCE in pure and co-cultures and to see the effect of corrinoid supply from S. multivorans, we collected proteome data of several different cultures. *D. mccartyi* BTF08 was cultivated with hydrogen as electron donor and either PCE ('P') or *cis*-DCE ('C') as electron acceptor, S. multivorans was cultivated with lactate as electron donor and PCE as electron acceptor ('S'), co-cultures were cultivated with lactate and PCE and with either cyanocobalamin supplied ('L') or without externally provided corrinoids, but with the lower ligand DMB amended ('D') (for an overview of all conditions, see Supplemental Table X). To better compare the protein values among all conditions, we decided to calculate indicator proteins for co-culture vs. pure cultures (Supplementary Table XY).

PART - NMDS plots of each of the four replicates of each conditions are shown in Fig. SXX. The data of one of each cultivation conditions from the *D. mccartyi* pure cultures were removed because they were outliers.

First of all, we noticed that only two RdhA proteins from BTF08 were detected among all samples, the gene products of btf_1393 and btf_1407 (VcrA). The two reductive dehalogenases PceA and TceA, putatively involved in degradation of PCE to *cis*-DCE were not identified under any condition. While VcrA was one of the most abundant proteins under any of the tested conditions, regardless of the electron donor, the gene product of btf_1393 was more abundant in the two co-culture conditions. A BLAST against the NCBI nr database revealed that the btf_1393 amino acid sequence was nearly identical (99% or 497/498 amino acid sequence identity over the whole length) to an RdhA from *D. mccartyi* 11a5, encoded by 11a5_1355 and characterized as a novel PCE reductive dehalogenase, PteA. Another set of indicator proteins more abundant in the co-cultures were proteins related to cell division.

Discussion

In this study, we investigated the dechlorination of PCE to ethene (or vinyl chloride) in co-cultures of *S. multivorans* and *Dehalococcoides mccartyi* strains 195 or BTF08. The syntrophic relationships include the generation of hydrogen and acetate during lactate-fermentation and synthesis of corrinoids by *Sulfurospirillum* and maintaining low hydrogen levels by hydrogen consumption and therefore favoring endergonic lactate oxidation by *Dehalococcoides*. The latter one relies on hydrogen as electron donor and utilizes acetate as sole carbon source. Produced corrinoids could be salvaged by *Dehalococcoides* and remodeled into functional corrinoid cofactors for the reductive dehalogenases.

Similar to other co-cultures, PCE was dechlorinated to mainly VC or ethene in Sm/195 and Sm/BTF08, respectively faster when compared with pure *D. mccartyi* cultures (He *et al.*, 2007; Mao *et al.*, 2015; Men *et al.*, 2012).PCE was required as a co-substrate for VC dechlorination and ethene production only started when PCE was converted to VC (Maymó-Gatell *et al.*, 2001). The same

process was found in association with *Desulfovibrio vulgaris* Hildenborough and *Syntrophomonas wolfei*, although TCE was the electron acceptor (Mao *et al.*, 2015; Men *et al.*, 2012). The fast dechlorination of PCE to *cis*-DCE with electrons from lactate oxidation is achieved mainly by *S. multivorans* alone as seen in the carbon stable isotopic fractionation pattern (Supplementary Figure 1). Enrichment factor of Sm/Dhc BTF08 co-culture are in the same range as the *S. multivorans* single culture which is additionally supported by PCE-fractionation pattern of crude extracts and purified PceA enzyme of *S. multivorans* (Cichocka *et al.*, 2007; Nijenhuis *et al.*, 2005; Renpenning *et al.*, 2014). *cis*-DCE is subsequently available for *D. mccartyi* as electron acceptor and presumably converted by VcrA to ethene

Dehalococcoides spp. are characterized by an incomplete *de novo* vitamin B_{12} biosynthesis and possesses corrinoid salvaging and remodeling pathways (Men *et al.*, 2014; Yan *et al.*, 2012; Yi *et al.*, 2012). Corrinoids required for the assembly of the various Rdh's have to be scavenged from the environment and therefore *D. mccartyi* strains rely directly on corrinoid production of other bacteria which highlights the importance of the microbial community. Despite the restriction to organohalide respiration and the dependence on corrinoid salvaging, only three different benzimidazolyl cobamides are known to be functional in *D. mccartyi* 195: cobalamin ([DMB]Cba), [5-OMeBza]Cba and [5-MeBza]Cba (Yi *et al.*, 2012). In contrast, *S. multivorans* exhibits all enzyme necessary for *de novo* biosynthesis and was shown to produce a special type of corrinoid restricted to this organism, norpseudovitamin B_{12} ([Ade]NCba) (Keller *et al.*, 2014; Kräutler *et al.*, 2003). The Sm/Dhc BTF08 co-culture was not capable of complete dechlorination of PCE to ethene without amendment of vitamin B_{12}, only PCE to *cis*-DCE dechlorination occurred, suggesting that *S. multivorans* assembled a cobamide with a lower ligand not functional in *D. mccartyi* BTF08. Complete dechlorination to ethene was restored by the addition of DMB indicating a remodeling of nonfunctional [Ade]NCba into functional cobalamin by *D. mccartyi* BTF08. DMB was shown to have a negative effect on *S. multivorans* and was chosen in concentrations allowing growth and dechlorination (Keller *et al.*, 2018). However, an inhibiting effect resulting in decreased dechlorination activity can be excluded since PCE-to-*cis*-DCE dechlorination rates were similar with and without the addition of DMB. Opposed to Sm/Dhc BTF08, the Sm/Dhc 195 co-culture showed an accumulation of VC without amendment of vitamin B_{12} indicating *cis*-DCE to VC conversion 195 which is in contrast to previous studies showing adenine-containing B_{12} as nonfunctional in *D. mccartyi*. The cofactor usage of the multiple RDases which might have a different is not completely understood so far and only a few RDases were found and could be clearly assigned for conversion of a certain organohalide. Moreover, transcription of the different RDases is dependent on the electron acceptor present and previous studies were conducted with TCE (Yi *et al.*, 2012). The presence of *cis*-DCE in the Sm/Dhc 195 co-culture might induce a RDase preferentially using [Ade]NCba. Addition of DMB was not enhancing the dechlorination rate. MS analysis of this culture revealed the presence of three different types of vitamin B_{12} which are available for *D. mccartyi* 195: [Ade]NCba, [DMB]NCba and [DMB]Cba. Lower base adenine was replaced by DMB and [Ade]NCba was converted through guided biosynthesis into [DMB]NCba by *S. multivorans*, since the organism is able to incorporate several benzimidazoles and generate the corresponding corrinoids. The ratio of modified [DMB]NCba to

[Ade]NCba is in accordance with previous studies (Keller *et al.*, 2014). [DMB]Cba was produced during salvaging and remodeling of available [Ade]NCba and [DMB]NCba by *D. mccartyi* 195. The usage of both B_{12}-types for the production of [DMB]Cba seems possible as the organism shows a general remodeling activity regardless of the provided cobamide being functional or nonfunctional (Yi *et al.*, 2012). Besides DMB, two other benzimidazoles are known to be attached to cobamide precursors or exchanged with the lower ligand of a nonfunctional cobamide by *D. mccartyi* 195: DMB, 5-OMeBza, 5-MeBza, and additionally benzimidazole (Bza) used by *D. mccartyi* BAV1 and GT (Men *et al.*, 2014; Yi *et al.*, 2012). The different benzimidazole-types are not functionally equal and DMB was added due to highest dechlorination rates among the others (Yan *et al.*, 2016). Remodeling of a complete cobamide includes the exchange of the nucleotide loop and lower ligand done by cbiZ as shown in *Rhodobacter sphaeroides* (Gray and Escalante-Semerena, 2009a; Gray and Escalante-Semerena, 2009b). CbiZ is predicted to fulfill the same role in *D. mccartyi* but no experimental data are available so far. The conversion of norpseudo-B_{12} or nor-B_{12} into cyanocobalamin, and hence the exchange of the ethanolamine linker into aminopropanol indicates an involvement in removing the nucleotide loop. This is the first experimental evidence of the physiological role of cbiZ in *D. mccartyi*, since guided biosynthesis was exclusively shown with aminopropanol-containing B_{12} and only the exchange of the lower ligand could be observed.

The cultivation of *D. mccartyi* in association with *S. multivorans* lead to the formation of cell aggregates which is a characteristic feature of obligate syntrophic interactions found in different acetogenic and methanogenic communities (Hulshoff Pol *et al.*, 2004; Ishii *et al.*, 2005; Stams *et al.*, 2012). FISH staining confirmed the close association of both organisms and provided first insights into the organization and spatial distribution of the organisms within aggregates of a dechlorinating mixed culture. Electron microscopic analysis of the co-cultures revealed cells in close physical contact embedded in extracellular polymer substances (EPS)-like structures and surrounding flagellum-like filaments. Decreasing of intermicrobial distances and establishing a cell-to-cell contact enables increased metabolite fluxes. According Fick's law, diffusion rates of hydrogen and formate are higher the lower the distances between cells which enhances growth and dechlorination rates (Schink and Thauer, 1988). The advantages of clustering and therefore a close physical contact was shown during syntrophic propionate oxidation of *Pelotomaculum thermopropionicum* SI and *Methanothermobacter thermoautotrophicus* ΔH. Interspecies hydrogen transfer was calculated being optimal during coaggregation (Ishii *et al.*, 2006). EPS-like substances might aid in exchanging metabolites and flagella could contribute to a stabilization of the aggregate by adhesion and attachment of the cells (Grotenhuis *et al.*, 1991; Ishii *et al.*, 2005). The role of pili in direct interspecies electron transfer (DIET) by acting as conductive nanowires was discussed recently as seen in *Geobacter* spp. co-cultures (Reguera *et al.*, 2005; Shrestha *et al.*, 2013). A pili formation was shown for *D. mccartyi* strain DCMB5 during organohalide respiration (Pöritz *et al.*, 2015). However, conductive analysis of *S. wolfei*/*D. mccartyi* 195 aggregates revealed low conductibility and suggests an electron transfer via hydrogen rather than DIET (Mao *et al.*, 2015). The involvement of *S. multivorans* flagella in DIET remains unclear and need more molecular and biochemical analysis. *D. mccartyi* showed in association with *S. multivorans* a barrel-like morphology with a septum-like

structure which is in contrast to the irregular disc-shaped coccus as found in single culture. This observation might be caused by the down-regulation of proteins involved in cell division in the co-culture. One of these proteins, FtsZ, localized at the cell division site, was shown to play a key role in cytokinesis in *E. coli*. It was shown to be responsible for septal invagination of the cell wall and cytoplasmic membrane by forming a ring-shaped septum following subsequent cell division (Bi and Lutkenhaus, 1991; de Boer *et al.*, 1992; Lutkenhaus, 1993). The down-regulation might hamper a complete membrane constriction resulting in slower cell division and therefore the observed barrel-like cell morphologies. However, the reason for this difference in cell division is subject to speculation and needs more investigation. Since electron micrographs of co-cultures with *D. mccartyi* are scarce, it is not possible to state whether the unusual cell morphology is specific for the partnership with *S. multivorans* or whether it is found frequently associated with co-cultures. With *S. wolfei* as syntrophic partner, *D. mccartyi* strain 195 was found to form cell structures typical for *D. mccartyi* in general (Mao *et al.*, 2015).

This study provided first insights into the syntrophic interactions of *S. multivorans* in association with other organohalide-respiring bacteria. Studies on the composition of dechlorinating microbial communities showed the presence of *S. multivorans* although in lower abundancies which might explain an unclear ecological of the organism and potential syntrophic relations with other OHRB could be overlooked so far. The ability of *S. multivorans* to produce hydrogen during fermentation and a complete *de novo* cobamide biosynthesis makes him an ideal syntrophic partner for *D. mccartyi*. An interspecies hydrogen and cobamide transfer was observed in the *Sulfurospirillum*/*Dehalococcoides* co-culture resulting in complete and rapid dechlorination of PCE to ethene compared to single cultures. Furthermore, *S. multivorans* supplied all growth requirements to the highly restricted *D. mccartyi*: hydrogen, *cis*-DCE, acetate as carbon source and corrinoids (Figure 5). It is the first study in which the syntrophic partner was shown to provide all nutrients required for growth of *D. mccartyi.* This is of high interest for bioremediation attempts using *Dehalococcoides*-containing mixed cultures since electron donor and cobalamin limitations often detract *Dehalococcoides* dechlorination activities. The here established co-culture capable of an efficient detoxification of PCE to ethene is a potential candidate for bioaugmentation processes.

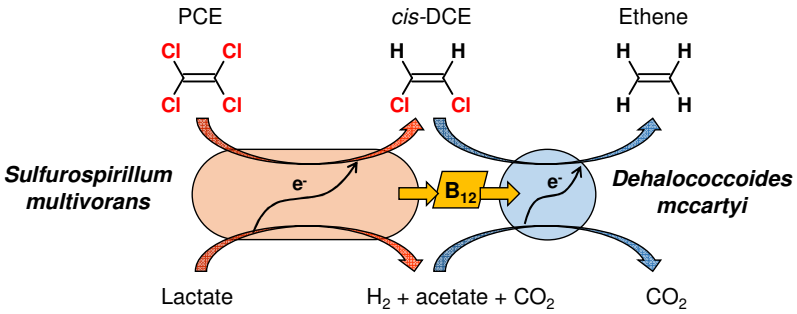

Figure 5: Syntrophic interactions between *Sulfurospirillum multivorans* and *Dehalococcoides mccartyi*. PCE is rapidly dechlorinated to *cis*-DCE by *S. multivorans* with reducing equivalents gained from lactate oxidation. After the depletion of the electron acceptor *S. multivorans* switches to lactate fermentation favoured by *D. mccartyi* consuming hydrogen and maintaining low hydrogen levels. *cis*-DCE serves subsequently as electron acceptor and is further dechlorinated to ethane by *D. mccartyi*. The interspecies cobamide transfer enables supply of the corrinoid cofactor.

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References

Bi, E., Lutkenhaus, J. 1991. FtsZ ring structure associated with division in *Escherichia coli*. *Nature*, 354, 161.

Cheng, D., Chow, W.L., He, J. 2010. A *Dehalococcoides*-containing co-culture that dechlorinates tetrachloroethene to *trans*-1,2-dichloroethene. *ISME J*, 4(1), 88-97.

Cichocka, D., Nikolausz, M., Haest, P., Nijenhuis, I. 2010. Tetrachloroethene conversion to ethene by a *Dehalococcoides*-containing enrichment culture from Bitterfeld. *Fems Microbiology Ecology*, 72(2), 297-310.

Cichocka, D., Siegert, M., Imfeld, G., Andert, J., Beck, K., Diekert, G., Richnow, H.-H., Nijenhuis, I. 2007. Factors controlling the carbon isotope fractionation of tetra- and trichloroethene during reductive dechlorination by *Sulfurospirillum* ssp. and *Desulfitobacterium* sp. strain PCE-S. *FEMS Microbiology Ecology*, 62(1), 98-107.

Coplen, T.B., Brand, W.A., Gehre, M., Gröning, M., Meijer, H.A., Toman, B., Verkouteren, R.M., Agency, I.A.E. 2006. After two decades a second anchor for the VPDB delta13C scale. *Rapid Commun Mass Spectrom*, 20(21), 3165-6.

de Boer, P., Crossley, R., Rothfield, L. 1992. The essential bacterial cell-division protein FtsZ is a GTPase. *Nature*, 359, 254.

DiStefano, T.D., Gossett, J.M., Zinder, S.H. 1992. Hydrogen as an electron donor for dechlorination of tetrachloroethene by an anaerobic mixed culture. *Appl Environ Microbiol*, 58(11), 3622-9.

Duhamel, M., Edwards, E.A. 2007. Growth and yields of dechlorinators, acetogens, and methanogens during reductive dechlorination of chlorinated ethenes and dihaloelimination of 1 ,2-dichloroethane. *Environ Sci Technol*, 41(7), 2303-10.

Elsner, M. 2010. Stable isotope fractionation to investigate natural transformation mechanisms of organic contaminants: principles, prospects and limitations. *J Environ Monit*, 12(11), 2005-31.

Elsner, M., Chartrand, M., Vanstone, N., Couloume, G.L., Lollar, B.S. 2008. Identifying abiotic chlorinated ethene degradation: characteristic isotope patterns in reaction products with nanoscale zero-valent iron. *Environ Sci Technol*, 42(16), 5963-70.

Fazi, S., Aulenta, F., Majone, M., Rossetti, S. 2008. Improved quantification of Dehalococcoides species by fluorescence in situ hybridization and catalyzed reporter deposition. *Syst Appl Microbiol*, 31(1), 62-7.

Gray, M.J., Escalante-Semerena, J.C. 2009a. The cobinamide amidohydrolase (cobyric acid-forming) CbiZ enzyme: A critical activity of the cobamide remodeling system of *Rhodobacter sphaeroides*. *Molecular microbiology*, 74(5), 1198-1210.

Gray, M.J., Escalante-Semerena, J.C. 2009b. In Vivo Analysis of Cobinamide Salvaging in *Rhodobacter sphaeroides* Strain 2.4.1. *Journal of Bacteriology*, 191(12), 3842-3851.

Grotenhuis, J.T.C., Smit, M., van Lammeren, A.A.M., Stams, A.J.M., Zehnder, A.J.B. 1991. Localization and quantification of extracellular polymers in methanogenic granular sludge. *Applied Microbiology and Biotechnology*, 36(1), 115-119.

Guimaraes, D., Weber, A., Klaiber, I., Vogler, B., Renz, P. 1994. Guanylcobamide and hypoxanthylcobamide: corrinoids formed by *Desulfovibrio vulgaris*. *Archives of Microbiology*, 162(4), 272-276.

He, J., Holmes, V.F., Lee, P.K., Alvarez-Cohen, L. 2007. Influence of vitamin B_{12} and cocultures on the growth of *Dehalococcoides* isolates in defined medium. *Appl Environ Microbiol*, 73(9), 2847-53.

He, J., Ritalahti, K.M., Aiello, M.R., Löffler, F.E. 2003a. Complete detoxification of vinyl chloride by an anaerobic enrichment culture and identification of the reductively dechlorinating population as a *Dehalococcoides* species. *Appl Environ Microbiol*, 69(2), 996-1003.

He, J., Ritalahti, K.M., Yang, K.L., Koenigsberg, S.S., Löffler, F.E. 2003b. Detoxification of vinyl chloride to ethene coupled to growth of an anaerobic bacterium. *Nature*, 424(6944), 62-5.

Henschler, D. 1994. Toxicity of Chlorinated Organic Compounds - Effects of the Introduction of Chlorine in Organic Molecules. *Angewandte Chemie-International Edition in English*, 33(19), 1920-1935.

Hulshoff Pol, L.W., de Castro Lopes, S.I., Lettinga, G., Lens, P.N.L. 2004. Anaerobic sludge granulation. *Water Research*, 38(6), 1376-1389.

Ishii, S., Kosaka, T., Hori, K., Hotta, Y., Watanabe, K. 2005. Coaggregation facilitates interspecies hydrogen transfer between *Pelotomaculum thermopropionicum* and *Methanothermobacter thermautotrophicus*. *Appl Environ Microbiol*, 71(12), 7838-45.

Ishii, S.i., Kosaka, T., Hotta, Y., Watanabe, K. 2006. Simulating the Contribution of Coaggregation to Interspecies Hydrogen Fluxes in Syntrophic Methanogenic Consortia. *Applied and Environmental Microbiology*, 72(7), 5093-5096.

Keller, S., Kunze, C., Bommer, M., Paetz, C., Menezes, R.C., Svatoš, A., Dobbek, H., Schubert, T. 2018. Selective utilization of benzimidazolyl-norcobamides as cofactors by the tetrachloroethene reductive dehalogenase of *Sulfurospirillum multivorans*. *Journal of Bacteriology*.

Keller, S., Ruetz, M., Kunze, C., Kräutler, B., Diekert, G., Schubert, T. 2014a. Exogenous 5,6-dimethylbenzimidazole caused production of a non-functional tetrachloroethene reductive dehalogenase in *Sulfurospirillum multivorans*. *Environmental Microbiology*, 16(11), 3361-3369.

Keller, S., Ruetz, M., Kunze, C., Kräutler, B., Diekert, G., Schubert, T. 2014b. Exogenous 5,6-dimethylbenzimidazole caused production of a non-functional tetrachloroethene reductive dehalogenase in *Sulfurospirillum multivorans*. *Environ Microbiol*, 16(11), 3361-9.

Kräutler, B., Fieber, W., Ostermann, S., Fasching, M., Ongania, K., Gruber, K., Kratky, C., Mikl, C. 2003. The cofactor of tetrachloroethene reductive dehalogenase of *Dehalospirillum multivorans* is norpseudo-B(12), a new type of a natural corrinoid. *Helv Chim Acta*, 86(11), 3698-3716.

Kruse, S., Goris, T., Wolf, M., Wei, X., Diekert, G. 2017. The NiFe Hydrogenases of the Tetrachloroethene-Respiring Epsilonproteobacterium *Sulfurospirillum multivorans*: Biochemical Studies and Transcription Analysis. *Front Microbiol*, 8, 444.

Kube, M., Beck, A., Zinder, S.H., Kuhl, H., Reinhardt, R., Adrian, L. 2005. Genome sequence of the chlorinated compound-respiring bacterium *Dehalococcoides* species strain CBDB1. *Nat Biotechnol*, 23(10), 1269-73.

Leys, D., Adrian, L., Smidt, H. 2013. Organohalide respiration: microbes breathing chlorinated molecules. *Philos Trans R Soc Lond B Biol Sci*, 368(1616), 20120316.

Löffler, F.E., Yan, J., Ritalahti, K.M., Adrian, L., Edwards, E.A., Konstantinidis, K.T., Muller, J.A., Fullerton, H., Zinder, S.H., Spormann, A.M. 2013. *Dehalococcoides mccartyi* gen. nov., sp. nov., obligately organohalide-respiring anaerobic bacteria relevant to halogen cycling and bioremediation, belong to a novel bacterial class, *Dehalococcoidia* classis nov., order *Dehalococcoidales* ord. nov. and family *Dehalococcoidaceae* fam. nov., within the phylum Chloroflexi. *Int J Syst Evol Microbiol*, 63(Pt 2), 625-35.

Lutkenhaus, J. 1993. FtsZ ring in bacterial cytokinesis. *Molecular Microbiology*, 9(3), 403-409.

Major, D.W., McMaster, M.L., Cox, E.E., Edwards, E.A., Dworatzek, S.M., Hendrickson, E.R., Starr, M.G., Payne, J.A., Buonamici, L.W. 2002. Field demonstration of successful bioaugmentation to achieve dechlorination of tetrachloroethene to ethene. *Environ Sci Technol*, 36(23), 5106-16.

Mao, X., Stenuit, B., Polasko, A., Alvarez-Cohen, L. 2015a. Efficient metabolic exchange and electron transfer within a syntrophic trichloroethene-degrading coculture of *Dehalococcoides mccartyi* 195 and *Syntrophomonas wolfei*. *Appl Environ Microbiol*, 81(6), 2015-24.

Mao, X., Stenuit, B., Polasko, A., Alvarez-Cohen, L. 2015b. Efficient Metabolic Exchange and Electron Transfer within a Syntrophic Trichloroethene-Degrading Coculture of *Dehalococcoides mccartyi* 195 and *Syntrophomonas wolfei*. *Applied and Environmental Microbiology*, 81(6), 2015-2024.

Matturro, B., Tandoi, V., Rossetti, S. 2013. Different activity levels of Dehalococcoides mccartyi revealed by FISH and CARD-FISH under non-steady and pseudo-steady state conditions. *N Biotechnol*, 30(6), 756-62.

Maymó-Gatell, X., Anguish, T., Zinder, S.H. 1999. Reductive dechlorination of chlorinated ethenes and 1, 2-dichloroethane by "Dehalococcoides ethenogenes" 195. *Appl Environ Microbiol*, 65(7), 3108-13.

Maymo-Gatell, X., Chien, Y., Gossett, J.M., Zinder, S.H. 1997. Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. *Science*, 276(5318), 1568-71.

Maymó-Gatell, X., Nijenhuis, I., Zinder, S.H. 2001. Reductive dechlorination of *cis*-1,2-dichloroethene and vinyl chloride by "*Dehalococcoides ethenogenes*" strain 195. *Environ Sci Technol*, 35(3), 516-21.

McMurdie, P.J., Behrens, S.F., Müller, J.A., Göke, J., Ritalahti, K.M., Wagner, R., Goltsman, E., Lapidus, A., Holmes, S., Löffler, F.E., Spormann, A.M. 2009. Localized plasticity in the streamlined genomes of vinyl chloride respiring *Dehalococcoides*. *PLoS Genet*, 5(11), e1000714.

Men, Y., Feil, H., Verberkmoes, N.C., Shah, M.B., Johnson, D.R., Lee, P.K., West, K.A., Zinder, S.H., Andersen, G.L., Alvarez-Cohen, L. 2012. Sustainable syntrophic growth of *Dehalococcoides ethenogenes* strain 195 with *Desulfovibrio vulgaris* Hildenborough and *Methanobacterium congolense*: global transcriptomic and proteomic analyses. *Isme j*, 6(2), 410-21.

Men, Y., Seth, E.C., Yi, S., Allen, R.H., Taga, M.E., Alvarez-Cohen, L. 2014a. Sustainable Growth of *Dehalococcoides mccartyi* 195 by Corrinoid Salvaging and Remodeling in Defined Lactate-Fermenting Consortia. *Applied and Environmental Microbiology*, 80(7), 2133-2141.

Men, Y., Seth, E.C., Yi, S., Allen, R.H., Taga, M.E., Alvarez-Cohen, L. 2014b. Sustainable growth of *Dehalococcoides mccartyi* 195 by corrinoid salvaging and remodeling in defined lactate-fermenting consortia. *Appl Environ Microbiol*, 80(7), 2133-41.

Morris, B.E., Henneberger, R., Huber, H., Moissl-Eichinger, C. 2013. Microbial syntrophy: Interaction for the common good. *FEMS Microbiol Rev*, 37(3), 384-406.

Neumann, A., Scholz-Muramatsu, H., Diekert, G. 1994. Tetrachloroethene metabolism of *Dehalospirillum multivorans*. *Arch Microbiol*, 162(4), 295-301.

Nijenhuis, I., Andert, J., Beck, K., Kästner, M., Diekert, G., Richnow, H.-H. 2005. Stable Isotope Fractionation of Tetrachloroethene during Reductive Dechlorination by *Sulfurospirillum multivorans* and *Desulfitobacterium* sp. Strain PCE-S and Abiotic Reactions with Cyanocobalamin. *Applied and Environmental Microbiology*, 71(7), 3413-3419.

Pöritz, M., Schiffmann, C.L., Hause, G., Heinemann, U., Seifert, J., Jehmlich, N., von Bergen, M., Nijenhuis, I., Lechner, U. 2015. *Dehalococcoides mccartyi* Strain DCMB5 Respires a Broad Spectrum of Chlorinated Aromatic Compounds. *Applied and Environmental Microbiology*, 81(2), 587-596.

Reguera, G., McCarthy, K.D., Mehta, T., Nicoll, J.S., Tuominen, M.T., Lovley, D.R. 2005. Extracellular electron transfer via microbial nanowires. *Nature*, 435, 1098.

Renpenning, J., Keller, S., Cretnik, S., Shouakar-Stash, O., Elsner, M., Schubert, T., Nijenhuis, I. 2014. Combined C and Cl Isotope Effects Indicate Differences between Corrinoids and Enzyme (*Sulfurospirillum multivorans* PceA) in Reductive Dehalogenation of Tetrachloroethene, But Not Trichloroethene. *Environmental Science & Technology*, 48(20), 11837-11845.

Richardson, R. 2016. Organohalide-Respiring Bacteria as Members of Microbial Communities: Catabolic Food Webs and Biochemical Interactions. in: *Organohalide-respiring Bacteria*, (Eds.) L. Adrian, F. Löffler, Springer. Berlin Heidelberg.

Richardson, R.E., Bhupathiraju, V.K., Song, D.L., Goulet, T.A., Alvarez-Cohen, L. 2002. Phylogenetic characterization of microbial communities that reductively dechlorinate TCE based upon a combination of molecular techniques. *Environ Sci Technol*, 36(12), 2652-62.

Rossetti, S., Aulenta, F., Majone, M., Crocetti, G., Tandoi, V. 2008. Structure analysis and performance of a microbial community from a contaminated aquifer involved in the complete reductive dechlorination of 1,1,2,2-tetrachloroethane to ethene. *Biotechnol Bioeng*, 100(2), 240-9.

Schink, B., Stams, A. 2013. Syntrophism Among Prokaryotes. in: *The Prokaryotes : Prokaryotic Communities and Ecophysiology*, (Eds.) E. Rosenberg, E. DeLong , S. Lory, E. Stackebrandt , F. Thompson, Springer. Berlin Heidelberg, pp. 471-493.

Schink, B., Thauer, R.K. 1988. Energetics of syntrophic methane formation and the influence of aggregation. in: *Granular anaerobic sludge: microbiology and technology*, (Eds.) L. G, Z. AJB, G. JTC, H. LW. Puduc, Wageningen, pp. 5-17.

Schipp, C.J., Marco-Urrea, E., Kublik, A., Seifert, J., Adrian, L. 2013. Organic cofactors in the metabolism of *Dehalococcoides mccartyi* strains. *Philos Trans R Soc Lond B Biol Sci*, 368(1616), 20120321.

Schmidt, M., Lege, S., Nijenhuis, I. 2014. Comparison of 1,2-dichloroethane, dichloroethene and vinyl chloride carbon stable isotope fractionation during dechlorination by two *Dehalococcoides strains*. *Water Res*, 52, 146-54.

Scholz-Muramatsu, H., Neumann, A., Messmer, M., Moore, E., Diekert, G. 1995. Isolation and characterization of *Dehalospirillum multivorans* gen. nov., sp. nov., a tetrachloroethene-utilizing, strictly anaerobic bacterium. *Arch Microbiol*, 163(1), 48-56.

Seshadri, R., Adrian, L., Fouts, D.E., Eisen, J.A., Phillippy, A.M., Methe, B.A., Ward, N.L., Nelson, W.C., Deboy, R.T., Khouri, H.M., Kolonay, J.F., Dodson, R.J., Daugherty, S.C., Brinkac, L.M., Sullivan, S.A., Madupu, R., Nelson, K.E., Kang, K.H., Impraim, M., Tran, K., Robinson, J.M., Forberger, H.A., Fraser, C.M., Zinder, S.H., Heidelberg, J.F. 2005. Genome sequence of the PCE-dechlorinating bacterium *Dehalococcoides ethenogenes*. *Science*, 307(5706), 105-8.

Shrestha, P.M., Rotaru, A.-E., Aklujkar, M., Liu, F., Shrestha, M., Summers, Z.M., Malvankar, N., Flores, D.C., Lovley, D.R. 2013. Syntrophic growth with direct interspecies electron transfer as the primary mechanism for energy exchange. *Environmental Microbiology Reports*, 5(6), 904-910.

Stams, A.J., Plugge, C.M. 2009. Electron transfer in syntrophic communities of anaerobic bacteria and archaea. *Nat Rev Microbiol*, 7(8), 568-77.

Stams, A.J.M., Worm, P., Sousa, D.Z., Alves, M.M., Plugge, C.M. 2012. Syntrophic Degradation of Fatty Acids by Methanogenic Communities. in: *Microbial Technologies in Advanced Biofuels Production*, (Ed.) P.C. Hallenbeck, Springer US. Boston, MA, pp. 127-142.

Stupperich, E., Eisinger, H.J., Krautler, B. 1988. Diversity of corrinoids in acetogenic bacteria. P-cresolylcobamide from *Sporomusa ovata*, 5-methoxy-6-methylbenzimidazolylcobamide from *Clostridium formicoaceticum* and vitamin B_{12} from *Acetobacterium woodii*. *Eur J Biochem*, 172(2), 459-64.

Stupperich, E., Kräutler, B. 1988. Pseudo vitamin B_{12} or 5-hydroxybenzimidazolyl cobamide are the corrinoids found in methanogenic bacteria. *Arch. Microbiol.*, 149(3), 268-271.

Yan, J., Im, J., Yang, Y., Löffler, F.E. 2013. Guided cobalamin biosynthesis supports *Dehalococcoides mccartyi* reductive dechlorination activity. *Philos Trans R Soc Lond B Biol Sci*, 368(1616), 20120320.

Yan, J., Ritalahti, K.M., Wagner, D.D., Löffler, F.E. 2012a. Unexpected Specificity of Interspecies Cobamide Transfer from *Geobacter* spp. to Organohalide-Respiring *Dehalococcoides mccartyi* Strains. *Applied and Environmental Microbiology*, 78(18), 6630-6636.

Yan, J., Ritalahti, K.M., Wagner, D.D., Löffler, F.E. 2012b. Unexpected specificity of interspecies cobamide transfer from *Geobacter* spp. to organohalide-respiring *Dehalococcoides mccartyi* strains. *Appl Environ Microbiol*, 78(18), 6630-6.

Yan, J., Simsir, B., Farmer, A.T., Bi, M., Yang, Y., Campagna, S.R., Loffler, F.E. 2016. The corrinoid cofactor of reductive dehalogenases affects dechlorination rates and extents in organohalide-respiring *Dehalococcoides mccartyi*. *Isme j*, 10(5), 1092-101.

Yi, S., Seth, E.C., Men, Y.-J., Stabler, S.P., Allen, R.H., Alvarez-Cohen, L., Taga, M.E. 2012a. Versatility in Corrinoid Salvaging and Remodeling Pathways Supports Corrinoid-Dependent Metabolism in *Dehalococcoides mccartyi*. *Applied and Environmental Microbiology*, 78(21), 7745-7752.

Yi, S., Seth, E.C., Men, Y.J., Stabler, S.P., Allen, R.H., Alvarez-Cohen, L., Taga, M.E. 2012b. Versatility in corrinoid salvaging and remodeling pathways supports corrinoid-dependent metabolism in *Dehalococcoides mccartyi*. *Appl Environ Microbiol*, 78(21), 7745-52.

Zinder, S. 2016. The Genus *Dehalococcoides*. in: *Organohalide-respiring Bacteria*, (Eds.) L. Adrian, F. Löffler, Springer. Berlin Heidelberg.

3 Discussion

While *Sulfurospirillum multivorans* has become a model organism for organohalide respiration, the research focus was on the reductive dehalogenase and the associated proteins of the organism. For example, the first solved crystal structure of a reductive dehalogenase was obtained from PceA of *S. multivorans* and gave new insights into the catalytic reaction mechanism of this group of enzymes. However, the hydrogen metabolism of the organism has never been investigated. Genome analyses revealed four different hydrogenase gene clusters which raised the question whether the organism has a more complex hydrogen metabolism and is presumably capable of hydrogen production. Additionally, the role of *Sulfurospirillum* spp. in the environment and especially in organohalide-respiring microbial communities is poorly understood. In the following chapters, the hydrogen metabolism and the impact of *S. multivorans* and in general of *Sulfurospirillum* spp. as a hydrogen-producing syntrophic partner in these communities will be discussed. Furthermore, an outlook on future application of mixed cultures containing organohalide-respiring bacteria for bioaugmentation and bioremediation processes will be given.

