Physiological and enzymatic studies of respiration in *Dehalococcoides* species strain CBDB1

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Jayachandran Gopalakrishnan

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Promotionsausschuss:
Vorsitzender: Prof. Dr. rer. nat. Roland Tressl
Berichter: Prof. Dr. rer. nat. Helmut Görisch
Berichter: Prof. Dr. rer. nat. Ulrich Szewzyk

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ABBREVIATIONS

A

A	adenine
A_{280}	absorbance at 280 nm
A_{450}	absorbance at 450 nm
ADP	adenosine diphosphate
AQDS	anthraquinone 2,4-disulphonic acid
ATP	adenosine triphosphate

C

C	cytosine
CLOHPA	3-chloro 4-hydroxy phenyl acetate
cDNA	complementary DNA
CB	chlorobenzoate

D

DBP	dibromophenol
DCA	dichloroethane
DCB	dichlorobenzene
DCCD	N,N´-dicyclohexylcarbodiimide
DCE	dichloroethene
DCP	dichlorophenol
DMN	2,3-dimethyl 1,4-napthoquinone
DNA	deoxy ribonucleic acid

E

E.coli	*Escherichia coli*
$E_o´$	redox potential

G

G	guanine

H

h	hour
HCB	hexachlorobenzene
HONOQ	2-n-heptyl-4-hydroxyquinoline N-oxide

K

kb	kilo base
kDa	kilo dalton

O

OD_{600nm}	optical density at 600 nanometer
OH-PCB	hydroxy-polychlorinated biphenyls
ORF	Open reading frame

P

PCB	polychlorobiphenyls
PCDDs	polychlorinated dibenzo- p-dioxins
PCDFs	polychlorinated dibenzofurans
PCE	perchloroethene
PCP	pentachlorophenol
PCR	polymerase chain reaction
PeCB	pentachlorobenzene
Pi	inorganic phosphate

R

RNA	ribonucleic acid
rpm	rotations per minute
RT-PCR	reverse transcriptase-polymerase chain reaction

T

	-amino methane
T	thymin
TCB	trichlorobenzene
TCE	trichloroethene
TCS	tetrachlorosalicylanilide
TeCB	tetrachlorobenzene
Tris	tris(hydroxymethyl)

U

U	units
UV	ultraviolet

V

v/v	volume/volume

W

w/v	weight/volume

1. Introduction

Due to the past and present application of halogenated hydrocarbons in industry and agriculture as solvents, pesticides and preservatives has led to their accumulation in the environment in high quantities. This requires attention towards environmental integrity and health (Ahlborg and Thunberg 1980; Jensen 1996). Apart from this anthropogenic origin, more than 2000 halogenated organic compounds are naturally produced at considerable levels (Gribble 1996). The United States Environmental Protection Agency (EPA) has identified more than 1,600 sites on its NPL (National Priorites List) requiring the clean up of pollutants.

1.1 Chlorobenzenes

Chlorobenzenes are cyclic aromatic compounds with one to six chlorine atoms attached to the benzene ring. This yields 12 compounds: monochlorobenzene, three isomeric forms each of di-, tri-, and tetrachlorobenzenes, as well as penta- and hexachlorobenzenes.

1.1.1 Occurrence and distribution

From 1980-1983, the global production was estimated to be 568×10^6 kg, though the use of chlorobenzenes has declined in some countries since then. About 50 % of this amount was manufactured within the USA and the remainder primarily in Western Europe and Japan (IPCS 1991). Out of this 50 %, about 60,000 to 70,000 tons/annum were produced in Germany. In a systematic survey of the Federal Environmental Agency of Germany, a mean concentration of 0.44 µg/l HCB was detected in human blood with a marked increase in its concentrations with age (Becker et al 2002). Although the production of HCB was stopped completely in Germany in 1993, HCB is still ubiquitous in the environment and enters the human body mainly via food (IPCS 1997).

1.1.2 Uses of chlorobenzenes

Chlorobenzenes are used mainly as intermediates in the synthesis of pesticides for crop protection and other chemicals; 1,4-DCB is used in deodorants and as a moth repellent. The higher chlorinated benzenes (1,2,3,4-TeCB to HCB) have been used as components of dielectric fluids.

1.1.3 Release of chlorobenzenes into the environment

The release of chlorobenzenes into the environment occurs primarily during manufacturing and through the dispersive nature of their uses. For example, in 1989 about 150 tons of chlorobenzenes were released into the environment in Germany. Release of chlorobenzenes from waste disposal including incineration of municipal waste is much lower. However, the incineration of chlorobenzenes may lead to the emission of PCDDs and PCDFs. Leachate

from a German waste dump contained chlorobenzenes about 8 mg/l and the ground water contaminated by landfill leachate contained about 300 µg/l. The United Nations Environment Program identified HCB as one of the twelve most persistent organic pollutants that requires immediate attention due to its threat to human and wildlife health on a global basis (Fisher 1999).

1.1.4 Effect of chlorobenzenes on humans

Most data on the effects of HCB on humans originate from the accidental poisonings in Turkey in 1955-1959, in which more than 600 cases of porphyria cutanea tarda were identified. In this incident, disturbances in porphyrin metabolism, dermatological lesions, hyperpigmentation, hypertrichosis, enlarged liver, enlarged thyroid gland and osteoporosis or arthritis were observed primarily in children (Gustafson et al 2002). Also, PeCB and HCB have been proven to show hepatocarcinogenic activity (Thomas et al 1998).

1.1.5 Physical and chemical properties of chlorobenzenes

The physical and chemical properties of the chlorobenzenes (mono- to hexachlorobenzene) are presented in Table 1.1

MCB, 1,2-DCB, 1,3-DCB, and 1,2,4-TCB are colourless liquids, while all other congeners are white crystalline solids at room temperature. In general, the solubility of chlorobenzenes in water is poor (decreasing with increasing chlorination), flammability is low, the octanol/water partition coefficients are moderate to high (increasing with increasing chlorination), and vapour pressures are low to moderate (decreasing with increasing chlorination).

Table 1.1 Physical and chemical properties of chlorobenzenes.

Compound	Relative molecular mass (g/mol)	Melting point (^oC)[a]	Boiling point (^oC)[a]	Vapor pressure at 25^oC (Pa)[b]	Density[c]	Log n-Octanol/water coefficient[d]	Solubility in water. mol/l (mg/l)[d]
MCB	112.6	-45.6	132.0	1665	$1.1058^{20/4}$	2.98	$2.6x10^{-3}$(293)
1,2-DCB	147.0	-17.0	180.5	197	$1.3048^{20/4}$	3.38	$6.2x10^{-4}$ (91.1)
1,3-DCB	147.0	-24.7	173.0	269	$1.2884^{20/4}$	3.48	$8.4x10^{-4}$ (123)
1,4-DCB	147.0	53.1	174.0	90.0	$1.2475^{20/4}$	3.38	$2.1x10^{-4}$ (30.9)
1,2,3-TCB	181.5	53.5	218.5	17.3	N.A	4.04	$6.7x10^{-5}$ (12.2)
1,2,4-TCB	181.5	17.0	213.5	45.3	$1.4542^{20/4}$	3.98	$2.5x10^{-4}$ (45.3)
1,3,5-TCB	181.5	63.5	208.7	24.0	N.A	4.02	$2.2x10^{-5}$ (3.99)
1,2,3,4-TeCB	215.9	47.5	254.0	5.2	N.A	4.55	$5.6x10^{-5}$ (12.1)
1,2,4,5-TeCB	215.9	139.5	243.6	0.72	N.A	4.51	$1.0x10^{-5}$ (2.16)
1,2,3,5-TeCB	215.9	54.5	246.0	9.8	N.A	4.65	$1.3x10^{-5}$ (2.81)
PeCB	250.3	86.0	277.0	0.88	N.A	5.03	$3.3x10^{-6}$ (0.83)
HCB	284.79	230.0	322.0	0.0023	N.A	5.5	(0.005)

[a](Weast 1986)
[b] (IPCS 1991; IPCS 1997)
[c]Density is relative to water, otherwise it has the dimensions g/ml. A superscript indicates the temperature of the liquid and a subscript indicates the temperature of water to which the density is referred (Weast 1986).
[d](Miller 1984)

1.2 Reductive dechlorination and dehalorespiration

Abundance of halogenated compounds in the environment might have resulted in the evolution of various microbial dehalogenating populations with high microbial capacity to dechlorinate different classes of xenobiotic haloorganics (Gribble 1996).

The biodegradation of halogenated organics mostly depends on their chemical structure and the environmental conditions at which they exist. Aerobic degradation of lower halogenated compounds has been described in detail at the physiological, biochemical and genetic level (Slater et al 1997; Fetzner 1998). Aerobic dehalogenating systems with monooxygenases involving molecular oxygen have selective preference to dehalogenate haloorganic compounds carrying a limited number of halogen substituents. For instance, mono or di-chloroethenes are dechlorinated by mono and dioxygenases from various bacteria but the highly chlorinated tetrachloroethene is persistent under these conditions (Arp 1995; Leisinger 1996).

Within the last two decades, several different haloaromatics and aliphatics have been shown to be degraded under anoxic conditions by reductive dechlorination.

Microbially mediated reductive dechlorination can be divided into co-metabolic and respiratory processes. In a co-metabolic process, the organism does not conserve energy from the transformation. One example of co-metabolism is the reductive dechlorination of PCE by sulphate reducers (Cole et al 1995), methanogenic archea (Fathepure and Boyd 1988) or acetogens (Terzenbach and Blaut 1994). The dechlorination rates in these co-metabolic processes are low and their contribution to the dechlorination reactions in natural environments is also thought to be low.

In contrast, in respiratory dechlorination, the organisms couple reductive dechlorination of chlorinated compounds to energy conservation and hence to microbial growth (Sanford et al 1996; Holliger et al 1999). This process is likely to be the major contribution to the microbially mediated reductive dechlorination in anoxic environment. Not like co-metabolic conversions, in these organisms specific enzymes catalyse dechlorination. This novel respiratory process in which halogenated hydrocarbons are used as terminal electron acceptors in anaerobic respiration is described as halorespiration (Sanford et al 1996) or dehalorespiration (Holliger et al 1999).

In a dechlorination reaction, hydrogen replaces chlorine from the chlorinated compound. The chlorinated compound acts as an electron acceptor as two electrons are added to the molecule and chloride is released.

1.3 Thermodynamic rationale in reductive dechlorination of chlorinated compounds

Halogenated aromatics and aliphatics could be good electron acceptors in anaerobic environments based on their redox potentials (Dolfing and Harrison 1992). For example, the change in Gibbs free energy ($\Delta G_o'$) accompanying the reductive dechlorination of HCB to PeCB by hydrogen has been calculated as -171.4 kJ. The redox potential (E_o') of the couple HCB/PeCB is +478 mV (Dolfing and Harrison 1992). In addition, this value is higher than those of the redox couples SO_4^{2-}/H_2S (E_o' = -217 mV) and fumarate/succinate ($\Delta E_o'$ = +30 mV) or NO_3/NO_2 (E_o' = +433 mV) (Dolfing and Harrison 1992). Reductive dechlorination reactions are thermodynamically possible even if the concentration of electron donor (eg, hydrogen) in the anaerobic environment is much below the standard conditions (Dolfing and Harrison 1992). The hydrogen threshold (minimum hydrogen concentration that can be consumed) is inversely correlated with changes in Gibbs free energy and the redox potential of the hydrogen consuming reaction (Lovley 1985; Lovley and Goodwin 1988). Therefore, dehalorespiring anaerobes can consume hydrogen to levels below the threshold concentrations observed for sulfidogens, acetogens or nitrate reducers.

The thermodynamic rationale can further be extended to predict that chlororespirers are excellent competitors for hydrogen in an anaerobic environment among other hydrogenotrophes. Therefore, dehalorespiring bacteria can dominate in anaerobic environments as long as reductive dechlorination is not limited by the concentration or availability of the chlorinated compounds as the electron acceptors.

1.4 Dehalorespiring bacteria

A rapidly increasing number of bacteria have been isolated based on their ability to couple dechlorination and growth with chlorinated compounds like PCE, TCE, chlorophenols or chlorobenzoates. These organisms have received attention because of their potential to bioremediate contaminated anoxic environments, their versatility to use both chloro aliphatics and aromatics and the novel respiratory mechanisms they possess. Most of these dehalorespirers have been grouped into distinctive phylogenetic branches of the bacterial domain namely, low G+C gram positives, ε- and δ- proteobacteria.

1.4.1 Low G-C gram positives

This group includes most of the dehalorespiring bacteria. *Desulfitobacterium* species include *D. dehalogenans* (Utkin et al 1994), *D. frappieri* (Bouchard et al 1996), *Desulfitobacterium* sp strain PCE-1 (Gerritse et al 1996) and *D. frappieri* TCE-1 (Gerritse et al 1999), *D. hafniense* (Christiansen and Ahring 1996), *D. chlororespirans* (Sanford et al 1996) and *Desulfitobacterium* sp. Strains Viet.1 (Löffler et al 1997) and PCE (Miller 1997 a). Varieties

of chlorinated compounds like PCE, TCE and chlorophenols have been identified to be electron acceptors for this group of bacteria. Bacteria in this group markedly differ in using electron donors.

1.4.2 Proteobacteria

Bacteria in the γ, ε and δ subphylums in the group of proteobacteria have been found to be capable of reductive dechlorination. *Desulfomonile tiedjei* (DeWeerd et al 1990), *Desulfuromonas chloroethenica* (Krumholz 1997) and *Desulfovibrio* TBP-1 (Boyle et al 1999) come under δ-proteobacteria. Chlorobenzoates, chloroethenes, chlorophenols or bromophenols are found to be electron acceptors for this group of microorganisms.

1.4.3 Chloroflexi (Green non-sulphur bacteria)

This group was not included in the chlororespiring bacteria containing phylogenetic branches until the isolation of *Dehalococcoides ethenogenes* strain 195 (Maymò-Gatell et al 1997). This organism was the first member of this phylum capable of dechlorinating PCE completely to ethene. Another two bacteria in this group have been described which are however not isolated in pure. The first one is bacterium o-17 capable of coupling its growth to reductive dechlorination of 2,3,5,6-chlorobiphenyl (PCB) having 89 % identity to *D. ethenogenes* based on its 16S rDNA (Cutter et al 2001). Secondly, strain DF-1, which dechlorinates doubly flanked chlorines from PCBs (Wu et al 2002 b). Recently the same organism was described to be responsible for HCB and PeCB dechlorination in a mixed culture (Wu et al 2002 a).

Although many bacteria have been isolated that dechlorinate a variety of chloroaromatics and aliphatics, no pure strain was described until the year 2000, that was capable of linking growth with reductive dechlorination of chlorobenzenes. Strain CBDB1 was isolated as a pure culture by Adrian et al (2000 a). The organism has the ability to reductively dechlorinate chlorobenzenes and it is the second species of genus *Dehalococcoides*. Very recently another *Dehalococcoides* strain BAV1, able to dechlorinate vinyl chloride, was also isolated (He et al 2003).

Table (1.2) summarises details of microorganisms isolated in pure capable of dehalorespiration.

Table 1.2 Summary of microorganisms isolated in pure capable of reductive dechlorination.