3.1 Hydrogen metabolism of *Sulfurospirillum multivorans*

The physiological roles of the four [NiFe] hydrogenases of *S. multivorans* were elucidated using quantitative real-time PCR and biochemical investigations. For the quantification of the transcript levels, each hydrogenase gene cluster was detected by their catalytic subunit The organism was cultivated with a broad range of different electron donor and acceptor combinations to cover most possible metabolic scenarios in which each hydrogenase gene cluster is more likely to be expressed. All in all, only two of the four catalytic subunit transcripts were found among the tested growth conditions: *hydB* of hydrogen-oxidizing

membrane-bound hydrogenase and *hyfG* of membrane-bound hydrogen-producing hydrogenase while transcripts of the cytoplasmic hydrogen-uptake hydrogenase (*hupL*) and the putative soluble cytoplasmic hydrogen-producing hydrogenase (*echE*) were not detected. Transcripts of the catalytic subunit *hydB* were detected regardless of the electron donor like hydrogen, pyruvate or formate. While an upregulation of the MBH gene expression only with hydrogen as electron donor would be useful, given the costly maturation of NiFe hydrogenases which use an array of maturation proteins responsible for correct synthesis of the complex NiFe active site with its non-proteinogenous ligands, this was not observed in *S. multivorans*. This is in contrast to the situation in *Ralstonia eutropha* and *Rhodobacter capsulates*, where an upregulation of an MBH was observed (Buhrke *et al.*, 2005; Duche *et al.*, 2005). The sensing of hydrogen in these organisms is achieved by a signal cascade composed of a response regulator HoxA, a histidine kinase HoxJ and a regulatory hydrogenase (Friedrich *et al.*, 2005). Hydrogen causes increased transcription levels and 10-fold higher activity (Toussaint *et al.*, 1997). Since *S. multivorans* lacks H_2 sensors and regulatory proteins adjacent to the hydrogenase gene clusters, a constitutive expression of the MBH is suggested. This is in accordance also on the protein level as enzyme activity assays with membrane extracts revealed similar H_2-oxidizing activities regardless of the growth condition. Moreover, hydrogen as electron donor had no inducing effect and enzyme activities were not significantly altered. The constitutive expression was already suggested in a proteomic study in which the MBH was detected in membranes of cells grown with pyruvate or formate as electron donor and PCE; fumarate or nitrate as electron acceptor in high abundance (Goris *et al.*, 2015). However, hydrogen was not tested as substrate and cultivation were limited in this study.

The *S. multivorans* MBH is closely related to the well characterized membrane-bound hydrogenase of *Wolinella succinogenes* (amino acid sequence identity of approximately 50 to 76%) which consists of a large subunit harbouring the [NiFe] catalytic site, a small subunit with three [FeS] cluster acting as an electron transfer relay and a cytochrome *b* subunit

(Dross *et al.*, 1992; Fritsch *et al.*, 2013; Fritsch *et al.*, 2011). In *W. succinogenes*, electrons gained from hydrogen oxidation at the [NiFe] catalytic centre are transferred via three [FeS] cluster of the small subunit to the membrane-integral subunit and enter the respiratory chain via the cytochrome *b* (Bernhard *et al.*, 1997; Frielingsdorf *et al.*, 2011; Gross *et al.*, 2004). The subunit composition of the MBH was elucidated by native polyacrylamide gel electrophoresis and revealed the same oligomeric structure. The presence of cytochrome *b* in the *S. multivorans* MBH was confirmed with UV/Vis difference spectra revealing characteristic cytochrome *b* absorption peaks as found in the *W: succinogenes* enzyme (Eguchi *et al.*, 2012; Unden and Kröger, 1981; Volbeda *et al.*, 2013). Additionally, hydrogen oxidation activity was only measured with the menaquinone analog 2,3-dimethyl-1,4-napthoquinone (DMN) suggesting a menaquinone as the physiological electron acceptor. This is supported by genes present in the genome encoding the futalosine pathway responsible for menaquinone biosynthesis and the detection of menaquinone in quinone analysis of membranes (Goris *et al.*, 2014; Hiratsuka *et al.*, 2008; Scholz-Muramatsu *et al.*, 1995). These findings strongly point towards the MBH mediating hydrogen oxidation in the organohalide respiration chain (Fig. 6).

The second hydrogenase detected at both, transcript and protein level, was the Hyf-like hydrogenase. A slight upregulation was seen in cells cultivated fermentatively on pyruvate and transcripts were preferently detected with pyruvate as the electron donor. This is in accordance with a previous proteomic study in which the Hyf enzyme was found in higher abundancies with pyruvate as electron donor (Goris *et al.*, 2015). The enzyme shares similarities with Hyd3 and Hyd4 of *E. coli*, forming the formate hydrogenlyase (FHL) complex together with a formate dehydrogenase (FdhF) in case of Hyd3 and Hyd4 presumably participates in a second FHL complex (FHL-2) with the FdhF homolog YdeP (Pinske and Sawers, 2016; Sawers, 1994; Sawers *et al.*, 2004). However, no experimental evidence confirmed this role, since the *hyf* operon is silent and therefore allows no biochemical analysis (Self *et al.*, 2004; Skibinski *et al.*, 2002).

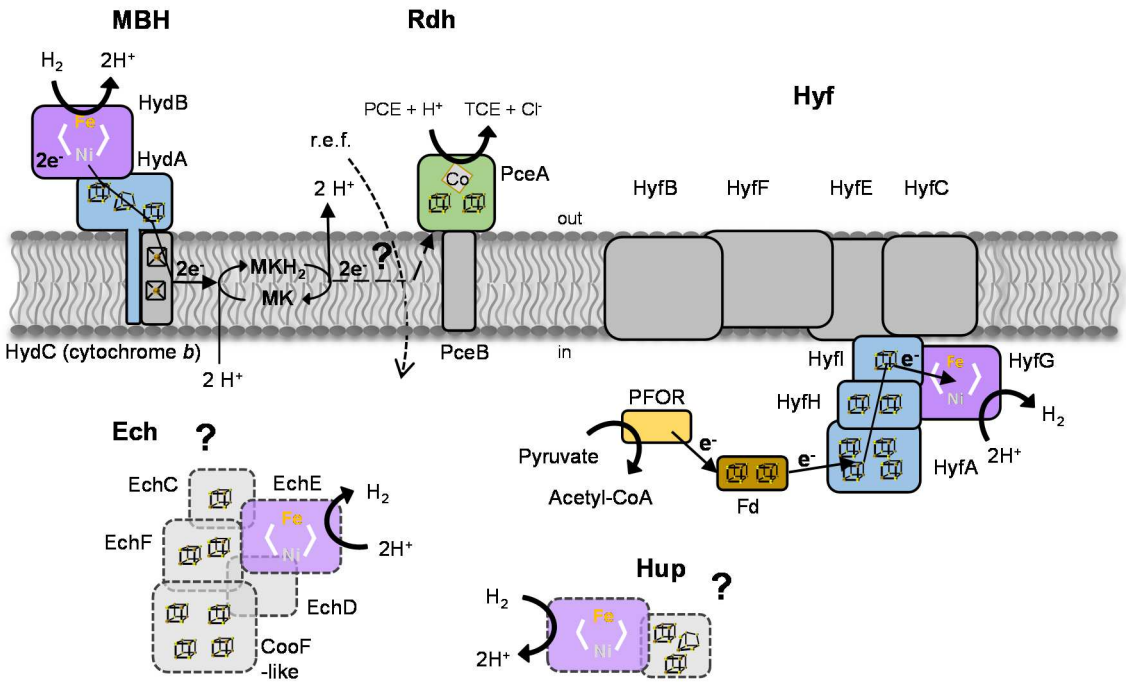


Figure 6: Hydrogen metabolism of *Sulfurospirillum multivorans*. The MBH is presumably involved in PCE respiratory chain by oxidizing electrons and the Hyf putatively produces hydrogen with electrons gained from pyruvate oxidation. Physiological roles of Hup and Ech hydrogenase remain unclear and therefore drawn transparent and in dashed boxes. Routes of electrons within the MBH and Hyf are shown by arrows. MBH - membrane-bound hydrogenase, Rdh - reductive dehalogenase, Hyf - Hyf-like hydrogenase, Ech - cytoplasmic hydrogen evolving hydrogenase, Hup - cytoplasmic uptake hydrogenase, MK/MKH_2 - menaquinone/reduced menaquinone, PCE - tetrachloroethene, TCE - trichloroethene, PFOR - pyruvate ferredoxin:oxidoreductase, Fd - ferredoxin, - [4Fe4S] cluster, - [3Fe4S] cluster, - heme *b*, $\langle {}^{Fe}_{Ni} \rangle$ - [NiFe] active site, Co - norpseudovitamin B_{12}.

A role in fermentative hydrogen production is discussed but not clarified so far (Mirzoyan *et al.*, 2017; Redwood *et al.*, 2008; Trchounian and Gary Sawers, 2014; Trchounian and Trchounian, 2014). The absence of a pyruvate formate lyase and a formate dehydrogenase showing only 30% amino acid sequence identity to FdhF of *E. coli* makes a participation of the Hyf in formate metabolism and formate oxidation unlikely (Goris *et al.*, 2015). Additionally, proteome studies and transcriptional analysis revealed an upregulation of the Hyf suggesting an involvement in disposing reducing equivalents rather than in formate metabolism (Fig. 6). The physiological roles of the other two hydrogenases remain elusive due to almost silent transcription levels among all tested groth conditions. Neither a role of the cytoplasmic uptake hydrogenase (Hup) in delivering low-potential electrons for the reductive TCA cycle nor recycling hydrogen generated by a nitrogenase seems to be the

case. A hydrogen production of the Ech hydrogenase from CO oxidation as in *Carboxydothermus hydrogenoformans* can be excluded, since a gene coding for a CO dehydrogenase is lacking.

In *S. multivorans*, the hydrogen metabolism involved in organohalide respiration is rather simple with the MBH being the main hydrogen-oxidizing enzyme and presumably the electron donating system in the PCE respiratory chain. This simplicity might be based on a general versatile metabolism not restricted to organohalide respiration and the utilization of also other electron donors besides hydrogen. Opposed to that are obligate OHRB for which hydrogen oxidation is an essential and central metabolic feature, since they rely on hydrogen as electron donor. In these organisms, a high number of RDase genes often correlate with a complex hydrogen metabolism and various [NiFe] and [FeFe] hydrogenases. The genome of *D. mccartyi* comprises genes encoding four [NiFe] hydrogenases and one [FeFe] hydrogenase (Kube *et al.*, 2005; Seshadri *et al.*, 2005). Of the four [NiFe] hydrogenases, three are membrane-bound of which only one (HupSL, referred to as 'Hup-type' in Table 2) is periplasmically oriented and therefore assumed to be responsible for delivering electrons into the organohalide respiratory chain. This is supported by several proteome studies showing highest transcript levels for HupSL among the other hydrogenases and the presence of a Tat signal peptide (Hartwig *et al.*, 2015; Rahm and Richardson, 2008; Türkowsky *et al.*, 2018). Moreover, the Hup hydrogenase is predicted to directly interact with the RDase by forming a supercomplex together with the RDase and formate dehydrogenase-like iron-sulfur molybdoenzyme (CSIM), due to missing cytochromes. Evidence for that was gained by isolating the complex from *D. mccartyi* strain CBDB1 and analyzing proteins of on native polyacrylamide gel electrophoresis (Hartwig *et al.*, 2017; Kublik *et al.*, 2016; Schubert *et al.*, 2018). A similar periplasmic [NiFe] uptake hydrogenase (HyaABC) was found in *Dehalobacter restrictus* strain PER-K23, harboring in total eight different hydrogenases which underscores the high specialization of the hydrogen metabolism assigning a central role for hydrogen in its metabolism. HyaABC

resembles a ‘standard-type’ membrane-bound hydrogenase containing a membrane-integral cytochrome *b*, unlike the Hup of *D. mccartyi*. It was the only hydrogen-oxidizing hydrogenase detected in the proteome of all strains suggesting it as the main hydrogen-oxidizing enzyme in *D. restrictus*. Besides [NiFe] hydrogenases, three [FeFe] were identified of which two were detected at the protein level although in not significantly altered abundancies during respiration. They are assumed to generate reducing equivalents by reduing NAD and FAD needed in the anabolism (Jugder *et al.*, 2016; Kruse *et al.*, 2013; Rupakula *et al.*, 2013). Opposed to these multiple sets of hydrogenases are the less complex hydrogenase sets of versatile OHRB containing several [NiFe] although often only one of the Hup-type (Tab. 2). *Geobacter lovleyi* strain SZ and *Anaeromyxobacter dehalogenans* strain 2CP-C harbor only one membrane-bound periplasmically oriented hydrogenase similar to *S. multivorans* and *S. halorespirans* (Butler *et al.*, 2010; Goris *et al.*, 2017; Neumann *et al.*, 1996; Thomas *et al.*, 2008).

Table 2: Overview of *rdhA* genes and hydrogenase sets of obligate and versatile organohalide-respiring bacteria. The ‘Hup-type’ resembles periplasmically oriented hydrogen uptake hydrogenase delivering electrons for organohalide respiration as the HupSL of *D. mccartyi.*

Organism[a]	Number of *rdhA* genes	[NiFe] hydrogenases		[FeFe] hydrogenases
		Hup-type	others	
obligate OHRB				
D. mccartyi strain 195	17	1	3	1
D. mccartyi strain CBDB1	32	1	3	1
D. mccartyi strain BTF08	20	1	3	1
D. alkenigignens	27	1	3	1
D. restrictus strain PER-K23	20	3	2	3
versatile OHRB				
D. hafniense strain Y51	1	4	1	4
D. hafniense strain DCB-2	5	4	1	4
G. lovleyi strain SZ	2	1	2	1
A. dehalogenans strain 2CP-P	2	1	1	-
S. multivorans	2	1	3	-
S. halorespirans	2	1	3	-

[a] *D. mccartyi - Dehalococcoides mccartyi*, *D. alkenigignens - Dehalogenimonas alkenigignens*, *D. restrictus - Dehalobacter restrictus*, *D. hafniense - Desulfitobacterium hafniense*, *G. lovleyi - Geobacter lovleyi*, *A. dehalogenans - Anaeromyxobacter dehalogenans*, *S. multivorans*/*halorespirans - Sulfurospirillum multivorans*/*halorespirans.*

An exception are *Desulfitobacterium hafniense* strains Y51 and DCB-2 which possess genes encoding nine hydrogenases (five [NiFe] and four [FeFe]) even though they are not restricted to hydrogen and utilize a broad range of electron donors (Kruse *et al.*, 2017b; Kruse *et al.*, 2013). Four of the five [NiFe] hydrogenases are membrane-bound and faced to the periplasm (Hup-type) of which only one (HydABC) was detected in the proteome under respiratory growth. Also here, the large subunit HydB is linked to the quinone-pool of the membrane by a membrane-integral cytochrome *b* (Kruse *et al.*, 2015; Türkowsky *et al.*, 2018). The other [NiFe] hydrogenase is similar to Hyd4 (Hyf) of *E. coli*, whereas an involvement in fermentation metabolism rather than in formate oxidiation is suggested since formate-related genes are not present in this operon. Experimental evidence for this is pending and protein detection failed so far which might be based on insufficient extraction of the membrane subunits. A role of the [FeFe] hydrogenases in the hydrogen metabolism can also be excluded due to missing maturation genes in *D. hafniense* strains Y51 and DCB-2 (Kruse *et al.*, 2017b).

Taken together, the hydrogen metabolism among the different OHRB differs in the complexity depending on an obligate organohalide respiring lifestyle or a versatile metabolism. Research on the hydrogenases of these organisms, especially of the obligate ones, is still at the beginning and needs more biochemical and enzymatic characterization. In this thesis, we provided deeper insights into the hydrogenase mainly responsible for delivering electrons for organohalide respiration and into the general hydrogen metabolism of the model OHRB *S. multivorans*.

3.2 Hydrogen production by *Sulfurospirillum* spp.

The MBH of *S. multivorans* was shown to be the primary hydrogen oxidizing hydrogenase and might be the electron donating system by delivering electron into the menaquinone pool of the organohalide respiration chain. The second hydrogenase found to be transcribed was the Hyf-like hydrogenase showing especially with pyruvate as electron

donor a transcription which was higher during pyruvate fermentation pointing towards an involvement the pyruvate and additional fermentation metabolism. To get deeper insights into the fermentation capabilities and to verify this role, *S. multivorans* and also other *Sulfurosprillum* spp. were tested for their ability to ferment pyruvate and to produce hydrogen via the Hyf hydrogenase. Scholz-Muramatsu and colleagues stated in 1994 that the organism was able to grow with pyruvate in the absence of the electron acceptor PCE resulting in the formation of the same products as with PCE and that hydrogen and/or lactate formation served as an electron sink for pyruvate oxidation. However, no data on growth curves or fermentation balances were shown (Scholz-Muramatsu *et al.*, 1995). We continued these studies and screened different *Sulfurospirillum spp.*, namely *S. multivorans*, *S. cavolei*, *S. arsenophilum* and *S. deleyianum*, for their capabilities to ferment pyruvate and dispose electrons by hydrogen production. Initial cultivation experiments showed only weak growth during first transfers on pyruvate alone as already described by Scholz-Muramatsu. Sequential subcultivation and transfers of the cultures lead to a continuous adaptation of all tested *Sulfurospirillum* spp. and gas chromatographic analysis of the gas phase revealed a significant hydrogen production. This was the first evidence of H_2 production of Epsilonproteobacteria, which are described and recognized as hydrogen consumers exclusively (Campbell *et al.*, 2006; Nakagawa *et al.*, 2005; Nakagawa and Takaki, 2001). The mechanisms behind this long-term adaptation process are unknown so far. A similar long-term regulatory effect was observed during continuous transfers without PCE. Transcript levels and enzyme activity of PceA were completely absent and not detectable after 35 transfers which corresponds to approximately 105 generations. Also here, the molecular basis for the long-term regulation of gene expression is not known so far (John *et al.*, 2009).

Screening of the four *Sulfurospirillum* spp. revealed two different pyruvate fermentation metabolisms. Highest hydrogen production rates were measured for the non-organohalide respiring *S. cavolei*, followed by *S. deleyianum* and *S. multivorans*. Hydrogen is a common

fermentation product and used as an electron sink by a broad range of fermentatively growing bacteria which can be classified into either strict anaerobes such as *Clostridium* and *Desulfovibrio* or into facultative anaerobes as *Enterobacter*, *Escherichia* and *Bacillus* (Axley *et al.*, 1990; Ren *et al.*, 2007; Thauer *et al.*, 1977; Wijffels and Janssen, 2003). The Hyf hydrogenase was identified being responsible for hydrogen production with electrons gained from pyruvate oxidation via pyruvate:ferredoxin oxidoreductase (PFOR) and transported by the low potential electron carrier ferredoxin (Fig. 7). Evidence for this function was found in proteome data in which all Hyf subunits, PFOR and ferredoxin showed a remarkable upregulation in cells of *S. multivorans* and *S. cavolei* grown fermentatively on pyruvate. A similar role of a group 4 hydrogenase was shown for the MBH in *Paracoccus furiosus* catalyzing proton reduction with electron from reduced ferredoxin and thereby generating a sodium ion gradient (McTernan *et al.*, 2015; Silva Pedro *et al.*, 2001). The same function was observed with Hyd3 of *E. coli*. An attachment of the enzyme to ferredoxin from *Thermotoga maritima* linked to PFOR resulted in hydrogen production with pyruvate as electron donor (Lamont *et al.*, 2017). Structural comparison of the Hyf-like hydrogenase of *S. multivorans* show high similarities to complex I of *Thermus thermophiles* and to the formate hydrogen lyase complex (FHL-1) forming Hyd-3 (Hyc) and Hyd-4 (Hyf), which might form a second FHL complex (FHL-2) in *E. coli* (Fig. 7) (Pinske and Sawers, 2016; Sargent, 2016). The homology of membrane subunits of group 4 hydrogenases to Na^+/H^+ or K^+/H^+ antiporters of complex I and therefore acting as a proton pump has been widely discussed (Brandt *et al.*, 2003; Hedderich, 2004; Hedderich and Forzi, 2005; Marreiros *et al.*, 2013; Pinske and Sawers, 2016). The high conservation grade of key amino acid residues in the membrane subunits found in an alignment with their counterparts of hydrogenase 3 (Hyc) and 4 (Hyf) of *E. coli* and complex I of *Thermus thermophilus* renders the involvement of *Sulfurospirillum* spp. Hyf in generating a proton gradient a possible scenario. A detailed discussion dealing with the hydrogen metabolism in *S. multivorans* and the Hyf as potential proton pump can be found in chapter 2.2 and Appendix 2.2 Supplementary information.

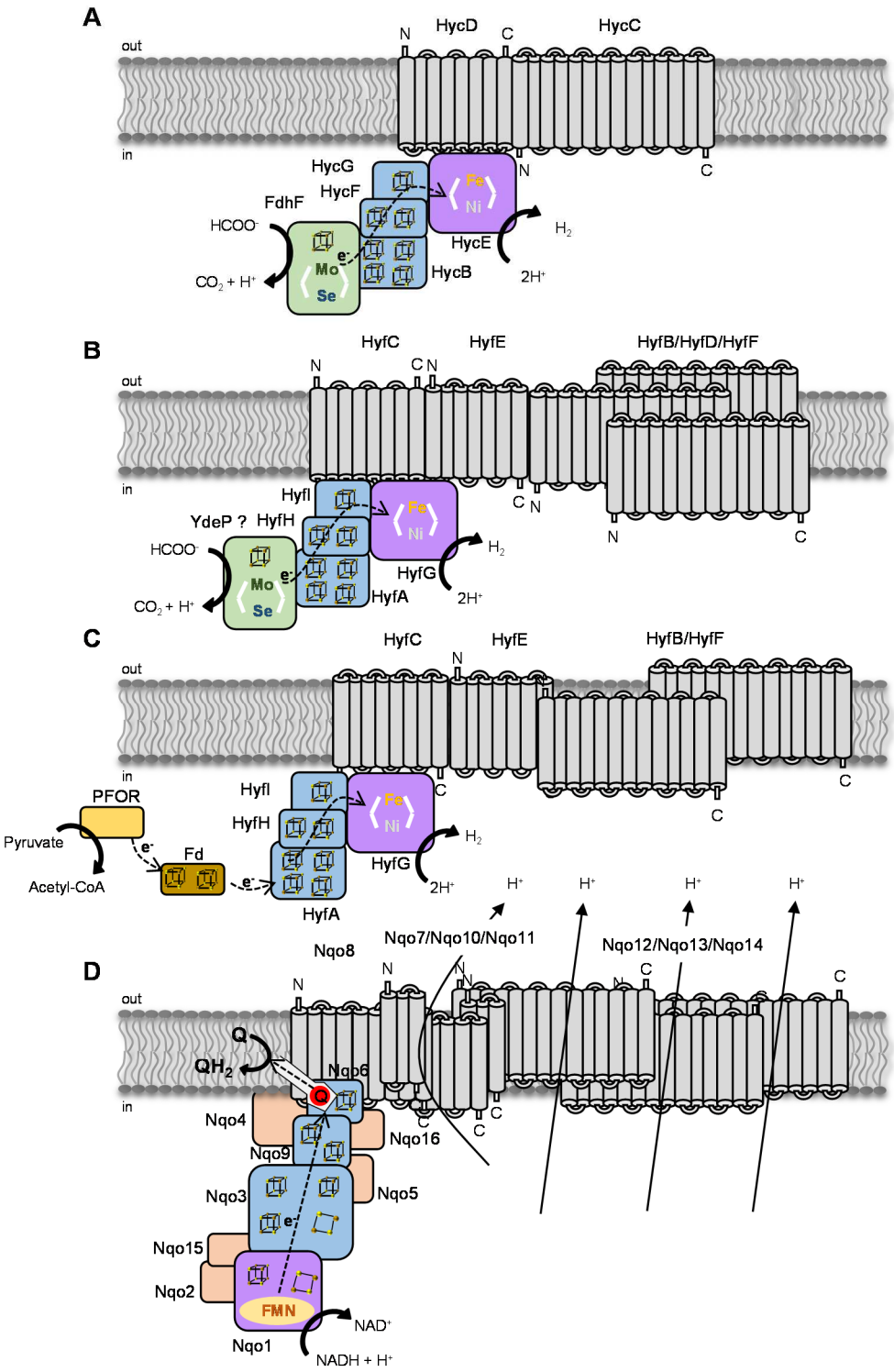


Figure 7: Architecture and comparison of homologous subunits of different group 4 [NiFe] hydrogenases. (A) Hyd3 of *E. coli*, (B) Hyd4 of *E. coli*, (C) Hyf-like hydrogenase of *S. multivorans* and (D) complex I of *Thermus thermophilus*. Color code resembles homologo+us subunits. Purple subunits represents catalytic subunits. Blue subunits represents [FeS] cluster containing subunits. Proton translocating sites are indicated by arrows. PFOR - pyruvate ferredoxin.oxidoreductase, FdhF - formate dehydrogenase H, YdeP - homolog to FdhH, Q - quinone, FMN, - Flavin mononucleotide. - [4Fe4S] cluster, - [2Fe2S] cluster, - [NiFe] active site, - molybdenum cofactor.

The Hyf hydrogenase was shown to be responsible for hydrogen production in *S. multivorans* and *S. cavolei*. Genomes of several other Epsilonproteobacteria contain also *hyf* gene clusters. In some *Arcobacter* spp. and *Campylobacter* spp., the hyf is co-located either with a formate dehydrogenase or a formate channel indicating an involvement in formate metabolism by forming a formate hydrogen lyase complex (FHL). However, a pyruvate formate lyase is missing in these genomes which makes a participation of the Hyf in hydrogen production more likely than in forming a FHL and hydrogen producing capabilities of also other Epsilonproteobacteria cannot be excluded.

3.3 *Sulfurospirillum multivorans* as a hydrogen and corrinoid producing syntrophic partner

The genus *Sulfurospirillum* and Epsilonproteobacteria in general, inhabit different ecological niches ranging from marine sediments and hydrothermal vents to terrestrial systems including sulfur- and nitrate-rich environments, limestone caves, sulphidic springs and organohalide-contaminated groundwater (Barton and Luiszer, 2005; Elshahed *et al.*, 2003; Engel *et al.*, 2003; Engel *et al.*, 2004; Gevertz *et al.*, 2000; Goris and Diekert, 2016; Voordouw *et al.*, 1996). Additionally to natural environments, *Helicobacter pylori* and *Campylobacter jejuni*, both belonging to the Epsilonproteobacteria, are pathogenic and colonize the intestine of the human body (Parkhill *et al.*, 2000; Tomb *et al.*, 1997). Despite the different metabolic features, all members are described to utilize hydrogen as electron donor so far and are considered as hydrogen consumers in microbial food webs exclusively (Campbell *et al.*, 2006; Nakagawa *et al.*, 2005; Nakagawa and Takaki, 2001). The ability of *Sulfurospirillum* spp. to produce hydrogen during fermentative growth sheds new light on the ecological role of Epsilonproteobacteria which was overlooked so far.

To get more insights into the possible roles of *Sulfurospirillum* spp. as hydrogen producer and syntrophic partner in microbial communities different co-cultures with hydrogen consuming bacteria were set up. In a first co-culture, *S. multivorans* was cultivated together

with the methanogen *Methanococcus voltae* (Fig. 8A). Lactate fermentation in pure cultures of *S. multivorans* was not possible and occurred only in the presence of a hydrogen consumer. This indicates an obligate syntrophic interaction in which *M. voltae* maintained low hydrogen levels favouring endergonic lactate fermentation by *S. multivorans* and received in turn hydrogen as electron donor. Aditionally, the fermentation product acetate served as carbon source and CO_2 was utilized as electron acceptor for methanogenesis in *M. voltae*.

The unrecognized role as syntrophic partner and the ability to interact also with other bacteria and to contribute to whole microbial communities as a hydrogen producer was supported in a second co-cultivation experiment with the obligate organohalide-respiring *D. mccartyi* (Fig. 8B). *S. multivorans* generated hydrogen and acetate during lactate fermentation and produced *de novo* corrinoids. Hydrogen and acetate were subsequently utilized by *D. mccartyi* since the organism relies on hydrogen as electron donor and acetate as carbon source due to an incomplete reductive acetyl-CoA pathway and the inability to fix CO_2 (Zinder, 2016).

The syntrophic interaction of *S. multivorans* and *D. mccarty* strain BTF08 led to a complete dechlorination of PCE to ethene with up to 5-fold higher rates compared to *D. mccarty* single cultures. Additionally, these observations are independent from the electron donor used during cultivation since amended hydrogen instead of lactate results in similar enhanced dechlorination rates which suggests also other stimulating and yet to be undiscovered growth factors or signals exchanged between both organisms. A positive effect on growth and dechlorination when co-cultivating *D. mccartyi* in association with fermenting bacteria, acetogens or methanogens was reported in several studies. The syntrophic association of *D. mccartyi* with butyrate-fermenting *Syntrophomonas wolfei* or lactate-fermenting *Desulfovibrio vulgaris* Hildenborough resulted in faster dechlorination and cell yields (He *et al.*, 2007; Mao *et al.*, 2015; Men *et al.*, 2012). The advantage of *S. multivorans* being the syntrophic partner for *D. mccartyi* compared to other fermenters is its ability to rapidly

dechlorinate high concentrations of PCE to *c*DCE. Carbon stable isotope analysis (CSIA) confirmed the conversion of PCE to *c*DCE exclusively done by *S. multivorans*. Under laboratory conditions, the organism is able to degrade 10 mM PCE in approximately 12 hours, which highlights its potential for bioremediation processes (Neumann *et al.*, 1996; Scholz-Muramatsu *et al.*, 1995). Other co-cultures with *D. mccartyi* were either conducted with non-dechlorinating partners (e.g. *S. wolfei, D. vulgaris* Hildenborough) or slow PCE degraders like *Geobacter sulfurreducens* or *G. lovleyi* which need several days to convert 40 µmol PCE to *c*DCE, while *S. multivorans* needed only several hours (Mao *et al.*, 2015; Men *et al.*, 2012; Yan *et al.*, 2013; Yan *et al.*, 2012). In addition, co-cultures with *Geobacter* species need additional amendment with hydrogen, since this genus is not known for hydrogen production. Besides the interspecies hydrogen transfer, an interspecies cobamide transfer was observed. Vitamin B_{12}-free cultures of *D. mccarty* strain BTF08 and *S. multivorans* showed an accumulation of *c*DCE indicating dechlorinating activity of only *S. multivorans* which assembled a corrinoid not functional in *D. mccartyi* resulting in no further dechlorination. Complete conversion to ethene can be restored by the addition of DMB. This phenomenon was also observed in co-cultures with *Geobacter* spp., *Pelosinus fermentans* strain R7, *Sporomusa ovata* strain KB-1 and *Methanosarcina barkeri* strain Fusaro. These organisms produced a corrinoid not functional in *D. mccartyi*: 5-hydroxybenzimidazolyl-cobamide (*G. sulfurreducens*, *M. barkeri*), phenolyl- and *p*-cresolyl-cobamide (*S. ovata*, *P. fermentans*), and dechlorination was restored when DMB was amended (Hazra *et al.*, 2015; Stupperich *et al.*, 1990; Stupperich *et al.*, 1989; Yan *et al.*, 2013).

In both, methanogenic and dechlorinating co-cultures, a formation of cell aggregates was observed which is a common feature of cells of an obligate syntrophic interaction and was already reported for different acetogenic and methanogenic mixed cultures (Hulshoff Pol *et al.*, 2004; Ishii *et al.*, 2005; Stams *et al.*, 2012).

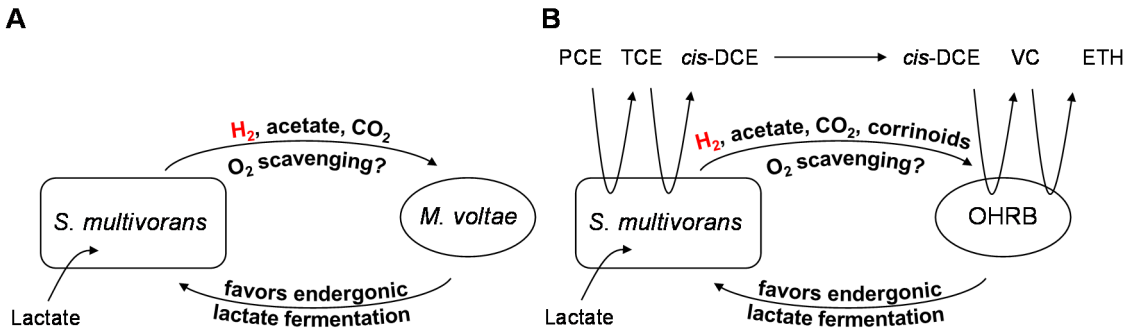


Figure 8: Overview of syntrophic interactions of *S. multivorans* in co-culture with *M. voltae* and *D. mccartyi*. Mechanisms of oxygen scavenging are unknown so far. *S. multivorans* - *Sulfurospirillum multivorans*, *M. voltae* - *Methanococcus voltae*, OHRB - organohalide-respiring bacteria.

Reduction of intermicrobial distances quarantee higher diffusion of metabolites which can directly influences growth and dechlorination rates (Schink and Thauer, 1988). Aggregation of OHRB with other bacteria was already reported. A bioreactor experiment revealed bioflocculation of *D. mccartyi* with *Desulfovibrio*, *Geobacter* and *Clostridia* forming microcolonies able to dechlorinate trichloroethene (TCE) to ethene at high rates (Delgado *et al.*, 2017; Delgado *et al.*, 2014). In addition, metabolite diffusion rates were calculated being optimal in a *D. mccartyi*/*S. wolfei* co-culture when forming aggregates. The close physical contact was for an efficient syntrophic butyrate fermentation required, since the cell-cell distances of dispersed cells would be to large for achieving an optimal hydrogen transfer (Mao *et al.*, 2015).

A further advantage of *S. multivorans* is the ability to grow also with oxygen as electron donor which makes him another ideal partner for strict anaerobic bacteria by eliminating toxic oxygen and corresponding radicals. One major challenge for strict anaerobes is the introduction of oxygen through geological or anthropogenic processes in their habitats. Especially *D. mccartyi* is characterized by an extremely low oxygen tolerance. Traces of oxygen and even a brief exposure to oxygen causes an immediate loss of dechlorination activity and inactivates the organism irreversible in pure culture (Adrian *et al.*, 2007; Amos *et al.*, 2008; Löffler *et al.*, 2013b). Metagenomic analysis of enrichment cultures revealed the presence of two oxygen free-radicals (ROS) scavenging mechanisms in *D. mccartyi*:

an [Mn]-superoxide dismutase (SOD) and a putative ruberythrin/rubredoxin system (Hug *et al.*, 2012). Multiple scavenging mechanisms including cytochrome c oxidases, catalases, peroxidases and different SODs and ruberythrin/rubredoxin systems to mitigate toxicity of oxygen were found in the metagenome and were assigned to non-dechlorinating community members (e.g. α/β/δ/γ-Proteobacteria, Firmicutes, Euryarchaeota, Actinobacteria). Especially the facultative anaerobic bacteria, which are capable of oxygen respiration, might therefore be involved in protection of *D. mccartyi*, since they diminish the oxygen and ROS. A *D. mccatyi*-containing microbial consortium was still able to dechlorinate trichloroethene to ethene when exposed to 20% oxygen (corresponds to 7.2 mg/L of dissolved oxygen in water) in the gas phase with increased lag phases before dechlorination started and reduced dechlorination rates compared to controls without the addition of oxygen (Liu *et al.*, 2017). The main oxygen scanvenging organisms in this community were identified to belong to the Proteobacteria and Bacteroidetes. Similar scavenging systems were found in *S. multivorans* (e.g. a *cbb*3-type cytochrome oxidase as the key enzyme for oxygen respiration, a SOD, superoxide reductase (SOR), catalase and cytochrome c551 peroxidase) which enable the organism to grow in the presence of 15% oxygen (Goris *et al.*, 2014). An upregulation of these enzymes during exposure to 5% oxygen was observed by comparative proteomics, while the cytochrome oxidase seems to be constitutively expressed (Gadkari *et al.*, 2018). From these findings it can be concluded that *S. multivorans* is also able to mitigate toxicity of ROS which is of advantage for *D. mccartyi* when grown in the corresponding co-culture.

The ability to completely dechlorinate PCE to ethene renders the co-culture as a potential candidate for bioaugmentation processes. The application of *Dehalococcoides*-containing mixed cultures for *in situ* bioremediation processes and the research still needed before applying the established *D. mccartyi*/*S. multivorans* co-culture on organohalide-contaminated sites will be discussed in the next chapter.

3.4 Application of *Dehalococcoides*-containing mixed cultures for bioaugmentation

The anthropogenic-caused environmental pollution with chlorinated hydrocarbons and other types of xenobiotics harmful to human and environmental health has become a globe-spanning problem over the last years. Industrial processes (e.g. dry cleaning of textiles, degreasing of metals) led to the release of enormous amounts of PCE into the atmosphere. The 2006 World Health Organization report documented an annual PCE production of 170.000 tons for the US and EU in the mid-90s (WHO, 2006). According estimates of the Agency for Toxic Substances and Disease Registry from the U.S. Department of Health and Human Services, 80 - 85% of the annually used PCE was released into the atmosphere at that time (ATSDR, 1997). These facts unambiguously highlight the importance of remediation processes to protect the environment for further pollution and a reduction of pollutant levels. Different strategies including physical, chemical and biological technologies have been developed, while bioremediation processes applying bacterial mixed cultures obtained from natural environments or artificially constructed *in vitro* are the most reliable strategies (Stroo *et al.*, 2013). Research on bioaugmentation strategies using dechlorinating cultures started in the late 1990s as a result of the massive release of chlorinated hydrocarbons into the environment. The discovery of the *D. mccartyi* with its unique capabilities was a breakthrough and made effective bioremediation possible (Duhamel *et al.*, 2002). *In situ* bioaugmentation using *Dehalococcoides*-containing cultures bears a remarkable high potential for further bioremediation attempts due to the facts that the organisms are able to completely degrade chlorinated solvents to benign ethene even at concentrations close to the limit of solubility. Several studies already demonstrated successful and promising bioremediation approaches. For example, complete dechlorination of TCE and *c*DCE to ethene in an organohalide-contaminated aquifer was achieved only after the injection of an ethene-forming microbial enrichment culture which was stable and survived more than 500 days. Additionally, the groundwater flow transport

the culture through the whole contaminated site (Ellis *et al.*, 2000). The application of microbial consortia has become a suitable practice and several inocula targeting different hydrocarbons (e.g. aromatic/poly aromatic, halogenated, BTEX, fuel oils) are commercially available so far which were already used at several hundred sites in North America and Europe (Lyon and Vogel, 2013). All dechlorinating mixed cultures have a complex community structure in common and contain different bacterial and archaeal genera. This complexity is of high importance for restricted OHRB (e.g. *Dehalococcoides*, *Dehalobacter*) which are dependent on other community members contributing to dechlorination by providing hydrogen as electron donor and other growth requirements (e.g. corrninoids). One of the best studied microbial inocula regarding bacterial composition and substrate spectrum including used electron donors and organic solvents is the KB-1® culture (SiREM lab, Guelph, Ontario, Canada, Fig. 9A). The consortium was originally obtained from a TCE-contaminated site and contains two *Dehlacoccoides* spp. as the main population and *Geobacter*, *Methanosarcina*, *Syntrophobacter*, *Syntrophus* and *Sulfurospirillum* in a minor portion (Duhamel and Edwards, 2006). The culture covers a broad range of chlorinated ethenes (e.g. PCE, TCE, all DCE isomers, VC), ethanes (e.g. 1,2-dichloroethane, 1,1,1-trichloroethane), methanes (e.g. chloroform, dichloromethane), chlorofluorocarbons (e.g. trichlorofluoromethane) and several chlorobenzenes (e.g. hexachlorobenzene, polychlorinated biphenyl and dioxins) and can be therefore applied for various decontamination purposes (Duhamel and Edwards, 2007; Duhamel *et al.*, 2004; Duhamel *et al.*, 2002; Grostern and Edwards, 2006b; Löffler *et al.*, 2013a). Additionally, the diverse community structure enables growth with many commonly used electron donors including sugars (e.g. glucose, molasses), organic acids (e.g. lactate, propionate), alcohols (e.g. methanol, ethanol) and oils (Aziz *et al.*, 2013; Duhamel *et al.*, 2002). The culture was already successfully in a number of field-scale studies via injection wells or push injection techniques applied (Fig. 9B, C).

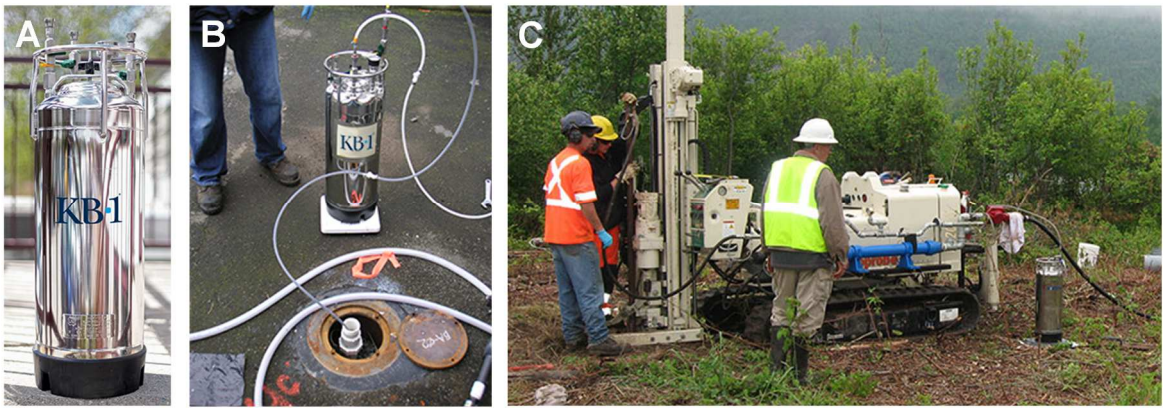


Fig. 9: KB-1® canister (A) and its application into an injection well (B) and via push injection into the contaminated soil (B). Picture courtesy and copyright by SiREM lab (with permission of Peter Dollar, SiREM lab, Guelph, Ontario, Canada).

For example, PCE-contaminated groundwater (1 mg/L) of an Air Force Base was almost completely bioremediated within 200 days (5 µg/L) with ethene as the end product (Major *et al.*, 2002). Another study at the Cape Canaveral Air Force Station reported a significant increase in ethene formation when using KB-1® for bioaugmentation and revealed a conversion of 98% TCE in 52 days. Furthermore, dechlorination and growth of the organisms was not inhibited even at high TCE concentrations (155 mg/L) (Hood *et al.*, 2008).

These examples demonstrate a remarkable progress of the field of bioaugmentation over the last 30 years which became a cost- and time-effective strategie for *in situ* remediation. Additionally, basic research investigating the biochemistry, physiology and ecology of microorganisms capable of organohalide respiration and the discovery of new organisms able to degrade PCE contributed enormous to the success of this technology. Bioaugmentation is still a young field and requires ongoing research in different areas: i) the molecular scale enabling a better understanding of gene regulation and enzymatic mechanisms, ii) the organismic scale, characterizing organohalide respiration capabilities of an isolate, iii) the community scale, identification of food webs, interspecies connections and signals for elucidation of the community dynamics and iv) the ecosystem scale combining the interaction of the community with the chemical and physical conditions of the

environment. Moreover, state of the art technologies such as metagenome sequencing and proteomic analysis enabling metabolic reconstruction and modelling will aid in the development of more efficient bioaugmentation processes and more accurate predictions of the success of bioremediation.

In contrast to KB-1®, the here established co-culture of *S. multivorans* and *D. mccartyi* is at a very early stage for considering being applied for bioremediation and needs much more characterization in terms of optimizing growth and dechlorination rates also in large-scale bioreactors and elucidating the substrate spectrum and the ability to degrade (poly-)chlorinated ethanes, aromatics, phenols and biphenyls. Another important feature to verify is the stability of the community which addresses the questions of how stable is the syntrophic interaction when other microorganisms like fermenters and/or methanogens come as competitors into play and how high the *in situ* survival rate is. This could be initially tested in constructed wetlands already used for the treatment or organohalide-contaminated groundwater (Imfeld *et al.*, 2010; Stottmeister *et al.*, 2003; Vymazal, 2011). A third community member would probably broaden the substrate spectrum of the electron donor by producing lactate from more complex organic substrates. This is a disadvantage of the co-culture utilizing only lactate and other short-chained organic acids compared to KB-1®.

However, advantageous is the simplicity of the co-culture with *S. multivorans* providing all nutrients required for growth and dechlorination of *D. mccartyi*. This makes an elucidation of the syntrophic relationship between *S. multivorans* and *D. mccartyi* much easier than in highly heterogenous mixed cultures which was the main focus of this doctoral thesis. These first insights could be used for the prediction of the general role of *Sulfurospirillum* spp. in OHRB-containing microbial communities and might be the basis for further investigations on the ecological role of the genus *Sulfurospirillum*.

4 References

Abrahamsson K., Ekdahl A., Collén J., Pedersén M. (1995). Marine algae-a source of trichloroethylene and perchloroethylene. *Limnology and Oceanography*, 40(7), 1321-1326.

Adams M.W. (1990). The structure and mechanism of iron-hydrogenases. *Biochim Biophys Acta*, 1020(2), 115-45.

Adrian L., Hansen S.K., Fung J.M., Görisch H., Zinder S.H. (2007). Growth of *Dehalococcoides* Strains with Chlorophenols as Electron Acceptors. *Environmental Science & Technology*, 41(7), 2318-2323.

Ahmann D., Roberts A.L., Krumholz L.R., Morel F.M.M. (1994). Microbe grows by reducing arsenic. *Nature*, 371, 750.

Albers S.V., Meyer B.H. (2011). The archaeal cell envelope. *Nat Rev Microbiol*, 9(6), 414-26.

Amos B.K., Ritalahti K.M., Cruz-Garcia C., Padilla-Crespo E., Löffler F.E. (2008). Oxygen Effect on *Dehalococcoides* Viability and Biomarker Quantification. *Environmental Science & Technology*, 42(15), 5718-5726.

ATSDR (1997). Toxicological profile for tetrachloroethylene, (Ed.) United States Department of Health and Human Services P.H.S., Agency for Toxic Substances and Disease Registry (ATSDR/TP-97/18) in de Raat 2003. Atlanta, GA, USA.

Axley M.J., Grahame D.A., Stadtman T.C. (1990). *Escherichia coli* formate-hydrogen lyase. Purification and properties of the selenium-dependent formate dehydrogenase component. *J Biol Chem*, 265(30), 18213-8.

Aziz C.E., Wymorc R.A., Steffan R.J. (2013). Bioaugmentation Considerations. in: *Bioaugmentation for Groundwater Remediation*, (Eds.) Stroo H.F., Leeson A., Ward C.H., Springer Verlag. New York, pp. 141-169.

Bagramyan K., Trchounian A. (2003). Structural and Functional Features of Formate Hydrogen Lyase, an Enzyme of Mixed-Acid Fermentation from *Escherichia coli*. *Biochemistry (Moscow)*, 68(11), 1159-1170.

Barton H., Luiszer F. (2005). *Microbial metabolic structure in a sulfidic cave hot spring: Potential mechanisms of biospeleogenesis*.

Bernhard M., Benelli B., Hochkoeppler A., Zannoni D., Friedrich B. (1997). Functional and structural role of the cytochrome *b* subunit of the membrane-bound hydrogenase complex of *Alcaligenes eutrophus* H16. *Eur J Biochem*, 248(1), 179-86.

Bi E., Lutkenhaus J. (1991). FtsZ ring structure associated with division in *Escherichia coli*. *Nature*, 354, 161.

Bommer M., Kunze C., Fesseler J., Schubert T., Diekert G., Dobbek H. (2014). Structural basis for organohalide respiration. *Science*, 2014, 455-458.

Brandt U., Kerscher S., Dröse S., Zwicker K., Zickermann V. (2003). Proton pumping by NADH:ubiquinone oxidoreductase. A redox driven conformational change mechanism? *FEBS Letters*, 545(1), 9-17.

Breznak J.A. (2006). The Genus *Sporomusa*. in: *The Prokaryotes: Volume 4: Bacteria: Firmicutes, Cyanobacteria*, (Eds.) Dworkin M., Falkow S., Rosenberg E., Schleifer K.-H., Stackebrandt E., Springer US. New York, NY, pp. 991-1001.

Buhrke T., Lenz O., Krauss N., Friedrich B. (2005). Oxygen tolerance of the H_2-sensing [NiFe] hydrogenase from *Ralstonia eutropha* H16 is based on limited access of oxygen to the active site. *J Biol Chem*, 280(25), 23791-6.

Bunge M., Lechner U. (2009). Anaerobic reductive dehalogenation of polychlorinated dioxins. *Applied Microbiology and Biotechnology*, 84(3), 429-444.

Butler J.E., Young N.D., Lovley D.R. (2010). Evolution of electron transfer out of the cell: comparative genomics of six *Geobacter* genomes. *BMC Genomics*, 11, 40.

Butler P., Ebert M.-O., Lyskowski A., Gruber K., Kratky C., Kräutler B. (2006). Vitamin B_{12}: A Methyl Group without a Job? *Angewandte Chemie International Edition*, 45(6), 989-993.

Buttet G.F., Murray A.M., Goris T., Burion M., Jin B., Rolle M., Holliger C., Maillard J. (2018). Coexistence of two distinct *Sulfurospirillum* populations respiring tetrachloroethene – genomic and kinetic considerations. *FEMS Microbiology Ecology*, fiy018-fiy018.

Campbell B.J., Engel A.S., Porter M.L., Takai K. (2006). The versatile epsilon-proteobacteria: key players in sulphidic habitats. *Nat Rev Microbiol*, 4(6), 458-68.

Chan C.H., Newmister S.A., Talyor K., Claas K.R., Rayment I., Escalante-Semerena J.C. (2014). Dissecting cobamide diversity through structural and functional analyses of the base-activating CobT enzyme of *Salmonella enterica*. *Biochimica et biophysica acta*, 1840(1), 464-475.

Chen M., Wolin M.J. (1977). Influence of CH_4 production by *Methanobacterium ruminantium* on the fermentation of glucose and lactate by *Selenomonas ruminantium*. *Applied and Environmental Microbiology*, 34(6), 756-759.

Cheng D., Chow W.L., He J. (2010). A *Dehalococcoides*-containing co-culture that dechlorinates tetrachloroethene to *trans*-1,2-dichloroethene. *ISME J*, 4(1), 88-97.

Cheong C.-G., Escalante-Semerena J.C., Rayment I. (2001). Structural Investigation of the Biosynthesis of Alternative Lower Ligands for Cobamides by Nicotinate Mononucleotide: 5,6-Dimethylbenzimidazole Phosphoribosyltransferase from *Salmonella enterica*. *Journal of Biological Chemistry*, 276(40), 37612-37620.

Cichocka D., Nikolausz M., Haest P.J., Nijenhuis I. (2010). Tetrachloroethene conversion to ethene by a *Dehalococcoides*-containing enrichment culture from Bitterfeld. *FEMS Microbiol Ecol*, 72(2), 297-310.

Cichocka D., Siegert M., Imfeld G., Andert J., Beck K., Diekert G., Richnow H.-H., Nijenhuis I. (2007). Factors controlling the carbon isotope fractionation of tetra- and trichloroethene during reductive dechlorination by *Sulfurospirillum* ssp. and *Desulfitobacterium* sp. strain PCE-S. *FEMS Microbiology Ecology*, 62(1), 98-107.

Claas K.R., Parrish J.R., Maggio-Hall L.A., Escalante-Semerena J.C. (2010). Functional Analysis of the Nicotinate Mononucleotide:5,6-Dimethylbenzimidazole Phosphoribosyltransferase (CobT) Enzyme, Involved in the Late Steps of Coenzyme B_{12} Biosynthesis in *Salmonella enterica*. *Journal of Bacteriology*, 192(1), 145-154.

Coplen T.B., Brand W.A., Gehre M., Gröning M., Meijer H.A., Toman B., Verkouteren R.M., Agency I.A.E. (2006). After two decades a second anchor for the VPDB delta13C scale. *Rapid Commun Mass Spectrom*, 20(21), 3165-6.

Coppi M.V. (2005). The hydrogenases of *Geobacter sulfurreducens*: a comparative genomic perspective. *Microbiology*, 151(Pt 4), 1239-54.

Corre E., Reysenbach A.L., Prieur D. (2001). Epsilon-proteobacterial diversity from a deep-sea hydrothermal vent on the Mid-Atlantic Ridge. *FEMS Microbiol Lett*, 205(2), 329-35.

de Boer P., Crossley R., Rothfield L. (1992). The essential bacterial cell-division protein FtsZ is a GTPase. *Nature*, 359, 254.

de Bok F.A.M., Luijten M.L.G.C., Stams A.J.M. (2002). Biochemical Evidence for Formate Transfer in Syntrophic Propionate-Oxidizing Cocultures of *Syntrophobacter fumaroxidans* and *Methanospirillum hungatei*. *Applied and Environmental Microbiology*, 68(9), 4247-4252.

Delgado A.G., Fajardo-Williams D., Bondank E., Esquivel-Elizondo S., Krajmalnik-Brown R. (2017). Coupling Bioflocculation of *Dehalococcoides mccartyi* to High-Rate Reductive Dehalogenation of Chlorinated Ethenes. *Environ Sci Technol*, 51(19), 11297-11307.

Delgado A.G., Kang D.-W., Nelson K.G., Fajardo-Williams D., Miceli J.F., Done H.Y., Popat S.C., Krajmalnik-Brown R. (2014). Selective Enrichment Yields Robust Ethene-Producing Dechlorinating Cultures from Microcosms Stalled at cis-Dichloroethene. *PLoS ONE*, 9(6), e100654.

Diekert G., Wohlfarth G. (1994). Metabolism of homoacetogens. *Antonie van Leeuwenhoek*, 66(1), 209-221.

DiStefano T.D., Gossett J.M., Zinder S.H. (1992). Hydrogen as an electron donor for dechlorination of tetrachloroethene by an anaerobic mixed culture. *Appl Environ Microbiol*, 58(11), 3622-9.

Doberenz C., Zorn M., Falke D., Nannemann D., Hunger D., Beyer L., Ihling C.H.*, et al.* (2014). Pyruvate Formate-Lyase Interacts Directly with the Formate Channel FocA to Regulate Formate Translocation. *Journal of molecular biology*, 426(15), 2827-2839.

Dross F., Geisler V., Lenger R., Theis F., Krafft T., Fahrenholz F., Kojro E.*, et al.* (1992). The quinone-reactive Ni/Fe-hydrogenase of *Wolinella succinogenes*. *European Journal of Biochemistry*, 206(1), 93-102.

Drzyzga O., Gottschal J.C. (2002). Tetrachloroethene Dehalorespiration and Growth of *Desulfitobacterium frappieri* TCE1 in Strict Dependence on the Activity of *Desulfovibrio fructosivorans*. *Applied and Environmental Microbiology*, 68(2), 642-649.

Dubini A., Pye R.L., Jack R.L., Palmer T., Sargent F. (2002). How bacteria get energy from hydrogen: a genetic analysis of periplasmic hydrogen oxidation in *Escherichia coli*. *International Journal of Hydrogen Energy*, 27(11), 1413-1420.

Duche O., Elsen S., Cournac L., Colbeau A. (2005). Enlarging the gas access channel to the active site renders the regulatory hydrogenase HupUV of *Rhodobacter capsulatus* O_2 sensitive without affecting its transductory activity. *Febs j*, 272(15), 3899-908.

Dugat-Bony E., Biderre-Petit C., Jaziri F., David Maude M., Denonfoux J., Lyon Delina Y., Richard J.Y.*, et al.* (2012). In situ TCE degradation mediated by complex dehalorespiring communities during biostimulation processes. *Microbial Biotechnology*, 5(5), 642-653.

Duhamel M., Edwards E.A. (2007). Growth and Yields of Dechlorinators, Acetogens, and Methanogens during Reductive Dechlorination of Chlorinated Ethenes and Dihaloelimination of 1,2-Dichloroethane. *Environmental Science & Technology*, 41(7), 2303-2310.

Duhamel M., Edwards E.A. (2006). Microbial composition of chlorinated ethene-degrading cultures dominated by *Dehalococcoides*. *FEMS Microbiology Ecology*, 58(3), 538-549.

Duhamel M., Mo K., Edwards E.A. (2004). Characterization of a Highly Enriched *Dehalococcoides*-Containing Culture That Grows on Vinyl Chloride and Trichloroethene. *Applied and Environmental Microbiology*, 70(9), 5538-5545.

Duhamel M., Wehr S.D., Yu L., Rizvi H., Seepersad D., Dworatzek S., Cox E.E., Edwards E.A. (2002). Comparison of anaerobic dechlorinating enrichment cultures maintained on tetrachloroethene, trichloroethene, cis-dichloroethene and vinyl chloride. *Water Res*, 36(17), 4193-202.

Eguchi S., Yoon K.S., Ogo S. (2012). O_2-stable membrane-bound [NiFe] hydrogenase from a newly isolated *Citrobacter* sp. S-77. *J Biosci Bioeng*, 114(5), 479-84.

Ellis D.E., Lutz E.J., Odom J.M., Buchanan R.J., Bartlett C.L., Lee M.D., Harkness M.R., DeWeerd K.A. (2000). Bioaugmentation for Accelerated *In Situ* Anaerobic Bioremediation. *Environmental Science & Technology*, 34(11), 2254-2260.

Elshahed M.S., Senko J.M., Najar F.Z., Kenton S.M., Roe B.A., Dewers T.A., Spear J.R., Krumholz L.R. (2003). Bacterial diversity and sulfur cycling in a mesophilic sulfide-rich spring. *Appl Environ Microbiol*, 69(9), 5609-21.

Elsner M. (2010). Stable isotope fractionation to investigate natural transformation mechanisms of organic contaminants: principles, prospects and limitations. *J Environ Monit*, 12(11), 2005-31.

Elsner M., Chartrand M., Vanstone N., Couloume G.L., Lollar B.S. (2008). Identifying abiotic chlorinated ethene degradation: characteristic isotope patterns in reaction products with nanoscale zero-valent iron. *Environ Sci Technol*, 42(16), 5963-70.

Engel A.S., Lee N., Porter M.L., Stern L.A., Bennett P.C., Wagner M. (2003). Filamentous “Epsilonproteobacteria” Dominate Microbial Mats from Sulfidic Cave Springs. *Applied and Environmental Microbiology*, 69(9), 5503-5511.

Engel A.S., Porter M.L., Stern L.A., Quinlan S., Bennett P.C. (2004). Bacterial diversity and ecosystem function of filamentous microbial mats from aphotic (cave) sulfidic springs dominated by chemolithoautotrophic "Epsilonproteobacteria". *FEMS Microbiol Ecol*, 51(1), 31-53.

Escalante-Semerena J.C. (2007). Conversion of Cobinamide into Adenosylcobamide in Bacteria and Archaea. *Journal of Bacteriology*, 189(13), 4555-4560.

Fauque G., Peck H.D., Jr., Moura J.J., Huynh B.H., Berlier Y., DerVartanian D.V., Teixeira M.*, et al.* (1988). The three classes of hydrogenases from sulfate-reducing bacteria of the genus *Desulfovibrio*. *FEMS Microbiol Rev*, 4(4), 299-344.

Fazi S., Aulenta F., Majone M., Rossetti S. (2008). Improved quantification of Dehalococcoides species by fluorescence in situ hybridization and catalyzed reporter deposition. *Syst Appl Microbiol*, 31(1), 62-7.

Fennell D.E., Gossett J.M. (1998). Modeling the Production of and Competition for Hydrogen in a Dechlorinating Culture. *Environmental Science & Technology*, 32(16), 2450-2460.

Finster K., Liesack W., Tindall B.J. (1997). *Sulfurospirillum arcachonense* sp. nov., a new microaerophilic sulfur-reducing bacterium. *Int J Syst Bacteriol*, 47(4), 1212-7.

Friedrich B., Buhrke T., Burgdorf T., Lenz O. (2005). A hydrogen-sensing multiprotein complex controls aerobic hydrogen metabolism in *Ralstonia eutropha*. *Biochem Soc Trans*, 33(Pt 1), 97-101.

Frielingsdorf S., Schubert T., Pohlmann A., Lenz O., Friedrich B. (2011). A Trimeric Supercomplex of the Oxygen-Tolerant Membrane-Bound [NiFe]-Hydrogenase from *Ralstonia eutropha* H16. *Biochemistry*, 50(50), 10836-10843.

Fritsch J., Lenz O., Friedrich B. (2013). Structure, function and biosynthesis of O_2-tolerant hydrogenases. *Nat Rev Microbiol*, 11(2), 106-14.

Fritsch J., Scheerer P., Frielingsdorf S., Kroschinsky S., Friedrich B., Lenz O., Spahn C.M. (2011). The crystal structure of an oxygen-tolerant hydrogenase uncovers a novel iron-sulphur centre. *Nature*, 479(7372), 249-52.

Futagami T., Goto M., Furukawa K. (2008). Biochemical and genetic bases of dehalorespiration. *Chem Rec*, 8(1), 1-12.

Gadkari J., Goris T., Schiffmann C.L., Rubick R., Adrian L., Schubert T., Diekert G. (2018). Reductive tetrachloroethene dehalogenation in the presence of oxygen by *Sulfurospirillum multivorans*: physiological studies and proteome analysis. *FEMS Microbiology Ecology*, 94(1), fix176-fix176.

Gevertz D., Telang A.J., Voordouw G., Jenneman G.E. (2000). Isolation and characterization of strains CVO and FWKO B, two novel nitrate-reducing, sulfide-oxidizing bacteria isolated from oil field brine. *Appl Environ Microbiol*, 66(6), 2491-501.

Goris T., Diekert G. (2016). The Genus *Sulfurospirillum*. in: *Organohalide-Respiring Bacteria*, (Eds.) Adrian L., Löffler F.E., Springer-Verlag. Berlin, Heidelberg, pp. 209-234.

Goris T., Schenz B., Zimmermann J., Lemos M., Hackermüller J., Schubert T., Diekert G. (2017). The complete genome of the tetrachloroethene-respiring Epsilonproteobacterium *Sulfurospirillum halorespirans*. *Journal of Biotechnology*, 255, 33-36.

Goris T., Schiffmann C.L., Gadkari J., Schubert T., Seifert J., Jehmlich N., von Bergen M., Diekert G. (2015). Proteomics of the organohalide-respiring Epsilonproteobacterium *Sulfurospirillum multivorans* adapted to tetrachloroethene and other energy substrates. *Scientific Reports*, 5, 13794.

Goris T., Schubert T., Gadkari J., Wubet T., Tarkka M., Buscot F., Adrian L., Diekert G. (2014). Insights into organohalide respiration and the versatile catabolism of *Sulfurospirillum multivorans* gained from comparative genomics and physiological studies. *Environmental Microbiology*, 16(11), 3562-3580.

Gray M.J., Escalante-Semerena J.C. (2009a). The cobinamide amidohydrolase (cobyric acid-forming) CbiZ enzyme: A critical activity of the cobamide remodeling system of *Rhodobacter sphaeroides*. *Molecular microbiology*, 74(5), 1198-1210.

Gray M.J., Escalante-Semerena J.C. (2009b). In Vivo Analysis of Cobinamide Salvaging in *Rhodobacter sphaeroides* Strain 2.4.1. *Journal of Bacteriology*, 191(12), 3842-3851.

Gribble G. (2012). *Recently Discovery Naturally Occurring Heterocyclic Organohalogen Compounds*.

Gross R., Pisa R., Sänger M., Lancaster C.R.D., Simon J. (2004). Characterization of the Menaquinone Reduction Site in the Diheme Cytochrome *b* Membrane Anchor of *Wolinella succinogenes* NiFe-hydrogenase. *Journal of Biological Chemistry*, 279(1), 274-281.

Grostern A., Chan W.W.M., Edwards E.A. (2009). 1,1,1-Trichloroethane and 1,1-Dichloroethane Reductive Dechlorination Kinetics and Co-Contaminant Effects in a *Dehalobacter*-Containing Mixed Culture. *Environmental Science & Technology*, 43(17), 6799-6807.

Grostern A., Edwards E.A. (2006a). A 1,1,1-Trichloroethane-Degrading Anaerobic Mixed Microbial Culture Enhances Biotransformation of Mixtures of Chlorinated Ethenes and Ethanes. *Applied and Environmental Microbiology*, 72(12), 7849-7856.

Grostern A., Edwards E.A. (2006b). Growth of Dehalobacter and *Dehalococcoides* spp. during Degradation of Chlorinated Ethanes. *Applied and Environmental Microbiology*, 72(1), 428-436.

Grotenhuis J.T.C., Smit M., van Lammeren A.A.M., Stams A.J.M., Zehnder A.J.B. (1991). Localization and quantification of extracellular polymers in methanogenic granular sludge. *Applied Microbiology and Biotechnology*, 36(1), 115-119.

Gruber K., Puffer B., Krautler B. (2011). Vitamin B_{12}-derivatives-enzyme cofactors and ligands of proteins and nucleic acids. *Chemical Society Reviews*, 40(8), 4346-4363.

Guimarães D.H., Weber A., Klaiber I., Vogler B., Renz P. (1994). Guanylcobamide and hypoxanthylcobamide-Corrinoids formed by *Desulfovibrio vulgaris*. *Archives of Microbiology*, 162(4), 272-276.

Guiral M., Aubert C., Giudici-Orticoni M.-T. (2005). Hydrogen metabolism in the hyperthermophilic bacterium *Aquifex aeolicus*. *Biochemical Society Transactions*, 33(1), 22-24.

Harper D.B., Hamilton J.T.G. (2003). The Global Cycles of the Naturally-Occurring Monohalomethanes. in: *Natural Production of Organohalogen Compounds*, (Ed.) Gribble G., Springer Berlin Heidelberg. Berlin, Heidelberg, pp. 17-41.

Hartwig S., Dragomirova N., Kublik A., Turkowsky D., von Bergen M., Lechner U., Adrian L., Sawers R.G. (2017). A H_2-oxidizing, 1,2,3-trichlorobenzene-reducing multienzyme complex isolated

from the obligately organohalide-respiring bacterium *Dehalococcoides mccartyi* strain CBDB1. *Environ Microbiol Rep*, 9(5), 618-625.

Hartwig S., Thomas C., Krumova N., Quitzke V., Turkowsky D., Jehmlich N., Adrian L., Sawers R.G. (2015). Heterologous complementation studies in *Escherichia coli* with the Hyp accessory protein machinery from Chloroflexi provide insight into [NiFe]-hydrogenase large subunit recognition by the HypC protein family. *Microbiology*, 161(11), 2204-19.

Hazra A.B., Han A.W., Mehta A.P., Mok K.C., Osadchiy V., Begley T.P., Taga M.E. (2015). Anaerobic biosynthesis of the lower ligand of vitamin B_{12}. *Proc Natl Acad Sci U S A*, 112(34), 10792-7.

Hazra A.B., Tran J.L.A., Crofts T.S., Taga M.E. (2013). Analysis of Substrate Specificity in CobT Homologs Reveals Widespread Preference for DMB, the Lower Axial Ligand of Vitamin B_{12}. *Chemistry & Biology*, 20(10), 1275-1285.

He J., Holmes V.F., Lee P.K.H., Alvarez-Cohen L. (2007). Influence of Vitamin B_{12} and Cocultures on the Growth of *Dehalococcoides* Isolates in Defined Medium. *Applied and Environmental Microbiology*, 73(9), 2847-2853.

He J., Ritalahti K.M., Aiello M.R., Löffler F.E. (2003a). Complete detoxification of vinyl chloride by an anaerobic enrichment culture and identification of the reductively dechlorinating population as a *Dehalococcoides* species. *Appl Environ Microbiol*, 69(2), 996-1003.

He J., Ritalahti K.M., Yang K.L., Koenigsberg S.S., Löffler F.E. (2003b). Detoxification of vinyl chloride to ethene coupled to growth of an anaerobic bacterium. *Nature*, 424(6944), 62-5.

He J., Sung Y., Krajmalnik-Brown R., Ritalahti K.M., Loffler F.E. (2005). Isolation and characterization of *Dehalococcoides* sp. strain FL2, a trichloroethene (TCE)- and 1,2-dichloroethene-respiring anaerobe. *Environ Microbiol*, 7(9), 1442-50.

Hedderich R. (2004). Energy-Converting [NiFe] Hydrogenases from Archaea and Extremophiles: Ancestors of Complex I. *Journal of Bioenergetics and Biomembranes*, 36(1), 65-75.

Hedderich R., Forzi L. (2005). Energy-Converting [NiFe] Hydrogenases: More than Just H_2 Activation. *Journal of Molecular Microbiology and Biotechnology*, 10(2-4), 92-104.

Heimann A.C., Batstone D.J., Jakobsen R. (2006). Methanosarcina spp. drive Vinyl Chloride Dechlorination via Interspecies Hydrogen Transfer. *Applied and Environmental Microbiology*, 72(4), 2942-2949.