Microorganism	Electron donor	Electron acceptor	Products of dechlorination
Low G+C gram Positives			
D. chlororespirans (Sanford et al 1996)	Butyrate, crotonate, formate, lactate, pyruvate, hydrogen	2,3-DCP, 2,4,6-TCP, PCP, ClOHPA	*o*-position dechlorinated
Desulfitobacterium dehalogenans (Utkin et al 1994)	Formate, pyruvate, lactate, hydrogen	2,4-DCP, 2,6-DCP, 2,4,6-TCP, ClOHPA, PCE, OH-PCB	*o*-position dechlorinated
D. frappieri PCP-1 (Bouchard 1996)	Pyruvate	PCP, 2,4,6-TCP, ClOHPA, PCE, OH-PCB, Cl-catechol	*o* and *p*-position dechlorinated
Desulfitobacterium PCE-1 (Gerritse et al 1996)	Butyrate, ethanol, formate, lactate, pyruvate, succinate	2-CP, 2,6-DCP, 2,4,6-TCP, ClOHPA, PCE	*o*-position dechlorinated TCE
D. frappieri TCE-1 (Gerritse et al 1999)	Butyrate, crotonate, ethanol, formate, lactate, hydrogen	PCE, TCE	*cis*-DCE
Desulfitobacterium hafniense (Christiansen and Ahring 1996)	Pyruvate	PCP, 2,4,5-TCP ClOHPA	*o*- and *m*-position dechlorinated
Desulfitobacterium Viet.1 (Löffler et al 1997)	Hydrogen	PCE	TCE
Desulfitobacterium PCE-S (Miller et al 1997 a)	Hydrogen, formate, pyruvate	PCE, TCE, 2,4,5-TCP, PCP	*o*-position dechlorinated
Dehalobacter restrictus (Wild 1996)	Hydrogen	PCE, TCE	*cis*-DCE
Dehalobacter sp. TEA-1 (Wild 1996)	Hydrogen	PCE, TCE	*cis*-DCE

Table cont.,

ε-subdivision proteobacteria			
Desulfurospirillum multivorans (Scholz-Muramatsu et al 1995)	Formate, ethanol, hydrogen, lactate pyruvate	PCE, TCE	*cis*-DCE
δ-subdivision proteobacteria			
Desufomonile tiedjei (DeWeerd et al 1990)	Formate, hydrogen, pyruvate	PCP, PCE, 3CB	*m*-position dechlorinated
Desulforomonas chloroethenica (Krumholz 1997)	Pyruvate, polysufide	PCE, TCE	*cis*-DCE
Desulfovibrio sp. TBP-1. (Boyle et al 1999)	Lactate, fumarate, hydrogen	2,6-DBP, 2,4,6-TCP	*m* and *o*-position dechlorinated.
γ-subdivision proteobacteria			
Enterobacter MS-1 (Sharma and McCarty 1996)	Aminoacids, acetates, formate, glucose, lactate, pyruvate, yeast extract	PCE, TCE	*cis*-DCE
Chloroflexi (Green non-sulfur bacteria)			
Dehalococcoides ethenogenes strain 195 (Maymò-Gatell et al 1997)	Hydrogen	PCE, TCE, *cis*-DCE, 1,1-DCE, 1,2-DCA	Ethene
Dehalococcoides strain CBDB1 (Adrian et al 2000 a; Bunge et al 2003)	Hydrogen	1,2,3-TCB, 1,2,4-TCB, 1,2,4,5-TeCB. 1,2,3,5-TeCB. Dioxins	DCBs, 1,3,5-TCB Less chlorinated dioxins
Dehalococcoides. sp BAV1 (He et al 2003)	Hydrogen	Vinyl chloride	Ethene

1.5 Reductive dechlorination of chlorobenzenes and strain CBDB1

Several enrichment cultures from sewage sludge, biofilm reactors, river sediment and soil (Fathepure et al 1988; Holliger et al 1992; Fathepure and Vogel 1991) have been described to reductively dechlorinate chlorobenzenes. *Dehalococcoides* sp strain CBDB1 is the first described pure strain that can conserve energy by the dehalorespiration with chlorobenzenes (Adrian et al 2000 a; 2000 b). Strain CBDB1 grows in a purely synthetic mineral medium containing trace elements, vitamins, hydrogen as the electron donor, acetate as carbon source, and chlorobenzenes as electron acceptor. Common sugars or small fatty acids did not support growth of strain CBDB1. Also, no growth occurred in complex media containing yeast extract or peptone.

Thus, using hydrogen as the electron donor and chlorobenzenes as electron acceptor, strain CBDB1 could be indefinitely subcultured yielding growth up to about 10^6 cells/ml.

All three isomers of tetrachlorobenzenes, 1,2,3-TCB, and 1,2,4-TCB were dechlorinated to less chlorinted benzenes (1,3-and 1,4-DCBs) while monochlorobenzenes, 1,3-DCB, 1,4-DCB and 1,3,5-TCB were not dechlorinated (Adrian et al 2000 a).

16S rRNA gene sequence of strain CBDB1 was determined and the sequence shows 98.3% identity with the 16S rRNA of *D. ethenogenes* indicating that strain CBDB1 has a close affiliation to *D. ethenogenes*. According to their 16S rRNA gene sequences, both strains CBDB1 and *D. ethenogenes* and several uncultivated bacteria form a new bacterial cluster (Adrian et al 2000 a).

Electron micrograph of strain CBDB1 (Fig 1.1) after negative staining with 1 % phosphotungstic acid shows notches lacking a typical cell wall on the surface. Instead of a typical bacterial cell wall, a S-layer –like structure covers the surface of the cell. Notches are arranged opposite to each other having roughly the same size.

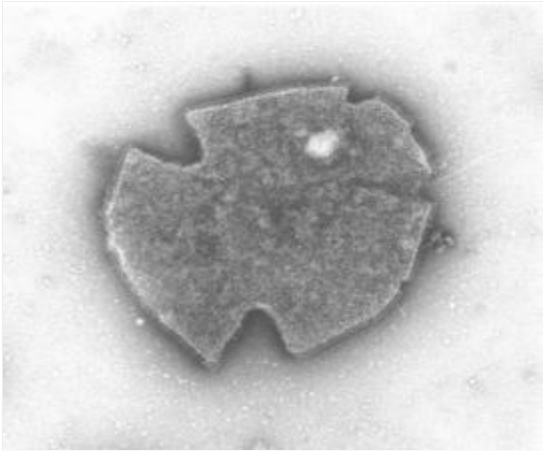


Fig.1.1 Electron micrograph of strain CBDB1 (Adrian et al 2000 a).

1.6 Biochemistry and physiology of dehalorespiration

Dehalogenases are the key enzymes involved in the process of reductive dechlorination. PCE dehalogenating enzymes from *Dehalobacter restrictus* (Schumacher et al 1997) *Desufurospirillum multivorans* (Neumann et al 1996; Miller et al 1998) have been isolated, purified partially or completely and characterised at the biochemical and/or genetic level.

The first described dehalogenating enzyme 3-chlorobenzoate reductive dehalogenase from *Desulfomonile tiedjei*, a sulphate reducing strain was characterised biochemically indicating that the enzyme is inducible. A quinone was involved in electron transport including cytochrome, which was found to be co-induced with the dehalogenase (Louie and Mohn 1999).

A dehalogenating enzyme from *Dehalobacter restrictus* facing the cytoplasmic side of the membrane with PCE dehalogenating activity was described (Schumacher and Holliger 1996). Similarly, PCE dehalogenating enzymes from *Desufurospirillum multivorans*, and *Desulfitobacterium frappieri* strain PCE-S have been isolated and studied (Neumann et al 1996; Miller et al 1998). All PCE dehalogenases contained corrinoids as the redox co-factor. Cobalamin and two [Fe-S] clusters as redox co-factors have also been found in *o*-chlorophenol reductive dehalogenase from *Desulfitobacterium dehalogenans* (Ni et al 1999).

In mixed culture containing *Dehalococcoides ethenogenes* strain 195, two membrane associated reductive dehalogenases were detected (Magnuson et al 1998). PCE reductive dehalogenase was responsible for PCE dechlorination and TCE reductive dehalogenase was responsible for the production of ethene from TCE. A corrinoid was found to be the redox co-factor in both enzymes.

1.6.1 Involvement of co-factors and [Fe-S] clusters in reductive dechlorination

Photoreversible inhibition studies by haloalkanes with dehalogenases from most of the dehalorespirers indicated that cob(I)alamin is involved in the reductive dechlorination reaction (Schumacher and Holliger 1996; Neumann et al 1996; Miller et al 1998; Magnuson et al 1998). In contrast, in *Desulfomonile tiedjei*, no corrinoid was involved but a heme seemed to be present in the small subunit of this enzyme (Louie and Mohn 1999).

Electron paramagnetic resonance spectroscopic (EPR) analysis of the purified *o*-chlorophenol reductive dehalogenase from *Desulfitobacterium dehalogenans* indicated the presence of a cobalamin, one [4Fe4S] cluster and one [3Fe4S] cluster. Similar results were obtained from purified PCE dehalogenase of *Dehalobacter restrictus*. But the latter enzyme contained an additional [4Fe4S] cluster (Schumacher et al 1997). In the dechlorination reaction, cobalt reduction in the cobalamin is proposed to be a two-step reaction. First, reduction of Co(III) to

Co(II) which requires a high-potential electron donor. Second, the reduction of Co(II) to Co(I) which requires a low- potential electron donor (Miller et al 1997). Therefore, this model assumes the splitting of electrons into high and low potential electron via two [Fe-S] clusters and hence cobalamin co-factor in reductive dehalogenases requires two different *in vivo* electron donors, which however, has not been demonstrated.

Most of the studies so far have been focused on the dehalogenases rather than on the primary dehydrogenases. To understand the molecular basis of this novel dehalorespiration process, studies have to be focused not only on the terminal reductases but also towards the primary dehydrogenaes. Dehydrogenases are also key enzymes of dehalorespiration to initiate the dechlorination reaction by oxidising the electron donor used by the strain.

In most of the isolated dehalorespirers, studies on hydrogenases were not demonstrated beyond the localisation of hydrogenase activities. With one exception, in *Desulfitobacterium dehalogenans* the genes coding for putative [Ni-Fe] hydrogenase subunits have been sequenced (Smidt 1999).

1.7 Thesis objectives

Within the last decade many *Dehalococcoides* species have been described to dechlorinate variety of highly persistent chlorinated compounds (Maymò-Gatell et al 1997; Adrian et al 2000 a; Wu et al 2002 b; He et al 2003; Bunge et al 2003). *Dehalococcoides* species are gradually recognised as one of the major groups among the dechlorinating bacteria. However, the molecular basis of dehalorespiration is still poorly understood. Previous studies in our laboratory mainly focused on the isolation of a bacterium in pure culture that is able to couple dechlorination of chlorobenzenes to growth. In this dissertation, the biochemistry and physiology of dehalorespiration is studied using the *Dehalococcoides* species strain CBDB1 as a model organism. Chapter 3 describes the development of a system for the cultivation of strain CBDB1 with highly chlorinated benzenes as electron acceptors. Chapter 4 describes experiments studying the localization of hydrogenase activities in strain CBDB1 and their biochemical properties. Development and optimisation of a cell disruption method for the strictly anaerobic strain CBDB1 is also with dealt with this chapter. Chapter 5 describes the electron transport in strain CBDB1. Chapter 6 explains the molecular characterisation of genes coding for a membrane bound [Ni-Fe] hydrogenase genes from strain CBDB1.

Finally, chapter 7 describes possible bioenergetics of chlorobenzene respiration by strain CBDB1 and probable reasons for the low growth yield of strain CBDB1 with relevant experiments.

2. Materials and methods

2.1 Source of chemicals

All chlorobenzenes in this study were obtained from Aldrich (Steinheim, Germany), Merck-Schuchardt (Hohenbrunn, Germany), Arcos (New Jersey, USA) and Fluka (Buchs, Switzerland). Titanium chloride (synthesis-grade solution) was obtained from Merck-Schuchardt. The reducing agent Titanium(III) citrate (0.1 M with respect to Ti(III)) was prepared as described by Zehnder and Wuhrmann (1976).

Benzyl viologen, methyl viologen, ethyl viologen, AQDS, DMN, PQQ and DCCD were obtained from Aldrich (Steinheim, Germany). TCS was obtained from Arcos (New Jersey, USA). All other chemicals used were at least of analytical grade and were purchased from Sigma (Deisenhofen, Germany) or Merck.

Gases, N_2, H_2 (99.999% vol/vol) and CO_2 (99.8% vol/vol) were obtained from Messer Griesheim (Berlin, Germany) and traces of oxygen were removed by a reduction column (Ochs, Göttingen, Germany).

2.2 Culture conditions

Strain CBDB1 was grown under strict anaerobic conditions. The bacteria were cultivated in 120 ml flasks containing 60 ml medium and 60 ml gas phase amended with 15 µM each of 1,2,3-TCB and 1,2,4-TCB as electron acceptors. A synthetic mineral medium with vitamins, trace elements, and 5 mM acetate as carbon source was used and reduced with 0.8 mM Titanium(III) citrate as described previously (Adrian et al 2000 a). The flasks were sealed with Teflon-lined butyl-rubber septa (Macherey and Nagel, Düren, Germany) and aluminium crimp caps and the headspace of each flask was exchanged by repeated evacuation and flushing with N_2-CO_2 (4:1, vol/vol) using an automatic gas exchanger. A nominal concentration of 7.5 mM hydrogen was added with a syringe to the headspace of the cultures. Inoculation was carried out with glass syringes previously flushed with sterile water. The standard inoculation volume was 200 µl of a active pure culture per flask. Cultures were incubated at 29^{o}C in the dark without shaking. Two weeks after inoculation, cultures were supplied with additional 10 mM 1,2,3-TCB (nominal concentration), added as a hexadecane solution (Adrian et al 2000 a; Holliger et al 1992).

To cultivate strain CBDB1 with PeCB or HCB, compounds were added either as a 0.2 M solution in hexadecane to a nominal concentration of 10 mM or directly to the cultures as crystals (HCB 50 µg/ml, PeCB 20 µg/ml equivalent to nominal concentrations of 175 and 80 µM, respectively) before the flasks were sealed with Teflon lined butyl rubber-septa and

aluminium crimp caps. The experiments were set up in duplicate or triplicate. Control experiments were done without inoculum or without electron acceptor.

2.3 Cell fractionation - Cell disruption using a ball mill - Application of response surface method

All fractionation steps were carried out anoxically. While cell disruption using the French press method completely inactivated the hydrogenase activity of strain CBDB1, a small-scale ball mill was used. Cultures were harvested when they reached about 1µg/ml of total protein, which was approximately after 10 days of incubation. Cells (10 ml of cell suspensions) were disrupted anaerobically using a ball mill to get an active crude extract.

Since the random selection of process variables for cell disruption (the ratio of culture volume to the grams of glass beads and shaking time) resulted in low enzyme activity, optimisation of these critical process variables were found to be necessary. Statistically designed experiments are a defined set of experiments to predict the optimum combination of variables to get the maximum response. Statistical methods vary from conventional methods where a single variable is changed keeping other variables unchanged thereby avoiding the interaction, squared effect among variables, which is an important aspect to be taken into consideration. Response surface methodology is one of the most widely used statistical procedures in bioprocess systems (Khuri and Cornell 1996).

The effect of these process variables on cell disruption [culture volume to grams of balls (x_1), shaking time (x_2)] were investigated using a second order central composite design (CCD). Table 2.1 shows the range of independent variables considered to get an optimal combination of variables.

Table 2.1 Independent variables (Ratio of culture volume to grams of balls, time of shaking) and their levels studied in the optimisation design.

S.No	Variables	Ranges				
		-α	-1	0	1	α
1	Ratio, x_1 (culture vol/grams of glass beads)	0.9417 (10ml/10.63g)	0.9 (10ml/11.1g)	0.8 (10ml/12.5g)	0.7 (10/14.2g)	0.66 (10ml/15.18g)
2	Shaking time, x_2 (min)	11.8	15	22.5	30	33.1

$X_{i\ (i=1-2)}$: real value of the independent variable.

- α, + α: Lowest and highest values in the range studied for each variable.

-1,+1: Intermediate values between the central and extreme levels of each variable.

0: Central level on the range studied for each variable.

The variables X_i were coded as x_i according to the following equation,

$x_i = (X_i - X_0)/\Delta X_i$ .....(1)

Where, x_i = dimensionless coded value of the variable X_i. X_0 = the variable of X_i at centre point, ΔX_i = step change.

The specific codes are: x_1 = (Ratio-0.8)/0.1, x_2 = (Time of shaking (min) - 22.5)/7.5

The levels of independent values according to the design are given in Table 2.2. The mean of hydrogenase activity at each experimental point with various combinations of variables as per the design was taken as response (Y_i).

The data obtained from CCD is usually used to fit a second order polynomial equation as it represents the behaviour of such systems more relevantly than the first order designs. Thus the following second order polynomial equation was fitted.

$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x^2 + \beta_{12} x_1 x_2$.....(2)

Where, Y= Predicted response (Hydrogenase activity)

β_0= offset term, β_1, β_2 = linear effect, β_{11}, β_{22} = squared effect and β_{12} = interaction effect.

A computer software Design-Expert 6 ("DX") was used to design the experiment with various combinations of variables as follows,

Table 2.2 Experimental design for two variables

Sample No	Variable 1 (Ratio)	Variable 2 (Time)
1	-1 (0.9)	-1 (15)
2	1 (0.7)	-1 (15)
3	-1 (0.9)	1 (30)
4	1 (0.7)	1 (30)
5	- α (0.94)	0 (22.5)
6	+ α (0.66)	0 (22.5)
7	0 (0.8)	- α (11.8)
8	0 (0.8)	+ α (33.1)
9	0 (0.8)	0 (22.5)
10	0 (0.8)	0 (22.5)

The optimum values of variables could be obtained by optimising the second order polynomial equation by equating the partial derivatives with respect to x_1 and x_2 to zero.

According to the experimental design (Table 2.2), the required volume of culture and glass beads were filled in 30 ml serum bottles previously flushed with nitrogen. The bottles were closed with Teflon lined aluminium crimp caps. The containers of the ball mill were replaced

by the serum bottles and were shaken to disrupt the cells for a particular time according to the experimental design (Table 2.2).

The crude extract obtained was centrifuged at 13,000 rpm for 20 min to remove intact cells. The supernatant was filled in a tube in an anaerobic chamber and was tightly closed with an air tight Teflon lined cap and was ultra centrifuged (37,500 rpm for 60 min). The resulted sediment was considered as membrane fraction and the supernatant was referred to as soluble fraction. The soluble fraction was further concentrated using either Sephadex G 25 coarse or centrifugal tubes equipped with a molecular cut off of 5 kd (Centricon Plus-20 Centrifugal Filter Units, Millipore)

2.4 Preparation of cell suspensions

Cell suspensions were prepared carefully under anoxic conditions to avoid contact with oxygen. Whenever necessary, the culture was concentrated by centrifugation. Nitrogen was used to flush cell suspensions to remove hydrogen and lower chlorinated benzenes, which would otherwise be considered to be dechlorinated products in the following dechlorination activity tests.

2.5 Analytical procedures

2.5.1 Chlorobenzene analysis

Chlorobenzene concentrations from cultures utilising TCBs were determined by removing 1 ml aliquots of bacterial suspension from culture flasks with a glass syringe, followed by extraction with 1 ml of hexane. Analysis of the extracts was done by gas chromatography with flame ionisation detection. 2,4-dichlorotoluene was used as an internal standard as described by Adrian et al (1998).

Chlorobenzenes concentrations from cultures utilising PeCB and HCB were quantified by extracting 1 ml of bacterial suspension with 1 ml of dichloromethane and successive capillary gas-chromatographic analysis. The use of dichloromethane for extraction resulted in a lower detection limit for HCB and PeCB compared to extraction with hexane. The temperature program was adapted to separate TeCBs, PeCB and HCB. Splitless injection was done at a column temperature of 55°C. After 1 min the split was opened and the column temperature was steadily increased to 225°C at a rate of 6°C/min. Chlorobenzene congeners were identified and quantified by injecting single compound standards.

2.5.2 Assay of dehalogenase activity

Dehalogenase activity was determined routinely with whole cells of strain CBDB1 using reduced methyl viologen as an artificial electron donor. The assay solution contained 100 mM

tris-HCl, pH 7.5, 1 mM methyl viologen, 2 mM Titanium(III) citrate and nominal concentrations of 50 µM 1,2,3-TCB, 50 µM 1,2,3,4-TeCB, 15 µM PeCB or 15 µM HCB (Hölscher et al 2003). The chlorobenzenes were added as 5 mM solutions in acetone. 800 µl of assay solution was filled in a 2 ml auto sampler vial in an anaerobic chamber and tightly closed with a Teflon-lined butyl septum and an aluminium crimp cap.

To test different redox compounds as artificial electron donors, methyl viologen in the assay solution was replaced by 1 mM concentrations of ethyl viologen, benzyl viologen, AQDS, DMN or pyrroloquinoline quinone.

200 µl of bacterial cultures pregrown with TCB, PeCB or HCB as electron acceptor and containing between $2x10^7$ and $5x10^7$cells/ml were added with a syringe and the test was incubated at 25°C. The reaction was stopped after 120 min (when necessary, incubation was continued to 10 h) by extracting the reaction mixture with 1 ml of hexane or dichloromethane (if PeCB or HCB was used as electron acceptor). Concentrations of the produced chlorobenzenes were determined by gas chromatography as described above. Reductive dechlorination of chlorinated benzenes results in the stoichiometric formation of lesser-chlorinated benzenes as end products. Therefore, enzymatic activities were calculated from the sum of all formed products and are expressed in nkat (nmol products per second at 25 °C).

2.5.3 Hydrogenase assay

Hydrogenase activity was assayed in a butyl rubber stoppered glass cuvette spectrophotometrically measuring the hydrogen dependent reduction of 1 mM benzyl viologen (ε = 9.75 $mM^{-1}cm^{-1}$) at 546 nm (Schneider and Schlegel 1976). The reaction mixture contained 1 mM benzyl viologen in nitrogen saturated 100 mM Tris-HCl (pH 7.5) buffer. The assay solution was filled in a 1 ml cuvette in an anaerobic chamber containing 5 % H_2.

The reaction was started by the addition of cell suspension or cellular fraction. The catalytic activity of hydrogenase was determined by selecting the linear region with the highest change in extinction from the absorbance curve. The increase in absorbance was measured over time with a spectrophotometer (Uvikon 923 Double beam UV/VIS). According to the experiments the hydrogenase activity was expressed as specific activity in nkat (nmol products per second at 25°C) per mg total protein or pkat (pmol products) per ml culture.

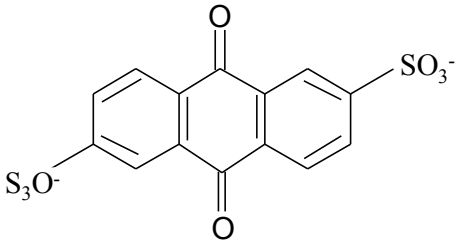
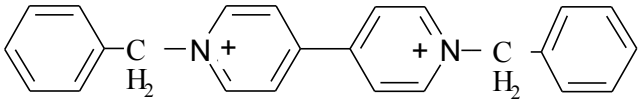
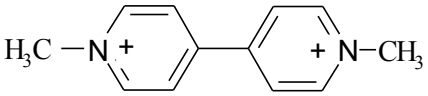
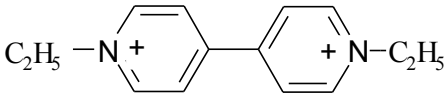
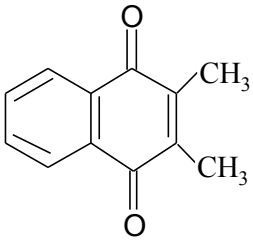
To test different redox active compounds as electron acceptors, benzyl viologen in the assay solution was replaced by 1 mM methyl viologen, ethyl viologen, AQDS or DMN.

A list of redox active compounds with their redox potentials and structures is given in table 2.3. Wavelengths used were: 450 nm for AQDS (ε = 1.8 $mM^{-1}cm^{-1}$), 578 nm for methyl

viologen (ε = 9.7 $mM^{-1}cm^{-1}$), and ethyl viologen (ε = 10 $mM^{-1}cm^{-1}$), 546 nm for benzyl viologen (ε = 9.75 $mM^{-1}cm^{-1}$), 270 nm for DNM (ε = 15.2 $mM^{-1}cm^{-1}$). To study the effect of 1,2,3,4-TeCB on hydrogenase activity of strain CBDB1, the compound AQDS was used as electron acceptor.

Diaphorase (NADH: benzyl viologen oxidoreductase) activity in the soluble fraction was assayed in a butyl rubber stoppered glass cuvette by spectrophotometrically measuring the reduction of 1 mM benzyl viologen using 1 mM NADH as electron donor in the presence of nitrogen in the head space (Schneider and Schlegel 1976).

Table 2.3 Structures and reduction potentials of redox compounds used in hydrogenase activity test.

Electron acceptor	Structure	E_o'(mV)
AQDS		-184
Benzyl viologen		-360
Methyl viologen		-450
Ethyl viologen		-480
DMN		-240

2.5.4 Cell count and total protein estimation

Direct counting of cells in a counting chamber was not possible, because cells of *Dehalococcoides* strain CBDB1 are very small (≤ 1 µm) and diffused rapidly out of focus. For estimating cell numbers 10 µl of a culture was placed on a microscopic slide and covered with a 20x20 mm cover glass. This procedure led to an even distribution of the culture volume underneath the total area of the cover glass, forming a thin film with a thickness of about 25 µm, in which the diffusion of cells out of focus was reduced compared to a counting chamber with a film thickness of 100 µm. The average number of cells observed in 20 different microscopic fields was used to calculate the estimated number of cells per ml. Counting was done on a phase contrast microscope (Axioskop 2 plus, Zeiss, Jena, Germany).

Total protein was quantified by a fluorescent test (NanoOrange-kit, Molecular Probes, Leiden, The Netherlands). According to the instructions of the manufacturer excitation/emission wavelengths of 485/590 nm were used. Fluorescence measurements were carried out on a RF-5001PC-fluorometer (Shimadzu, Tokyo, Japan).

2.6 Biochemical methods

2.6.1 Optimum pH and temperature stability of whole cell hydrogenase activity

The optimum pH for the whole cell hydrogenase activity was determined by estimating the hydrogenase activity using phosphate or Tris-HCl buffers with pH from 6 to pH 8.5. The temperature stability of hydrogenase activity was determined by incubating 2 ml of bacterial suspension anoxically in individual vials stoppered with Teflon lined butyl rubber septa. The vials were incubated for 2 hours at various temperatures ranging from 4°C to 70°C prior to the determination of hydrogenase activity.

2.6.2 Effect of metal ions on hydrogenase activity

Effect of metal ions (Ni^{2+}, Cu^{2+} and Hg^{2+} as $NiCl_2$, $CuCl_2$ and $HgCl_2$) on hydrogenase activity was tested by measuring the enzyme activity with bacterial suspensions, membrane or soluble fractions in the presence of metal ions (75 µM). The metal ion stock solutions were previously flushed with nitrogen for 30 min.

2.6.3 Oxygen sensitivity of hydrogenase activity and stability of the enzymatic activity with respect to the redox potential

Stability of hydrogenase activity at a positive redox potential in the presence of oxygen was investigated by stirring 5 ml of culture aerobically. The change in redox potential upon exposure of culture to air was measured simultaneously with a platinum- Ag/AgCl electrode (Pt 4800 M5-S7/80; Mettler-Toledo, Steinbach, Germany). The measured values were

expressed relative to the standard hydrogen electrode by adding +200 mV (E_h of saturated Ag-AgCl electrode).

Hydrogenase activities were estimated with samples taken every 30 seconds and the corresponding redox potentials were recorded. The redox indicator resazurin was used (150 µl of 5 mg/ml) to alter the redox potential of the culture anoxically in the glove box, when a change in redox potential was needed in the absence of oxygen. Titanium(III) citrate (approximately up to the absorbance of 0.4 at 546 nm due to the reduction of benzyl viologen) was added in the above samples before hydrogenase activity could be determined.

2.6.4 Effect of electron transport inhibitors on reductive dechlorination reaction

The effect of electron transport inhibitors on reductive dechlorination reactions was studied with cell suspensions prepared as described above. The assay solution contained 200 µl cell suspension containing about $5x10^7$ cells/ml, 50 µM 1,2,3-TCB, 2 mM Titanium(III) citrate and inhibitor or ionophores in 100 mM Tris-HCl buffer at pH 7.5. The assay solution was filled in 2 ml vials in the anaerobic chamber containing 5% H_2. The vials were stoppered with Teflon lined butyl rubber septa and aluminium crimp caps. The tests were incubated overnight at 25^oC. Where indicated, HOQNO, TCS or DCCD were added. Table 2.4 summarises the details about the compounds, their assigned action and their concentrations used.

Table. 2.4 Inhibitors, electron donors and ionophore used in reductive dechlorination reaction.

Compound and concentration used	Function	Head space of the activity test vial containing intact cells, 1,2,3-TCB in Tris-HCl buffer.
HOQNO; 1µM	A menaquinone antagonist	Hydrogen
TCS; 1µM	Protonophore	Hydrogen
DMN; 0.1 mM	Menaquinone analogue (used as electron donor)	Nitrogen
Pyrroloquinoline quinone; 0.1mM	Used as electron donor	Nitrogen
DCCD; 0.5 mM	ATP-synthase inhibitor	Hydrogen

2.6.5 Effect of 1,2,3,4-TeCB on reductive dechlorination reaction

The effect of 1,2,3,4-TeCB on reductive dechlorination of other chlorobenzenes catalysed by whole cells of strain CBDB1 was also studied using cell suspensions prepared as described above. The assay solution contained 200 µl of cell suspension, chlorobenzenes (nominal concentrations of 50 µM 1,2,3-TCB, 50 µM 1,2,4,5-TeCB, 50 µM 1,2,3,5-TeCB or 15 µM PeCB), 2 mM Titanium(III) citrate in Tris-HCl buffer at pH 7.5. The chlorobenzenes were

added as 5 mM solutions in acetone. 800 µl of reaction mixture was transferred into 2 ml vials which were tightly closed with Teflon lined butyl rubber septa under an anaerobic chamber containing 95 % N_2 and 5 % H_2. The tests were incubated overnight at 25^{o}C with or without 10 µM of 1,2,3,4-TeCB and the dechlorinated products were analysed by gas chromatography as described above.

2.7 Molecular biological methods

2.7.1 Computer programs used

Several different computer programs were used for sequence comparison, homology searches, multiple sequence alignments, phylogenetic analyses and secondary structure predictions (Table 2.5).

Table. 2.5 Summary of computer programs and data bases used.

Internet program	Internet address	Remarks
Genome data of PCE dechlorinating *Dehalococcoides ethenogenes*	www.tigr.org	Data base for blast-homology search to identify ORFs coding for membrane bound and soluble hydrogenases
Blast search (National Center for Biotechnology Information, USA)	www.ncbi.nlm.nih.gov/BLAST/	BLASTX for DNA-data bank BLASTP for protein data bank
Clustalx-1.83 (MacOS-X.tar.gz)	http://www.embl-heidelberg.de/~chenna/clustal/darwin	Multiple sequence alignment
ExPASy Molecular Biology Server (Swiss Institute of Bioinformatics, Switzerland) A-Compute pI/MW tool B-PROSITE C-Transeq D-PredictProtein	www.expasy.org	Molecular biology data base A- Isoelectric point and Molecular weight estimation tool B- Swiss-Prot and TrEMBL, protein database C- Nucleotide to protein translation D-Secondary structure determination tool
Prediction of hydrophobicity of protein	http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html	To analyse and estimate number of hydrophobic segments in a protein
QAlign	http://www.ridom.de/qalign.shtml	Multiple sequence alignment and phylogenetic analysis
NEBcutter V2.0	http://tools.neb.com/NEBcutter2/index.php	Screening of restriction sites in DNA
Chromas 1.45	http://www.technelysium.com.au/chromas14x.html	Verification of DNA sequence and its correction

2.7.2 Homology search for the identification of putative hydrogenase encoding genes

Since strain CBDB1 has a close affiliation to the PCE dechlorinating strain *Dehalococcoides ethenogenes* strain 195 based on 16S rRNA sequence (Adrian et al 2000 a), the genome of *Dehalococcoides ethenogenes* strain 195 (www.tigr.org) was screened for [Ni-Fe] hydrogenase coding regions.

2.7.3 DNA isolation and quantification

For isolating DNA, a 10 fold concentrated cell suspension was used. Isolation of DNA was performed according to the manufacturer's instruction using the Qiagen DNeasy kit (QIAGEN GmbH, Hilden, Germany).

After the isolation of DNA, concentration was measured with a Uvikon 923 Double beam UV/VIS spectrophotometer. The absorption at 260 nm and 280 nm was measured with MQ water as reference, and the ratio of OD_{260}/OD_{280} and the concentration of DNA in the solution were calculated (Sambrook et al 1989).

2.7.4 Polymerase chain reactions and manipulation

Amplification of a putative hydrogenase gene containing region from DNA of strain CBDB1 was obtained by PCR (Coen 1996) using specific oligonucleotides. Specific oligonucleotides were designed from the genome data of the PCE dechlorinating bacterium *Dehalococcoides ethenogenes* (www.tigr.org) by homology search and multiple alignments of different hydrogenase genes. Oligonucleotides were obtained from Metabion GmbH, Martinsried, Germany. Table 2.6 summarises the list of primers used in this study.

Table 2.6 List of primers used.