Henschler D. (1994). Toxicity of Chlorinated Organic Compounds: Effects of the Introduction of Chlorine in Organic Molecules. *Angewandte Chemie International Edition in English*, 33(19), 1920-1935.

Hiratsuka T., Furihata K., Ishikawa J., Yamashita H., Itoh N., Seto H., Dairi T. (2008). An Alternative Menaquinone Biosynthetic Pathway Operating in Microorganisms. *Science*, 321(5896), 1670-1673.

Hood E.D., Major D.W., Quinn J.W., Yoon W.S., Gavaskar A., Edwards E.A. (2008). Demonstration of Enhanced Bioremediation in a TCE Source Area at Launch Complex 34, Cape Canaveral Air Force Station. *Ground Water Monitoring & Remediation*, 28(2), 98-107.

Huber H., Hohn M.J., Rachel R., Fuchs T., Wimmer V.C., Stetter K.O. (2002). A new phylum of Archaea represented by a nanosized hyperthermophilic symbiont. *Nature*, 417, 63.

Hug L.A., Beiko R.G., Rowe A.R., Richardson R.E., Edwards E.A. (2012). Comparative metagenomics of three *Dehalococcoides*-containing enrichment cultures: the role of the non-dechlorinating community. *BMC Genomics*, 13, 327.

Hulshoff Pol L.W., de Castro Lopes S.I., Lettinga G., Lens P.N.L. (2004). Anaerobic sludge granulation. *Water Research*, 38(6), 1376-1389.

Imfeld G., Aragonés C.E., Fetzer I., Mészáros É., Zeiger S., Nijenhuis I., Nikolausz M., Delerce S., Richnow H.H. (2010). Characterization of microbial communities in the aqueous phase of a constructed model wetland treating 1,2-dichloroethene-contaminated groundwater. *FEMS Microbiology Ecology*, 72(1), 74-88.

Ishii S., Kosaka T., Hori K., Hotta Y., Watanabe K. (2005). Coaggregation facilitates interspecies hydrogen transfer between *Pelotomaculum thermopropionicum* and *Methanothermobacter thermautotrophicus*. *Appl Environ Microbiol*, 71(12), 7838-45.

Ishii S.i., Kosaka T., Hotta Y., Watanabe K. (2006). Simulating the Contribution of Coaggregation to Interspecies Hydrogen Fluxes in Syntrophic Methanogenic Consortia. *Applied and Environmental Microbiology*, 72(7), 5093-5096.

Jahn U., Gallenberger M., Paper W., Junglas B., Eisenreich W., Stetter K.O., Rachel R., Huber H. (2008). *Nanoarchaeum equitans* and *Ignicoccus hospitalis*: New Insights into a Unique, Intimate Association of Two Archaea. *Journal of Bacteriology*, 190(5), 1743-1750.

Jensen A., Finster K. (2005). Isolation and characterization of *Sulfurospirillum carboxydovorans* sp. nov., a new microaerophilic carbon monoxide oxidizing Epsilonproteobacterium. *Antonie Van Leeuwenhoek*, 87(4), 339-53.

John M., Rubick R., Schmitz R.P.H., Rakoczy J., Schubert T., Diekert G. (2009). Retentive Memory of Bacteria: Long-Term Regulation of Dehalorespiration in *Sulfurospirillum multivorans*. *Journal of Bacteriology*, 191(5), 1650-1655.

John M., Schmitz R.P.H., Westermann M., Richter W., Diekert G. (2006). Growth substrate dependent localization of tetrachloroethene reductive dehalogenase in *Sulfurospirillum multivorans*. *Archives of Microbiology*, 186(2), 99-106.

Jordan A., Harnisch J., Borchers R., Le Guern F., Shinohara H. (2000). Volcanogenic Halocarbons. *Environmental Science & Technology*, 34(6), 1122-1124.

Ju X., Zhao L., Sun B. (2007). Nitrogen fixation by reductively dechlorinating bacteria. *Environmental Microbiology*, 9(4), 1078-1083.

Jugder B.E., Ertan H., Wong Y.K., Braidy N., Manefield M., Marquis C.P., Lee M. (2016). Genomic, transcriptomic and proteomic analyses of *Dehalobacter* UNSWDHB in response to chloroform. *Environ Microbiol Rep*.

Keller S., Kunze C., Bommer M., Paetz C., Menezes R.C., Svatoš A., Dobbek H., Schubert T. (2018). Selective utilization of benzimidazolyl-norcobamides as cofactors by the tetrachloroethene reductive dehalogenase of *Sulfurospirillum multivorans*. *Journal of Bacteriology*.

Keller S., Ruetz M., Kunze C., Kräutler B., Diekert G., Schubert T. (2014). Exogenous 5,6-dimethylbenzimidazole caused production of a non-functional tetrachloroethene reductive dehalogenase in *Sulfurospirillum multivorans*. *Environmental Microbiology*, 16(11), 3361-3369.

Knappe J., Sawers G. (1990). A radical-chemical route to acetyl-CoA: the anaerobically induced pyruvate formate-lyase system of *Escherichia coli*. *FEMS Microbiology Letters*, 75(4), 383-398.

Krajmalnik-Brown R., Holscher T., Thomson I.N., Saunders F.M., Ritalahti K.M., Loffler F.E. (2004). Genetic identification of a putative vinyl chloride reductase in *Dehalococcoides* sp. strain BAV1. *Appl Environ Microbiol*, 70(10), 6347-51.

Krauter H. (2006). Untersuchungen zur Beteiligung membranstandiger redoxaktiver Komponenten bei der Dehalorespiration in *Sulfurospirillum multivorans*, Diploma Thesis, Vol. Diploma, Friedrich Schiller University Jena, Jena, Germany.

Kräutler B., Fieber W., Ostermann S., Fasching M., Ongania K.-H., Gruber K., Kratky C.*, et al.* (2003). The Cofactor of Tetrachloroethene Reductive Dehalogenase of *Dehalospirillum multivorans* Is Norpseudo-B_{12}, a New Type of a Natural Corrinoid. *Helvetica Chimica Acta*, 86(11), 3698-3716.

Kruse S., Goris T., Wolf M., Wei X., Diekert G. (2017a). The NiFe Hydrogenases of the Tetrachloroethene-Respiring Epsilonproteobacterium *Sulfurospirillum multivorans*: Biochemical Studies and Transcription Analysis. *Frontiers in Microbiology*, 8, 444.

Kruse T., Goris T., Maillard J., Woyke T., Lechner U., de Vos W., Smidt H. (2017b). Comparative genomics of the genus *Desulfitobacterium*. *FEMS Microbiology Ecology*, 93(12), fix135-fix135.

Kruse T., Maillard J., Goodwin L., Woyke T., Teshima H., Bruce D., Detter C.*, et al.* (2013). Complete genome sequence of *Dehalobacter restrictus* PER-K23(T.). *Stand Genomic Sci*, 8(3), 375-88.

Kruse T., van de Pas B.A., Atteia A., Krab K., Hagen W.R., Goodwin L., Chain P.*, et al.* (2015). Genomic, proteomic, and biochemical analysis of the organohalide respiratory pathway in *Desulfitobacterium dehalogenans*. *J Bacteriol*, 197(5), 893-904.

Kube M., Beck A., Zinder S.H., Kuhl H., Reinhardt R., Adrian L. (2005). Genome sequence of the chlorinated compound–respiring bacterium *Dehalococcoides* species strain CBDB1. *Nature Biotechnology*, 23, 1269.

Kublik A., Deobald D., Hartwig S., Schiffmann C.L., Andrades A., Bergen M., Sawers R.G., Adrian L. (2016). Identification of a multi-protein reductive dehalogenase complex in *Dehalococcoides mccartyi* strain CBDB1 suggests a protein□dependent respiratory electron transport chain obviating quinone involvement. *Environmental Microbiology*, 18(9), 3044-3056.

Kunze C., Bommer M., Hagen W.R., Uksa M., Dobbek H., Schubert T., Diekert G. (2017). Cobamide-mediated enzymatic reductive dehalogenation via long-range electron transfer. *Nature Communications*, 8, 15858.

Lamont C.M., Kelly C.L., Pinske C., Buchanan G., Palmer T., Sargent F. (2017). Expanding the substrates for a bacterial hydrogenlyase reaction. *Microbiology*.

Latham M.J., Wolin M.J. (1977). Fermentation of cellulose by *Ruminococcus flavefaciens* in the presence and absence of *Methanobacterium ruminantium*. *Applied and Environmental Microbiology*, 34(3), 297-301.

Lee L.K., Ding C., Yang K.-L., He J. (2011). Complete Debromination of Tetra- and Penta-Brominated Diphenyl Ethers by a Coculture Consisting of *Dehalococcoides* and *Desulfovibrio* Species. *Environmental Science & Technology*, 45(19), 8475-8482.

Lendvay J.M., Löffler F.E., Dollhopf M., Aiello M.R., Daniels G., Fathepure B.Z., Gebhard M.*, et al.* (2003). Bioreactive Barriers: A Comparison of Bioaugmentation and Biostimulation for Chlorinated Solvent Remediation. *Environmental Science & Technology*, 37(7), 1422-1431.

Lettinga G., van Velsen A.F.M., Hobma S.W., de Zeeuw W., Klapwijk A. (1980). Use of the upflow sludge blanket (USB) reactor concept for biological wastewater treatment, especially for anaerobic treatment. *Biotechnology and Bioengineering*, 22(4), 699-734.

Leys D., Adrian L., Smidt H. (2013). Organohalide respiration: microbes breathing chlorinated molecules. *Philos Trans R Soc Lond B Biol Sci*, 368(1616), 20120316.

Liu N., Li H., Li M., Ding L., Weng C.-H., Dong C.-D. (2017). Oxygen exposure effects on the dechlorinating activities of a trichloroethene-dechlorination microbial consortium. *Bioresource Technology*, 240, 98-105.

Löffler F.E., Ritalahti K.M., Zinder S.H. (2013a). *Dehalococcoides* and reductive dechlorination of chlorinated solvents. in: *Bioaugmentation for Groundwater Remediation*, (Eds.) Stroo H.F., Leeson A., Ward C.H., Springer Verlag. New York, pp. 39-88.

Löffler F.E., Tiedje J.M., Sanford R.A. (1999). Fraction of Electrons Consumed in Electron Acceptor Reduction and Hydrogen Thresholds as Indicators of Halorespiratory Physiology. *Applied and Environmental Microbiology*, 65(9), 4049-4056.

Löffler F.E., Yan J., Ritalahti K.M., Adrian L., Edwards E.A., Konstantinidis K.T., Muller J.A.*, et al.* (2013b). *Dehalococcoides mccartyi* gen. nov., sp. nov., obligately organohalide-respiring anaerobic bacteria relevant to halogen cycling and bioremediation, belong to a novel bacterial class, *Dehalococcoidia* classis nov., order *Dehalococcoidales* ord. nov. and family *Dehalococcoidaceae* fam. nov., within the phylum Chloroflexi. *Int J Syst Evol Microbiol*, 63(Pt 2), 625-35.

Lorenz A., Löffler F.E. (2016). *Organohalide-Respiring Bacteria*. Springer-Verlag, Berlin Heidelberg.

Lovley D.R., Ferry J.G. (1985). Production and Consumption of H(2) during Growth of *Methanosarcina* spp. on Acetate. *Applied and Environmental Microbiology*, 49(1), 247-249.

Lovley D.R., Goodwin S. (1988). Hydrogen concentrations as an indicator of the predominant terminal electron-accepting reactions in aquatic sediments. *Geochimica et Cosmochimica Acta*, 52(12), 2993-3003.

Luijten M.L.G.C., de Weert J., Smidt H., Boschker H.T.S., de Vos W.M., Schraa G., Stams A.J.M. (2003). Description of *Sulfurospirillum halorespirans* sp. nov., an anaerobic, tetrachloroethene-respiring bacterium, and transfer of *Dehalospirillum multivorans* to the genus Sulfurospirillum as *Sulfurospirillum multivorans* comb. nov. *International Journal of Systematic and Evolutionary Microbiology*, 53(3), 787-793.

Luijten M.L.G.C., Weelink S.A.B., Godschalk B., Langenhoff A.A.M., van Eekert M.H.A., Schraa G., Stams A.J.M. (2004). Anaerobic reduction and oxidation of quinone moieties and the reduction of oxidized metals by halorespiring and related organisms. *FEMS Microbiology Ecology*, 49(1), 145-150.

Lutkenhaus J. (1993). FtsZ ring in bacterial cytokinesis. *Molecular Microbiology*, 9(3), 403-409.

Lyon D.Y., Vogel T.M. (2013). Bioaugmentation for Groundwater Remediation: An Overview. in: *Bioremediation for Groundwater Remediation*, (Eds.) Stroo H.F., Leeson A., Ward C.H., Springer-Verlag. New York, pp. 1-37.

Magnuson J.K., Romine M.F., Burris D.R., Kingsley M.T. (2000). Trichloroethene reductive dehalogenase from *Dehalococcoides ethenogenes*: sequence of *tceA* and substrate range characterization. *Appl Environ Microbiol*, 66(12), 5141-7.

Maillard J., Charnay M.P., Regeard C., Rohrbach-Brandt E., Rouzeau-Szynalski K., Rossi P., Holliger C. (2011). Reductive dechlorination of tetrachloroethene by a stepwise catalysis of different organohalide respiring bacteria and reductive dehalogenases. *Biodegradation*, 22(5), 949-60.

Major D.W., McMaster M.L., Cox E.E., Edwards E.A., Dworatzek S.M., Hendrickson E.R., Starr M.G., Payne J.A., Buonamici L.W. (2002). Field Demonstration of Successful Bioaugmentation To

Achieve Dechlorination of Tetrachloroethene To Ethene. *Environmental Science & Technology*, 36(23), 5106-5116.

Mao X., Stenuit B., Polasko A., Alvarez-Cohen L. (2015). Efficient Metabolic Exchange and Electron Transfer within a Syntrophic Trichloroethene-Degrading Coculture of *Dehalococcoides mccartyi* 195 and *Syntrophomonas wolfei*. *Applied and Environmental Microbiology*, 81(6), 2015-2024.

Maphosa F., de Vos W.M., Smidt H. (2010). Exploiting the ecogenomics toolbox for environmental diagnostics of organohalide-respiring bacteria. *Trends in Biotechnology*, 28(6), 308-316.

Marreiros B.C., Batista A.P., Duarte A.M., Pereira M.M. (2013). A missing link between complex I and group 4 membrane-bound [NiFe] hydrogenases. *Biochim Biophys Acta*, 1827(2), 198-209.

Matturro B., Tandoi V., Rossetti S. (2013). Different activity levels of Dehalococcoides mccartyi revealed by FISH and CARD-FISH under non-steady and pseudo-steady state conditions. *N Biotechnol*, 30(6), 756-62.

Maymó-Gatell X., Anguish T., Zinder S.H. (1999). Reductive dechlorination of chlorinated ethenes and 1, 2-dichloroethane by "Dehalococcoides ethenogenes" 195. *Appl Environ Microbiol*, 65(7), 3108-13.

Maymó-Gatell X., Chien Y., Gossett J.M., Zinder S.H. (1997). Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. *Science*, 276(5318), 1568-71.

Maymó-Gatell X., Nijenhuis I., Zinder S.H. (2001). Reductive Dechlorination of cis-1,2-Dichloroethene and Vinyl Chloride by "*Dehalococcoides ethenogenes*". *Environmental Science & Technology*, 35(3), 516-521.

McCarty P.L. (2010). Groundwater Contamination by Chlorinated Solvents: History, Remediation Technologies and Strategies. in: *In Situ Remediation of Chlorinated Solvent Plumes*, (Eds.) Stroo H.F., Ward C.H., Springer New York. New York, NY, pp. 1-28.

McInerney M.J., Sieber J.R., Gunsalus R.P. (2011). Microbial Syntrophy: Ecosystem-Level Biochemical Cooperation-Genomic sequences reveal systems required to produce hydrogen and formate, plus other hallmarks of the syntrophic lifestyle. *Microbe*, 6(11), 479–485.

McInerney M.J., Sieber J.R., Gunsalus R.P. (2009). Syntrophy in anaerobic global carbon cycles. *Curr Opin Biotechnol*, 20(6), 623-32.

McInerney M.J., Struchtemeyer C.G., Sieber J., Mouttaki H., Stams A.J.M., Schink B., Rohlin L., Gunsalus R.P. (2008). Physiology, Ecology, Phylogeny, and Genomics of Microorganisms Capable of Syntrophic Metabolism. *Annals of the New York Academy of Sciences*, 1125(1), 58-72.

McMurdie P.J., Behrens S.F., Müller J.A., Göke J., Ritalahti K.M., Wagner R., Goltsman E.*, et al.* (2009). Localized Plasticity in the Streamlined Genomes of Vinyl Chloride Respiring *Dehalococcoides*. *PLOS Genetics*, 5(11), e1000714.

McTernan P.M., Chandrayan S.K., Wu C.-H., Vaccaro B.J., Lancaster W.A., Adams M.W.W. (2015). Engineering the respiratory membrane-bound hydrogenase of the hyperthermophilic archaeon *Pyrococcus furiosus* and characterization of the catalytically active cytoplasmic subcomplex. *Protein Engineering, Design and Selection*, 28(1), 1-8.

Men Y., Feil H., Verberkmoes N.C., Shah M.B., Johnson D.R., Lee P.K., West K.A.*, et al.* (2012). Sustainable syntrophic growth of *Dehalococcoides ethenogenes* strain 195 with *Desulfovibrio vulgaris* Hildenborough and *Methanobacterium congolense*: global transcriptomic and proteomic analyses. *Isme j*, 6(2), 410-21.

Men Y., Seth E.C., Yi S., Allen R.H., Taga M.E., Alvarez-Cohen L. (2014). Sustainable Growth of *Dehalococcoides mccartyi* 195 by Corrinoid Salvaging and Remodeling in Defined Lactate-Fermenting Consortia. *Applied and Environmental Microbiology*, 80(7), 2133-2141.

Meuer J., Kuettner H.C., Zhang J.K., Hedderich R., Metcalf W.W. (2002). Genetic analysis of the archaeon *Methanosarcina barkeri* Fusaro reveals a central role for Ech hydrogenase and ferredoxin in methanogenesis and carbon fixation. *Proc Natl Acad Sci U S A*, 99(8), 5632-7.

Miller E., Wohlfarth G., Diekert G. (1996). Studies on tetrachloroethene respiration in *Dehalospirillum multivorans*. *Archives of Microbiology*, 166(6), 379-387.

Mirzoyan S., Romero-Pareja P.M., Coello M.D., Trchounian A., Trchounian K. (2017). Evidence for hydrogenase-4 catalyzed biohydrogen production in *Escherichia coli*. *International Journal of Hydrogen Energy*, 42(34), 21697-21703.

Mitchell P. (2011). Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1807(12), 1507-1538.

Mohn W.W., Tiedje J.M. (1992). Microbial reductive dehalogenation. *Microbiological Reviews*, 56(3), 482-507.

Morris B.E.L., Henneberger R., Huber H., Moissl-Eichinger C. (2013). Microbial syntrophy: interaction for the common good. *FEMS Microbiology Reviews*, 37(3), 384-406.

Moulis J.M., Sieker L.C., Wilson K.S., Dauter Z. (1996). Crystal structure of the 2[4Fe-4S] ferredoxin from *Chromatium vinosum*: Evolutionary and mechanistic inferences for [3/4Fe□4S] ferredoxins. *Protein Science*, 5(9), 1765-1775.

Nakagawa S., Takai K., Inagaki F., Hirayama H., Nunoura T., Horikoshi K., Sako Y. (2005). Distribution, phylogenetic diversity and physiological characteristics of Epsilonproteobacteria in a deep-sea hydrothermal field. *Environ Microbiol*, 7(10), 1619-32.

Nakagawa S., Takaki Y. (2001). Nonpathogenic Epsilonproteobacteria. in: *eLS*, John Wiley & Sons, Ltd.

Neumann A., Scholz-Muramatsu H., Diekert G. (1994). Tetrachloroethene metabolism of *Dehalospirillum multivorans*. *Arch Microbiol*, 162(4), 295-301.

Neumann A., Wohlfarth G., Diekert G. (1996). Purification and Characterization of Tetrachloroethene Reductive Dehalogenase from *Dehalospirillum multivorans*. *Journal of Biological Chemistry*, 271(28), 16515-16519.

Neumann A., Wohlfarth G., Diekert G. (1998). Tetrachloroethene Dehalogenase from *Dehalospirillum multivorans*: Cloning, Sequencing of the Encoding Genes, and Expression of the pceA Gene in *Escherichia coli*. *Journal of Bacteriology*, 180(16), 4140-4145.

Nijenhuis I., Andert J., Beck K., Kästner M., Diekert G., Richnow H.-H. (2005). Stable Isotope Fractionation of Tetrachloroethene during Reductive Dechlorination by *Sulfurospirillum multivorans* and *Desulfitobacterium* sp. Strain PCE-S and Abiotic Reactions with Cyanocobalamin. *Applied and Environmental Microbiology*, 71(7), 3413-3419.

Parkhill J., Wren B.W., Mungall K., Ketley J.M., Churcher C., Basham D., Chillingworth T.*, et al.* (2000). The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature*, 403, 665.

Parthasarathy A., Stich T.A., Lohner S.T., Lesnefsky A., Britt R.D., Spormann A.M. (2015). Biochemical and EPR-Spectroscopic Investigation into Heterologously Expressed Vinyl Chloride Reductive Dehalogenase (VcrA) from *Dehalococcoides mccartyi* Strain VS. *Journal of the American Chemical Society*, 137(10), 3525-3532.

Pinske C., Sawers R.G. (2016). Anaerobic Formate and Hydrogen Metabolism. *EcoSal Plus*, 7(1).

Platen H., Janssen P.H., Schink B. (1994). Fermentative degradation of acetone by an enrichment culture in membrane-separated culture devices and in cell suspensions. *FEMS Microbiology Letters*, 122(1), 27-32.

Platen H., Schink B. (1987). Methanogenic degradation of acetone by an enrichment culture. *Archives of Microbiology*, 149(2), 136-141.

Pöritz M., Goris T., Wubet T., Tarkka M.T., Buscot F., Nijenhuis I., Lechner U., Adrian L. (2013). Genome sequences of two dehalogenation specialists - *Dehalococcoides mccartyi* strains BTF08 and DCMB5 enriched from the highly polluted Bitterfeld region. *FEMS Microbiol Lett*, 343(2), 101-4.

Pöritz M., Schiffmann C.L., Hause G., Heinemann U., Seifert J., Jehmlich N., von Bergen M., Nijenhuis I., Lechner U. (2015). *Dehalococcoides mccartyi* Strain DCMB5 Respires a Broad Spectrum of Chlorinated Aromatic Compounds. *Applied and Environmental Microbiology*, 81(2), 587-596.

Rahm B.G., Richardson R.E. (2008). *Dehalococcoides*' Gene Transcripts As Quantitative Bioindicators of Tetrachloroethene, Trichloroethene, and cis-1,2-Dichloroethene Dehalorespiration Rates. *Environmental Science & Technology*, 42(14), 5099-5105.

Redwood M.D., Mikheenko I.P., Sargent F., Macaskie L.E. (2008). Dissecting the roles of *Escherichia coli* hydrogenases in biohydrogen production. *FEMS Microbiology Letters*, 278(1), 48-55.

Reguera G., McCarthy K.D., Mehta T., Nicoll J.S., Tuominen M.T., Lovley D.R. (2005). Extracellular electron transfer via microbial nanowires. *Nature*, 435, 1098.

Reinhold A., Westermann M., Seifert J., von Bergen M., Schubert T., Diekert G. (2012). Impact of Vitamin B(12) on Formation of the Tetrachloroethene Reductive Dehalogenase in *Desulfitobacterium hafniense* Strain Y51. *Applied and Environmental Microbiology*, 78(22), 8025-8032.

Ren N., Xing D., Rittmann B.E., Zhao L., Xie T., Zhao X. (2007). Microbial community structure of ethanol type fermentation in bio-hydrogen production. *Environ Microbiol*, 9(5), 1112-25.

Renpenning J., Keller S., Cretnik S., Shouakar-Stash O., Elsner M., Schubert T., Nijenhuis I. (2014). Combined C and Cl Isotope Effects Indicate Differences between Corrinoids and Enzyme (*Sulfurospirillum multivorans* PceA) in Reductive Dehalogenation of Tetrachloroethene, But Not Trichloroethene. *Environmental Science & Technology*, 48(20), 11837-11845.

Renz P. (1999). Biosynthesis of the 5,6-Dimetyhlbenzimidazole Moiety of Cobalamin and of the Other Bases Found in Natural Corrinoids. in: *Chemistry and Biochemistry of B_{12}*, (Ed.) Banerjee R., John Wiley & Sons, Inc. New York, pp. 558-575.

Richardson R. (2016). Organohalide-Respiring Bacteria as Members of Microbial Communities: Catabolic Food Webs and Biochemical Interactions. in: *Organohalide-respiring Bacteria*, (Eds.) Adrian L., Löffler F., Springer. Berlin Heidelberg.

Richardson R.E., Bhupathiraju V.K., Song D.L., Goulet T.A., Alvarez-Cohen L. (2002). Phylogenetic characterization of microbial communities that reductively dechlorinate TCE based upon a combination of molecular techniques. *Environ Sci Technol*, 36(12), 2652-62.

Rossetti S., Aulenta F., Majone M., Crocetti G., Tandoi V. (2008). Structure analysis and performance of a microbial community from a contaminated aquifer involved in the complete reductive dechlorination of 1,1,2,2-tetrachloroethane to ethene. *Biotechnology and Bioengineering*, 100(2), 240-249.

Rupakula A., Kruse T., Boeren S., Holliger C., Smidt H., Maillard J. (2013). The restricted metabolism of the obligate organohalide respiring bacterium *Dehalobacter restrictus*: lessons from tiered functional genomics. *Philos Trans R Soc Lond B Biol Sci*, 368(1616), 20120325.

Sargent F. (2016). The Model [NiFe]-Hydrogenases of Escherichia coli. in: *Advances in Microbial Physiology*, (Ed.) Poole R.K., Vol. 68, Academic Press, pp. 433-507.

Sawers G. (1994). The hydrogenases and formate dehydrogenases of *Escherichia coli*. *Antonie van Leeuwenhoek*, 66(1), 57-88.

Sawers R.G., Blokesch M., Bock A. (2004). Anaerobic Formate and Hydrogen Metabolism. *EcoSal Plus*, 1(1).

Schink B. (1990). Conservation of small amounts of energy in fermenting bacteria. in: *Biotechnology focus*, (Eds.) RK F., P P., Vol. 2, Hanser. Munich, Vienna, New York, pp. 63-89.

Schink B. (2002). Synergistic interactions in the microbial world. *Antonie van Leeuwenhoek*, 81(1), 257-261.

Schink B. (1991). Syntrophism among Prokaryotes. in: *The Prokaryotes*, (Eds.) A B., HG T., M D., KH S., Vol. 2nd edition, Springer-Verlag New York, pp. 276-299.

Schink B., Stams A.J.M. (2006). Syntrophism among Prokaryotes. in: *The Prokaryotes*, (Eds.) Dworkin M., Falkow S., Rosenberg E., Schleifer K.H., Stackebrandt E., Vol. 2, Springer-Verlag. New York, pp. 309-335.

Schink B., Stams A.J.M. (2013). Syntrophism among Prokaryotes. in: *The Prokaryotes: Prokaryotic Communities and Ecophysiology*, (Eds.) Rosenberg E., DeLong E.F., Lory S., Stackebrandt E., Thompson F., Springer Berlin Heidelberg. Berlin, Heidelberg, pp. 471-493.

Schink B., Thauer R.K. (1988). Energetics of syntrophic methane formation and the influence of aggregation. in: *Granular anaerobic sludge: microbiology and technology*, (Eds.) G L., AJB Z., JTC G., LW H. Puduc, Wageningen, pp. 5-17.

Schipp C.J., Marco-Urrea E., Kublik A., Seifert J., Adrian L. (2013). Organic cofactors in the metabolism of *Dehalococcoides mccartyi* strains. *Philos Trans R Soc Lond B Biol Sci*, 368(1616), 20120321.

Schmidt M., Lege S., Nijenhuis I. (2014). Comparison of 1,2-dichloroethane, dichloroethene and vinyl chloride carbon stable isotope fractionation during dechlorination by two *Dehalococcoides strains*. *Water Res*, 52, 146-54.

Schmitz R.P.H., Diekert G. (2004). The fdh operon of *Sulfurospirillum multivorans*. *FEMS Microbiology Letters*, 237(2), 235-242.

Schmitz R.P.H., Diekert G. (2003). Purification and properties of the formate dehydrogenase and characterization of the fdhA gene of *Sulfurospirillum multivorans*. *Archives of Microbiology*, 180(6), 394-401.

Scholz-Muramatsu H., Neumann A., Meßmer M., Moore E., Diekert G. (1995). Isolation and characterization of *Dehalospirillum multivorans* gen. nov., sp. nov., a tetrachloroethene-utilizing, strictly anaerobic bacterium. *Archives of Microbiology*, 163(1), 48-56.

Schubert T., Adrian L., Sawers R.G., Diekert G. (2018). Organohalide respiratory chains: composition, topology and key enzymes. *FEMS Microbiology Ecology*, 94(4), fiy035-fiy035.

Schut G.J., Adams M.W. (2009). The iron-hydrogenase of *Thermotoga maritima* utilizes ferredoxin and NADH synergistically: a new perspective on anaerobic hydrogen production. *J Bacteriol*, 191(13), 4451-7.

Schut G.J., Boyd E.S., Peters J.W., Adams M.W.W. (2013). The modular respiratory complexes involved in hydrogen and sulfur metabolism by heterotrophic hyperthermophilic archaea and their evolutionary implications. *FEMS Microbiology Reviews*, 37(2), 182-203.

Schwartz E., Fritsch J., Friedrich B. (2013). H_2-Metabolizing Prokaryotes. in: *The Prokaryotes: Prokaryotic Physiology and Biochemistry*, (Eds.) Rosenberg E., DeLong E.F., Lory S., Stackebrandt E., Thompson F., Springer Berlin Heidelberg. Berlin, Heidelberg, pp. 119-199.

Seelert H., Poetsch A., Dencher N.A., Engel A., Stahlberg H., Müller D.J. (2000). Proton-powered turbine of a plant motor. *Nature*, 405, 418.

Self W.T., Hasona A., Shanmugam K.T. (2004). Expression and Regulation of a Silent Operon, *hyf*, Coding for Hydrogenase 4 Isoenzyme in *Escherichia coli*. *Journal of Bacteriology*, 186(2), 580-587.

Seshadri R., Adrian L., Fouts D.E., Eisen J.A., Phillippy A.M., Methe B.A., Ward N.L.*, et al.* (2005). Genome sequence of the PCE-dechlorinating bacterium *Dehalococcoides ethenogenes*. *Science*, 307(5706), 105-8.

Shrestha P.M., Rotaru A.-E., Aklujkar M., Liu F., Shrestha M., Summers Z.M., Malvankar N., Flores D.C., Lovley D.R. (2013). Syntrophic growth with direct interspecies electron transfer as the primary mechanism for energy exchange. *Environmental Microbiology Reports*, 5(6), 904-910.

Sieber J.R., Sims D.R., Han C., Kim E., Lykidis A., Lapidus A.L., McDonnald E.*, et al.* (2010). The genome of *Syntrophomonas wolfei*: new insights into syntrophic metabolism and biohydrogen production. *Environ Microbiol*, 12(8), 2289-301.

Silva Pedro J., van den Ban Eyke C.D., Wassink H., Haaker H., de Castro B., Robb Frank T., Hagen Wilfred R. (2001). Enzymes of hydrogen metabolism in *Pyrococcus furiosus*. *European Journal of Biochemistry*, 267(22), 6541-6551.

Skibinski D.A., Golby P., Chang Y.S., Sargent F., Hoffman R., Harper R., Guest J.R.*, et al.* (2002). Regulation of the hydrogenase-4 operon of *Escherichia coli* by the sigma(54)-dependent transcriptional activators *FhlA* and *HyfR*. *J Bacteriol*, 184(23), 6642-53.

Smatlak C.R., Gossett J.M., Zinder S.H. (1996). Comparative Kinetics of Hydrogen Utilization for Reductive Dechlorination of Tetrachloroethene and Methanogenesis in an Anaerobic Enrichment Culture. *Environmental Science & Technology*, 30(9), 2850-2858.

Soboh B., Linder D., Hedderich R. (2002). Purification and catalytic properties of a CO-oxidizing:H_2-evolving enzyme complex from *Carboxydothermus hydrogenoformans*. *European Journal of Biochemistry*, 269(22), 5712-5721.

Stams A.J.M. (1994). Metabolic interactions between anaerobic bacteria in methanogenic environments. *Antonie van Leeuwenhoek*, 66(1), 271-294.

Stams A.J.M., Plugge C.M. (2009). Electron transfer in syntrophic communities of anaerobic bacteria and archaea. *Nature Reviews Microbiology*, 7, 568.

Stams A.J.M., Worm P., Sousa D.Z., Alves M.M., Plugge C.M. (2012). Syntrophic Degradation of Fatty Acids by Methanogenic Communities. in: *Microbial Technologies in Advanced Biofuels Production*, (Ed.) Hallenbeck P.C., Springer US. Boston, MA, pp. 127-142.

Stock D., Leslie A.G.W., Walker J.E. (1999). Molecular Architecture of the Rotary Motor in ATP Synthase. *Science*, 286(5445), 1700.

Stolz J.F., Ellis D.J., Blum J.S., Ahmann D., Lovley D.R., Oremland R.S. (1999). *Sulfurospirillum barnesii* sp. nov. and Sulfurospirillum arsenophilum sp. nov., new members of the *Sulfurospirillum* clade of the Epsilonproteobacteria. *Int J Syst Bacteriol*, 49 Pt 3, 1177-80.

Stottmeister U., Wießner A., Kuschk P., Kappelmeyer U., Kästner M., Bederski O., Müller R.A., Moormann H. (2003). Effects of plants and microorganisms in constructed wetlands for wastewater treatment. *Biotechnology Advances*, 22(1), 93-117.

Stroo H.F., Leeson A., Ward C.H. (2013). *Bioaugmentation for Groundwater Remediation*. Springer-Verlag, New York.

Stupperich E., Eisinger H.-J., Schurr S. (1990). Corrinoids in anaerobic bacteria. *FEMS Microbiology Letters*, 87(3), 355-359.

Stupperich E., Eisinger H.J., Krautler B. (1989). Identification of phenolyl cobamide from the homoacetogenic bacterium *Sporomusa ovata*. *Eur J Biochem*, 186(3), 657-61.