Primers	Sequences (5'end to 3'end)
DCH1	Forward; 5'-GCGGGTTTAGACAAAAGAACCA-3' (Forward primer used for the PCR amplification of putative membrane bound [Ni-Fe] hydrogenase structural and accessory genes)
DCH3	Reverse; 5'-GTGGTGGAAATGATGAAGGC-3' (Reverse primer used for the PCR amplification of putative membrane bound [Ni-Fe] hydrogenase structural and accessory genes, nested PCR and for reverse transcription)
DCHK	Forward; 5'-ATGTTTAATACTAAACTTACTA-3' (Forward primer used for RT-PCR)
DCH2	Forward; 5'-CGCCTTTCTACCCCAGATAC-3' (Forward primer used for nested PCR)
hupS1	Forward; 5'-GGCAATCTGAAGGAGTGTGC-3' (Forward primer used for the amplification of hupS upstream)
hupS2	Reverse; 5'-TTTATGCTGAGGTAGGCGAA-3' (Reverse primer used for the amplification of hupS upstream)
hupD1	Forward; 5'-ATATTGGTCCTGGGTACAGGC-3' (Forward primer used for the amplification of hupD downstream)
hupD2	Reverse; 5'-GTGGTGGAAATGATGAAGGC-3' (Reverse primer used for the amplification of hupD downstream)

Taq polymerase and dNTPs were obtained from MBI fermentas. The relevant coding regions of DNA were amplified using an Eppendorf Master cycler (Hamburg, Germany).

25 µl of each PCR mixture contained, genomic DNA 5 µl (2 µg), 10X PCR-buffer 2.5 µl, dNTPs (each 200 µM) 2 µl, primer forward (25 pmol/µl) 1 µl, primer reverse (25 pmol/µl) 1 µl, DMSO 0.5 µl, *Taq* polymerase (4 U/µl) 0.5 µl and PCR water 12.5 µl.

The reaction mixture was subjected to 30 amplification cycles consisting of: denaturation at 92 ^{o}C for 3 min, primer annealing at 56^{o}C for 30 sec and elongation at 72^{o}C for 1 min. A final extension of 6 min at 72^{o}C was performed.

PCR amplified products were purified prior to subsequent manipulations using the Invisorb Spin PCRapid Kit (Invitek, Berlin, Germany) and analysed by electrophoresis using 1 % agarose gels. Standard DNA marker, 2-log DNA ladder was obtained from New England Biolabs.

2.7.5 Isolation of total RNA

Total RNA was isolated from concentrated culture having hydrogenase activity. According to the instructions of the manufacturer, 1 ml of TRI reagent (Molecular Research Center, Inc) was used for 1 ml of 10 fold concentrated cell suspensions.

After 5 min incubation at room temperature, phase separation was done by adding 0.2 ml chloroform per 1 ml of TRI reagent and the sample was shaken vigorously for 15 seconds. The resulting mixture was stored at room temperature for 15 minutes and centrifuged at 12,000g for 15 minutes at 4^{o}C. Following centrifugation, the colourless upper aqueous phase, where RNA remains exclusively was collected carefully.

RNA precipitation was done by adding 0.5 ml isopropanol to the aqueous phase and the mixture was stored for 10 minutes at room temperature and centrifuged at 12,000g for 8 minutes at 4^{o}C. Finally the supernatant was removed and the RNA was washed with 1 ml of 75 % ethanol followed by centrifugation at 7,500g for 5 minutes at 4^{o}C. The resulted sediment was air dried for 2-3 min and redissolved in 50 µl diethyl pyrocarbonate (DEPC)-treated water (Applichem, Darmstadt, Germany).

2.7.6 RT-PCR

Reverse transcriptase PCR (RT-PCR) was performed by using 5 µl (1 µg) of the total RNA in a 25 µl reaction containing the following: 25 pmol of specific primer DCH3, 10 mM dNTP mix, 5 µl 5X reaction buffer, 1 µl (20U) ribonuclease inhibitor and 1 µl (200U) RevertAid M-MuLV reverse transcriptase (RevertAid First Strand cDNA Synthesis Kit, MBI fermentas). cDNA synthesis was done by incubating the above mixture at 42^{o}C for 60 min. The reaction was stopped by raising the temperature to 70^{o}C for 10 min and chilled on ice. The first strand cDNA thus synthesised was subsequently used for the PCR amplification.

25 µl of PCR reaction mixture contained 3 µl cDNA, 1 µl of 200 µM dATP, dCTP, dGTP, and dTTP, 0.5 µl (2.5U) Taq polymerase, 1 µl of 25 pmol each primer DCHK, DCH3 and 2.5 µl 10X PCR reaction buffer. The reaction mixture was subjected to 30 amplification cycles consisting of: denaturation at 92^{o}C for 3 min, primer annealing at 56^{o}C for 30 sec and elongation at 72^{o}C

for 1 min. A final extension of 6 min at 72^{o}C was performed. In the negative control, RNA instead of cDNA or RNA sample incubated under the conditions used for cDNA synthesis without reverse transcriptase was used as a template under the same conditions.

2.7.7 Nested PCR

To amplify a fragment inside the gene cluster, nested PCR was performed with RT-PCR product using the primers DCH2 (an internal primer) and DCH3. Same primer pairs were used in a control experiment in which, genomic DNA of strain CBDB1 was used as a template instead of the RT-PCR product.

2.7.8 Restriction digestion analysis

Restriction digestion analysis was performed with PCR amplified fragments as well as with RT-PCR products to check the sequence similarity and the arrangement of hydrogenase genes in the amplified locus. A restriction enzyme (BsaA1) with recognition site 5'-Y(C/T)ACGTR(A/G)-3' was obtained from New England Biolabs and was used according to the recommendations of the manufacturer.

After the digestion reaction, fragments of DNA were analysed by the agarose gel electrophoresis as described above.

Sequencing of the amplified DNA fragments was done by the sequencing service, Martin Meixner (Humboldt University, Berlin, Germany).

3. Dehalorespiration with hexa- and pentachlorobenzene by *Dehalococcoides* species strain CBDB1

Abstract

The dehalorespiring anaerobe *Dehalococcoides* sp. strain CBDB1 used HCB and PeCB as electron acceptors in an energy-conserving process with hydrogen as electron donor. Previous attempts to grow *Dehalococcoides* sp. strain CBDB1 with HCB or PeCB as electron acceptors failed if these compounds were provided as solutions in hexadecane. However, *Dehalococcoides* sp. strain CBDB1 was able to grow with HCB or PeCB when added in crystalline form directly to cultures. Growth of *Dehalococcoides* sp. strain CBDB1 by dehalorespiration resulted in a growth yield (*Y*) of 2.1±0.24 g protein/mol Cl^- released with HCB as electron acceptor; with PeCB, the growth yield was 2.9±0.15 g/mol Cl^-. HCB was reductively dechlorinated to PeCB, which was converted to a mixture of 1,2,3,5- and 1,2,4,5-TeCB. Formation of 1,2,3,4-TeCB was not detected. The final end-products of HCB and PeCB dechlorination were 1,3,5-TCB, 1,3- and 1,4-DCB, which were formed in a ratio of about 3:2:5. As reported previously, *Dehalococcoides* sp. strain CBDB1 converted 1,2,3,5-TeCB exclusively to 1,3,5-TCB, and 1,2,4,5-TeCB exclusively to 1,2,4-TCB. The organism therefore catalyses two different pathways to dechlorinate highly chlorinated benzenes. In the route leading to 1,3,5-TCB, only doubly flanked chlorine substituents were removed, while in the route leading to 1,3-and 1,4-DCB via 1,2,4-TCB singly flanked chlorine substituents were also removed.

Reductive dehalogenase activity measurements using whole cells pregrown with different chlorobenzene congeners as electron acceptors indicated that different reductive dehalogenases might be induced by the different electron acceptors. This is the first demonstration of dehalorespiration with a pure culture depending on PeCB/HCB.

3.1 Introduction

The environmental and health perspectives of PeCB and HCB contamination have already been dealt in Chapter 1. Under natural conditions, HCB and PeCB can persist for many decades (Oliver and Nicol 1992). However, some mixed cultures have been reported that are able to reductively dechlorinate these highly chlorinated benzenes under anaerobic conditions to less chlorinated benzenes (Fathepure et al 1988; Holliger et al 1992, Beurskens et al 1994; Chang et al 1998; Yeh and Pavlostathis 2001). Before this study, no pure culture that transforms PeCB or HCB had been described. Several previous attempts to demonstrate dechlorination of PeCB and HCB with strain CBDB1 were failed. However, PeCB dechlorination was observed in a mixed culture containing strain CBDB1 (Adrian et al 2000 a). In this chapter, reductive dechlorination of PeCB and HCB by strain CBDB1 after adding these compounds as crystals to the growth medium is described. Furthermore, the work in this chapter demonstrates dehalorespiratory growth of strain CBDB1 with both PeCB and HCB. The results in this chapter were recently published (Jayachandran et al 2003).

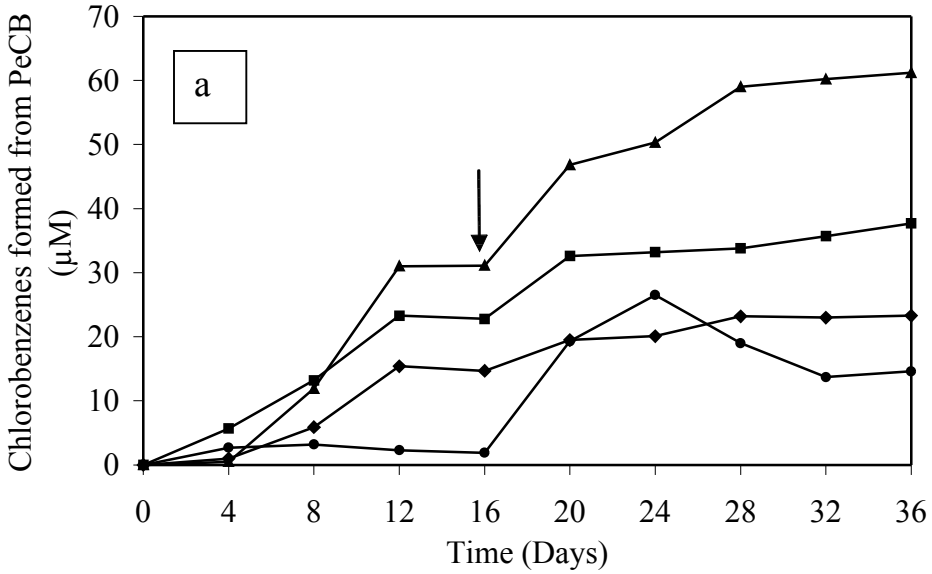
3.2 Results

3.2.1 Dechlorination of PeCB and HCB

Pure cultures of *Dehalococcoides* strain CBDB1 which had been grown with 15 µM 1,2,3- and 15 µM 1,2,4-TCB as electron acceptor were used as an inoculum to asses the dechlorination potential towards highly chlorinated benzenes. Addition of PeCB or HCB to a nominal concentration of 10 mM as a solution in hexadecane did not result in the reproducible production of less chlorinated benzenes even after 3-5 months of incubation. Only trace concentrations below 2 µM of dechlorination products could be detected. However, in cultures in which PeCB or HCB were added as crystals, significant concentrations of less chlorinated products (> 3 µM) could be detected within 4 days of incubation (Fig. 3.1). The cultures were subcultured eight times using 0.2 % inocula within one year in medium containing acetate, hydrogen and crystals of PeCB or HCB. Reproducible growth of strain CBDB1 was observed. Strain CBDB1 dechlorinated both HCB and PeCB to the products 1,3,5-TCB, 1,3-DCB and 1,4-DCB in a ratio of about 3:2:5 (Fig. 3.1). These products were not further dechlorinated. Product formation was much faster in cultures with PeCB crystals than in cultures with HCB crystals (Fig. 3.1). Only trace concentrations below 2 µM of PeCB could be detected in cultures incubated with HCB within the first days of incubation. Intermediates detected both in cultures with HCB and in

cultures with PeCB were 1,2,4,5-TeCB, 1,2,3,5-TeCB, and 1,2,4-TCB. However, TeCBs could only be detected during the first days of incubation at concentrations below 2 µM. As reported previously, strain CBDB1 converted 1,2,3,5-TeCB exclusively to 1,3,5-TCB and 1,2,4,5-TeCB to 1,2,4-TCB (Adian et al 2000 a). Formation of 1,2,3,4-TeCB, 1,2,3-TCB, 1,2-DCB or monochlorobenzene was not detected. Control cultures not containing inocula or containing heat-sterilised inocula did not form dechlorination products of HCB or PeCB.

After 16 days of incubation PeCB concentrations in the aqueous phase were below the detection limit of about 0.5 µM and no crystals could be seen in the medium indicating that the crystals were completely consumed. Also, no further increase in the concentration of the dechlorination products was found between day 12 and 16. Therefore, further PeCB crystals were added to the cultures at day 16 leading to an increase of the product concentration at day 20 (Fig. 3.1a). Cultures containing HCB crystals showed only low initial aqueous concentrations of HCB below 3 µM. In contrast to cultures with PeCB, cultures with HCB contained these low concentrations of the substrate even at the end of the incubation period indicating a steady dissolution of the crystals throughout the incubation time. Accordingly, the concentrations of the dechlorinated products increased steadily throughout the experiment (Fig. 3.1b).



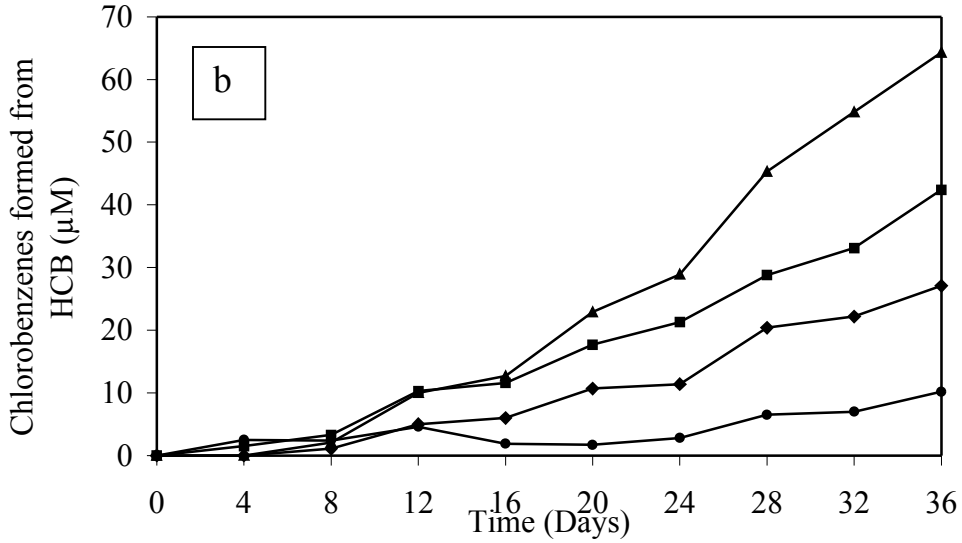


Fig. 3.1 Product formation in cultures with *Dehalococcoides* sp strain CBDB1 amended with crystals of PeCB (**a**) or HCB (**b**). (■) 1,3,5-TCB, (p) 1,4-DCB, (¿) 1,3-DCB, (˜) 1,2,4-TCB. No dechlorination was found in uninoculated controls or with autoclaved inocula. Arrow marks further addition of PeCB crystals.

3.2.2 Growth of strain CBDB1 based on HCB or PeCB dechlorination

Growth of strain CBDB1 with hydrogen as the electron donor and acetate as carbon source required the presence of HCB or PeCB as electron acceptor. Without chlorinated benzenes no increase in cell number or protein concentration was found. Both protein concentration and cell number indicated faster growth of strain CBDB1 with PeCB compared to growth with HCB as electron acceptor (Fig. 3.2). After 12 days, no crystals of PeCB were detectable visually and the PeCB concentration in the liquid phase was below the detection limit (< 0.5 µM). Also, no further growth of strain CBDB1 was observed. Cultures with HCB did show only slow growth, indicated by both growth parameters monitored. However, this growth was constant over the whole incubation time leading to 4.2×10^7 cells/ml and 0.98 µg/ml protein at day 36.

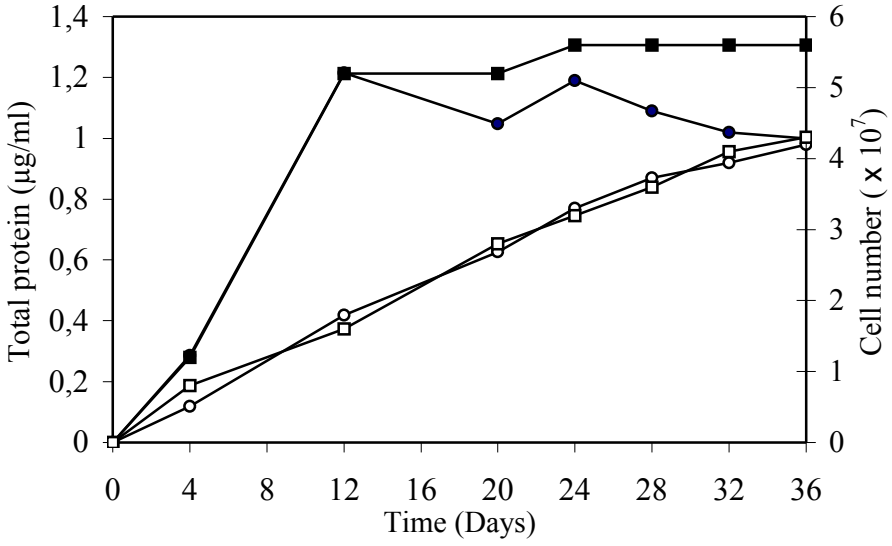


Fig. 3.2 Growth of *Dehalococcoides* sp strain CBDB1 on H_2/PeCB or H_2/HCB. Cell numbers of strain CBDB1 consuming PeCB (■), and HCB (£). Total protein yield of strain CBDB1 consuming PeCB (˜), and HCB (•). In inoculated controls containing no chlorobenzenes, no cells could be found and protein concentrations were constantly below the detection limit of about 0.1 µg/ml.

From the data shown in Fig. 3.1, molar growth yields were calculated for days 28, 32 and 36. With PeCB as electron acceptor an average molar growth yield of Y = 2.9±0.15 (g of protein/mole Cl^-) was found, while for HCB a value of Y = 2.1±0.24 (g of protein/ mole Cl^-) was calculated.

1,2,3,4-TeCB did not support growth of *Dehalococcoides* strain CBDB1 even when the compound was supplied in crystal form. When crystals of 1,2,3,4-TeCB were added to cultures amended with HCB, PeCB, or TCB as electron acceptors, no further increase in cell number or protein concentration occurred and dechlorination was completely inhibited.

3.2.3 Measurements of dehalogenase activities

Cultures grown on HCB, PeCB or 1,2,3-TCB were used to measure specific dehalogenase activities towards different chlorobenzene congeners. 1,2,3,4-TeCB and 1,2,3-TCB were tested because they have previously been shown to be good electron acceptors for strain CBDB1 reductive dehalogenases (Hölscher et al 2003). Cells grown on HCB or PeCB showed the highest specific dechlorination activities towards 1,2,3,4-TeCB followed by the specific activity towards PeCB and 1,2,3-TCB, whereas activity towards HCB was low (Table. 3.1). The highest specific

activity towards HCB was found in cultures grown on HCB. In contrast, cultures grown on 1,2,3-TCB did not show detectable HCB dechlorinating activity.

Table 3.1 Specific activities (nkat/mg) towards different chlorobenzene congeners with cultures grown on 1,2,3-TCB, PeCB or HCB.

Culture grown on	Specific activity towards (nkat/mg)[a]			
	1,2,3-TCB	1,2,3,4-TeCB	PeCB	HCB
1,2,3-TCB	6.7	64	19	≤ 0.1
PeCB	8.1	310	22	0.7
HCB	8.9	200	183	3.36

[a]nkat=nmol s^{-1}; tests were incubated for 2 h (TCB, TeCB, PeCB) or 4 h (HCB) at 25 °C; values are relative to protein. Numbers given are means of duplicate determinations.

3.3 Discussion

Dehalococcoides strain CBDB1 is the first isolated pure bacterial strain respires with PeCB and HCB. The solubility in water for the used chlorobenzene congeners (in µM at 22°C) differ widely as summarised by Holliger et al. (Holliger et al 1992): HCB 0.4; PeCB 1.0; 1,2,3,4-TeCB 16; 1,2,3-TCB 66; 1,4-DCB 333; 1,3-DCB 469. While TCBs and DCBs are toxic for chlorobenzene dechlorinating bacteria below their saturation level in aqueous solution (Holliger et al 1992; Adrian et al 1998) solutions containing crystals of HCB and PeCB are non-toxic to strain CBDB1 and were dechlorinated efficiently.

In contrast to HCB and PeCB, 1,2,3,4-TeCB appeared to be toxic for strain CBDB1 when added in crystal form since it inhibited completely growth and dechlorination of other chlorobenzenes. The molecular basis for the inhibitory effect of 1,2,3,4-TeCB is not known. However, the reductive dehalogenase activity was not affected as whole cells of strain CBDB1 quickly dechlorinated 1,2,3,4-TeCB when methyl viologen was used as electron donor (Table 1 and Hölscher 2003).

Recently, a mixed culture containing the polychlorinated biphenyl-dechlorinating bacterium DF-1 was described to dechlorinate exclusively doubly flanked chlorine substituents from PeCB and HCB added as a solution in acetone (Wu et al 2002 a). The culture dechlorinated about 36 µM of PeCB to 1,2,3,5-TeCB and 1,3,5-TCB within 14 days of incubation. After 56 days, about 85 % of the added PeCB accounting for about 150 µM was dechlorinated to the only final product 1,3,5-TCB (Wu et al 2002 a). Strain CBDB1 formed about 75 µM dechlorination products from PeCB crystals within 12 days (Fig. 3.1a).

The sole product formed from 1,2,3,5-TeCB is 1,3,5-TCB for both strain CBDB1 (Adrian et al 2000 a) and the mixed culture containing strain DF-1 (Wu et al 2002 a). Whereas the sole dechlorination product formed by the mixed culture containing strain DF-1 from PeCB is 1,2,3,5-TeCB, strain CBDB1 in addition forms 1,2,4,5-TeCB from PeCB. 1,2,4,5-TeCB is exclusively converted to 1,2,4-TCB by strain CBDB1 and finally leads to the formation 1,3- and 1,4-DCB as reported earlier (Adrian et al 2000 a). In this second pathway singly flanked chlorine substituents are also removed (Fig. 3.3).

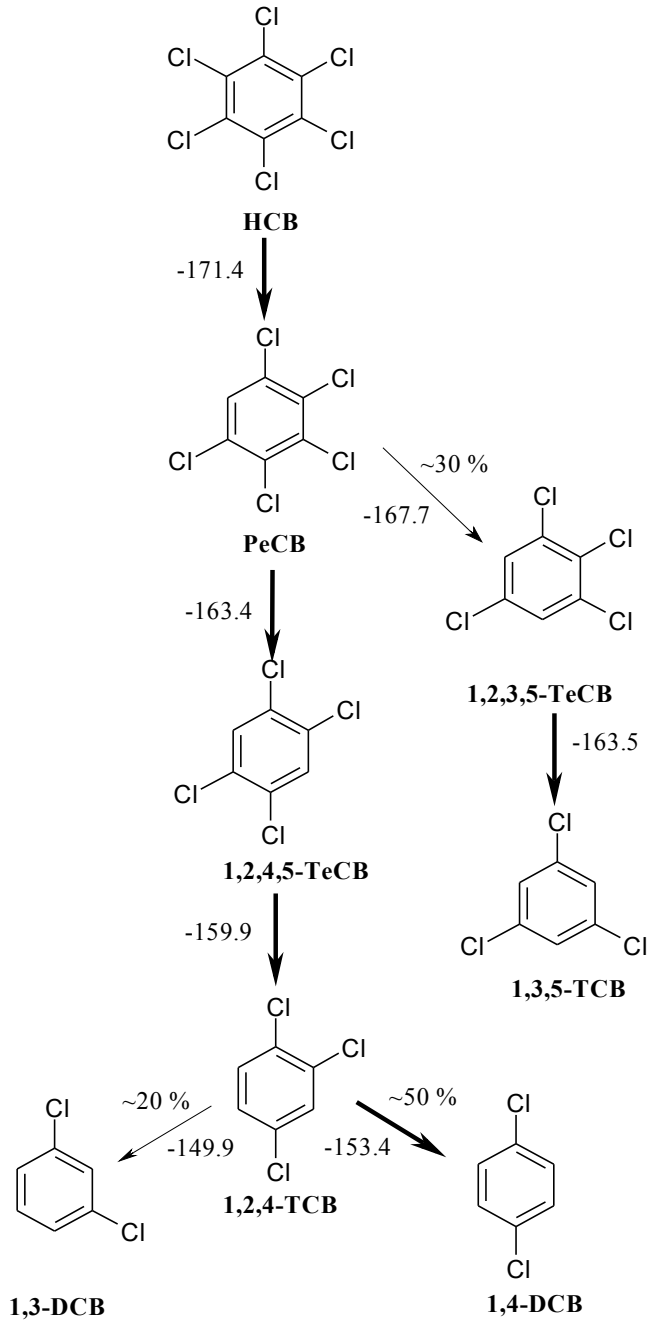


Fig. 3.3 Observed pathway of HCB and PeCB reductive dechlorination by *Dehalococcoides* sp strain CBDB1. The values indicate G_o' of the reaction in kJ/mol and the relative amounts of product formation, which are related to the total amount of products detected. Other cholorobenzene dehalogenation reactions are not catalysed by strain CBDB1 as determined using the different chlorobenzene congeners separately. Bold arrows indicate the major dechlorination pathways.

Dolfing and Harrison (Dolfing and Harrison 1992) provided values for the Gibbs free energy changes for the reductive dechlorination of polychlorinated benzenes with hydrogen as electron donor. According to these values it can be hypothesised that microbially catalysed dechlorination pathways of polychlorinated benzene congeners follow those steps, which are accompanied by the largest change in $\Delta G_o'$. For example, from PeCB the most likely dechlorination product would be 1,2,3,5-TeCB $\Delta G_o'$ = -167.7 kJ/mol) while conversion of PeCB to 1,2,4,5-TeCB $\Delta G_o'$ = -163.4 kJ/mol) or PeCB to 1,2,3,4-TeCB ($\Delta G_o'$ = -161.1 kJ/mol) would be less favourable. Conversion of PeCB to 1,2,3,5-TeCB and finally to the dead end product 1,3,5-TCB was observed in mixed cultures by Fathepure et al (1988), Holliger et al (1992), Chang et al (1998), and Wu et al (2002 a). This dechlorination sequence is accompanied by a total change in $\Delta G_o'$ of -331.2 (kJ/mol). Dechlorination of PeCB to DCBs via 1,2,4,5-TeCB and 1,2,4-TCB was observed as a minor pathway (below 10 %) by Fathepure et al (1988). In contrast, strain CBDB1 converted about 65 % of added PeCB to DCBs via 1,2,4,5-TeCB and 1,2,4-TCB which is accompanied by a total $\Delta G_o'$ of -473.2 (kJ/mol) for 1,3-DCB and -476.7 (kJ/mol) for 1,4-DCB (Fig. 3.3).

Strain CBDB1 could be indefinitely subcultured in a medium containing acetate, hydrogen, and PeCB or HCB as electron acceptors indicating that PeCB and HCB are used in a respiratory process for energy conservation. Increase of cell numbers and cell protein (Fig. 3.2) concurrent with dechlorination of PeCB (Fig. 3.1a) or HCB (Fig. 3.1b) as well as the cessation of growth after depletion of PeCB further supports the conclusion that growth is coupled to PeCB or HCB dechlorination.

The observed growth yields with HCB and PeCB of 2-3 g of protein per mole chloride released are similar to those described previously for other bacteria growing by dehalorespiration. Reported growth yields range from 0.2 g/mol Cl^- for a vinyl chloride respiring *Dehalococcoides* species (He et al 2003) to about 5 g/mol Cl^- for two other chloroethene respiring *Dehalococcoides* species (Maymò-Gatell et al 1997; Cupples et al 2003).

The dechlorination reactions observed with strain CBDB1 could be catalysed by a single enzyme with broad substrate specificity. However, taking also into account the results of Holliger et al (1992), Tiedje et al (1987), Chang et al (1998), and Wu et al (2002 a), where mixed cultures dechlorinated HCB and/or PeCB exclusively to 1,3,5-TCB, it could be that the two different identified pathways for PeCB dechlorination in strain CBDB1 are catalysed by different

enzymes. One enzyme would dechlorinate exclusively doubly flanked substituents catalysing the dechlorination of PeCB via 1,2,3,5-TeCB to 1,3,5-TCB. Other enzymes present in strain CBDB1, but not in the DF-1 containing culture, could dechlorinate PeCB to 1,4- and 1,3-DCB via 1,2,4,5-TeCB and 1,2,4-TCB. Our results indicate that the specific activity of strain CBDB1 towards HCB is highest in cultures grown with HCB and that the specific activity towards 1,2,3,4-TeCB is three to five times higher in cultures grown with PeCB or HCB than with 1,2,3-TCB. This suggests that different dehalogenase activities are inducible by different chlorobenzene congeners. Wu et al. (2002 a) found a longer adaptation time for the dechlorination of HCB than for PeCB possibly also indicating the need for an induction for the responsible dehalogenases. In contrast, 1,2,3-TCB dehalogenase activity is present in cells of *Dehalococcoides* strain CBDB1 irrespective of whether growth occurred with 1,2,3-TCB, PeCB or HCB although 1,2,3-TCB is not produced as an intermediate in HCB and PeCB dechlorination.

In summary, strain CBDB1 is capable of dehalorespiration using PeCB and HCB as electron acceptors and hydrogen as electron donor. We expect that the faster growth of strain CBDB1 utilising PeCB or HCB as electron acceptors compared to TCBs will enable us to investigate in more detail the physiology of strain CBDB1 to allow a valid strain description. Therefore, further experiments in the following chapters were carried out with strain CBDB1 growing on PeCB or HCB.

4. Hydrogenases of strain CBDB1 and their biochemical properties

Abstract

In this chapter the presence of an oxygen sensitive hydrogenase in the dehalorespiring *Dehalococcoides* species strain CBDB1 and some of its biochemical properties are described. Hydrogenase activity was detected in both membrane and soluble fractions. In addition to hydrogenase activity, diaphorase activity (NADH:benzyl viologen oxidoreductase) was detected in the soluble fraction. Metal ions Cu^{2+} and Hg^{2+} irreversibly inhibited hydrogenase activity in intact cells. Whereas Ni^{2+} ions reversibly inhibited the catalytic activity of hydrogenase both in membrane and soluble fractions. Inhibition of hydrogenase activity in intact cells by membrane non-permeable Cu^{2+} ions suggest that the active site of the membrane bound hydrogenase of strain CBDB1 is periplasmically oriented. Hydrogenase of strain CBDB1 was found to be highly sensitive to oxygen. About 80 % of the initial hydrogenase activity was inactivated irreversibly within 30 seconds when the cells were exposed to air even if the redox potential was still at -100 mV. In contrast, hydrogenase was found to be active at a positive redox potential of about +10 mV when this redox potential was established anoxically with the redox indicator.

4.1 Introduction

Within the last decade several *Dehalococcoides* species have been described to couple dechlorination and growth becoming one of the major genus among the reductively dechlorinating anaerobes identified to date (Maymò-Gatell et al 1997; Adrian et al 2000 a; Wu et al 2002 b; He et al 2003). Among *Dehalococcoides* species, the pure strain CBDB1 uses a wide spectrum of chlorinated organic compounds for energy conservation with hydrogen as electron donor (Adrian et al 2000 a; Bunge et al 2003; Jayachandran et al 2003). Strain CBDB1 allowed to study the molecular basis of this novel respiration process with respect to the dehalogenase and its properties (Hölscher et al 2003). Studies on primary dehydrogenases are also needed to understand the dehalorespiration process. As hydrogen is the only electron donor known to support growth of strain CBDB1 (Adrian et al 2000 a), we investigated the presence of hydrogenase, which should be one of the key enzymes.

Hydrogenases are a family of metalloenzymes of different constitution that catalyse the reversible oxidation of molecular hydrogen (Vignais et al 1995) (enzyme classification EC 1.18.99.1). Hydrogenases are found in a wide variety of microorganisms. Hydrogenase enables organisms to use hydrogen as a source of reducing equivalents under either aerobic or anaerobic conditions. Fermentative organisms can also use hydrogenases to dispose off reducing equivalents without the need of terminal electron acceptors other than protons (Adams and Martension 1984; Adams 1990). Depending on the metal contents of their active sites, hydrogenases can be divided into [Ni-Fe], [NiFeSe] (Albracht 1994), [Fe]-only (Adams 1990) and transition metal-free (Thauer et al 1996) hydrogenases.

The [Fe]-only containing hydrogenases have high specific activities and usually have the function to evolve hydrogen. On the other hand, [Ni-Fe] hydrogenases consist of a binuclear [Ni-Fe] center (Volbeda et al 1995) and are less active than [Fe]-only hydrogenases. The physiological role of [Ni-Fe] hydrogenases is usually restricted to the oxidation of hydrogen (Adams 1984). In aerobic hydrogen oxidising bacteria, [Ni-Fe] hydrogenases can function both as cytoplasmic NAD-reducing enzymes and as membrane bound parts of a respiratory chain where oxygen is the terminal electron acceptor (Bowien and Schlegel 1981; Albrecht 1993).