Stupperich E., Eisinger H.J., Kräutler B. (1988). Diversity of corrinoids in acetogenic bacteria. P-cresolylcobamide from *Sporomusa ovata*, 5-methoxy-6-methylbenzimidazolylcobamide from *Clostridium formicoaceticum* and vitamin B_{12} from *Acetobacterium woodii*. *Eur J Biochem*, 172(2), 459-64.

Stupperich E., Kräutler B. (1988). Pseudo vitamin B_{12} or 5-hydroxybenzimidazolyl-cobamide are the corrinoids found in methanogenic bacteria. *Archives of Microbiology*, 149(3), 268-271.

Swarts H.J., Verhagen F.J.M., Field J.A., Wijnberg J.B.P.A. (1998). Trichlorinated phenols from *Hypholoma elongatum*. *Phytochemistry*, 49(1), 203-206.

Tanner A., Paster B.J. (1992). The Genus *Wolinella*. in: *The Prokaryotes: A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications*, (Eds.) Balows A., Trüper H.G., Dworkin M., Harder W., Schleifer K.-H., Springer New York. New York, NY, pp. 3512-3523.

Thauer R.K., Jungermann K., Decker K. (1977). Energy conservation in chemotrophic anaerobic bacteria. *Bacteriological Reviews*, 41(1), 100-180.

Therien J.B., Artz J.H., Poudel S., Hamilton T.L., Liu Z., Noone S.M., Adams M.W.W.*, et al.* (2017). The Physiological Functions and Structural Determinants of Catalytic Bias in the [FeFe]-Hydrogenases CpI and CpII of *Clostridium pasteurianum* Strain W5. *Frontiers in Microbiology*, 8(1305).

Thomas S.H., Wagner R.D., Arakaki A.K., Skolnick J., Kirby J.R., Shimkets L.J., Sanford R.A., Loffler F.E. (2008). The mosaic genome of *Anaeromyxobacter dehalogenans* strain 2CP-C suggests an aerobic common ancestor to the delta-proteobacteria. *PLoS One*, 3(5), e2103.

Tiedje J.M., Stevens T.O. (1988). The Ecology of an Anaerobic Dechlorinating Consortium. in: *Environmental Biotechnology: Reducing Risks from Environmental Chemicals through Biotechnology*, (Ed.) Omenn G.S., Springer US. Boston, MA, pp. 3-14.

Tomb J.-F., White O., Kerlavage A.R., Clayton R.A., Sutton G.G., Fleischmann R.D., Ketchum K.A.*, et al.* (1997). The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature*, 389, 412.

Toussaint B., De Sury d'Aspremont R., Delic-Attree I., Berchet V., Elsen S., Colbeau A., Dischert W., Lazzaroni Y., Vignais P.M. (1997). The *Rhodobacter capsulatus hupSLC* promoter: identification of *cis*-regulatory elements and of trans-activating factors involved in H_2 activation of *hupSLC* transcription. *Molecular Microbiology*, 26(5), 927-937.

Trchounian A., Gary Sawers R. (2014). Novel insights into the bioenergetics of mixed-acid fermentation: can hydrogen and proton cycles combine to help maintain a proton motive force? *IUBMB Life*, 66(1), 1-7.

Trchounian K., Trchounian A. (2014). Hydrogen producing activity by *Escherichia coli* hydrogenase 4 (Hyf) depends on glucose concentration. *International Journal of Hydrogen Energy*, 39(30), 16914-16918.

Türkowsky D., Jehmlich N., Diekert G., Adrian L., von Bergen M., Goris T. (2018). An integrative overview of genomic, transcriptomic and proteomic analyses in organohalide respiration research. *FEMS Microbiol Ecol*, 94(3).

Unden G., Kröger A. (1981). The function of the subunits of the fumarate reductase complex of *Vibrio succinogenes*. *Eur J Biochem*, 120(3), 577-84.

van der Zaan B., de Weert J., Rijnaarts H., de Vos W.M., Smidt H., Gerritse J. (2009). Degradation of 1,2-dichloroethane by microbial communities from river sediment at various redox conditions. *Water Research*, 43(13), 3207-3216.

Vignais P.M., Billoud B. (2007). Occurrence, Classification, and Biological Function of Hydrogenases: An Overview. *Chemical Reviews*, 107(10), 4206-4272.

Vignais P.M., Billoud B., Meyer J. (2001). Classification and phylogeny of hydrogenases. *FEMS Microbiol Rev*, 25(4), 455-501.

Volbeda A., Darnault C., Parkin A., Sargent F., Armstrong Fraser A., Fontecilla-Camps Juan C. (2013). Crystal Structure of the O_2-Tolerant Membrane-Bound Hydrogenase 1 from *Escherichia coli* in Complex with Its Cognate Cytochrome *b*. *Structure*, 21(1), 184-190.

Voordouw G., Armstrong S.M., Reimer M.F., Fouts B., Telang A.J., Shen Y., Gevertz D. (1996). Characterization of 16S rRNA genes from oil field microbial communities indicates the presence of a variety of sulfate-reducing, fermentative, and sulfide-oxidizing bacteria. *Appl Environ Microbiol*, 62(5), 1623-9.

Vymazal J. (2011). Constructed wetlands for wastewater treatment: five decades of experience. *Environ Sci Technol*, 45(1), 61-9.

WHO (2006). Concise International Chemical Assessment Document, World Health Organization (WHO), Vol. 68. Geneva, Switzerland.

Wijffels R., Janssen M. (2003). *Bio-methane & Bio-hydrogen. Status and perspectives of biological methane and hydrogen production.*, The Hague, The Netherlands.

Yan J., Im J., Yang Y., Löffler F.E. (2013). Guided cobalamin biosynthesis supports *Dehalococcoides mccartyi* reductive dechlorination activity. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 368(1616), 20120320.

Yan J., Ritalahti K.M., Wagner D.D., Löffler F.E. (2012). Unexpected Specificity of Interspecies Cobamide Transfer from *Geobacter* spp. to Organohalide-Respiring *Dehalococcoides mccartyi* Strains. *Applied and Environmental Microbiology*, 78(18), 6630-6636.

Yan J., Simsir B., Farmer A.T., Bi M., Yang Y., Campagna S.R., Loffler F.E. (2016). The corrinoid cofactor of reductive dehalogenases affects dechlorination rates and extents in organohalide-respiring *Dehalococcoides mccartyi*. *Isme j*, 10(5), 1092-101.

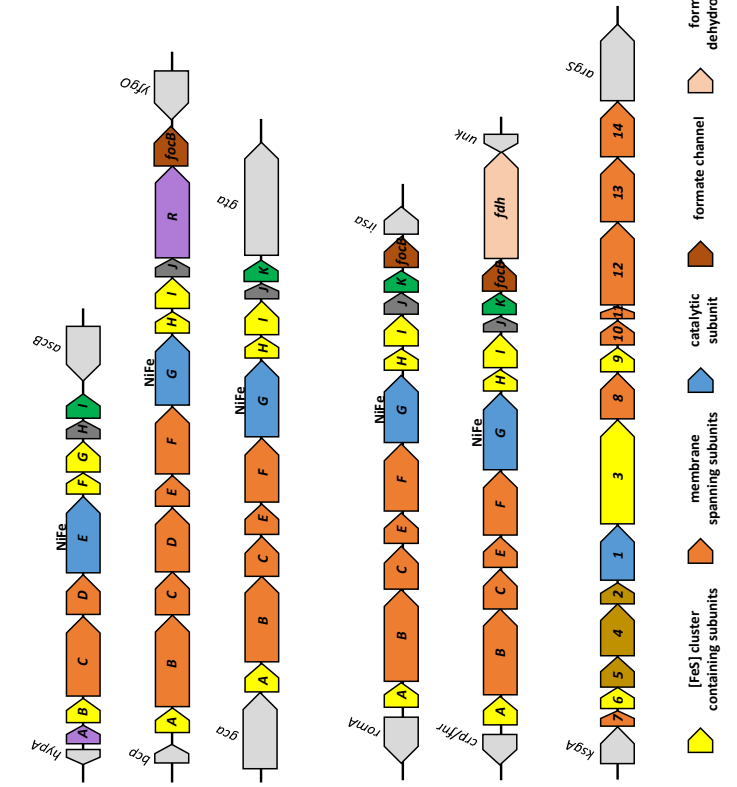
Yang Y., McCarty P.L. (1998). Competition for Hydrogen within a Chlorinated Solvent Dehalogenating Anaerobic Mixed Culture. *Environmental Science & Technology*, 32(22), 3591-3597.

Yi S., Seth E.C., Men Y.-J., Stabler S.P., Allen R.H., Alvarez-Cohen L., Taga M.E. (2012). Versatility in Corrinoid Salvaging and Remodeling Pathways Supports Corrinoid-Dependent Metabolism in *Dehalococcoides mccartyi*. *Applied and Environmental Microbiology*, 78(21), 7745-7752.

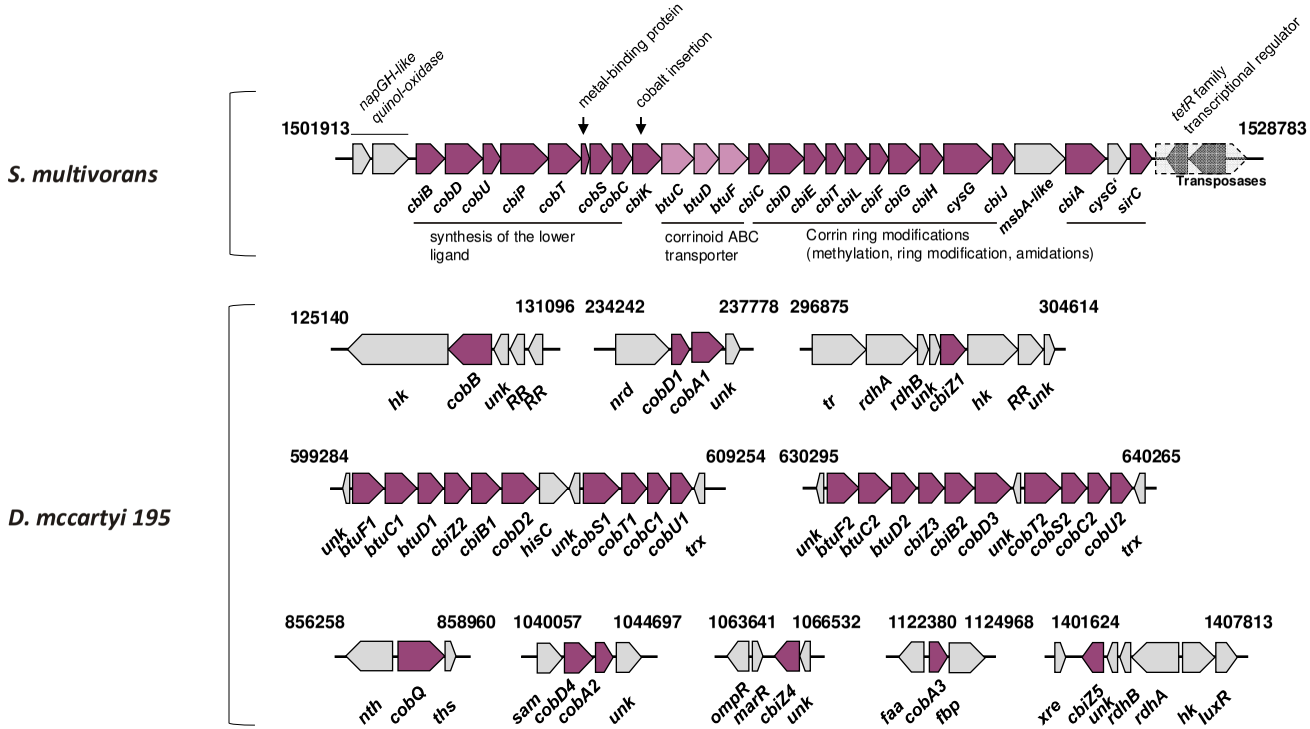
Yohda M., Yagi O., Takechi A., Kitajima M., Matsuda H., Miyamura N., Aizawa T.*, et al.* (2015). Genome sequence determination and metagenomic characterization of a *Dehalococcoides* mixed culture grown on cis-1,2-dichloroethene. *J Biosci Bioeng*, 120(1), 69-77.

Zinder S.H. (2016). The Genus *Dehalococcoides*. in: *Organohalide-Respiring Bacteria*, (Eds.) Adrian L., Löffler F.E., Springer-Verlag. Berlin, Heidelberg, pp. 107-136.

Appendix Introduction



Appendix Figure 1: Organization and comparison of the *S. multivorans hyf* gene cluster to related cluster encoding Hyd-3 and Hyd-4 of *E. coli* (A), other epsilonproteobacterial *hyf* clusters (B) and to complex I of *Thermus thermophilus*. Genes coding for proteins with the same functions are depicted in the same colour. Colour code below the cluster. *C. concisus* - *Campylobacter concisus*, *A. nitrofigilis* - *Arcobacter nitrofigilis*, *T. thermophilus* - *Thermus thermophilus*. NiFe - catalytic subunit, *ascB* - β-glucosidase, *argS* - arginyl-tRNA synthetase, *bcp* - putative thiol peroxidase, *crp/fnr* - putative transcriptional regulator, *gca* - diguanylate cyclase, *gta* - putative β-glucosyltransferase, *hypA* - NiFe hydrogenase maturation protein, *irsa* - putative membrane protein, *ksgA* - adenine dimethyltransferase, *romA* - outer membrane protein, *unk* - unknown gene, *yfgO* - putative permease (PerM).



Appendix Figure 2: Organization and comparison of the B_{12} biosynthesis cluster of *S. multivora.ns*, *D. mccartyi* strain 195 and strain BTF08. Genes related to biosynthesis and transport of vitamin B_{12} are coloured purple, flanking genes are coloured grey. Descriptions of *cbi* and *cob* genes are listed in Appendix Table 1. *act* - acetyl transferase, *cheY* - chemotaxis protein, *faa* - fumarylacetoacetate hydrolase, *fbp* - fructose 1-6 bisphosphatase, *hisC* - histidinol phosphate aminotransferase, *hk* - histidine kinase, *luxR* - response regulator, *hyl* - carbon nitrogen hydrolase, *marR* - MarR-type transcriptional regulator, *nrd* - ribonucleotide reductase, *nth* - endonuclease III, *ompR* - transcriptional regulator, *paaI* - thioesterase family protein, *rdhA* - reductive dehalogenase subunit A, *rdhB* - reductive dehalogenase subunit B (membrane anchor), *RR* - response regulator, *sam* - radical S-adenosyl-L-methionine enzyme, *ths* - thioesterase, *tr* - transcriptional regulator, *trx* - thioredoxin, *unk* - unknown gene, *xre* - DNA binding protein

Appendix Figure 2: continued

Appendix Table 1: Summary of genes involved in corrinoid biosynthesis in *S. multivorans* (Sm), *D. mccaryti* strain 195 and *D. mccaryti* strain BTF08.

Gene	EC number	Annotation	Function	present in		
				Sm	195	BTF08
*btuC*1	3.6.3.11	corrinoid ABC transporter, permease component	corrinoid ABC transport	x	x	x
*btuC*2					x	x
*btuD*1	3.6.3.11	corrinoid ABC transporter, ATPase component		x	x	x
*btuD*2					x	x
*btuF*1	3.6.3.11	corrinoid ABC transporter, corrinoid-binding protein		x	x	x
*btuF*2					x	x
cbiA	6.3.5.11	cobyrinic acid A,C-diamide synthase	corrin ring modifications			x
cbiC	5.4.1.2	cobalt-precorrin-8x methylmutase		x		
cbiD	2.1.1.195	cobalt-precorrin-6 synthas		x		
cbiE	2.1.1.132	cobalt-precorrin-6y C5-methyltransferase		x		
cbiF	2.1.1.133	cobalt-precorrin-4 C11-methyltransferase		x		
cbiG	3.7.1.12	cobalamin biosynthesis protein		x		
cbiH	2.1.1.131	cobalt-precorrin-3b C17-methyltransferase		x		
cbiJ	1.3.1.54	cobalt-precorrin-6x reductase		x		
cbiK	4.99.1.3	sirohydrochlorin cobaltochelatase		x		
cbiL	2.1.1.130	cobalt-precorrin-2 C20-methyltransferase		x		
cbiT	2.1.1.196	cobalt-precorrin-6y C15-methyltransferase		x		
cobB	6.3.5.9	cobyrinic acid a,c-diamide synthase		x	x	
cobQ	6.3.5.10	cobyric acid synthase		x	x	
cysG	1.3.1.76	precorrin-2 oxidase / Sirohydrochlorin ferrochelatase /		x		
sirC	1.3.1.76	precorrin-2 dehydrogenase		x		
*cbiB*1	6.3.1.10	adenosylcobinamide-phosphate synthase	lower ligand synthesis	x	x	x
*cbiB*2					x	x
cbiP	6.3.5.10	cobyric acid synthase		x		
*cbiZ*1	3.5.1.90	adenosylcobinamide amidohydrolase			x	x
*cbiZ*2					x	x
*cbiZ*3					x	
*cbiZ*4					x	
*cbiZ*5					x	
*cobA*1	2.5.1.17	cob(I)alamin adenosyltransferase			x	x
*cobA*2					x	x
*cobA*3					x	
*cobC*1	3.1.3.73	cobalamin-5´-phosphate phosphatase		x	x	x
*cobC*2					x	
*cobD*1	4.1.1.81	threonine-phosphate decarboxylase		x	x	x
*cobD*2					x	
*cobD*3					x	
*cobD*4					x	
*cobS*1	2.7.8.26	cobalamin synthase		x	x	x
*cobS*2					x	
*cobT*1	2.4.2.21	nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase		x	x	x
*cobT*2					x	
*cobU*1	2.7.1.156	cobinamide kinase/adenosylcobinamide phosphate guanylyltransferase		x	x	x
*cobU*2					x	
msbA-like	3.6.3.25	lipid A export ATP-binding/permease protein	sulfate ABC transport	x		

2.1 Supplementary Information

Supplementary Material

The NiFe Hydrogenases of the Tetrachloroethene-Respiring Epsilonproteobacterium *Sulfurospirillum multivorans*: Biochemical and Transcription Studies

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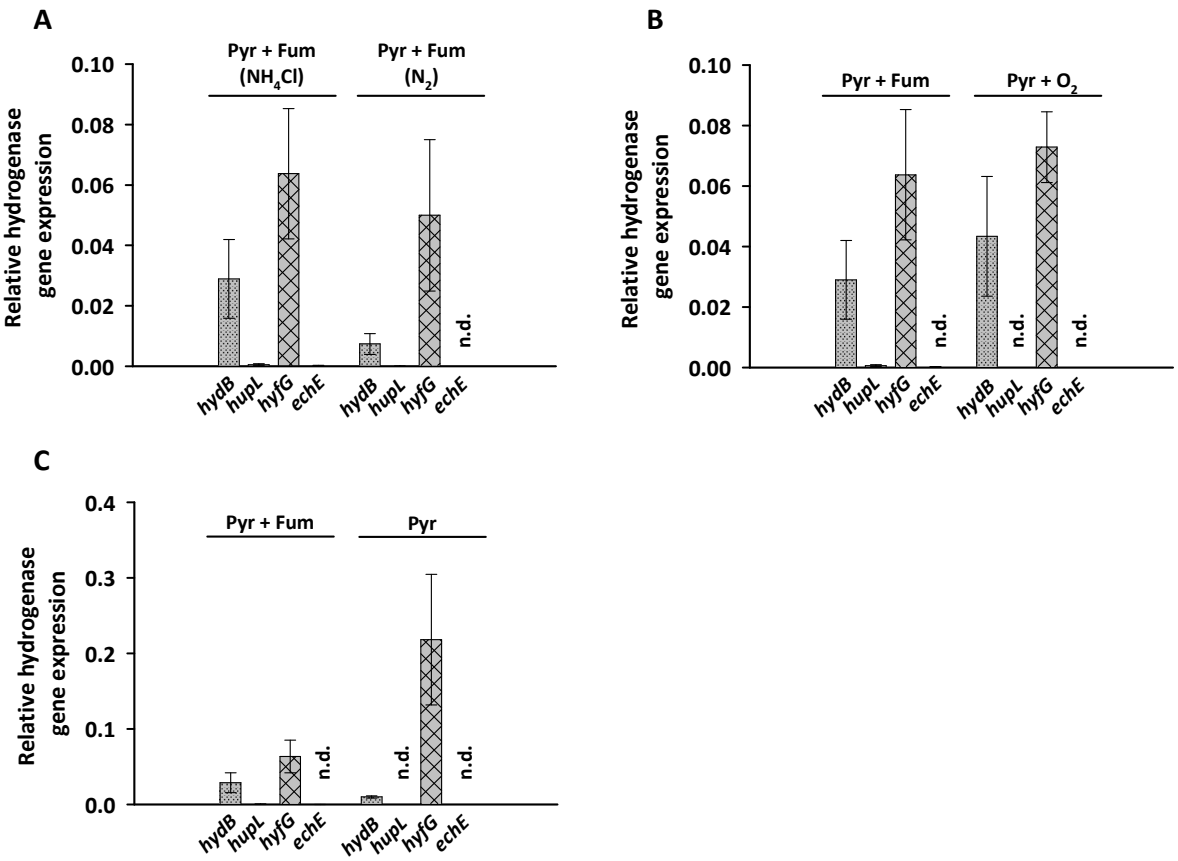
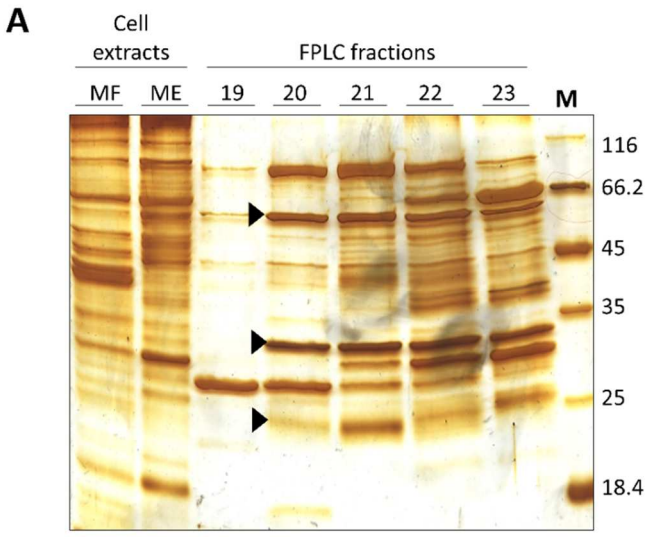
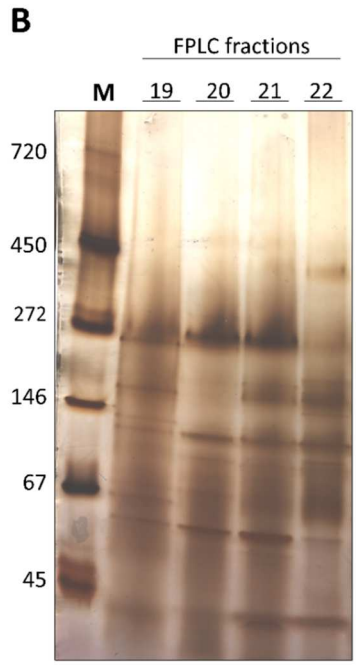
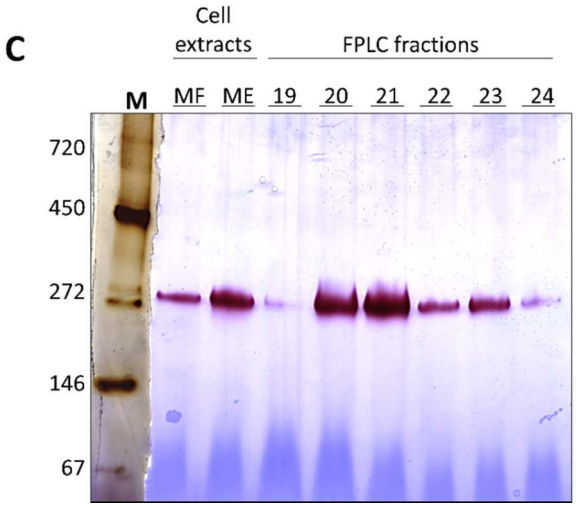


Supplementary Figure 1: Transcription pattern of hydrogenase catalytic subunit genes of *S. multivorans* with N_2 as sole N-source (A) and O_2 (B) as electron acceptor and under pyruvate fermentation (C). Transcript levels are normalized to the *16S* rRNA gene. All data were obtained from three biological replicates and three technical replicates. When amplification was detected only in one biological replicate, the hydrogenase gene was designated as not detected (n.d.).*hydB* - membrane-bound hydrogenase (MBH), *hupL* - cytoplasmic uptake hydrogenase, *hyfG* - Hyf-hydrogenase, *echE* - Ech-like hydrogenase. Pyr - pyruvate; Fum - fumarate; NH_4Cl - with ammonium chloride; N_2 - N_2 as sole N-source.

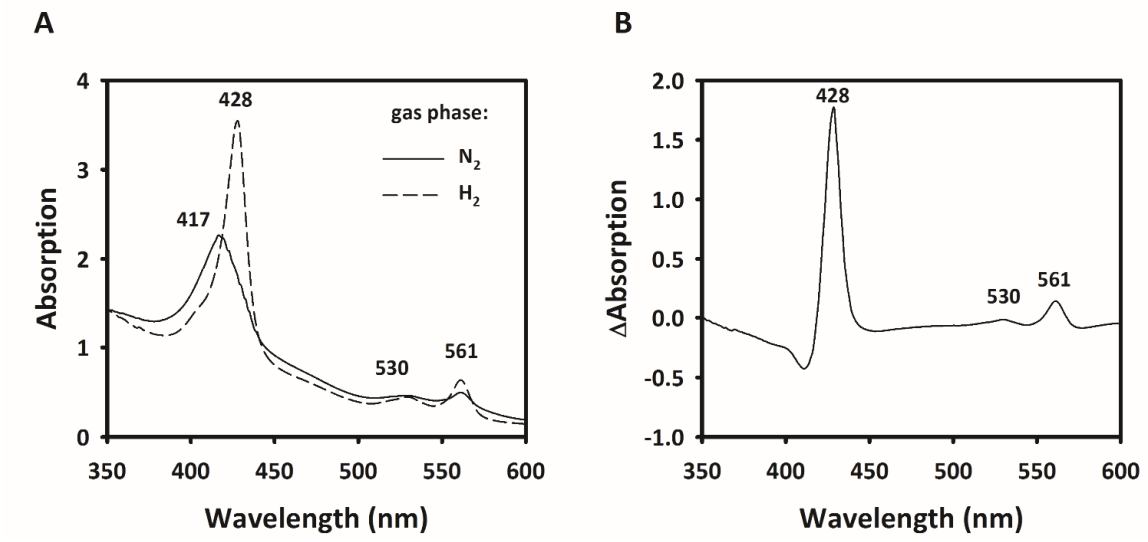


Specific activity (nkat/mg)							
H_2 → BV	5	57	290	1020	1031	527	310
H_2 → DMN	n.d.	n.d.	17	38	86	44	27





Supplementary Figure 2: Analysis of fractions obtained after FPLC purification according enrichment and oligomeric organization of the hydrogen oxidizing enzyme of *S. multivorans*. (A) Silver stained SDS polyacrylamide gel electrophoresis of cell extracts and FPLC fractions. 10 µg of protein was applied. Specific activities of fractions with BV and DMN are depicted in the table below. Arrows are indicating sizes/bands of the predicted MBH subunits, from top to bottom: HydB, HydA, HydC. HydB and HydA could be detected via MS in a different purification not shown here. (B) Non denaturing PAGE of FPLC fractions. 5 µg protein was applied. (C) Hydrogenase activity stained Blue Native PAGE of cell extracts and FPLC fractions. Marker band was cut off and silver stained. 1 µg of protein was applied. MF: membrane fraction, ME: membrane extract, M: marker lane, BV: benzyl viologen, DMN: 2,3-dimethyl-1,4-naphthoquinone, n.d.: not determined.



Supplementary Figure 3: Absorption spectra (A) and difference spectra (B) of the enriched MBH of *S. multivorans*. Cells were grown with pyruvate and fumarate. Spectra were obtained from fraction 20.

Supplementary Table 1: Bacterial strains, plasmids and oligonucleotides used in this study. Km^r - kanamycin resistance, DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen.

Strain, oligonucleotides	Characteristics or sequence (5'-3')	Amplicon size (bp)	Source or reference
Strain			
Sulfurospirillum multivorans			DSMZ 12446
Wild type			
Oligonucleotides			
*hydB*_fw	GTT GAA GAT GCG CTT GGA	234	This work
*hydB*_rev	TTT GGG TTG CAA CAA GAT		This work
*hupL*_fw	GCG TTT GGA AGA GTT ATT GGA G	214	This work
*hupL*_rev	TAC GTA TTT GAC ATC CGC ACT C		This work
*echE*_fw	AGC GTT GAT GAC CCA GTT T	249	This work
*echE*_rev	ATA GCT CAA AAC GCC CAC		This work
*hyfG*_fw	TGA CGT GCC TCT AGG ACC TT	205	This work
*hyfG*_rev	CAT GGG CAT AAC CAC AGA TG		This work
*recA*_fw	TAA AGT GGC ACC TCC GTT TC	266	This work
*recA*_rev	CGC CAC ATG TCA TAA CCA TC		This work
*16S rRNA*_fw	GAG ACA CGG TCC AGA CTC CTA C	255	This work
*16S rRNA*_rev	CTC GAC TTG ATT TCC AGC CTA C		This work

Supplementary Table 2: Hydrogen oxidizing activity of *S. multivorans* crude extract with different electron acceptors. Cells were grown on Pyr/PCE.

Electron Acceptor	Redox potential (mV)	Wavelength (nm)	Specific activity (nkat mg^{-1})
Benzylviologen[1] (BV)	-374	578	56.2 ± 5.2
Methylviologen[1] (MV)	-446	578	19.6 ± 4.6
NAD^{+2}	-320	365	<0.01
Methylene blue[1] (MB)	+11	570	8.6 ± 1.1
Nitroblue tetrazoliumchloride[2] (NBT)	+50	593	<0.01
Phenazine methosulfate[2] (PMS)	+65	388	<0.01

[1]Enzyme activity assays with BV, MV and MB are described in Materials & Methods

[2]NAD^+, NBT and PMS were prepared in concentrations of 0.2 mM in 50 mM Tris-HCl (pH 8.0)

2.2 Supplementary Information

Hydrogen production by *Sulfurospirillum* spp. enables syntrophic interactions of Epsilonproteobacteria

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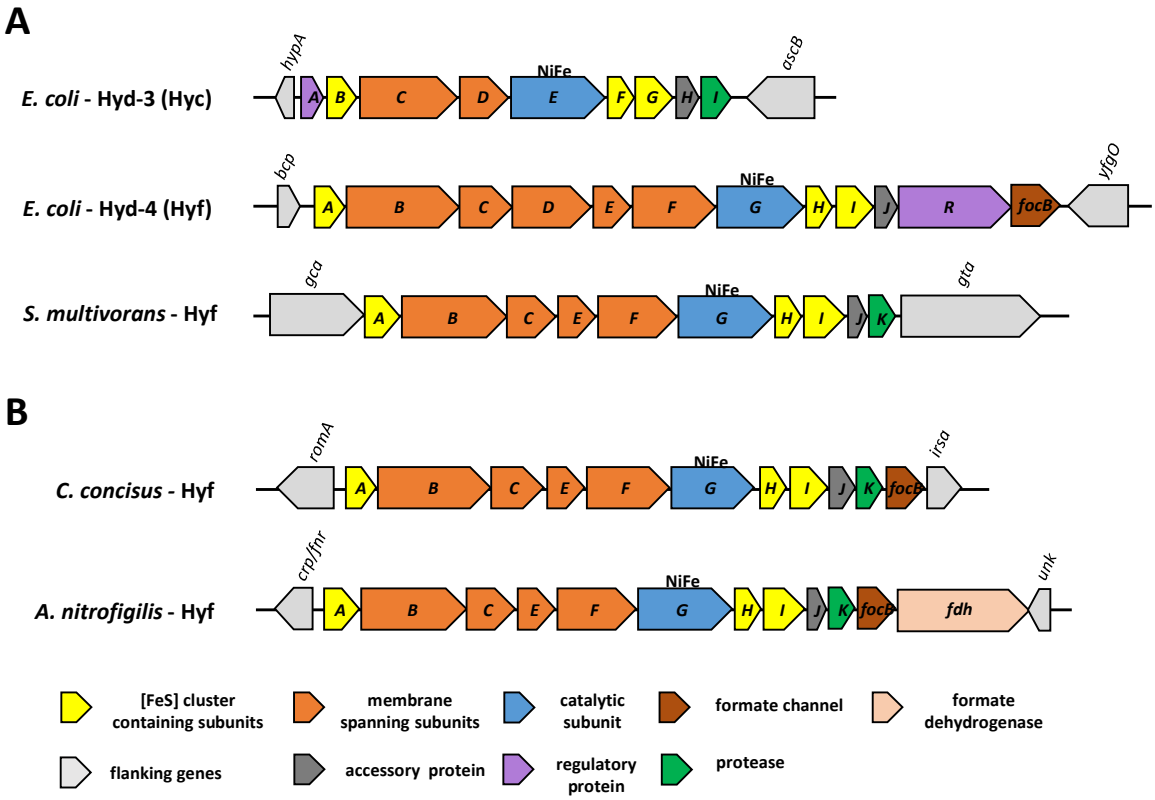
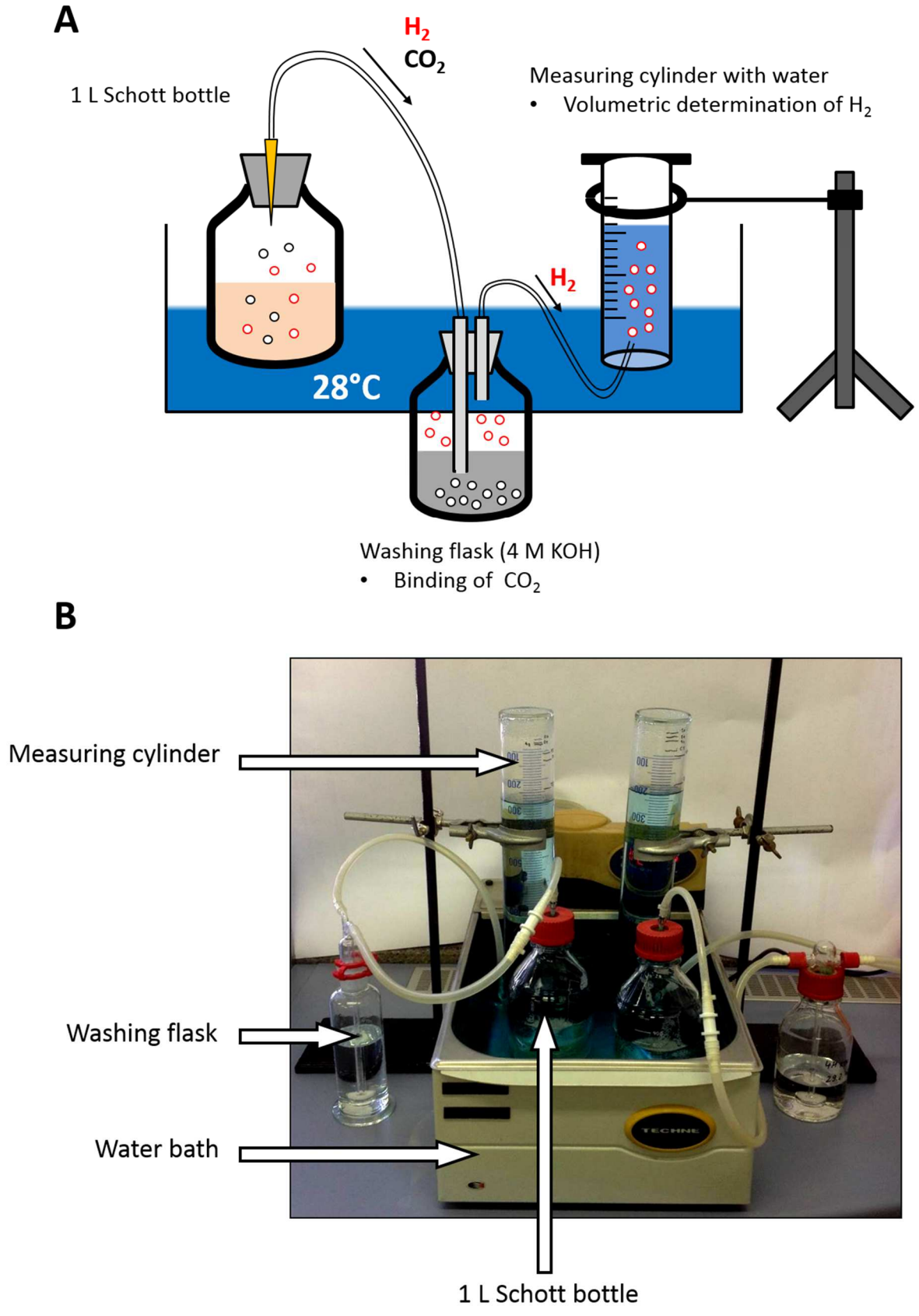
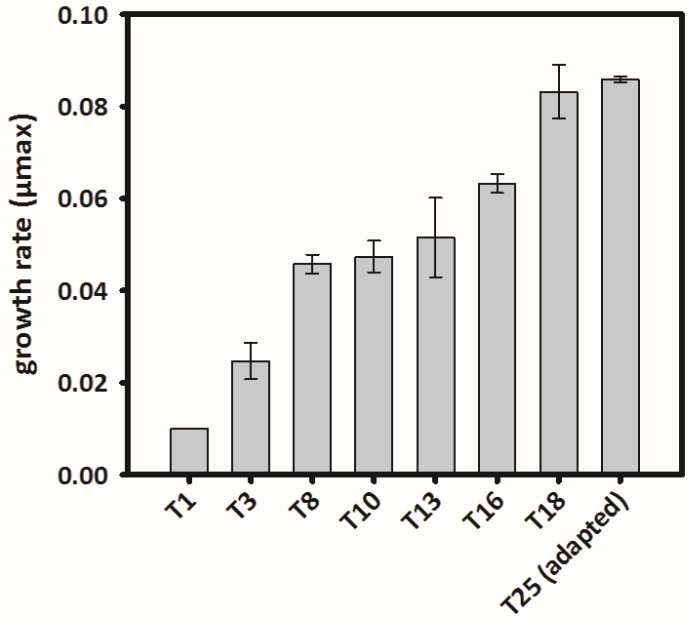
Supplementary Figures and Tables