All membrane bound [Ni-Fe] hydrogenases, which have been sequenced so far, possess two highly conserved sequences Cys–x–x–Cys in the large subunit, one at the N and one at the C-terminus. In [NiFeSe] hydrogenases the first cysteine of the second motif is replaced by a

selenocysteine. The conservation of amino acids is less pronounced in the small subunit, which is responsible for electron transfer. A varying number of [Fe-S] clusters of different composition may participate in the electron transfer chain.

Comparing [Ni-Fe] and [Fe] hydrogenases, there is no significant homology between the polypeptides encoding the active sites of these enzymes. Therefore, it can be concluded that the two families have evolved independently.

In *Dehalococcoides* species, the function of hydrogenases is poorly understood. The membrane bound systems are thought to be involved in energy conservation but, the precise role of the enzymes and their biochemical properties are unclear. Therefore, we focused on the metabolism of hydrogen coupled to reductive dechlorination of chlorobenzenes by the anaerobic *Dehalococcoides* species strain CBDB1. This study addresses the presence of both membrane bound and soluble hydrogenases in strain CBDB1 and some of their biochemical properties.

4.2 Results

4.2.1 Hydrogenase activity of strain CBDB1

Hydrogenase activity of strain CBDB1 was detected in cultures growing with PeCB or HCB and hydrogen. Hydrogenase activity could be detected with whole cells of strain CBDB1 by the reduction of redox active compounds. Among the compounds tested as electron acceptors, the highest activity of hydrogenase was detected with benzyl viologen (Table. 4.1). Therefore this compound was used for estimating hydrogenase activity throughout this study. The hydrogenase activity measured with whole cells of strain CBDB1 also reduced methyl viologen, ethyl viologen and AQDS but not DMN (Table. 4.1). Reduction of benzyl viologen did not occur if the headspace of the cuvettes did not contain hydrogen.

Table. 4.1 Hydrogenase activity in whole cells of strain CBDB1 measured with various artificial electron acceptors. Enzyme activity was determined in 100 mM Tris-HCl buffer, pH 7.5 in the presence of a nominal concentration of 0.4 mM H_2.

Redox active compound		Hydrogenase activity[a] (nkat/mg protein)
Added as electron acceptor (1 mM)	Reduction potential $\Delta E_o'$ (mV)	
Benzyl viologen	-360	178±8.6
Methyl viologen	-450	130±7
Ethyl viologen	-480	110±10.7
AQDS	-184	150±8
DMN	-230	≤1

[a]Numbers are means of triplicate determinations ± standard deviation.

4.2.2 Optimum pH and temperature stability of whole cell hydrogenase activity

Hydrogenase activity of whole cells was determined between pH 6.5 and pH 8. The optimum of hydrogenase activity was found at pH 7.5 (Fig. 4.1). The thermal stability of the enzyme activity was determined after storage of samples for two hours at temperatures from 4°C to 70°C. Hydrogenase activity was most stable at 4°C, while already at 10°C the activity decreased within two hours by 15 %. A decrease of about 70 % and 90 % of the enzyme activity was detected with the samples incubated at 50°C and 70 °C (Fig. 4.2).

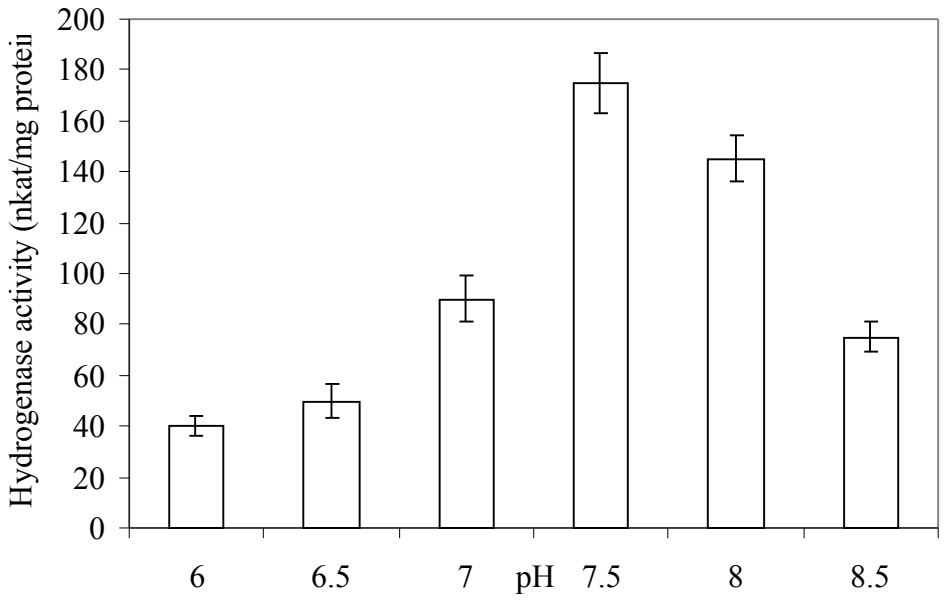


Fig. 4.1 Optimum pH for hydrogenase activity in whole cells of strain CBDB1. Enzyme activity was measured in a reaction mixture containing 100 mM Tris-HCl buffer at pH 7.5 in the presence of a nominal concentration of 0.4 mM H_2

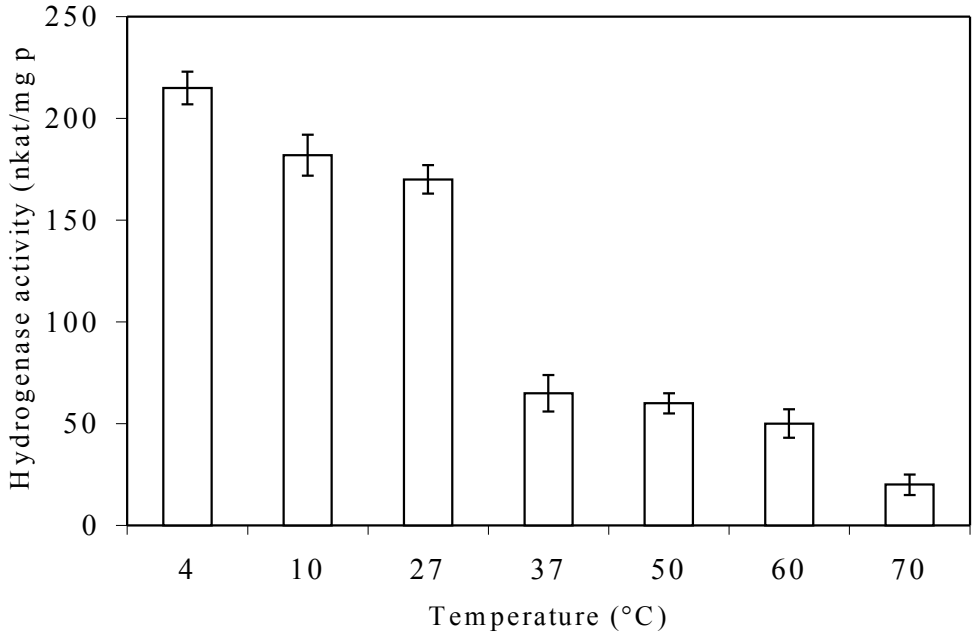


Fig. 4.2 Thermal stability of hydrogenase activity in whole cells of strain CBDB1. Enzyme activity was measured in a reaction mixture containing 100 mM Tris-HCl buffer at pH 7.5 in the presence of a nominal concentration of 0.4 mM H_2. The tests were incubated for 2 hours at temperatures ranging from 4^{o}C to 70^{o}C prior to the determination of hydrogenase activity

4.2.3 Localisation of hydrogenase activities in strain CBDB1

In order to localise the hydrogenase activity in strain CBDB1, cells of strain CBDB1 were disrupted with a small-scale ball mill under anoxic conditions.

4.2.3.1 Optimisation of cell disruption

To optimise the cell disruption, experiments were carried out according to central composite design (CCD) with various combinations of variables as given in Table. 2.2. The crude extract obtained after the cell disruption at each experiment was centrifuged to remove intact cells and hydrogenase activity was detected in the resulted cell free extract. The values obtained at each experimental point were analysed statistically to predict a response equation.

The following table gives experimental values along with the values predicted by the model. It is seen that the model predicts the actual response to a satisfactory extent. The model has a coefficient of multiple determinations 0.92 and the model is given as equation 3. The value of R^2 is a measure of total variation of observed values about the mean explained by the fitted model and it shows a good agreement between experimental and predicted values.

Table 4.2 Experimental design along with experimental and predicted values of hydrogenase activity in cell free extract of strain CBDB1.

S.No	Variable 1 Ratio (x_1) (culture vol/grams of glass beads)	Variable 2 (x_2) Shaking time (min)	Response (Hydrogenase activity in cell free crude extract, nkat/ml)	
			Experimental	Predicted
1	-1 (0.9)	-1 (15)	0.017	0.013
2	+1 (0.7)	-1 (15)	0.013	0.010
3	-1 (0.9)	1 (30)	0	-0.001
4	-1 (0.7)	1 (30)	0	-0.000
5	- α (0.9417)	0 (22.5)	0	0.003
6	+ α (0.6586)	0 (22.5)	0	0.001
7	0 (0.8)	- α (11.895)	0.013	0.016
8	0 (0.8)	+ α (33.1)	0	0.000
9	0 (0.8)	0 (22.5)	0.0014	0.014
10	0 (0.8)	0 (22.5)	0.0014	0.014

$$Y=0.004199-0.000525x_1-0.006169x_2-0.006038x_1^2-0.002687x_2^2+0.001050x_1x_2 \quad \text{.....(3)}$$

Optimum variables were obtained by solving the equation (3) and were, x_1 (ratio of culture volume to grams of glass beads) 0.61 and x_2 (time of shaking) 12.75 min.

4.2.3.2 Validation of the model by an experiment

To validate the predicted model, an experiment was performed with optimised variables. Simultaneously, two more experiments were carried out with two extremes of variables (un-optimised) (Table 4.3).

Table. 4.3 Cell disruption with optimised and unoptimised variables.

Experimental condition	Ratio (Culture vol/grams of beads)	Time of Shaking (mins)	Hydrogenase activity in cell free crude extract (nkat/ml)
Unoptimised variables (Exp1)	1	20	0.049
Unoptimised variables (Exp2)	0.65	35	0
Optimised variables	0.611	12.75	0.070

Therefore, the variables x_1(ratio) 0.61 and x_2 (time of shaking) 12.75 min could be the best set of combinations to disrupt cells of strain CBDB1 to maximise the yield of active hydrogenase to a possible extent.

The active cell free crude extract was further fractionated into membrane and soluble fractions by ultracentrifugation. Hydrogenase activity was detected in the membrane as well as in the soluble fraction (Table. 4.4). The total activity of both fractions, however, amounted to only 20 % of the activity found with whole cells. In the soluble fraction in addition to hydrogenase activity, diaphorase activity (NADH: benzyl viologen oxidoreductase) was also detected. The diaphorase activity was about 2.5-fold higher than the hydrogenase activity (Table. 4.4).

Table. 4.4 Localisation of hydrogenase activity in strain CBDB1.

Preparation of enzyme activity	Enzyme activity[a] (pkat/ml)
Intact cell suspension	180±8.6
Membrane fraction	16±0.8
Soluble fraction	21±1.1
NADH:benzyl viologen oxidoreductase in soluble fraction	51.3±2.7

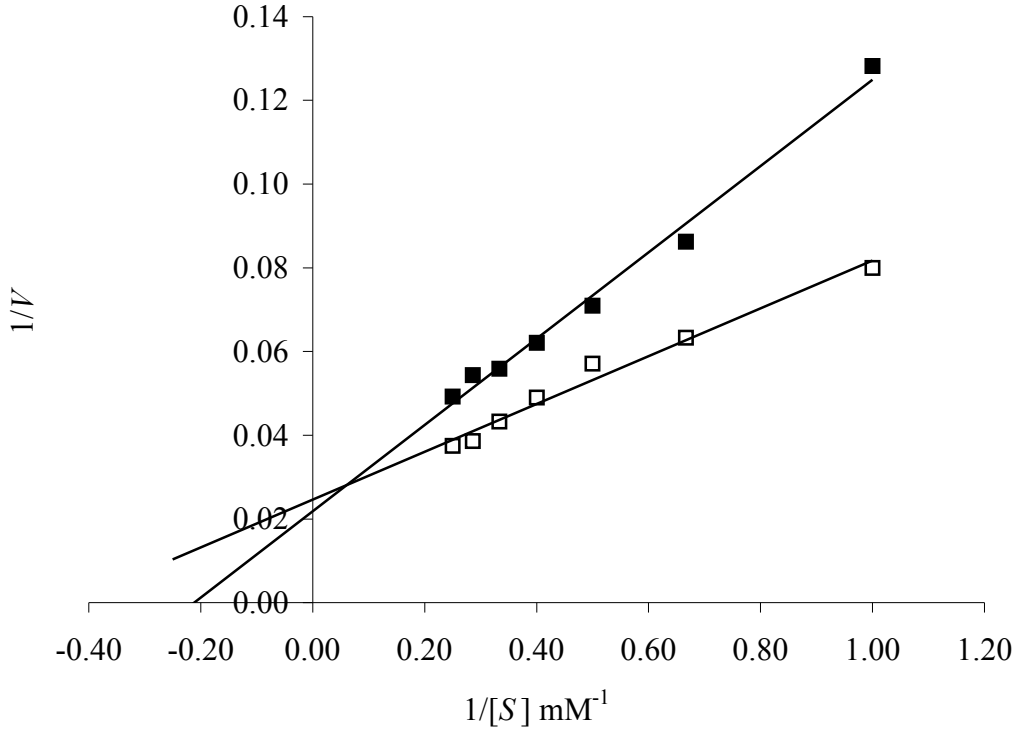
Enzyme activity was measured in a reaction mixture containing 100 mM Tris-HCl buffer at pH 7.5 in the presence of a nominal concentration of 0.4 mM H_2

[a] Values are means of triplicate determinations ± standard deviation; the activity is given in pmol s^{-1} ml^{-1}.

4.2.4 Effect of metal ions on hydrogenase activity

Whole cell hydrogenase activity was irreversibly inhibited by 75 µM Cu^{2+} and Hg^{2+}. The inhibitory effect of Ni^{2+} on the hydrogenase activity of both membrane and soluble fractions appeared to be reversible because a competitive effect was observed with respect to the substrate benzyl viologen. The inhibitory effect of Ni^{2+} on the catalytic activity of cell fractions was decreased substantially as the concentration of benzyl viologen increased (Fig. 4.3).

A



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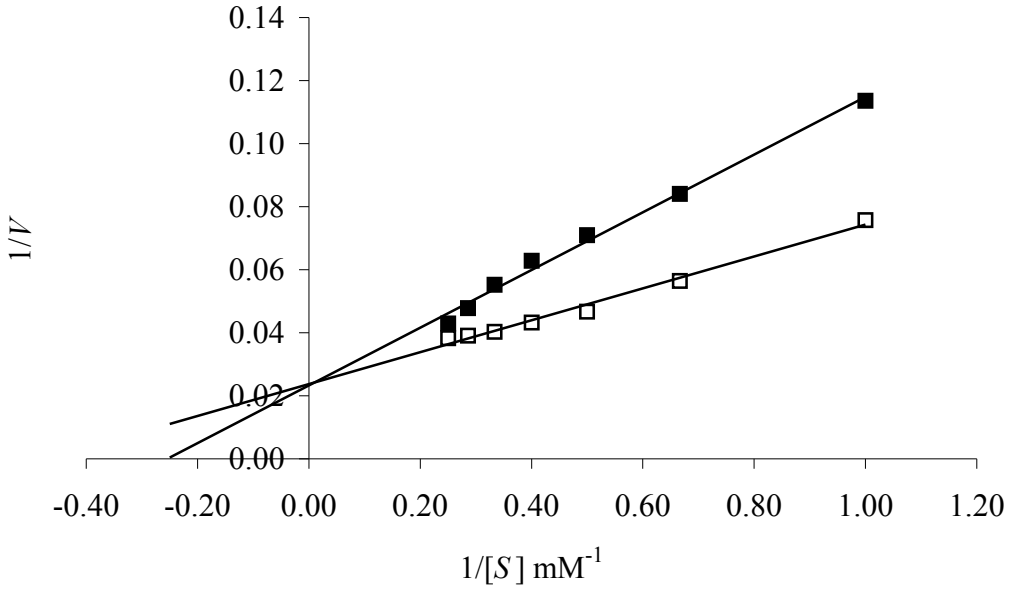


Fig. 4.3 Double reciprocal plots of the rate of hydrogen oxidation catalysed by membrane **(A)** and soluble fractions **(B)** of strain CBDB1 in the presence (■) and absence (□) of 75 μM Ni^{2+}. The reaction mixture contained 100 mM Tris-HCl buffer at pH 7.5 in the presence of a nominal concentration of 0.4 mM H_2.