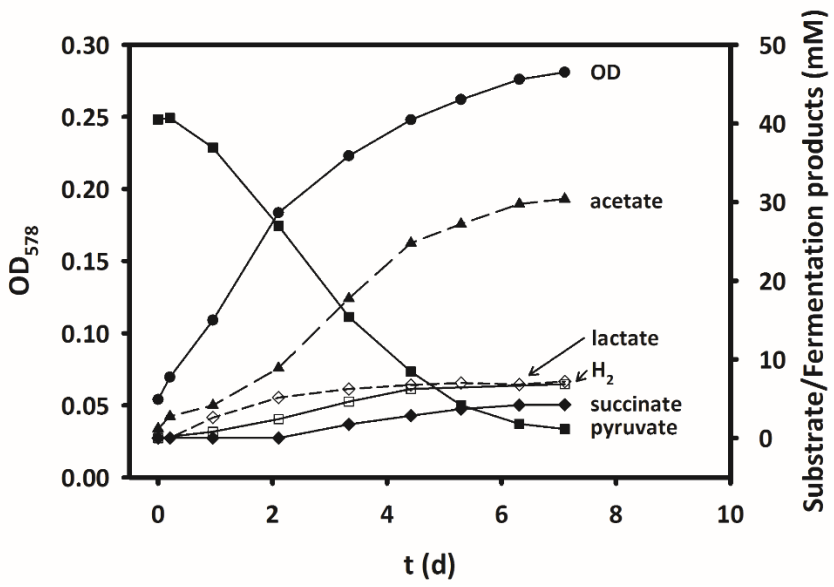
Supplementary Figure 1: Comparison of the *S. multivoranshyf* gene cluster to those encoding Hyd-3 and Hyd-4 of *E. coli* (A) and to other epsilonprotobacterial *hyf* clusters (B). Genetic organization and flanking genes around the operons are depicted. *C. concisus* - *Campylobacter concisus*, *A. nitrofigilis* - *Arcobacter nitrofigilis.* NiFe - catalytic subunit, - *hypA* - NiFe hydrogenase maturation protein, *ascB* - β-glucosidase, *bcp* - putative thiol peroxidase, *yfgO* - putative permease (PerM), *gca* - diguanylate cyclase, *gta* - putative β-glucosyltransferase, *romA* - outer membrane protein, *irsa* - putative membrane protein, *crp/fnr* - putative transcriptional regulator, *unk* - unknown protein.



Supplementary Figure 2: Scheme (A) and image (B) of the experimental setup of the fermentation apparatus. The washing flask with 4 M KOH is placed outside of the waterbath.



Supplementary Figure 3: Adaptation to pyruvate fermentation of *S. multivorans*. Increase of growth rate during continuous transfer to pyruvate medium without an electron acceptor. Data are representatives of two biological independent replicates. T - transfer step.



Supplementary Figure 4: Growth, substrate concentration and fermentation products of *S. deleyianum* during fermentative growth on pyruvate. Organic acids were measured via HPLC and H_2 was determined volumetrically (for details see materials and methods).

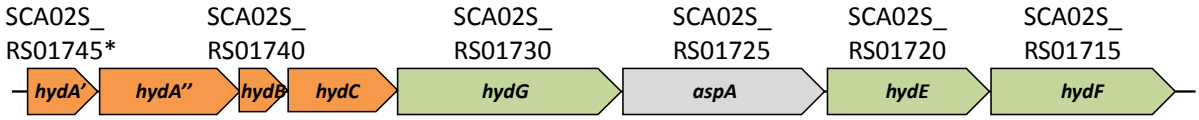
Supplementary Table 1: Oxidation/Reduction (O/R) balance and carbon recovery of pyruvate fermentation in *S. multivorans* and *S. cavolei*.

		O/R balance *S. multivorans*			O/R balance *S. cavolei*				Carbon recovery[b]	
Substrate or Product	O/R value[a]	mol/40 mol of substrate	O/R value mol reduced	O/R value mol oxidized	mol/40 mol of substrate	O/R value mol reduced	O/R value mol oxidized	Sum formula	*S. multivorans* carbon (mol)	*S. cavolei* carbon (mol)
Substrate										
pyruvate	0	40	-	-	40	-	-	$C_3H_4O_3$	120	120
Products										
acetate	0	27	-	-	38	-		$C_2H_4O_2$	54	76
lactate	-1	10	-10	-	-	-	-	$C_3H_6O_2$	30	-
succinate	-2	3	-6	-	-	-	-	$C_4H_6O_2$	12	-
H_2	-1	10	-10	-	36	-36	-	H_2	0	0
CO_2	+1	27	-	+27	38	-	+38	CO_2	27	38
Total			-26	+27		-36	+38		123	114
O/R balance		$\frac{\Sigma\ oxidized}{\Sigma\ reduced} = \frac{27}{26} = \underline{1.03}$			$\frac{\Sigma\ oxidized}{\Sigma\ reduced} = \frac{38}{36} = \underline{1.05}$					
Carbon recovery								$\frac{\Sigma\ C\ products}{\Sigma\ C\ substrate} \cdot 100$	<u>102.5%</u>	<u>95%</u>

[a]each excess of 2[H] is counted as -1; each deficiency of 2[H] is counted as +1; the O/R values for substrate oxidation and product reduction are in both organisms the same.
[b]total carbon was calculated by multiplying the number of carbons by the number of moles of each compound.

Supplementary Table 2: Hydrogen production and oxidizing activities of cell suspensions and subcellular fractions of *C. pasteurianum* W5 grown with glucose. Data are derived from three independent biological replicates. MV - methyl viologen, BV - benzyl viologen, n.d. - not determined.

Cellular fraction	Hydrogenase activity (nkat/mg)		
	MV → H_2	H_2→ BV	H_2→ MV
Cell suspensions	< 0.01	3.5 ± 0.7	0.8 ± 0.1
Membrane fraction	40.3 ± 3.9	38.4 ± 0.1	n.d.
Soluble fraction	241.3 ± 25.7	314.3 ± 12.6	n.d.



Supplementary Figure 5: [FeFe] hydrogenase gene cluster of *S. cavolei*. Point mutation in *hydA* leads to disruption of the gene into *hydA'* and *hydA''*. Locus tags of genes are given above the genes. * - hydA is annotated as pseudogene. Orange – [FeFe] hydrogenase structural genes, green - hydrogenase maturation genes, grey - not related to hydrogenase.

Supplementary Table 3: Protein abundance ratios in *S. multivorans* and *S. cavolei* cultivated with pyruvate compared to pyruvate/fumarat
Sorted after putative functional category, locus tags (hydrogenases) and abundance (other proteins). The complete dataset of proteins is given Supplementary Dataset 1. n.d. - not quantified in the respective proteome. Red: significantly higher abundance, green: significantly lower abundance the proteomes of pyruvate-cultivated cells when compared to pyruvate/fumarate-cultivated cells.

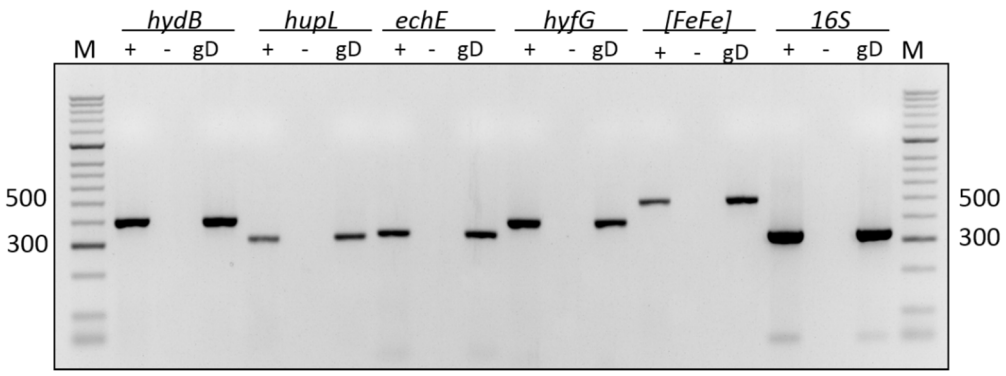
S. multivorans Locus tag	*S. cavolei* Locus tag	Description	*S. multivorans* Fold-change	*S. multivorans* p-value	*S. cavolei* Fold-change	*S. cavolei* p-value
Hydrogenase and hydrogenase accessory proteins						
Ech gene cluster						
SMUL_1306	SCA02S_RS12020	soluble, cytoplasmic Ech-like hydrogenase electron transfer subunit CooF-like	only Pyr/Fum		n.d.	
SMUL_1307	SCA02S_RS12030	soluble, cytoplasmic Ech-like hydrogenase large subunit CooH-like	only Pyr/-		+ 1.0	0.807
SMUL_1308	SCA02S_RS12025	soluble, cytoplasmic Ech-like hydrogenase CooU-like	n.d.		+ 1.5	0.631
SMUL_1309	SCA02S_RS12035	soluble, cytoplasmic Ech-like hydrogenase FeS protein CooX-like	n.d.		only in Pyr/-	
SMUL_1310	SCA02S_RS12015	soluble, cytoplasmic Ech-like hydrogenase electron transfer subunit CooL-like	n.d.		only in Pyr/-	
MBH and accessory gene cluster						
SMUL_1423	SCA02S_RS01360	membrane-bound NiFe hydrogenase small chain precursor	- 2.0	0.400	- 3.4	<0.001
SMUL_1424	SCA02S_RS01355	membrane-bound NiFe hydrogenase large subunit	- 1.7	0.070	- 2.2	<0.001
SMUL_1425	SCA02S_RS01350	membrane-bound NiFe hydrogenase, cytochrome b subunit	+ 2.8	0.011	- 2.8	0.007
SMUL_1426	SCA02S_RS01345	NiFe hydrogenase maturation protease HydD	- 4.7	0.070	- 1.3	0.129
SMUL_1427	SCA02S_RS01340	NiFe hydrogenase accessory protein HydE	+ 1.4	0.347	- 1.5	0.081
SMUL_1428	SCA02S_RS01335	NiFe hydrogenase metallocenter assembly protein HypF	n.d.		n.d.	
SMUL_1433	SCA02S_RS00525	maturation protein HypB	- 1.0	0.718	- 1.0	0.186
SMUL_1434	SCA02S_RS00520	maturation protein HypC	only Pyr/Fum		n.d.	
SMUL_1435	SCA02S_RS00515	maturation protein HypD	- 1.6	0.027	- 1.2	0.379
SMUL_1436	SCA02S_RS00510	maturation protein HypE	- 1.6	0.123	+ 1.1	0.836
SMUL_1437	SCA02S_RS00505	maturation protein HypA	n.d.		n.d.	
Hyf and accesory gene cluster						
SMUL_2383	SCA02S_RS01920	hydrogenase-4 component A, iron-sulfur cluster containing subunit	+ 9.5	<0.001	+ 4.9	0.002
SMUL_2384	SCA02S_RS01925	hydrogenase-4 component B, membrane subunit	+ 27.4	<0.001	+ 4.2	0.028
SMUL_2385	SCA02S_RS01930	hydrogenase-4 component C, membrane subunit	+ 10.4	<0.001	+ 2.7	0.079
SMUL_2386	SCA02S_RS01935	hydrogenase-4 component E, membrane-subunit	only Pyr/-		n.d.	
SMUL_2387	SCA02S_RS01940	hydrogenase-4 component F, membrane-subunit	only Pyr/-		n.d.	
SMUL_2388	SCA02S_RS01945	hydrogenase-4 component G, large subunit	+ 5.5	<0.001	+ 4.3	<0.001
SMUL_2389	SCA02S_RS01950	hydrogenase-4 component H	+ 13.5	<0.001	+ 4.9	0.035
SMUL_2390	SCA02S_RS01955	hydrogenase-4 component I, small subunit	+ 7.9	<0.001	+ 4.6	<0.001
SMUL_2391	SCA02S_RS01960	hydrogenase-4 component J, accessory protein	+ 5.9	0.006	+ 1.8	0.023
SMUL_2392	SCA02S_RS01965	hydrogenase-4 component K, maturation protease	+ 4.0	0.020	+ 7.2	<0.001
Other proteins possibly involved in the energy metabolism						
			Sorted by *S. multivorans*			
SMUL_2101	SCA02S_RS10975	aldehyde oxidoreductase	+ 141.6	<0.001	+ 6.6	<0.001
SMUL_2394	SCA02S_RS05380	aldehyde ferredoxin oxidoreductase	+ 8.2	<0.001	- 3.2	0.062
SMUL_0303	SCA02S_RS12260	Ferredoxin	+ 6.5	0.008	+ 2.4	0.013
SMUL_1602	/	phosphoenolpyruvate synthase	+ 6.3	0.021	/	

Supplementary Table 3: continued

SMUL_2522	SCA02S_RS04100	ubiquinol--cytochrome c reductase, cytochrome B subunit		+ 4.6	0.042	+ 1.5	0.043
SMUL_2901	SCA02S_RS08980	formate dehydrogenase, catalytic subunit alpha FdhA		+ 4.5	0.037	n.d.	<0.001
SMUL_3158	SCA02S_RS00475	NADP-dependent malic enzyme		+ 3.7	<0.001	/	/
SMUL_2630	SCA02S_RS04525	pyruvate-ferredoxin/flavodoxin oxidoreductase		+ 2.0	0.007	+ 3.8	<0.001
			Sorted by *S. cavolei*				
SMUL_1318	SCA02S_RS11745	(Fe-S)-binding protein		+ 1.7	0.027	+ 8.9	<0.001
SMUL_1317	SCA02S_RS11750	lactate utilization protein		+ 1.2	0.475	+ 8.4	<0.001
SMUL_3281	SCA02S_RS07425	molybdopterin oxidoreductase		- 3.3	0.179	+ 3.9	<0.001
SMUL_0787	SCA02S_RS02530	FAD-binding oxidoreductase		- 1.9	0.002	+ 3.2	<0.001
SMUL_1484	SCA02S_RS00240	acetate kinase		+ 1.7	0.005	+ 3.2	<0.001
SMUL_1483	SCA02S_RS00245	phosphate acetyltransferase		+ 2.0	0.003	+ 3.1	<0.001
SMUL_1698	SCA02S_RS08870	LLM class flavin-dependent oxidoreductase		1.0	0.801	+ 2.6	0.001
/	SCA02S_RS01635	NAD(P)-dependent alcohol dehydrogenase		/		+ 2.2	0.006
			Sorted by *S. multivorans*				
SMUL_1640	SCA02S_RS00755	FAD-dependent oxidoreductase		- 12,1	<0.001	- 1.2	0.062
SMUL_1703	/	pyruvate dehydrogenase [ubiquinone]		- 6.8	0.016	/	
SMUL_1531	/	tetrachloroethene reductive dehalogenase catalytic subunit PceA		- 6.4	0.022	/	
SMUL_1365	SCA02S_RS09935	putative NAD(P)H nitroreductase		- 6.3	<0.001	+ 1.1	0.993
SMUL_2873	SCA02S_RS05345	formate dehydrogenase, subunit alpha FdhA		- 5.1	<0.001	- 12.9	<0.001
SMUL_2872	SCA02S_RS05340	formate dehydrogenase, iron-sulfur subunit FdhB		- 4.3	<0.001	- 15.7	<0.001
SMUL_0200	SCA02S_RS07925	epsilonproteobacterial nuoF-like protein		- 4.3	<0.001	+ 1.5	0.143
SMUL_1459	SCA02S_RS08715	fumarate hydratase class II		- 4.2	0.001	+ 2.5	0.072
SMUL_3037	SCA02S_RS10055	acetaldehyde dehydrogenase		- 3.9	0.002	+ 1.3	0.976
SMUL_0196	SCA02S_RS06700	NADH-ubiquinone oxidoreductase chain B		- 2.9	<0.001	- 1.2	0.047
SMUL_0203	SCA02S_RS06665	NADH-ubiquinone oxidoreductase chain I		- 2.8	0.011	- 1.6	0.017
SMUL_0197	SCA02S_RS06695	NADH-ubiquinone oxidoreductase chain C		- 2.5	<0.001	- 1.4	0.053
SMUL_0195	SCA02S_RS06705	NADH-ubiquinone oxidoreductase chain A		- 2.5	<0.001	n.d.	
SMUL_0198	SCA02S_RS06690	NADH-ubiquinone oxidoreductase chain D		- 2.2	0.002	- 1.5	0.006
			Sorted by *S. cavolei*				
SMUL_0552	SCA02S_RS07735	fumarate reductase		- 1.2	0.213	- 6.9	<0.001
SMUL_1680	SCA02S_RS00615	fumarate hydratase		- 1.8	0.015	- 6.0	<0.001
SMUL_1679	SCA02S_RS00620	fumarate hydratase		- 2.2	0.013	- 5.7	<0.001
SMUL_0551	SCA02S_RS07740	fumarate reductase flavoprotein subunit		- 1.1	0.285	- 5.3	<0.001
SMUL_0199	SCA02S_RS06685	NADH-ubiquinone oxidoreductase subunit E		n.d.		- 2.9	0.002
Other proteins							
			Sorted by *S. multivorans*				
SMUL_1016	SCA02S_RS06075	heavy metal resistance P-type ATPase		+ 39.8	<0.001	- 2.8	0.023
SMUL_1017	SCA02S_RS09930	heavy metal efflux system accessory protein		+ 19.2	<0.001	only Pyr/-	
SMUL_2587	SCA02S_RS04335	phosphate ABC transporter, periplasmic phosphate-binding protein		+ 17.5	0.002	n.d.	
SMUL_0955	SCA02S_RS01815	putative two-component sensor histidine kinase		+ 13.5	<0.001	only Pyr/-	
SMUL_0188	SCA02S_RS06740	manganese/zinc/iron chelate uptake transporter (MZT) family		+ 8.9	<0.001	n.d.	
SMUL_1512	SCA02S_RS00550	PHP/AAA ATPase domain-containing protein		+ 7.3	<0.001	+ 1.5	0.269
SMUL_3232	SCA02S_RS09470	NosL family protein		+ 5.9	0.042	+ 6.1	<0.001
SMUL_0547	SCA02S_RS07760	heat shock protein Hsp20		+ 5.5	0.004	- 1.2	0.232

Supplementary Table 3: continued

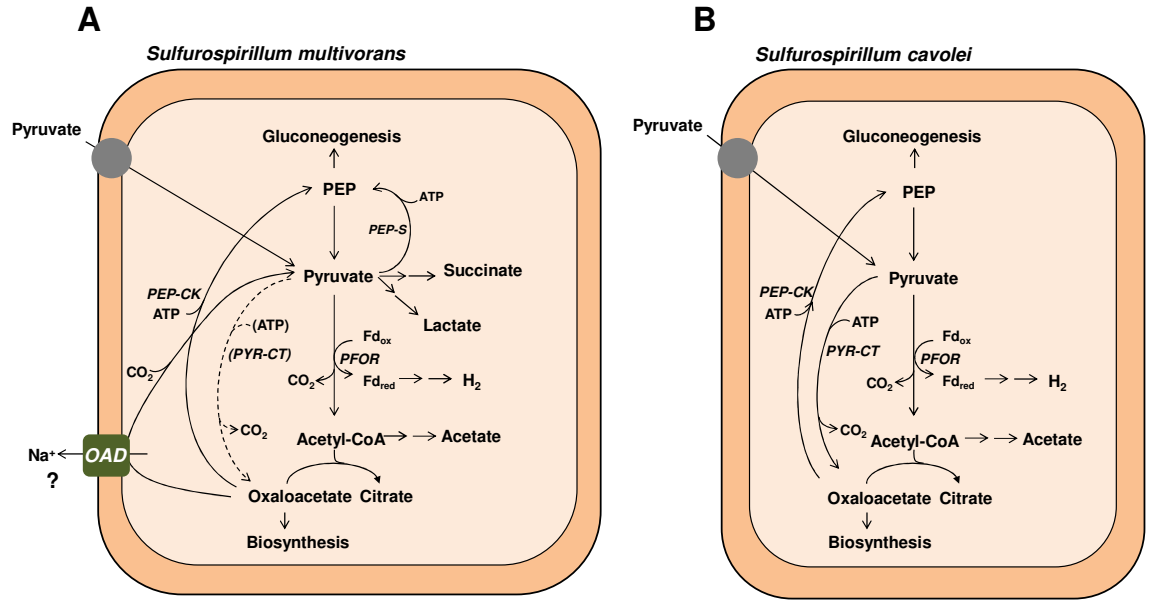
			Sorted by *S. cavolei*				
SMUL_2124	SCA02S_RS05290	cytochrome c		only Pyr/-		+ 92.9	<0.001
SMUL_0325	SCA02S_RS12370	homoserine kinase		+ 1.5	0.277	+ 10.6	0.039
SMUL_0337	SCA02S_RS12425	cytochrome c family protein		+ 0.7	0.132	+ 5.2	<0.001
SMUL_2091	SCA02S_RS11025	HlyC/CorC family transporter		- 1.2	0.251	+ 4.6	0.002
SMUL_1239	SCA02S_RS12085	Hydrolase		n.d.		+ 4.3	0.008
SMUL_0313	SCA02S_RS12310	sensor histidine kinase		+ 1.7	0.310	+ 4.2	<0.001
SMUL_2235	SCA02S_RS12725	type II secretion system protein		n.d.		+ 3.9	0.021
SMUL_0529	SCA02S_RS07855	superoxide dismutase		- 1.4	0.121	+ 3.2	<0.001
			Sorted by *S. multivorans*				
SMUL_0622	SCA02S_RS03235	TRAP-type C4-dicarboxylate transport system, periplasmic component		- 12.3	<0.001	- 3.4	<0.001
SMUL_1639	SCA02S_RS00760	cytochrome c		- 10.2	<0.001	- 2.3	0.654
SMUL_0306	SCA02S_RS12275	50S ribosomal protein L32		- 6.6	<0.001	- 2.2	0.005
SMUL_0892	SCA02S_RS02045	nitrite reductase cytochrome c biogenesis protein NrfJ		- 6.1	0.017	n.d.	
SMUL_0802	SCA02S_RS02460	RND family protein		- 4.6	0.010	only Pyr/Fum	
SMUL_0804	SCA02S_RS02450	RND family protein		- 4.2	<0.001	only Pyr/Fum	
SMUL_0224	SCA02S_RS06575	LSU ribosomal protein L31p		- 4.1	0.002	- 2.3	0.006
SMUL_2691	SCA02S_RS04835	SSU ribosomal protein S14p (S29e), Zinc-independent		- 3.8	0.012	- 1.9	<0.001
SMUL_0477	SCA02S_RS08145	LSU ribosomal protein L33p		- 3.8	0.001	- 1.6	0.003
SMUL_3236	SCA02S_RS09455	RNP-1 like RNA-binding protein		- 3.3	0.001	- 1.5	<0.001
SMUL_0987	SCA02S_RS14055	heat shock protein 60 family co-chaperone GroES		- 3.6	0.003	- 2.0	0.002
SMUL_1429	SCA02S_RS01330	nickel responsive regulator NikR		- 2.9	<0.001	+ 1.1	0.679
SMUL_2699	SCA02S_RS04875	LSU ribosomal protein L22p (L17e)		- 2.9	<0.001	- 2.0	<0.001
SMUL_2696	SCA02S_RS04860	LSU ribosomal protein L29p (L35e)		- 2.8	0.004	- 2.4	0.004
SMUL_2290	SCA02S_RS12980	SSU ribosomal protein S16p		- 2.7	0.006	- 1.4	0.085
			Sorted by *S. cavolei*				
SMUL_1277	SCA02S_RS12160	molybdopterin guanine dinucleotide-containing S/N-oxide reductase		n.d.		- 35.0	<0.001
SMUL_2591	SCA02S_RS00610	citrate:succinate antiporter		n.d.		- 32.6	<0.001
SMUL_0963	SCA02S_RS01780	phosphatidylserine decarboxylase		- 1.1	0.843	- 3.6	0.002
SMUL_2857	SCA02S_RS08830	sulfurtransferase-like selenium metabolism protein YedF		- 3.3	0.001	- 3.6	<0.001
SMUL_0851	SCA02S_RS02250	DNA-directed RNA polymerase subunit omega		- 4.0	0.004	- 3.1	0.034
SMUL_0141	SCA02S_RS06970	30S ribosomal protein S21		- 1.9	0.015	- 2.6	<0.001
SMUL_1944	SCA02S_RS11615	30S ribosomal protein S15		- 1.9	0.002	- 2.3	<0.001
SMUL_0482	SCA02S_RS08125	50S ribosomal protein L1		- 1.5	0.020	- 2.1	<0.001



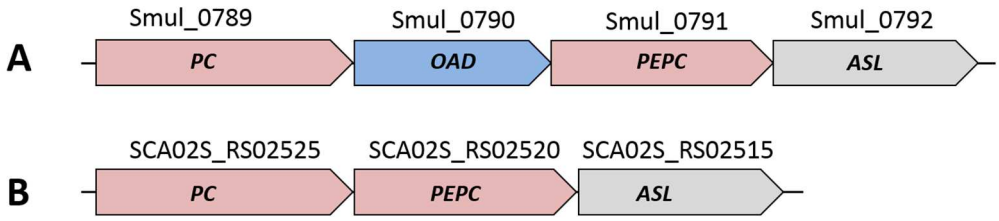
Supplementary Figure 6: Transcriptional analysis of catalytic subunits of [NiFe] hydrogenases in *S. cavolei*. *hydB* - membrane-bound hydrogenase (MBH), *hupL* - cytoplasmic uptake hydrogenase, *echE* - Ech-like hydrogenase, *hyfG* - Hyf hydrogenase, [FeFe] - [FeFe] hydrogenase *hydA*, 16S - 16S rRNA, + with reverse transcriptase, - without reverse transcriptase (negative control), gD - genomic DNA, M - DNA marker.

Supplementary Table 4: Oligonucleotides used for transcriptional analysis of hydrogenase catalytic subunits in *S. cavolei* (Supplementary Figure 6) and PCR of *hyfA* and *hyfB* of *S. multivorans* and *S. halorespirans* (Supplementary Figure 8).

Primer	Sequence (5' – 3')	Amplicon size (bp)
S. cavolei		
***hydB*_fw**	GTATAAGTTAACGCCTGAGCAA	402
***hydB*_rev**	CCACTCACGAGATTAATGACG	
***hupL*_fw**	GGATTTGATTCCGCCGTA	324
***hupL*_rev**	TCCCTCTTTGGCTACCATC	
***echE*_fw**	ACCGACGAGATCAAACGTATC	339
***echE*_rev**	GAGTTTGAGGGCATCTTCATAC	
***hyfG*_fw**	CGCTTGGTCTTAAGCGAC	398
***hyfG*_rev**	GGAATTTCCAAGTTGATCGC	
***[FeFe]*_fw**	CTAACAAATGCGTCGCGT	504
***[FeFe]*_rev**	TCCATACAAACTCCGCCG	
***16S*_fw**	GAGACACGGTCCAGACTCCTAC	334
***16S*_rev**	CACCAATTCCATCTACCTCTCCC	
S. multivorans		
***hyfA*_fw**	CCCAACCAGTGTCGTCAA	285
***hyfA*_rev**	CGTGTATAGCGTAAACTACC	
***hyfB*_fw**	ATGACATTAATATCCGCACT	672
***hyfB*_rev**	CATATCGCGTGTTTTGGTTG	
S. halorespirans		
***hyfA*_fw**	CCCAACCAGTGTCGTCAA	285
***hyfA*_rev**	CGTGTATAGCGTAAACTACC	
***hyfB*_fw**	ATGACATTAATATCCGCACT	1585
***hyfB*_rev**	CATATCGCGTGTTTTGGTTG	

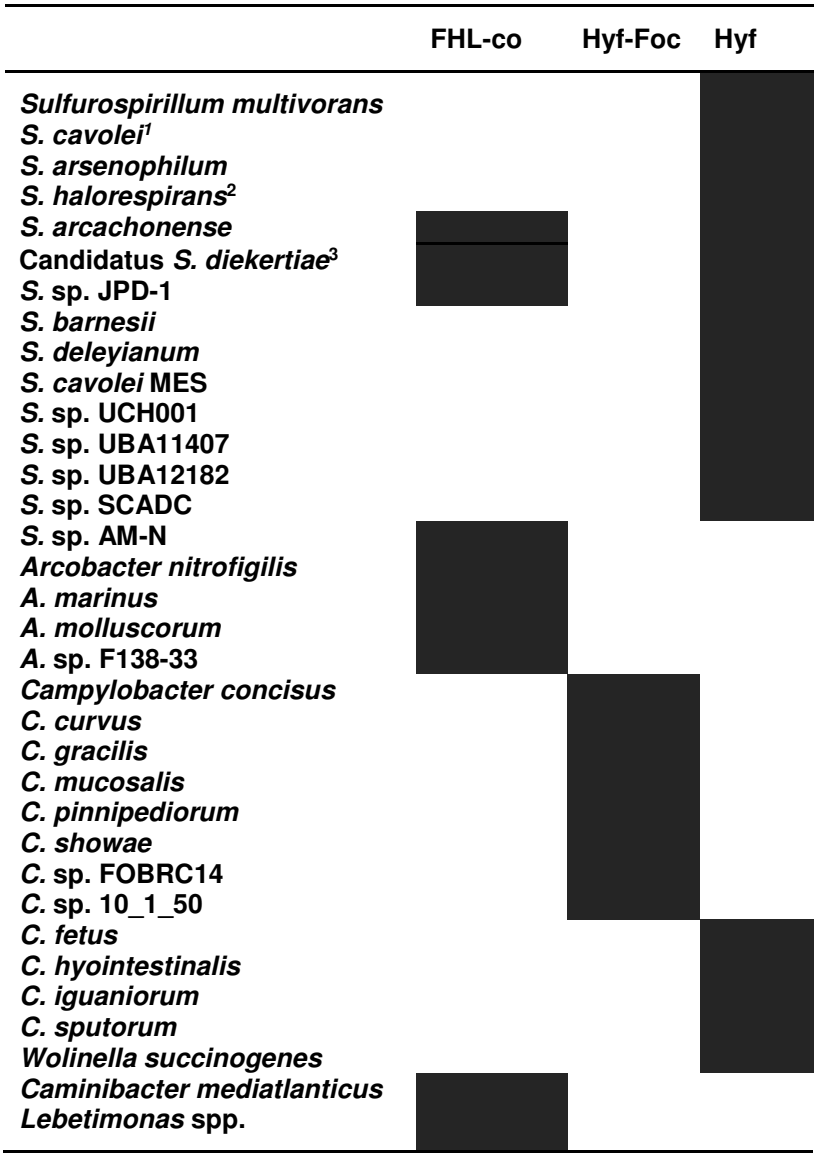


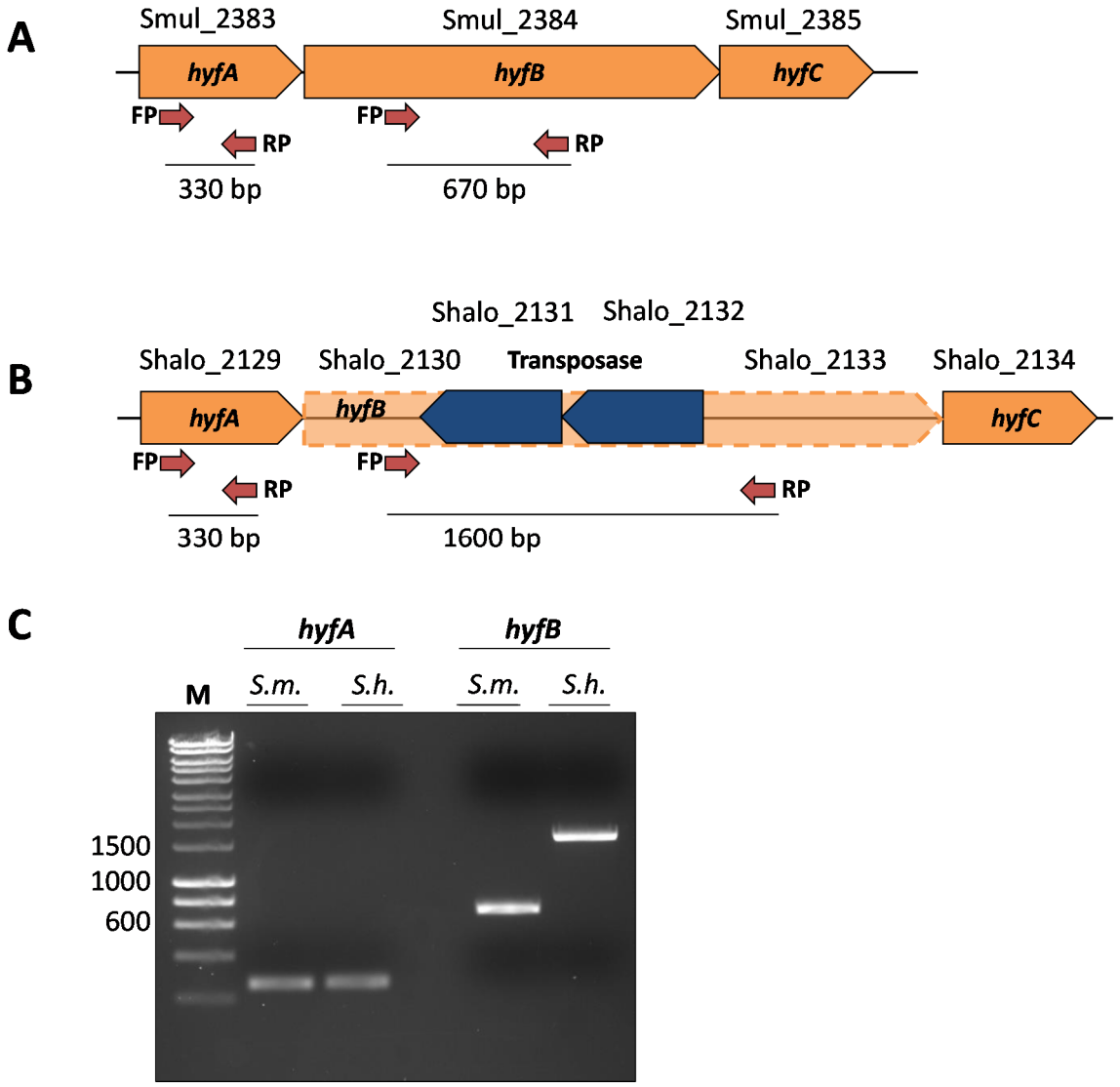
Supplementary Figure 7: Biosynthetic reactions including gluconeogenesis from pyruvate in (A) *Sulfurospirillum multivorans* and (B) *Sulfurospirillum cavolei*. Pyruvate carboxylation via pyruvate carboxylase in *S. cavolei* proceeds presumably in the reverse direction via oxaloacetate decarboxylase in *S. multivorans*, since an oxaloacetate decarboxylase subunit is encoded in *S. multivorans* (see also Supplementary Figure 8). PEP-S - PEP-synthetase, PEP-CK - PEP-carboxykinase, PYR-CT - pyruvate carboxylase, OAD - oxaloacetate decarboxylase, PFOR - pyruvate:ferredoxin oxidoreductase, Fd - ferredoxin.



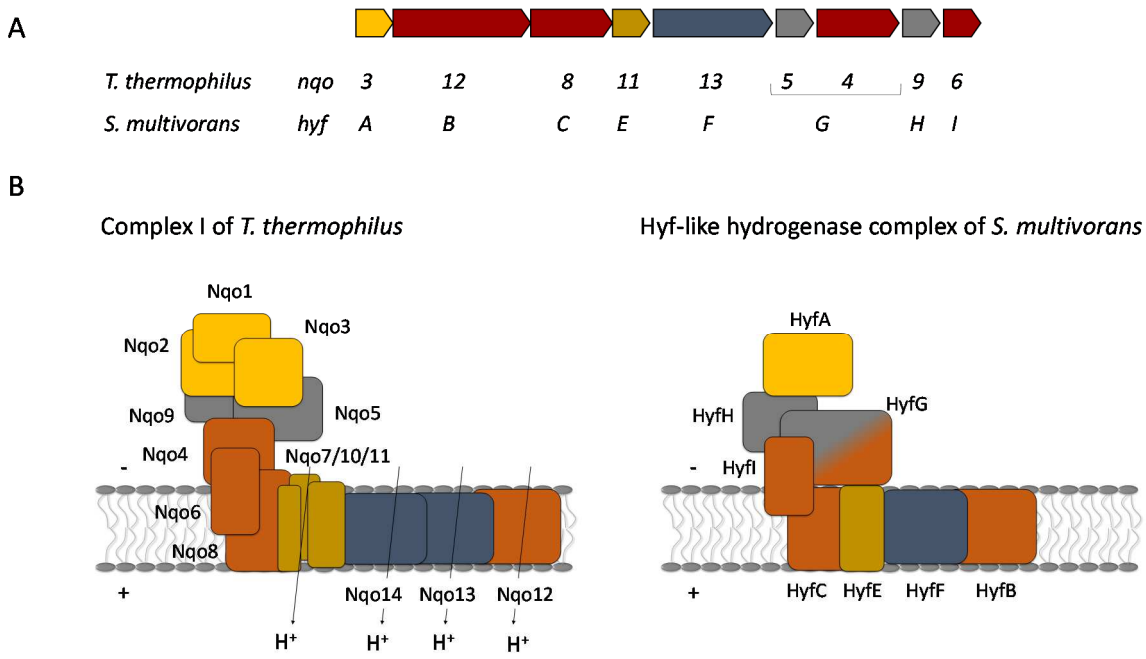
Supplementary Figure 8: Cluster of genes related to pyruvate metabolism in (A) *S. multivorans and (B) *S. cavolei**.** Locus tags of genes are given above the genes. PC - pyruvate carboxylase, OAD - oxaloacetate decarboxylase, PEPC - phosphoenolpyruvate carboxykinase ASL, - argininosuccinate lyase. A*: Identified in *S. deleyianum*, *S. halorespirans*, S. sp. SCADC, *S. barnesii*, S. sp. UBA12182, S. sp. UBA11407, *S. arcachonense*, S. sp. AM-N. B*: Identified in S. UCH001, *S. cavolei* strains MES and UCH003, *S. arsenophilum*, S. sp. JPD-1.

Supplementary Table 5: Distribution of the *hyf* operon among different Epsilonproteobacteria and possible formation of a formate dehydrogenase lyase complex. FHL-co - *hyf* operon associated with a formate dehydrogenase, Hyf-foc - *hyf* operon associated with a formate channel (*focB*). For an overview of the organization of different *hyf* operons see Supplementary Figure 1.





Supplementary Figure 9: Hyf-like hydrogenase gene cluster of *S. multivorans* (A) and *S. halorespirans* (B) and confirmation of transposon integration in *hyfB* of *S. halorespirans* (C). (A, B) Locus tags are given above the genes and primer binding sites are indicated by red arrows. Transposase in *hyfB* of *S. halorespirans* coloured dark blue. Structural genes are coloured orange. (C) PCR with primers binding in *hyfA* and *hyfB* of *S. multivorans* and *S. halorespirans*. M - marker lane, *S.m.* - *S. multivorans*, *S.h.* - *S. halorespirans*, NiFe - catalytic subunit. Primer used are listed in Supplementary Table 4.



Supplementary Figure 10: Comparison of Hyf-like hydrogenase of *S. multivorans* to complex I of *Thermus thermophilus*. (A) Schematic representation of genetic organization. Subunits of complex I (Nqo) are directly compared to subunits of Hyf-like hydrogenase (hyf). Nqo5 and Nqo4 are fused to HyfG. (B) Hypothetical scheme of the structural organization of both complexes. Arrows indicate putative proton-translocation channels in *T. thermophiles* complex I. Color code resembles corresponding homologues.

SUPPLEMENTARY NOTE 1

Hyf-like hydrogenase of *Sulfurospirillum* spp. as a proton pump

The relationship of the subunit composition and amino acid sequences of group 4 hydrogenases to the respiratory complex I and the possible involvement of these membrane-bound hydrogenases in energy conservation via the generation of a proton motive force has been discussed previously. In *S. multivorans* and other *Sulfurospirillum* spp., the eight subunits of Hyf (HyfABCEFGHI) are homologs to the subunits of complex I (Supplementary Figure 10, Supplementary Table 6). The most likely candidate for potential H^+-transport HyfF, four putative key amino acids for proton transfer, Glu148, Lys237, Lys268, and Glu424 are present (Supplementary Figures 11-13). The high conservation grade of key amino acid residues possibly involved in proton transfer especially in HyfB renders the involvement of *Sulfurospirillum* spp. Hyf in energy conservation via a chemosmotic generation of ATP a possible scenario.

Supplementary Table 6: Comparison of complex I subunits to homologs in bacteria.

Gene order	Complex I (Eukaryotes)	*E. coli* complex I	*T. thermophilus* complex I	*S. multivorans* (Hyf)	*E. coli* (Hyf/Hyc)
1	ND3	NuoA	Nqo7	-	-
2	IP21K	NuoB	Nqo6	HyfI	HyfI/HycG
3	IP30K	NuoC	Nqo5[1]	HyfG	HyfG/HycE
4	IP49K	NuoD	Nqo4[1]		
5	IP24K	NuoE	Nqo2	-	-
6	IP51K	NuoF	Nqo1	-	-
7	IP75K	NuoG	Nqo3	HyfA	HyfA/HycB
8	ND1	NuoH	Nqo8	HyfC	HyfC/HycD
9	IP23K	NuoI	Nqo9	HyfH	HyfH/HycF
10	ND6	NuoJ	Nqo10	-	-
11	ND4L	NuoK	Nqo11	HyfE	HyfE/-
12	ND5	NuoL	Nqo12	HyfB	HyfB/HyfD/HycC
13	ND4	NuoM	Nqo13	HyfF	HyfF/HycC
14	ND2	NuoN	Nqo14	-	-

[1]Nqo5 and Nqo4-similar proteins are fused to HyfG.