4.2.5 Oxygen sensitivity of hydrogenase activity and stability of the enzymatic activity with respect to the redox potential

Hydrogenase activity was not detected in cell suspensions of strain CBDB1 in the absence of Titanium(III) citrate even under strict anoxic conditions. This suggested that the presence of reducing agent is necessary to maintain the stability of the enzyme. Early experiments in assessing the oxygen sensitivity of enzymes from strain CBDB1 showed that hydrogenase activity of intact cells was lost within 5 min of aerobic storage. Whereas, dehalogenase activity catalysing the reductive dechlorination of chlorobenzenes in crude extract of strain CBDB1 could still be detected even after 20 h of aerobic storage (Holscher et al 2003). In addition, air exposed cells (5 mins) inoculated into a fresh medium did not show any growth.

Therefore, investigations for redox dependence of hydrogenase activity in the absence or presence of air were needed. About 80 % of hydrogenase activity was inactivated irreversibly within 30 seconds when the cells were exposed to air. During this time the redox potential of the medium increased from -190 mV to -100 mV (Fig. 4.4).

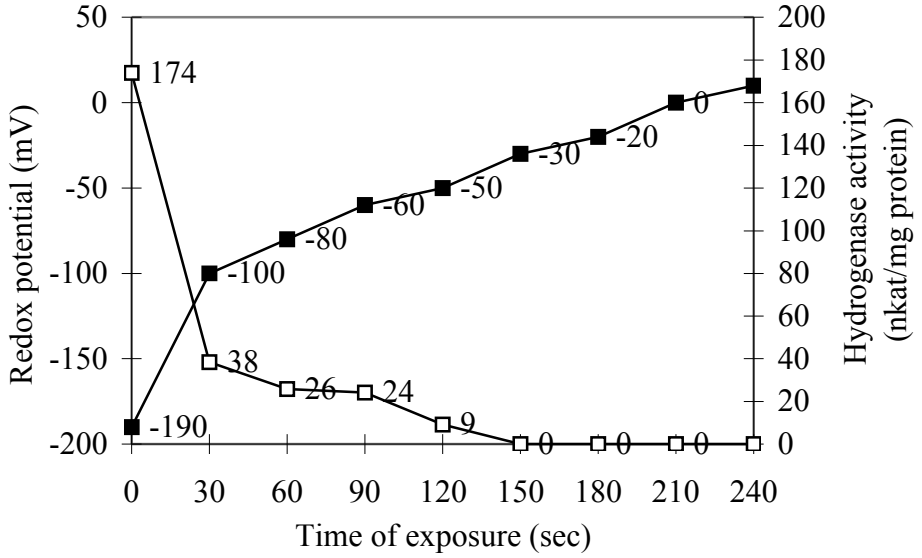


Fig. 4.4 Hydrogenase activity in whole cells of strain CBDB1 at various redox potentials in the presence of oxygen. Hydrogenase activity and redox potential were measured at every 30 seconds while the culture was stirred aerobically as described in Materials and methods.
(■) Redox potential, (□) hydrogenase activity. Values are means of two independent experiments.

In contrast, when the redox potential of the culture was altered in the absence of oxygen by successive addition of the redox indicator resazurin in an anaerobic chamber (till the appearance of slight pink where the redox potential was +10 mV) showed hydrogenase activity about 80 % of the control, which was not added with resazurin (Fig. 4.5).

This indicated that, hydrogenase of strain CBDB1 could be stable even at a positive redox potential of about +10 mV in the absence of oxygen (compare with Fig. 4.4). However, after 30 minutes of interval, loss of hydrogenase activity (where the redox potential of the sample and the control were +470 and +110 mV respectively) in both of the samples was detected.

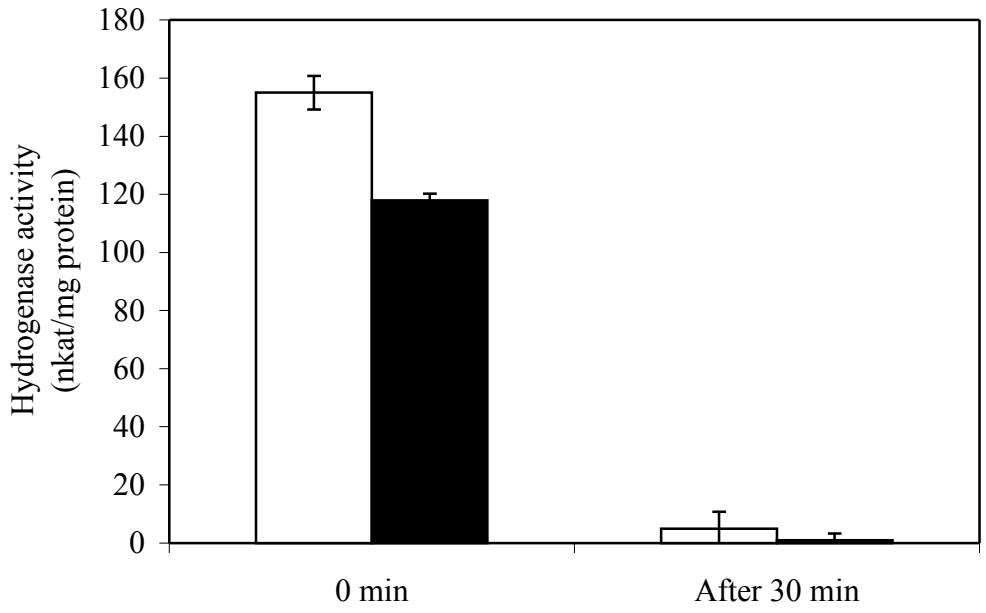


Fig. 4.5 Stability of hydrogenase at a positive redox potential in the absence of oxygen. (□) control with a redox potential of –190 mV, (■) redox potential adjusted to +10 mV by resazurin. The activity was determined as nkat/mg protein. Means of triplicate determinations are shown ± standard deviation.

4.3 Discussion

Hydrogenase activity was detected with whole cells, soluble and membrane fractions of strain CBDB1 by the reduction of benzyl viologen using hydrogen as electron donor.

Complete inhibition of hydrogenase activity in intact cells of strain CBDB1 by 75 µM Cu^{2+} and Hg^{2+} ions even at various increasing concentrations of benzyl viologen indicated that both of the metal ions inhibit hydrogenase activity irreversibly. In contrast, Ni^{2+} ions competitively inhibited the hydrogenase activity in cell fractions (Fig. 4.3).

Cu^{2+} and Hg^{2+}have been described to inactivate [Ni-Fe] hydrogenases irreversibly (Llama et al 1979; Cypionka and Dilling 1986). Inactivation of the enzyme could be due to the interaction of metal ions with functional groups of the protein molecule that are necessary for the catalytic action of hydrogenase. Competitive inhibition of hydrogenase activity by Ni^{2+} and non-competitive inhibition by Cu^{2+} and Hg^{2+} has also been demonstrated in the [Ni-Fe] hydrogenase of the purple sulphur bacterium *Thiocapsa roseopersicina* (Zadvorny et al 2000).

The dehalogenase activity in crude extract of strain CBDB1 was found to be stable even after 20 h of aerobic storage (Hölscher et al 2003). In contrast, hydrogenase activity was found to be highly sensitive towards oxygen (Fig. 4.4). Therefore, the instability of hydrogenase towards oxygen could be the main reason that the strain looses almost instantly its viability when exposed to air.

Complete inhibition of hydrogenase activity in intact cells by membrane-nonpermeating Cu^{2+} ions (Cypionka and Dilling 1986) suggests that the active site of membrane bound hydrogenase in strain CBDB1 is oriented to the outside of the cell. Rapid inactivation of hydrogenase activity in intact cells by air (Fig. 4.4) additionally supported the above observation.

The differences between the activities measured with whole cells, membrane and soluble fractions (Table 4.4) might be caused by several factors due to the method of cell disruption. Cell disruption by ball mill was carried out, because it was found to be the only reliable method to avoid inactivation of hydrogenase by oxygen. During the centrifugation steps involved in the preparation of cell fractions, a contamination with air might not be completely avoided. This could probably have resulted in loss of activity.

In summary, this chapter describes the presence of an oxygen sensitive hydrogenase in the dehalorespiring anaerobe strain CBDB1 and some of its biochemical properties. This preliminary

study is the first report on the hydrogenases of strain CBDB1 and it will be useful for further studies of the dehalorespiration process among *Dehalococcoides* species.

5. Electron transport in *Dehalococcoides* species strain CBDB1

Abstract

Chlorobenzene respiration was studied in *Dehalococcoides* species strain CBDB1. In the chlorobenzene dehalogenase assay with intact cells, only redox compounds with a standard redox potential of ≤-360 mV were found to be effective electron donors. This suggested that a low potential physiological electron donor is required for chlorobenzene reduction. Astonishingly, reduced pyrroloquinoline quinone (E_o' = +80 mV) also served as an artificial electron donor for 1,2,3-TCB reduction. DMN (a menaquinone analogue, E_o' = -240 mV) served neither as electron acceptor for the hydrogenase nor electron donor for the dehalogenase of strain CBDB1. Whole cells catalysed the reductive dechlorination of 1,2,3-TCB with hydrogen as electron donor. HOQNO, an antagonist of menaquinone dependent redox reactions had no effect on chlorobenzene dechlorination in cell suspensions. The ionophore TCS also did not inhibit the dechlorination reaction by cell suspensions. This suggests that strain CBDB1 does not require reverse electron transport for reductive dechlorination depending on a proton gradient.

1,2,3,4-TeCB at a nominal concentration of 10 µM strongly inhibited the reductive dechlorination of other chlorobenzenes by whole cells of strain CBDB1 with hydrogen as electron donor. However, the same concentration of 1,2,3,4-TeCB did not interfere with either hydrogenase or dehalogenase activity. A nominal concentration of 50 µM 1,2,3,4-TeCB inhibited the hydrogenase activity by about 25 %.

5.1 Introduction

Dehalococcoides species strain CBDB1 is the first pure isolate that is able to reductively dechlorinate chlorobenzenes and dioxins (Adrian et al 2000 a; Bunge et al 2003; Jayachandran et al 2003). The recent results of Fennell et al (2004) describe that the PCE dechlorinating *Dehalococcoides ethenegenes* strain 195 is also able to dechlorinate HCB, PeCB, TeCBs and dioxins. Energy metabolism and electron transport mechanism of dehalorespiration processes have been studied in PCE dechlorinating strains *Dehalobacter restrictus* (Schaumacher and Holliger 1996), *Desufurospirillum multivorans* (Miller et al 1997 b) and 3-CB dechlorinating bacterium *Desulfomonile tiedjei* DCB-1 (Louie and Mohn 1999). But, these aspects are yet to be investigated in strain CBDB1.

Hölscher et al (2003) reported that the chlorobenzene dehalogenase activity of strain CBDB1 is membrane associated. Results presented in Chapter 4 showed that the hydrogenase activity of strain CBDB1 is also membrane associated. Therefore, the membrane bound systems in strain CBDB1 are believed to be involved in the dehalorespiration process. An *in vitro* assay of dehalogenase using various artificial electron donors showed that only compounds having redox potentials of ≤-360 mV can be used by the enzyme (Hölscher 2003). This suggested that a low redox potential electron donor is required for the process of reductive dechlorination of chlorobenzenes although the standard redox potential of the chlorobenzene couples is always positive (eg, hexachlorobenzene/pentachlorobenzene = +478 mV).

The results of Miller et al (1997 b) indicate that reverse electron transport was required for the reductive dechlorination of PCE and that quinones were not involved in the electron transport chain of *Desufurospirillum multivorans*. In contrast, in another anaerobe, *Dehalobacter restrictus,* which is also capable of dehalorespiration with PCE and hydrogen, menaquinone was involved in PCE reduction (Schumacher and Holliger 1996).

In this chapter, experiments on the dehalorespiratory electron transport of strain CBDB1 are described. The aim was to find indications for the carriers that mediate electron transport in strain CBDB1.

5.2 Results

5.2.1 Artificial electron donors for the dehalogenase activity of strain CBDB1

Dehalogenase activity of whole cells of strain CBDB1 was assayed by the dechlorination of 1,2,3-TCB with an artificial electron donor as described in Materials and methods. Among the redox active compounds tested as artificial electron donors for the dehalogenase, only viologens with a redox potential of ≤-360 mV were effective electron donors (Table. 5.1). The highest enzyme activity was measured with methyl viologen (E_o' = -450 mV). The quinone analogue, AQDS did not serve as electron donor for the dehalogenase of strain CBDB1. However, PQQ in reduced form did support the reductive dechlorination but the activity was relatively low (Table. 5.1) $DMNH_2$, a menaquinone derivative, did not serve as electron donor for the 1,2,3-TCB dehalogenase reaction. No dechlorination was observed in the absence of any added electron donor.

Table. 5.1 1,2,3-Trichlorobenzene dehalogenase activity in intact cells of strain CBDB1 with various artificial electron donors. The concentrations of the electron donors were 1 mM. A nominal concentration of 50 µM 1,2,3-TCB was used as electron acceptor.

Electron donor	E_o' (V)	Specific activity (nkat/mg)[a]
Ethyl viologen	-0.48	5.8±0.4
Methyl viologen	-0.45	7.2±0.6
Benzyl viologen	-0.36	2.1±0.3
DMN	-0.24	≤ 0.1
AQDS	-0.18	≤ 0.1
PQQ	+0.5	0.7±0.2

[a] Numbers are means of triplicate determinations ± standard deviation.
One nanokatal is defined as 1 nmol of dechlorination product formed per s at 25 °C.

5.2.2 Effect of HOQNO and TCS on reductive dechlorination of 1,2,3-TCB with cell suspensions and hydrogen as electron donor

Dechlorination of 1,2,3-TCB to 1,3-DCB could be demonstrated using cell suspensions of strain CBDB1 with hydrogen as electron donor and 1,2,3-TCB as electron acceptor. HOQNO (1 µM), an antagonist of menaquinone dependent redox reactions (Zhao and Weiner 1998), did not inhibit the dechlorination reaction in intact cell suspensions (Fig. 5.1).

Also, TCS (1 μM), a protonophore that dissipates both membrane potential and the proton gradient did not inhibit the dechlorination reaction catalysed by cell suspensions in the presence of hydrogen (Fig. 5.1).

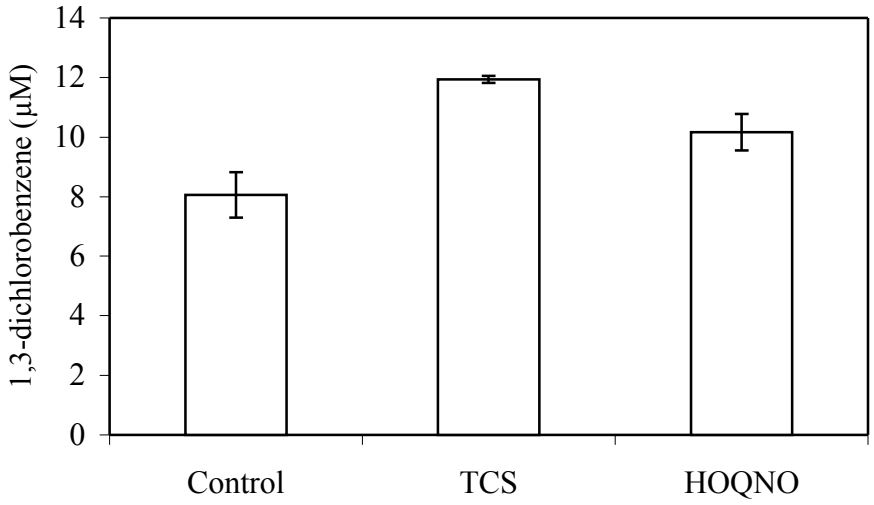


Fig. 5.1 Effect of TCS and HOQNO on reductive dechlorination of 1,2,3-TCB by cell suspensions of strain CBDB1 in the presence of a nominal concentration of 2 mM H_2 as electron donor. The dechlorination reaction was carried out as described in Materials and methods.