Alignment HyfB/Nqo12/ND5

```
                10        20        30        40        50        60        70
           ....|....|....|....|....|....|....|....|....|....|....|....|....|
S. mul     ------MQTIYTLFLLTSLLSLALYKKPLLAQKIGFG---LSSLISLYAAIFFFSHLG-ETMTWQLPGNF
E. coli4   MDALQLLTWSLILYLFASLASLFLLGLDRLAIKLSGITSLVGGVIG-IISGITQLHAGVTLVARFAPPFE
E. coli3   MSAISLINSGVAWFVAAAVLAFLFSFQKALSGWIAGIGGAVGSLYT-AAAGFTVLTGAVGVSG----ALS
T. ther    ------MALLGTILLPLLGFALLGLFGKRMREPLPGVLASGLVLASFLLGAGLLLSGGARFQAEWLPGIP
B. tau     MNMFSSLSLVTLLLLTMPIMMMSFNTYKPSNYPLYVKTAISYAFITSMIPTMMFIHSGQELIISNWHWLT
O. aries   MNLFSSLTLVTLILLTMPIAAINFNTHKFTNYPLYVKTTISCAFITSMIPTMMFIHTGQEMIISNWHWLT

                80        90        100       110       120       130       140
           ....|....|....|....|....|....|....|....|....|....|....|....|....|
S. mul     ISSPLFRLDSIEMFFSFLVSLIAFAVSLFSFDYAKFYEKKAN-LAVFASLFNAFILSMLLVIASDNVFSF
E. coli4   FADLTLRMDSLSAFMVLVISLLVVVCSLYSLTYMREYEGKGAAAMGFFMN--IFIASMVALLVMDNAFWF
E. coli3   LVSYDVQISPLNAIWLITLGLCGLFVSLYNIDWHRHAQVK---CNGLQIN--MLMAAAVCAVIASNLGMF
T. ther    FS---LLLDNLSGFMLLIVTGVGFLIHVYAIG----YMGGDPGYSRFFAYFNLFIAMMLTLVLADSYPVM
B. tau     IQTLKLSLSFKMDYFSMMFIPVALFVTWSIMEFSMWYMYSDPNINKFFKYLLLFLITMLILVTANNLFQL
O. aries   IQTLKLSLSFKMDFFSMMFVPVALFVTWSIMEFSMWYMHSDPNINQFFKYLLLFLITMLILVTANNLFQL

                150       160       170       180       190       200       210
           ....|....|....|....|....|....|....|....|....|....|....|....|....|
S. mul     MLLWEMMTLISALLILINDGEGAGKKVMIYLGIA-QIGASCLMVALLIMASFAGSFEFSKFADLNIGFGM
E. coli4   IVLFEMMSLSSWFLVIARQDKTSINAGMLYFFIA-HAGSVLIMIAFLLMGRESGSLDFASFRTLSLSPGL
E. coli3   VVMAEIMALCAVFLTSNSKE------GKLWFALG-RLGTLLLAIACWLLWQRYGTLDLRLLDMRMQQLPL
T. ther    FIGWEGVGLASFLLIGFWYKNPQYADSARKAFIVNRIGDLGFMLGMAILWALYGTLSISELKEAMEGPLK
B. tau     FIGWEGVGIMSFLLIGWWYGRADANTAALQAILYNRIGDIGFILAMAWFLTNLNTWDLQQIFMLNPSD--
O. aries   FIGWEGVGIMSFLLIGWWYGRTDANTAALQAILYNRIGDIGFILAMAWFLINLNTWDLQQIFMLNPND--

                220       230       240       250       260       270       280
           ....|....|....|....|....|....|....|....|....|....|....|....|....|
S. mul     SITLFTLL----LVGLGSKAGMFPFHVWLPLAYCQSPSNASALMSGVMIKVALFAFIKFSLLLPQFA---
E. coli4   ASAVFLLA----FFGFGAKAGMMPLHSWLPRAHPAAPSHASALMSGVMVKIGIFGILKVAMDLLAQTGLP
E. coli3   GSDIWLLG----VIGFGLLAGIIPLHGWVPQAHANASTPAAALFSTVVMKIGLLGILTLS--LLGGN-AP
T. ther    NPDLLALAGLLLFLGAVGKSAQIPLMVWLPDAMAGPTPVSALIHAATMVTAGVYLIARSSFLYSVLP---
B. tau     SNMPLIGL----ALAATGKSAQFGLHPWLPSAMEGPTPVSALLHSSTMVVAGIFLLIRFYPLTENNK---
O. aries   SNLPLMGL----ILAATGKSAQFGLHPWLPSAMEGPTPVSALLHSSTMVVAGIFLLIRFYPLTENNK---
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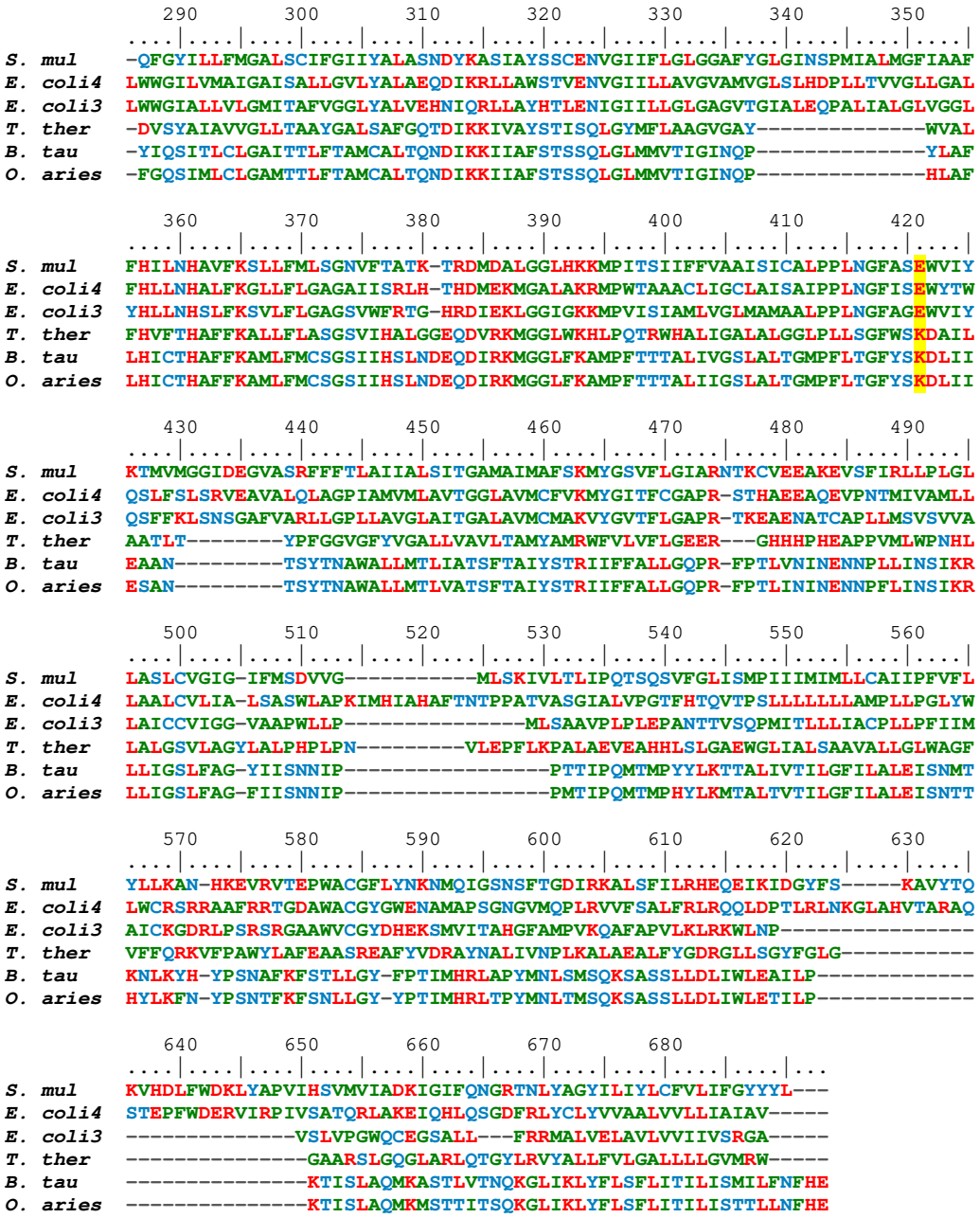
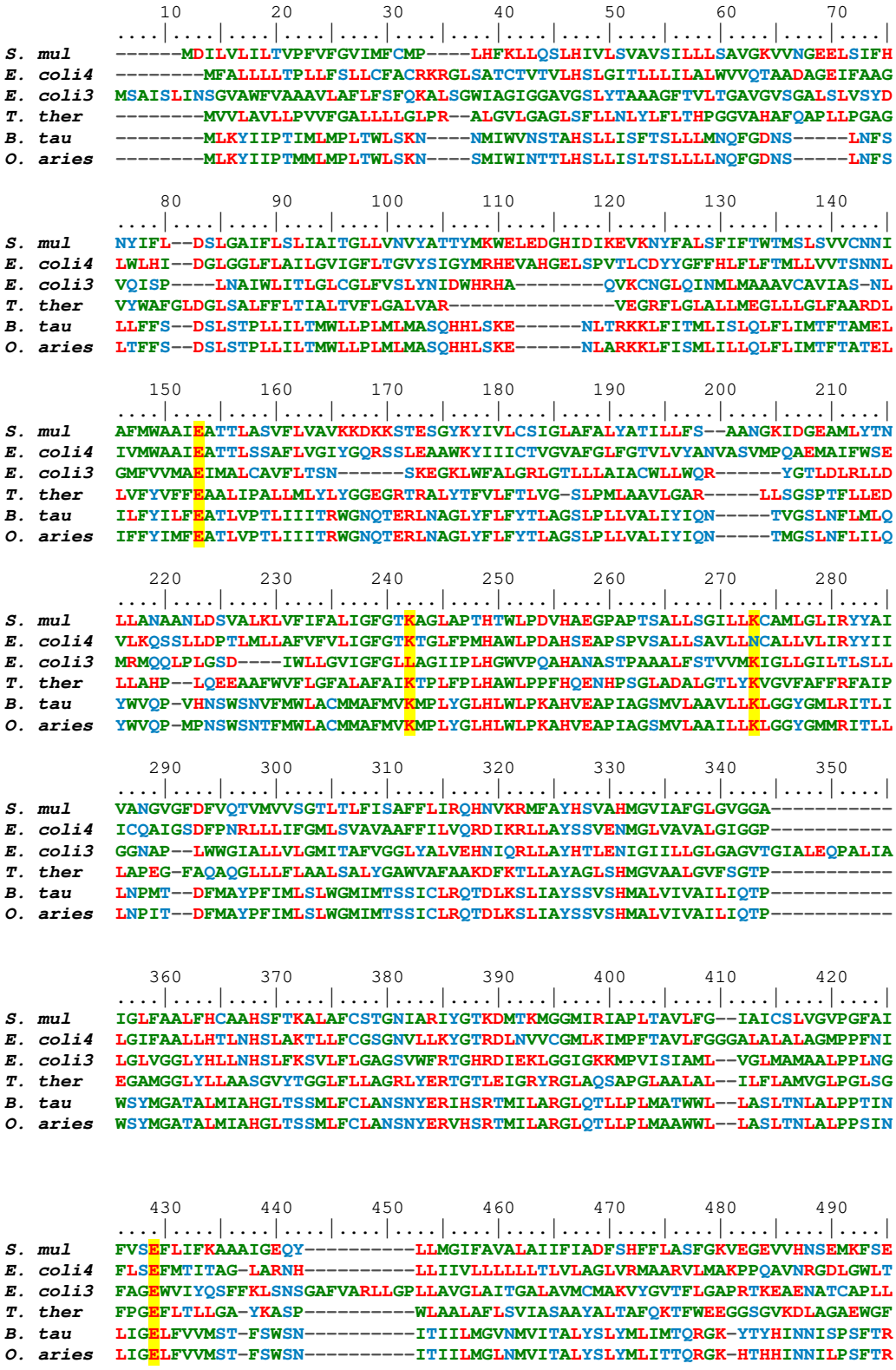
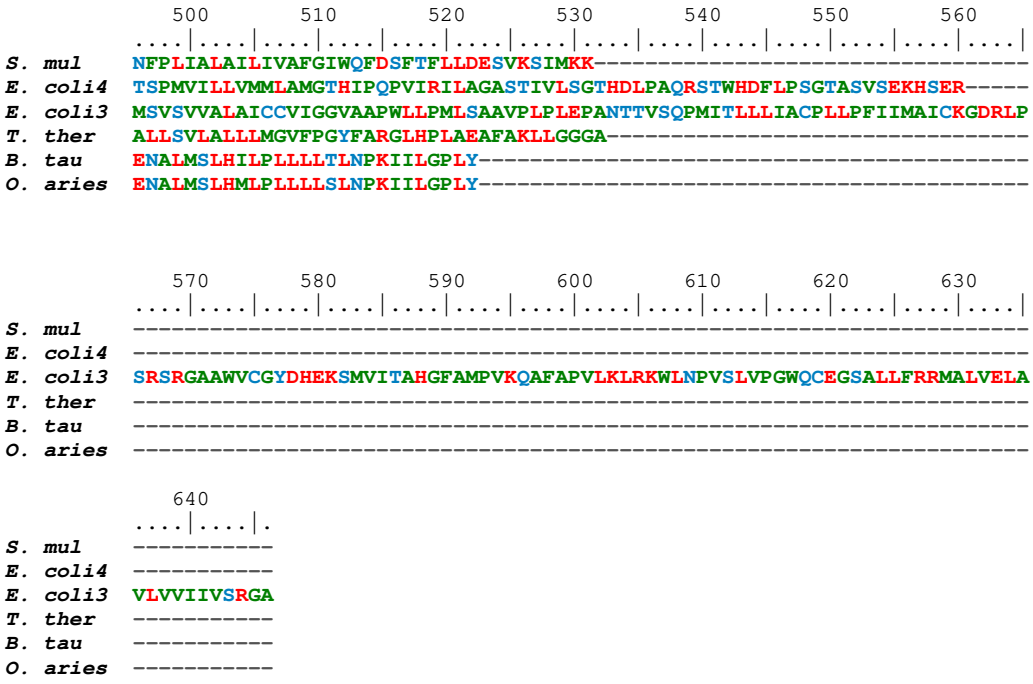


Supplementary Figure 11: Amino acid sequence alignment of HyfB/Nqo12/ND5. *S. mul* - *S. multivorans* AHJ13630.1 Hyf-like hydrogenase, membrane subunit B, *E. coli*4 - *E. coli* AAB88564.1 Hyf hydrogenase, membrane subunit B, *E. coli*3 - *E. coli* CAA35548.1 - Hyc hydrogenase, membrane subunit 3, *T. ther* - *T. thermophilus* Q56227.1 NADH-quinone oxidoreductase subunit 12 (Nqo12), *B. tau* - *Bos taurus* P03920.2 NADH-ubiquinone oxidoreductase chain 5 (ND5), *O. aries* - *Ovis aries* O78756.1 NADH-ubiquinone oxidoreductase chain 5 (ND5). Amino acid color code: green - hydrophobic, red - charged, blue - polar/uncharged. Key charged residues are highlighted in yellow.

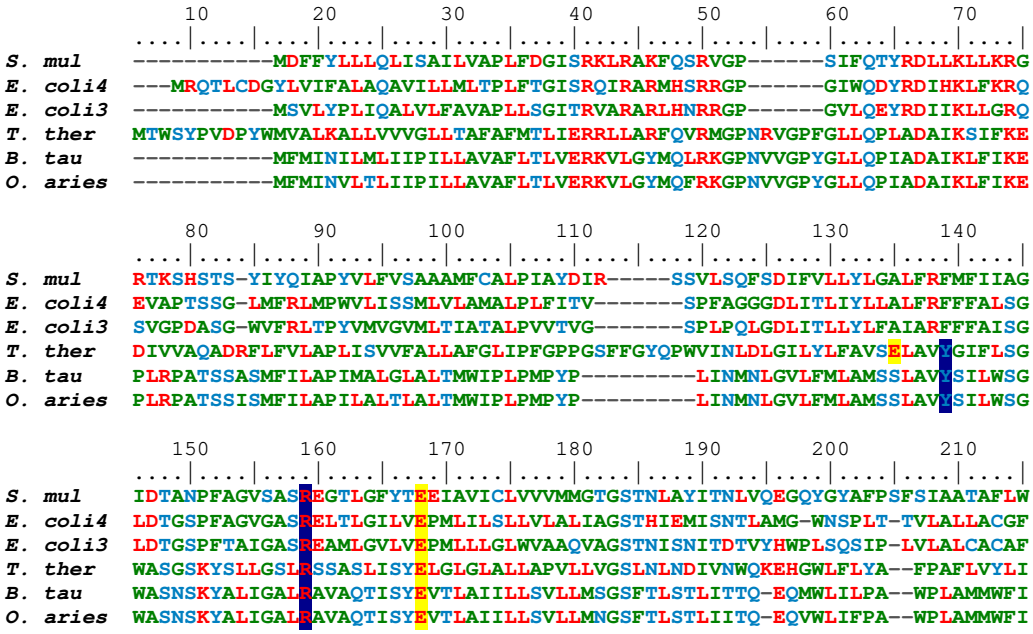
Alignment HyfF/Nqo13/ND4

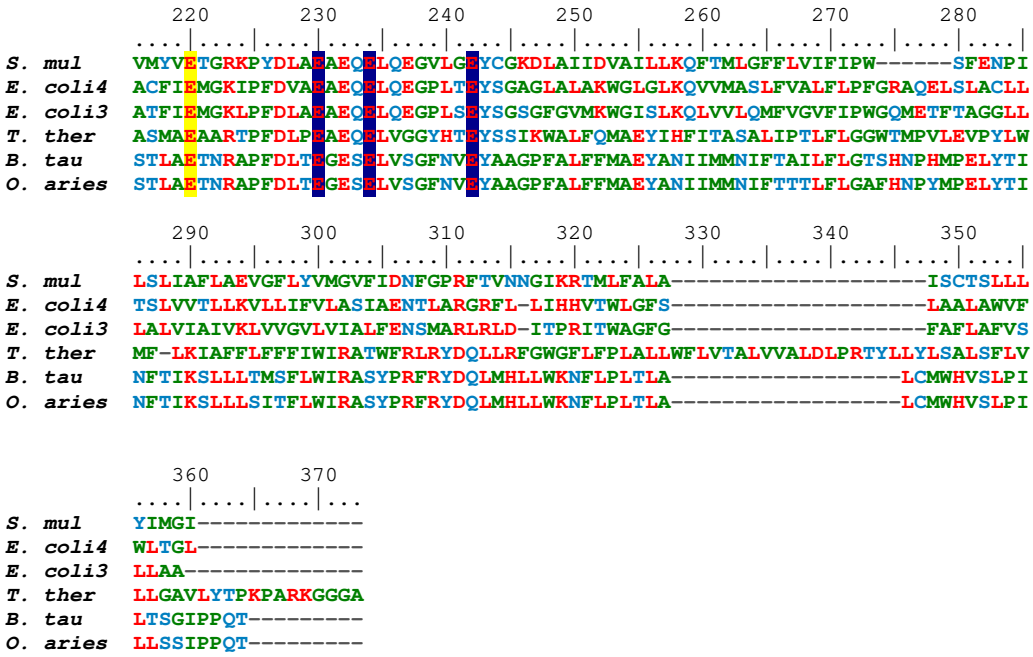




Supplementary Figure 12: Amino acid sequence alignment of HyfF/Nqo13/ND4. *S. mul* - *S. multivorans* AHJ13633.1 Hyf-like hydrogenase, membrane subunit F, *E. coli*4 - *E. coli* CTT40508.1 Hyf hydrogenase, membrane subunit F, *E. coli*3 - *E. coli* CAA35548.1 Hyc hydrogenase, membrane subunit 3, *T. ther* - *Thermus thermophilus* Q56228.2 NADH-quinone oxidoreductase subunit 13 (Nqo13), *B. tau* - *Bos taurus* P03910.1 NADH-ubiquinone oxidoreductase chain 4 (ND4), *O. aries* - *Ovis aries* O78755.1 NADH-ubiquinone oxidoreductase chain 4 (ND4). Amino acid color code: green - hydrophobic, red - charged, blue - polar/uncharged. Key charged residues are highlighted in yellow.

Alignment HyfC/Nqo8/ND1





Supplementary Figure 13: Amino acid sequence alignment of HyfC/Nqo8/ND1. *S. mul* - *S. multivorans* AHJ13631.1 Hyf-like hydrogenase, membrane subunit C, *E. coli*4 - *E. coli* AAB88565.1 Hyf hydrogenase, membrane subunit C, *E. coli*3 - *E. coli* CAA35549.1 Hyc hydrogenase, membrane subunit 4, *T. ther* - *Thermus thermophilus* Q60019.1 NADH-quinone oxidoreductase subunit 8 (Nqo8), *B. tau* - *Bos taurus* P03887.1 NADH-ubiquinone oxidoreductase chain 1 (ND1), *O. aries* - *Ovis aries* O78747.1 NADH-ubiquinone oxidoreductase chain 1 (ND1). Amino acid color code: green - hydrophobic, red - charged, blue - polar/uncharged. Key charged residues of the connection of the two half-channels are highlighted in yellow. Key charged residues from the first-half channel are highlighted in dark blue.

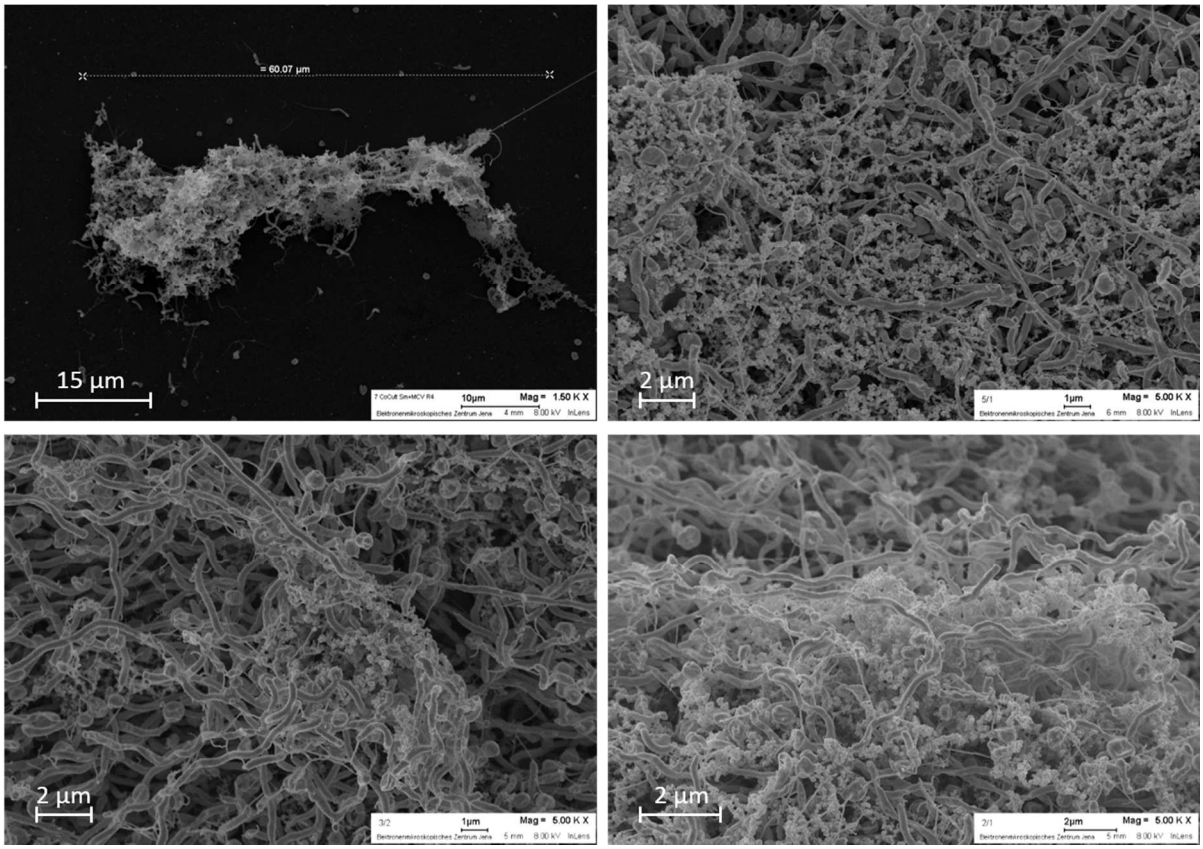
SUPPLEMENTARY NOTE 2

Determination of lactate dehydrogenase activity in crude extracts of *S. multivorans*

Lactate-producing and lactate-oxidizing enzyme activity was measured anoxically in quartz cuvettes using a Cary 100 spectrophotometer (Agilent Technologies, Waldbronn, Germany) with NADH and NAD^+ as electron donor/acceptor. Lactate-producing activity was followed by the decrease of absorbance at 340 nm of NADH. The assay mixture contained: 50 mM Tris-HCl (pH 7.5), 0.2 mM NADH, 1 mM KCN and 30 mM sodium pyruvate (pH 7.5). The reaction was started by the addition of crude extracts to 1 ml of the reaction mixture and the assay was incubated at room temperature. Lactate oxidation activity with NAD^+ was monitored by the increase of absorbance at 340 nm in a hydrazine-containing buffer; hydrazine was applied to trap pyruvate and thus shift the equilibrium of the normally thermodynamically unfavorable LDH-catalyzed oxidation of lactate with NAD^+ towards pyruvate formation. In addition, an excess of NAD^+ was applied to aid in overcoming the

thermodynamic barrier. The reaction mixture contained 50 mM Tris-HCl (pH 9.0), 0.5 mM NAD^+, 1 mM KCN, 30 mM sodium pyruvate (pH 7.5) and 30 mM hydrazine. Commercially available lactate dehydrogenase (~95% purity, 1.100 U/mg, Sigma Aldrich, Steinheim, Germany) served as positive control. No NAD^+- or NADH-dependent enzyme activity was measured in crude extracts.

Additionally, artificial electron donors such as sodium dithionite, methyl viologen and duroquinone (2,3,5,6-Tetramethyl-1,4-benzoquinone) were tested for enzyme-mediated lactate formation from pyruvate. NADH was replaced by 20 mM sodium dithionite, 10 mM reduced (with sodium dithionite) methyl viologen (98% purity, Sigma Aldrich, Steinheim, Germany) or 10 mM duroquinol (midpoint redox potential E°' = -240 mV) (97% purity, Sigma Aldrich, Steinheim, Germany). Lactate was measured by HPLC (AMINEX HPX-87H column, 7.8x300 mm, BioRad, Munich, Germany).



Supplementary Figure 14: Syntrophic co-culture of *S. multivorans* and *M. voltae*. Field emission scanning electron microscopic (FE-SEM) analysis of the formed aggregates. Different areas and magnifications are depicted in the images.

2.3 Supplementary Information

Syntrophy between *Dehalococcoides* and *Sulfurospirillum* leads to rapid and complete dechlorination of tetrachloroethene

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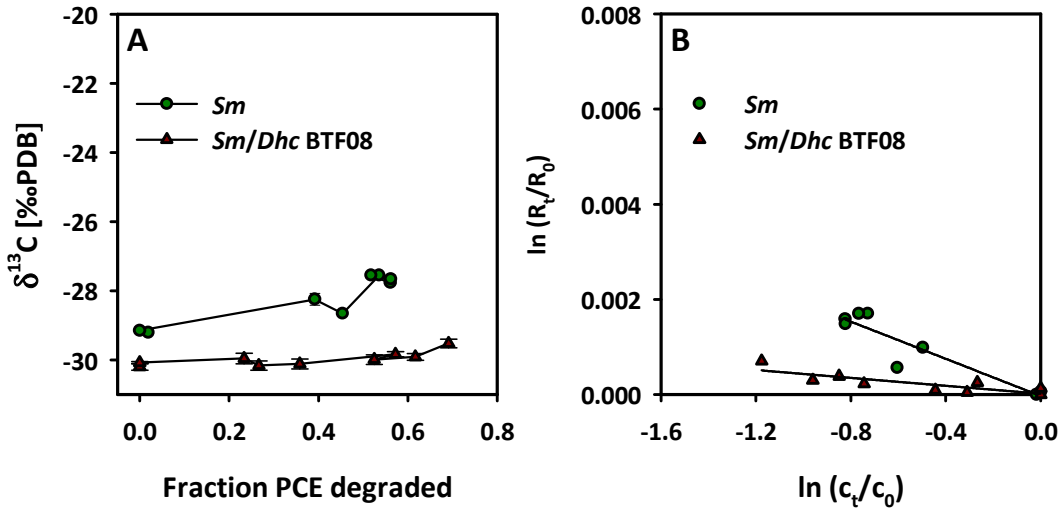
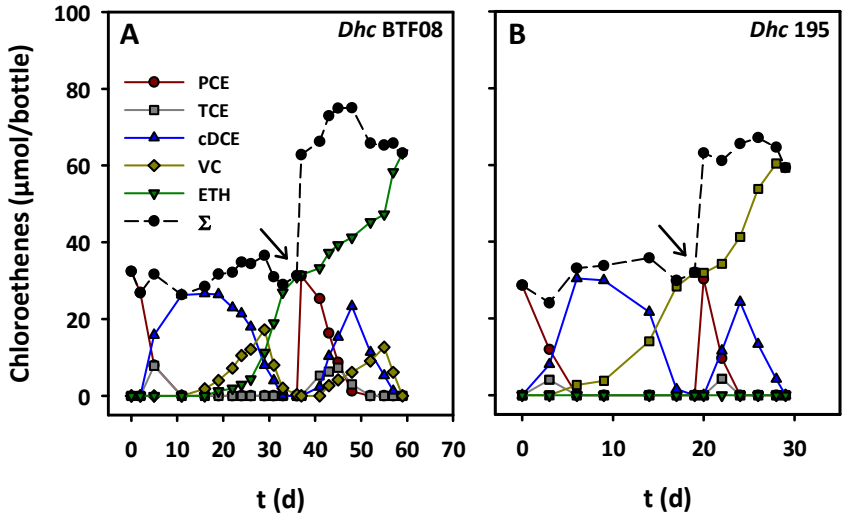
[4]Department of Isotope Biogeochemistry, Helmholtz Centre for Environmental Research – UFZ, Leipzig, Germany

[5]Center for Electron Microscopy of the University Hospital Jena, Jena, Germany

Supplementary Figures and Tables



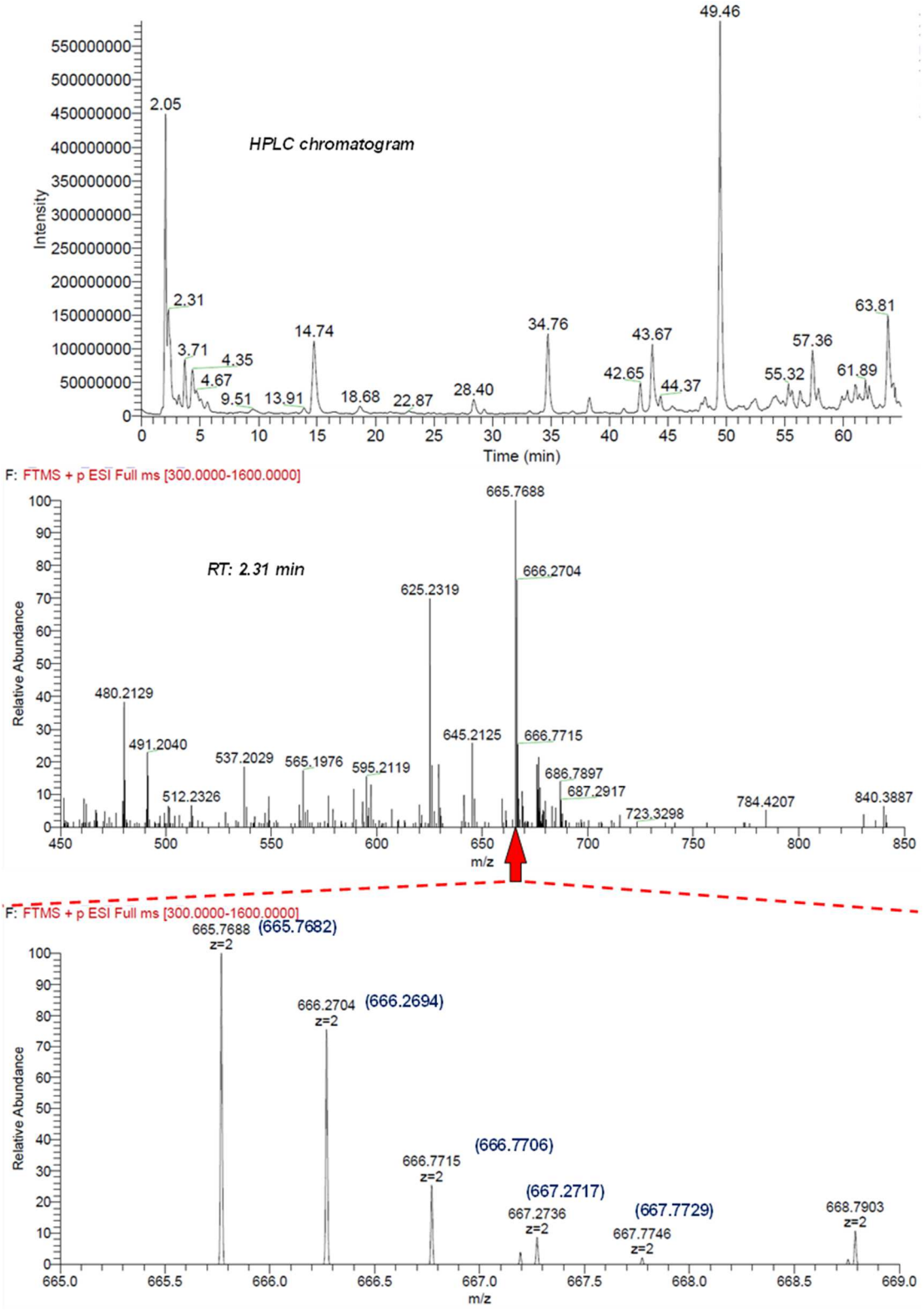
Supplementary Figure 1: Change in carbon isotope composition (A) and stable isotope fractionation (B) during reductive dechlorination of PCE by growing cells of *S. multivorans* and Sm/BTF08 co-culture. (A) SD was calculated from three technical replicates and is for <0.5 δ-units smaller than the symbols. (B) Correlation of stable isotope fractionation is R^2 = 0.859 for *S. multivorans* and R^2 = 0.684 for Sm/BTF08 co-culture.



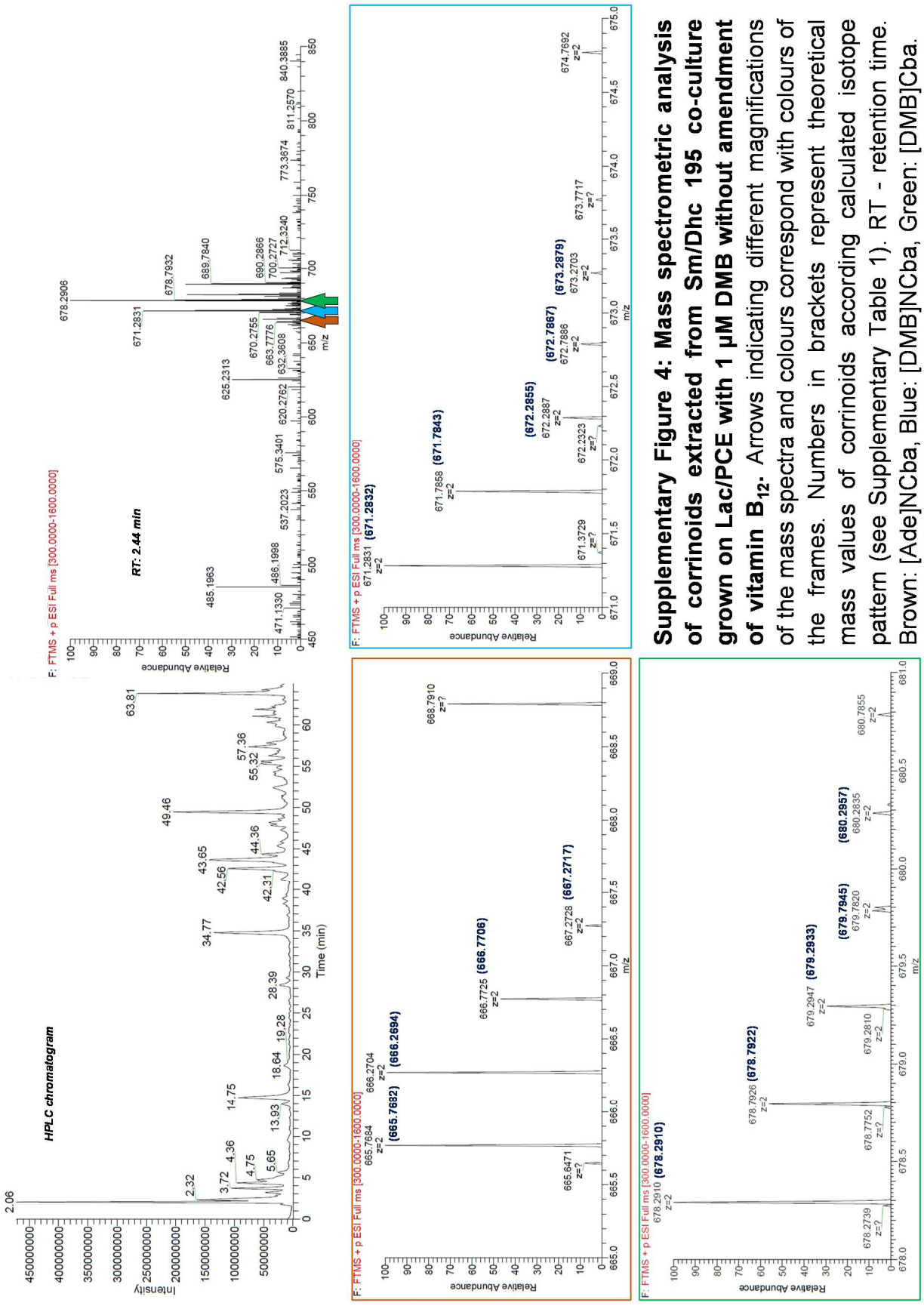
Supplementary Figure 2: Dechlorination of PCE with H_2 as electron donor by pure cultures of (A) *D. mccartyi* strain BTF08 and (B) *D. mccartyi* strain 195. Arrow indicates refeeding of PCE. ∑ = mass balance; sum of PCE, TCE, *cis*-DCE, VC and ethene.

Supplementary Table 1: Calculation of theoretical masses of the isotope distribution of different corrinoid types according to IDCalc.

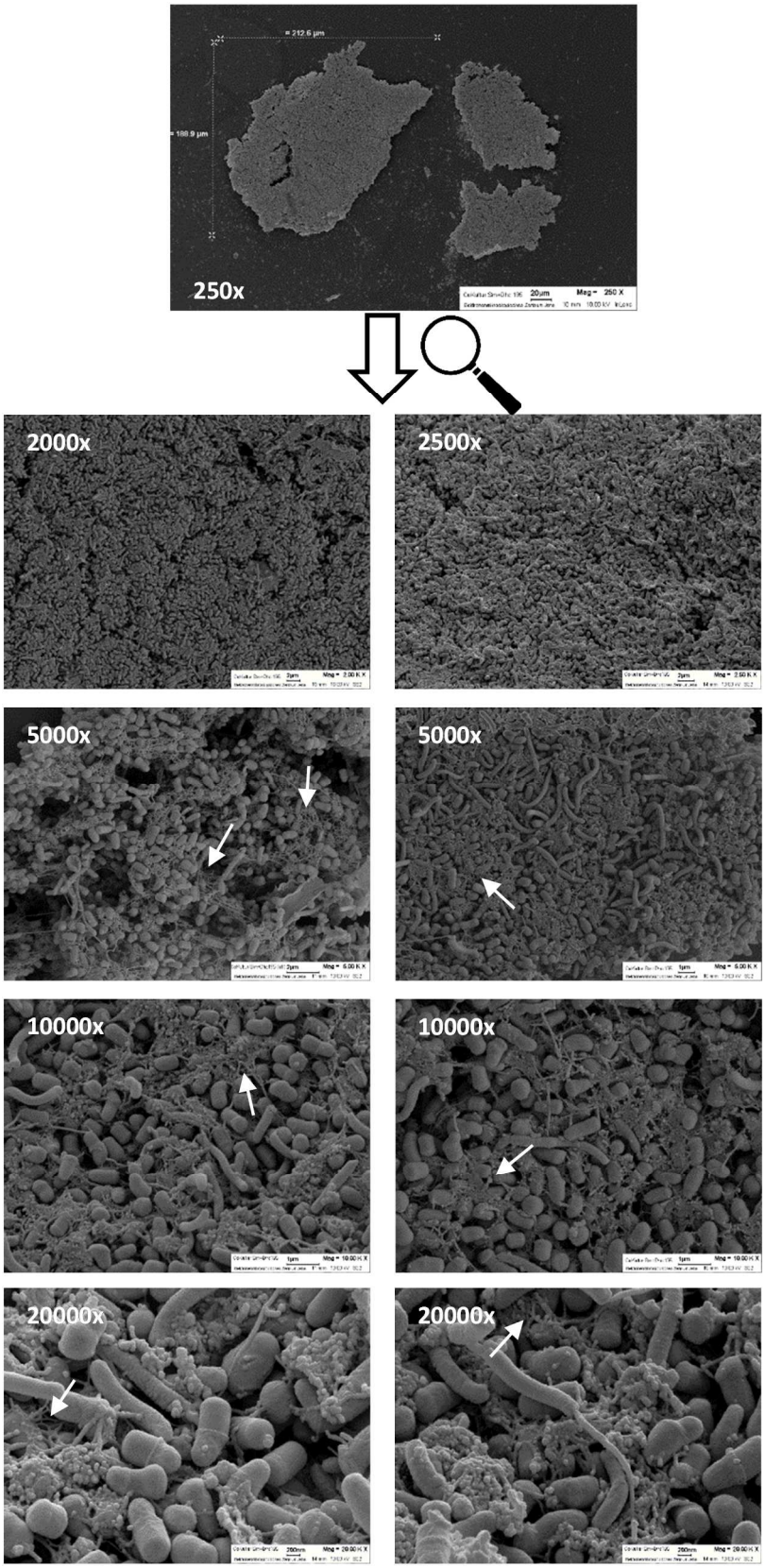
B_{12} type	Lower ligand	Linker	Formula	Rel. abundance	m/z z = 2
Cyanocobalamin [DMB]Cba	DMB	Aminopropan-2-ol O-2-phosphate	$C_{63}H_{88}N_{14}O_{14}PCo$	100.00	678.2910
				75.98	678.7922
				31.35	679.2933
				9.20	679.7945
				2.13	680.2957
5-methoxybenzimidazolyl-cobamide [5-OMeBza]Cba	5-OMeBza	Aminopropan-2-ol O-2-phosphate	$C_{61}H_{84}N_{14}O_{15}PCo$	100.00	672.2728
				73.77	672.7740
				29.91	673.2751
				8.68	673.7763
				2.00	674.2775
Norpseudovitamin B_{12} [Ade]NCba	Adenine	Ethanolamine O-phosphate	$C_{58}H_{81}N_{17}O_{14}PCo$	100.00	665.7682
				71.51	666.2694
				28.07	666.7706
				7.89	667.2717
				1.76	667.7729
Norvitamin B_{12} [DMB]NCba	DMB	Ethanolamine O-phosphate	$C_{62}H_{86}N_{14}O_{14}PCo$	100.00	671.2832
				74.85	671.7843
				30.50	672.2855
				8.85	672.7867
				2.03	673.2879



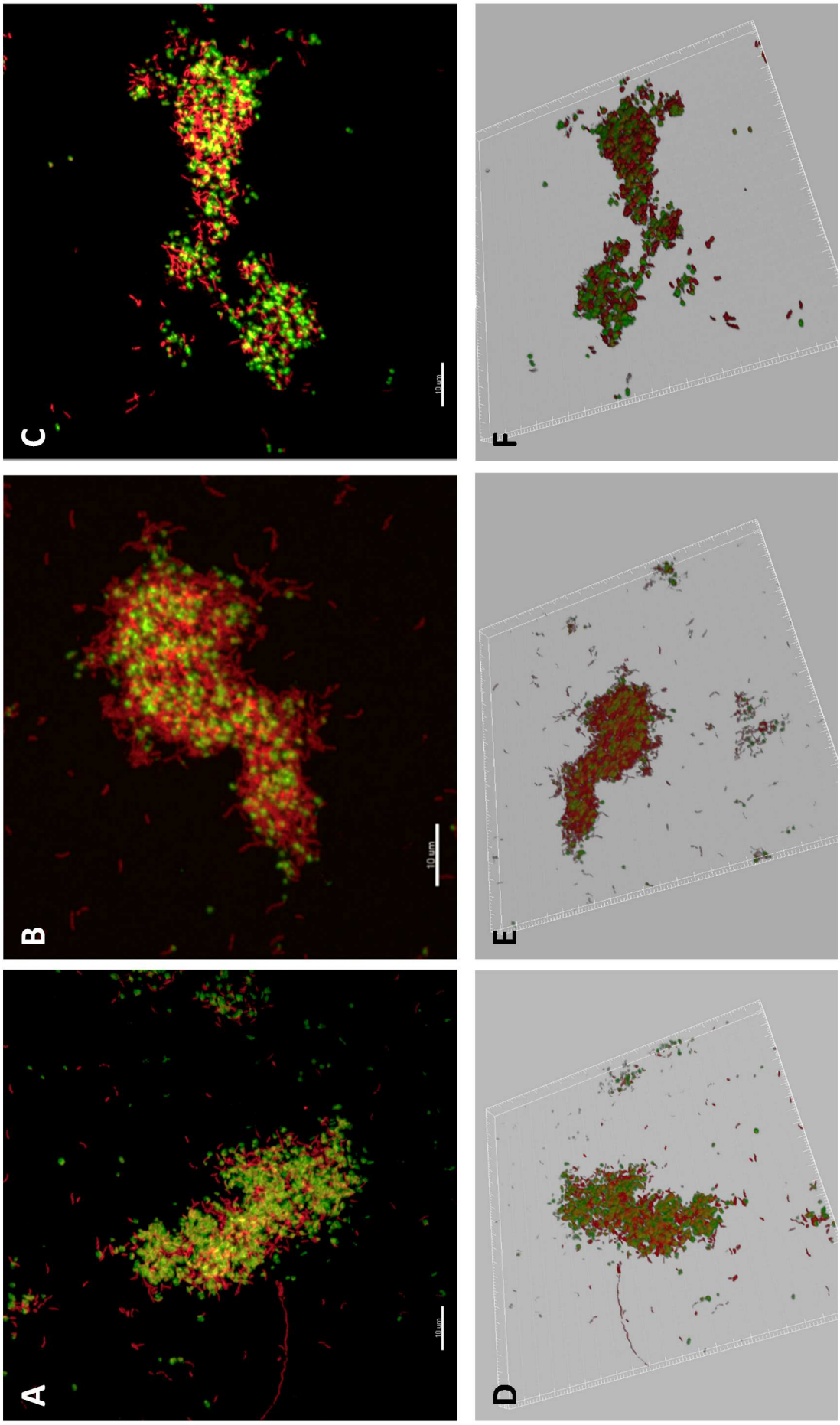
Supplementary Figure 3: Mass spectrometric analysis of corrinoids extracted from Sm/Dhc 195 co-culture grown on Lac/PCE without DMB and without amendment of vitamin B_{12}. Arrow indicates zoom-in and numbers in brackets represent theoretical mass values of corrinoids according calculated isotope pattern (see Supplementary Table 1). RT - retention time.



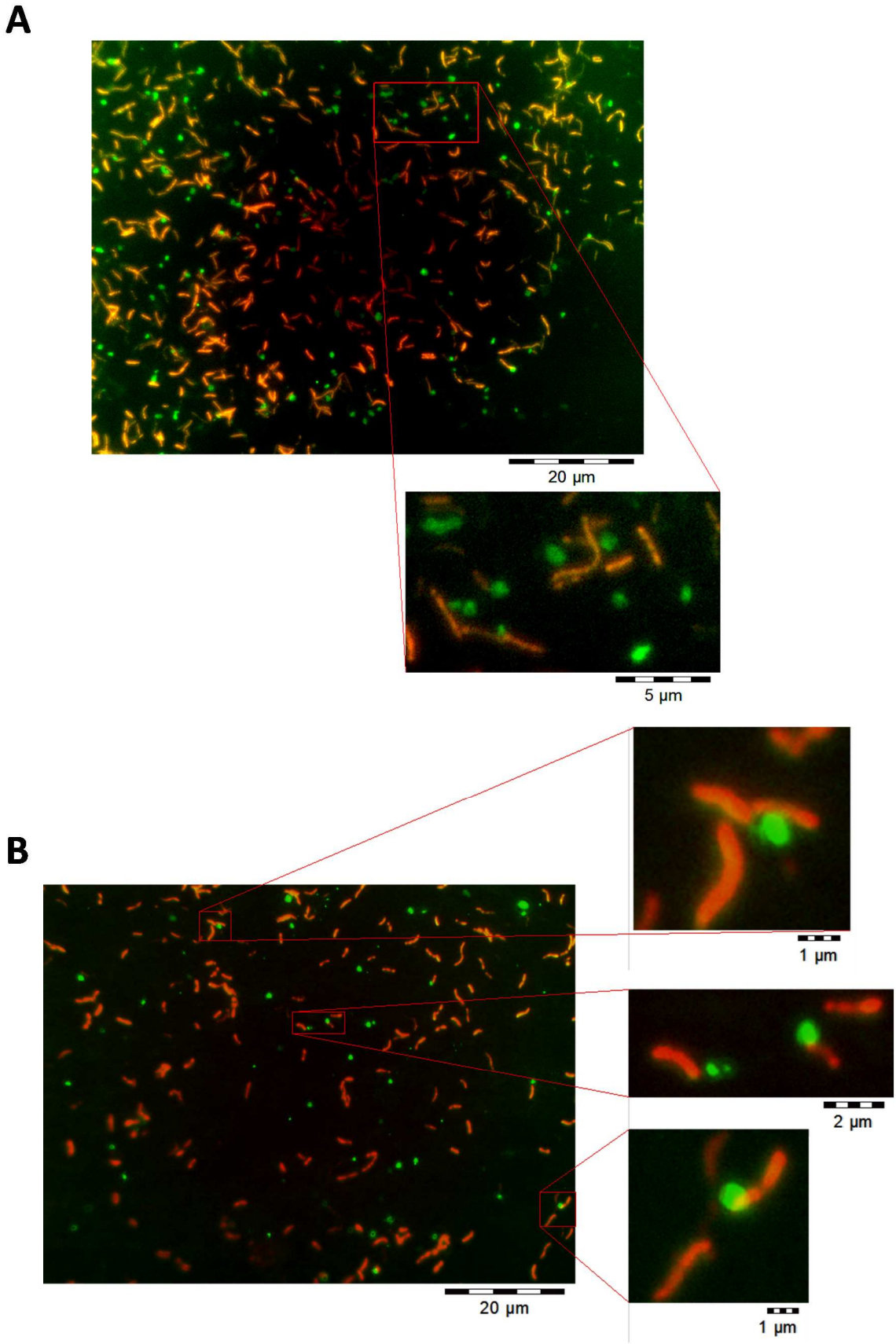
Supplementary Figure 4: Mass spectrometric analysis of corrinoids extracted from Sm/Dhc 195 co-culture grown on Lac/PCE with 1 µM DMB without amendment of vitamin B_{12}. Arrows indicating different magnifications of the mass spectra and colours correspond with colours of the frames. Numbers in brackets represent theoretical mass values of corrinoids according calculated isotope pattern (see Supplementary Table 1). RT - retention time. Brown: [Ade]NCba, Blue: [DMB]NCba, Green: [DMB]Cba.



Supplementary Figure 5: A detailed view and zoom-in an aggregate of *S. multivorans* and *D. mccartyi* strain 195. Arrows are indicating nets of flagellum-like filaments. Micrographs were taken from different parts of the aggregate. Primary magnifications are given in upper left corner of each micrograph



Supplementary Figure 6: FISH stained aggregates of co-cultures of *S. multivorans* and *D. mccartyi* strain BTF08 (A, D) and 195 (B, C, E, F) and corresponding 3-dimensional imaging (D-F). red: *S. multivorans*, green: *D. mccartyi*.



Supplementary Figure 7: FISH stained co-cultures of *S. multivorans* and *D. mccartyi* strain BTF08 (A) and 195 (B). red: *S. multivorans*, green: *D. mccartyi*.

Acknowledgements

„Leider läßt sich eine wahrhafte Dankbarkeit mit Worten nicht ausdrücken.“

Johann Wolfgang von Goethe (1749 - 1832)

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Author's declaration of originality

I, Stefan Kruse, born on 7th March 1989 in Gera (Germany), hereby declare that I am aware of the official doctoral regulations for doctoral candidates of the Faculty of Biological Sciences of the Friedrich Schiller University Jena. The here presented thesis originated entirely from me and published and/or unpublished work from others has been cited. All supporting information including assistance from cooperations are acknowledged in this thesis. The help of a doctoral advisor was not used and no commercial or monetary activities are related to the content and submission of this work. This dissertation has only been submitted to the Council of the Faculty of Biological Sciences of the Friedrich Schiller University Jena, and not to any other university for academic examination or dissertation.

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Place, date

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Stefan Kruse

Scientific publications

Peer-reviewed Journal Articles

Kruse S*, Goris T*, Wolf M, Wei X, Diekert G (2017)
The NiFe Hydrogenases of the Tetrachloroethene-Respiring Epsilonproteobacterium *Sulfurospirillum multivorans*: Biochemical Studies and Transcription analysis.
Frontiers in Microbiology 8:444
*These authors contributed equally to this work.

Kruse S*, Goris T*, Adrian L, Westermann M, Diekert G (2018)
Hydrogen production by *Sulfurospirillum* spp. enables syntrophic interactions of Epsilonproteobacteria.
published on bioRxiv (2017), under revision in *Nature Communications*
*These authors contributed equally to this work.

Kruse S, Türkowsky D, Matturro B, Franke S, Birkigt J, Nijenhuis I, Westermann M, Rossetti S, Jehmlich N, Adrian L, Diekert G, Goris T (2018)
Syntrophy between *Dehalococcoides* and *Sulfurospirillum* leads to rapid and complete dechlorination of tetrachloroethene
in preparation for submission in the *The ISME Journal*

Oral presentations at national and international conferences

2017 Complete dechlorination of tetrachloroethene to ethene by a co-culture of the organohalide-respiring organisms *Sulfurospirillum multivorans* and *Dehalococcoides mccartyi*

Stefan. Kruse, Tobias. Goris, Dominique Türkowsky, Nico Jehmlich, Lorenz Adrian, Martin Westermann and Gabriele Diekert. DehaloCon II – A Conference on Anaerobic Biological Dehalogenation. March 26-29, 2017, Leipzig (Germany).

2017 Syntrophic interaction of *Sulfurospirillum multivorans* and *Dehalococcoides mccartyi* lead to complete dechlorination of tetrachloroethene to ethene

Stefan. Kruse, Tobias. Goris, Dominique Türkowsky, Nico Jehmlich, Lorenz Adrian, Martin Westermann and Gabriele Diekert. 5th International Students' Conference on Microbial Communication (MICOM). March 20-23, 2017, Jena (Germany).

2017 Complete dechlorination of tetrachloroethene to ethene by a co-culture of the organohalide-respiring organisms *Sulfurospirillum multivorans* and *Dehalococcoides mccartyi*

Stefan. Kruse, Tobias. Goris, Dominique Türkowsky, Nico Jehmlich, Lorenz Adrian, Martin Westermann and Gabriele Diekert. Annual Conference of the Association for General and Applied Microbiology (VAAM). March 5-8, 2017. Würzburg (Germany).

2016 Interspecies hydrogen transfer in a dechlorinating Co-culture of *Sulfurospirillum multivorans* and *Dehalococcoides mccartyi* strain BTF08

Stefan Kruse. Symposium of the Graduate School Jena School for Microbial Communication (JSMC). December 15-16, 2016, Jena (Germany).

2016 Unprecedented H_2 production of free-living Epsilonproteobacteria (*Sulfurospirillum* spp.)

Stefan Kruse, Tobias Goris, Lorenz Adrian, Maria Gutsche and Gabriele Diekert. 11th International H2ase Conference. July 10-14, 2016, Marseille (France).

2016 Unprecedented hydrogen production of free-living Epsilonproteobacteria (*Sulfurospirillum* spp.)

Stefan Kruse, Tobias Goris, Maria Gutsche and Gabriele Diekert. Annual Conference of the Association for General and Applied Microbiology (VAAM). March 13-16, 2016, Jena (Germany).

2015 Syntrophism among Prokaryotes - Communication based on an exchange of hydrogen

Stefan Kruse. Retreat of the Graduate School Jena School for Microbial Communication (JSMC). November 26-27, 2015, Bad Sulza (Germany).

Poster presentations at national and international conferences

2015 Syntrophic relations of *Sulfurospirillum multivorans* - interspecies hydrogen transfer in a dechlorinating Co-culture

Stefan Kruse, Tobias Goris, Maria Gutsche, Xi Wei and Gabriele Diekert. Retreat of the Graduate School 'Jena School for Microbial Communication (JSMC). November 26-27, 2015, Bad Sulza (Germany).

2015 Hydrogen metabolism and hydrogen production of *Sulfurospirillum* spp. (Epsilonproteobacteria)

Stefan Kruse, Tobias Goris, Maria Gutsche, Xi Wei and Gabriele Diekert. Annual Conference of the Association for General and Applied Microbiology (VAAM). March 1-4, 2015, Marburg (Germany).

2014 Insights into the hydrogen metabolism and syntrophic relations of *Sulfurospirillum multivorans*, an organohalide-respiring bacterium.

Stefan Kruse, Tobias Goris, Maria Gutsche, Xi Wei and Gabriele Diekert. Symposium of the Graduate School Jena School for Microbial Communication (JSMC). September 2-3, 2014, Jena (Germany).

2014 The four [NiFe] hydrogenases of *Sulfurospirillum multivorans*: Insights into hydrogen metabolism of an organohalide-respiring bacterium

Stefan Kruse, Tobias Goris, Maria Gutsche, Xi Wei, Christian Schiffmann, Jana Seifert and Gabriele Diekert. DehaloCon I - A Conference on Anaerobic Biological Dehalogenation. March 23-26, 2014, Jena (Germany).