5.2.3 Inhibition of the dechlorination reaction by 1,2,3,4-TeCB

With reduced methyl viologen as artificial electron donor, reductive dechlorination of various chlorobenzenes could be demonstrated using whole cells of strain CBDB1. With this artificial test the highest specific activity was found with 1,2,3,4-TeCB (Jayachandran et al 2003). This indicated that 1,2,3,4-TeCB is a good substrate for the reductive dehalogenase activity. However, with strain CBDB1 inoculated in a medium containing 1,2,3,4-TeCB as electron acceptor and hydrogen as electron donor, no increase in cell number or protein concentration was observed (Jayachandran et al 2003), indicating that this compound cannot be used for growth. In addition, a nominal concentration of 10 μM 1,2,3,4-TeCB completely inhibited the dechlorination of other chlorobenzenes catalysed by whole cells of strain CBDB1 with hydrogen as electron donor. Significant inhibition of 1,2,3-TCB dechlorination was observed even at a 1 μM concentration of 1,2,3,4-TeCB (Table 5.2).

Table. 5.2 Inhibitory effect of 1,2,3,4-TeCB on the dechlorination reaction catalysed by whole cells of strain CBDB1 in the presence of a nominal concentration of 2 mM H_2 as electron donor.

Chlorobenzene used as electron acceptor	Dechlorination			
		(μM product/over night)[a]		
	Product determined	Without 1,2,3,4-TeCB	With 10 μM 1,2,3,4-TeCB	With 1 μM 1,2,3,4-TeCB
1,2,3-TCB (50 μM)	1,3-DCB	5.25±0.5	≤ 0.1	2.4±0.2
1,2,4,5-TeCB (50 μM)	1,2,4-TCB	2.96±0.2	≤ 0.1	n.d
1,2,3,5-TeCB (50 μM)	1,3,5-TCB	9.65±0.7	≤ 0.1	n.d
PeCB (15 μM)	1,2,4,5-TeCB	5.4±0.4	≤ 0.1	n.d

[a]Numbers (μM concentrations of dechlorinated products) are means of triplicate determinations ± standard deviation determined after overnight incubation.
Numbers in parentheses are the nominal concentration of chlorobenzenes as electron acceptors used. n.d; Not determined.

While 1,2,3,4-TeCB did not interfere with the dehalogenase activity, the effect of 1,2,3,4-TeCB on hydrogenase activity was investigated. Hydrogenase activity of strain CBDB1 could be demonstrated by the reduction of AQDS using intact cells in the presence of hydrogen as electron donor (Chapter. 4).

Figure 5.2 shows the change in absorbance of AQDS reduction by whole cells of strain CBDB1 in the presence and absence of 1,2,3,4-TeCB. However, 1,2,3,4-TeCB at a nominal concentration of 50 μM inhibited the hydrogenase activity of strain CBDB1 about 25 %.

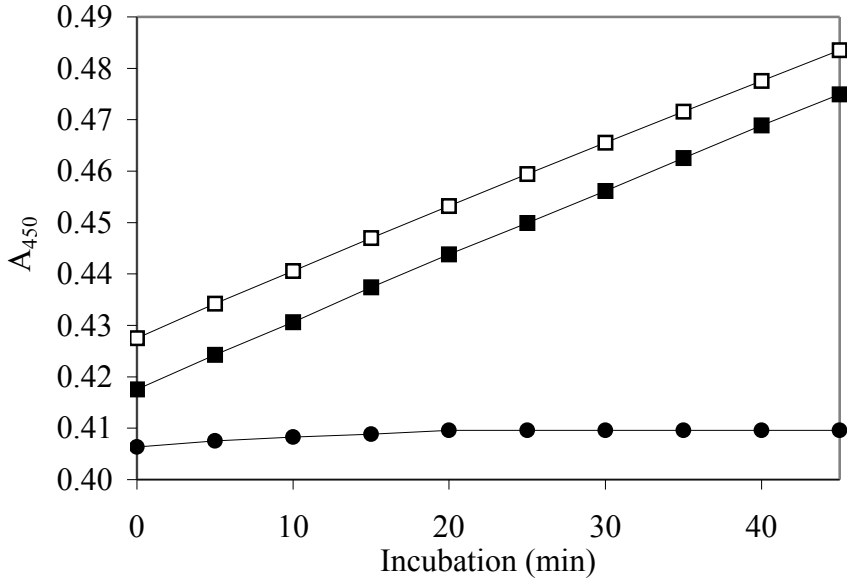


Fig. 5.2. Change in absorbance of hydrogen dependent AQDS reduction catalysed by whole cells of strain CBDB1 in the presence and absence of 1,2,3,4-TeCB. The reaction mixture in butyl rubber stoppered cuvettes contained 100 mM Tris-HCl buffer, pH 7.5, and nominal concentrations of 0.4 mM H_2 with (■) or without (□) 10 µM 1,2,3,4-TeCB. In the control experiment H_2 was omitted from the headspace (●).

5.2.4 Localisation of electron mediating components

To demonstrate that all necessary components of the electron transport chain in strain CBDB1 were associated with the membrane, membrane fractions were incubated with hydrogen and 1,2,3-TCB. Membranes could reductively dechlorinate 1,2,3-TCB to 1,3-DCB (6.3±0.4 µM after overnight incubation) indicating that all essential components needed for the dechlorination reaction are present in or associated with the membrane. Addition of soluble fraction of strain CBDB1 did not increase the dechlorination rate. The soluble fraction alone also did not support reductive dechlorination.

5.3 Discussion

5.3.1 Putative electron mediators

Although we could demonstrate both hydrogenase and dehalogenase activities of strain CBDB1 using artificial electron donors or acceptors, we were unable to identify the *in vivo* electron donor for the dehalogenase of strain CBDB1. The high rate of 1,2,3-TCB dechlorination measured with viologen dyes (Table. 5.1) having redox potentials of <-360 mV indicated that the dehalogenase needs a low potential electron donor. This could be due to the involvement of a low redox potential corrinoid co-factor in the dechlorination reaction since the cob(II)alamin/cob(I)alamin pair of corrinoid also has a low E_o' of -500 mV (Harder et al 1989).

Hölscher et al (2003) described that the dechlorination reaction catalysed by the dehalogenase of strain CBDB1 is corrinoid dependent. Corrinoid dependent dechlorination reaction has also been described in *Dehalobacter restrictus* (Schumacher and Holliger 1996), *Dehalococcoides ethenogenes* (Magnuson et al 1998) and *Desufurospirillum multivorans* (Neumann et al 1995; Neumann et al 1996).

Considering this low redox potential of the couple cob(II)alamin/cob(I)alamin, it is unlikely for a functional electron donor for the dehalogenase of strain CBDB1 having a redox potential less negative than -500 mV. Therefore, it is unlikely that a quinone, like menaquinone (E_o' = -74 mV) could be the electron donor for the reduction of cob(II)alamin to cob(I)alamin in the dehalogenase of strain CBDB1. Indeed HONOQ, an inhibitor of menaquinone dependent redox reactions did not inhibit the reductive dechlorination reaction catalysed by intact cells of strain CBDB1 with hydrogen as electron donor (Fig. 5.1). In addition, $DMNH_2$ a reduced menaquinone analogue did not support 1,2,3-TCB dechlorination (Table. 5.1). These two results indicate therefore, a quinoid is not a respiratory component in the electron transport of strain CBDB1. However, Schumacher and Holliger (1996) observed the involvement of menaquinone in PCE reduction by *Dehalobacter restrictus*. For thermodynamic reasons, use of menaquinone (E_o' = -74 mV) as the only electron donor for the reduction of cob(II)alamin to cob(I)alamin is unlikely.

Miller et al (1997 b) proposed a reaction mechanism for PCE reductive dehalogenase of *Desulfurospirillum multivorans,* which requires the endergonic transfer of a high-potential electron for the reduction of cob(III)alamin to cob(II)alamin. This suggested the involvement of a reverse electron flow driven by electrochemical proton potential.

Therefore, we performed experiments using proton gradient dissipating agents to investigate the involvement of reverse electron transport in strain CBDB1. If a proton gradient is required for reductive dechlorination, the protonophore TCS used here should have inhibited the dechlorination reaction. Rather, TCS promoted the reaction (Fig. 5.1) indicating that a pH gradient or membrane potential is apparently not required for the reductive dechlorination in strain CBDB1. This rules out that the involvement of reversed electron transport in reductive dehalogenation in strain CBDB1. This indication is also observed in PCE utilising *Dehalococcoides ethenogenes* strain 195 (Nijenhuis 2002).

Based on the *in vitro* dehalogenase activities measured with benzyl viologen as electron donor, the results of Hölscher et al (2003) suggested that the co-factor of the dehalogenase from strain CBDB1 has a more positive redox potential than the co-factor of the dehalogenase from *Desufurospirillum multivorans*. If the co-factor of dehalogenase of strain CBDB1 has a more positive redox potential than that of *Desufurospirillum multivorans*, this could explain why a reversed electron transport is needed in *Desufurospirillum multivorans* for the reduction of corrinoid but not in strain CBDB1. The results described above are thus in need of further studies to explain about the corrinoids and redox potentials of free and protein bound corrinoids.

The higher reaction rate of dehalogenase activity measured with methyl viologen (E_o'= -450 mV) than with ethyl viologen (E_o'= -480 mV) (Table. 5.1) indicates that the dehalogenase activity was not solely determined by the redox potential of the compound but also by the chemical structure of the substrate. A higher activity with methyl viologen as the electron donor than with ethyl viologen was also described for the PCE dehalogenases of *Desufurospirillum multivorans* (Miller et al 1997 b) and *Desulfitobacterium* sp. strain PCE-S (Miller et al 1997 a).

For *Desufurospirillum multivorans*, a hampered access of the larger molecule ethyl viologen to the electron-accepting site of the dehalogenase was assumed (Miller et al 1997 b). Dehalogenase activity supported by PQQ (E_o' = +80 mV) additionally suggests that the steric effect of the compound has an important influence on the dehalogenase activity of strain CBDB1.

Although the pathway of electron transport in the reductive dechlorination in strain CBDB1 is not yet clear, the available evidence in this study is consistent with the assumption that electrons are transported in the membrane during dehalorespiration involving hydrogenase, dehalogenase and an electron carrier which we have not yet identified.

5.3.2 Inhibition of electron transport by 1,2,3,4-TeCB

Inhibition of whole cells catalysed dechlorination reactions by 1,2,3,4-TeCB in the presence of hydrogen as electron donor indicated that 1,2,3,4-TeCB has some interference in the dehalorespiration process. While reductive dechlorination of 1,2,3,4-TeCB with methyl viologen as electron donor excluded the possibility that the dehalogenase was inactivated by 1,2,3,4-TeCB, its effect on hydrogenase was tested. Reduced AQDS did not serve as an artificial electron donor in estimating the dehalogenase activity in cell suspension (Table 5.1) or in crude extract (Hölscher et al 2003) of strain CBDB1.

Therefore, AQDS was used as electron acceptor in the hydrogenase activity test in the presence of 1,2,3,4-TeCB. AQDS is a hydrophilic compound, which can accept electrons directly from hydrogenase and not through electron transport components, which would be located in the membrane. Hydrogen dependent reduction of AQDS by whole cells of strain CBDB1 in the presence of 1,2,3,4-TeCB (Fig. 5.2) indicated that 10 µM 1,2,3,4-TeCB does not interfere with hydrogenase activity.

Therefore, the results indicate that 1,2,3,4-TeCB somehow interferes with the electron transport in strain CBDB1 without inhibiting hydrogenase or dehalogenase activity.

As 1,2,3,4-TeCB is lipophilic it could easily be taken up by the membrane where electron-mediating components are located and thus might interfere with electron transport. It was found that 1,2,3,4-TeCB is the only interfering chlorobenzene congener whereas, 1,2,4,5 and 1,2,3,5-TeCB were not. Rather, the later two TeCB congeners are produced as less chlorinated intermediate products during the dechlorination of PeCB and HCB by strain CBDB1 (Jayachandran et al 2003). The interference by 1,2,3,4-TeCB might be due to its chemical structure with all chlorine atoms in this congener are sequentially arranged one after the other. This is significantly different from other TeCB isomers. Significant inhibition of dechlorination at 1 µM of 1,2,3,4-TeCB excludes a non-specific inhibition caused by high concentration of the lipophilic compound (Table 5.2).

So far we are unable to elucidate the mechanism of 1,2,3,4-TeCB interference with electron transport. It might be that the chemical structure of 1,2,3,4-TeCB resembles one of the electron mediating quinones and competes at the binding site with a putative quinone mediator that we have not yet identified. Due to the low cell numbers and oxygen sensitivity of strain CBDB1 that are general biochemical limitations discussed in this dissertation, it is difficult to study this effect

in more detail. However, it will be of interest to see if 1,2,3,4-TeCB inhibits chlororespiration in dehalorespiratory organisms other than strain CBDB1. Inhibition of hydrogenase activity by 50 µM of 1,2,3,4-TeCB indicates that 1,2,3,4-TeCB at higher concentration could cause an additional inhibitory effect.

5.3.3 Topology of respiratory enzymes involved in the energy metabolism

Enzymes involved in electron transport in dehalorespiring strains have been characterised and were found to be an electron donating primary dehydrogenase and a terminal reductase. In all cases, membrane-associated extra-cytoplasmic location of a primary dehydrogenase and a membrane bound terminal reductase has been reported except the PCE reductive dehalogenase from *Desufurospirillum multivorans* that was isolated from the cytoplasm (Miller at al 1997 b).

The dehalogenase activity of strain CBDB1 was found to be membrane associated (Hölscher et al 2003). The ability of reduced methyl viologen, which is a monovalent membrane non permeable cationic radical (Jones and Garland 1977) to function as artificial electron donor for the reduction of chlorobenzenes with intact cells of strain CBDB1 suggested that the active site of dehalogenase in strain CBDB1 could be oriented to the outside of the cytoplasmic membrane. Complete inhibition of hydrogenase activity in intact cells of strain CBDB1 by membrane non-permeable Cu^{2+} ions (Chapter. 4), an inhibitor of hydrogenases (Cypionka and Dilling 1986) suggested that the active site of hydrogenase is also oriented to the outside of the membrane.

Therefore, the results indicate that both of the key enzymes of dehalorespiration, the dehalogenase and the hydrogenase are membrane bound with their active site oriented to the outside of the membrane.

6. Molecular analysis of the putative [Ni-Fe] hydrogenase genes from *Dehalococcoides* sp strain CBDB1 reveals an unusual subunit composition

Abstract

The gene cluster putatively coding for a membrane bound group-1 [Ni-Fe] hydrogenase was amplified and sequenced from strain CBDB1. The cluster consisted of the [Ni-Fe] hydrogenase structural genes hupS, hupL and a gene, which codes for an accessory protein for hydrogenase maturation, hupD. The transcript obtained by reverse trancriptase PCR from mRNA isolated from strain CBDB1 cultivated with hydrogen as electron donor confirms that the investigated gene cluster is expressed under the growth conditions studied. The analysis of the length and the restriction pattern of the cDNA confirmed that the operon was transcribed as a polycistronic messenger. A putative rho-independent transcription terminator was found immediately downstream of hupD. The amplified operon lacks a gene coding for a membrane spanning hydrophobic cytochrome b that is found in all other membrane bound [Ni-Fe] hydrogenases known so far, but is not present in soluble periplasmic or cytoplasmic hydrogenases. However, hydrogenase activity of strain CBDB1 is believed to be bound to the membrane based on the enzymatic activity detected in the membrane fraction. A unique hydrophobic segment found in the small subunit (HupS) of the hydrogenase could be responsible for attaching the complex to the membrane. Therefore, the membrane bound hydrogenase of strain CBDB1 differs from all other membrane bound hydrogenases described so far.

6.1 Introduction

Molecular biological studies on hydrogenases of *Dehalococcoides* species have not been performed. The available genome data of the PCE dechlorinating bacterium *Dehalococcoides ethenogenes* 195 (www.tigr.org) show open reading frames coding for subunits of putative membrane bound and soluble hydrogenases. Some of the biochemical properties of hydrogenases from strain CBDB1 are described in Chapter 4. Studies on hydrogenase genes, their genetic organisation and their expression are needed to understand the molecular basis of dehalorespiration.

The work in this chapter describes the molecular analysis of a hydrogenase gene cluster from strain CBDB1 with special focus to the membrane bound systems, because they are believed to be involved in the energy conservation with dehalorespiration.

6.2 Results

6.2.1 [Ni-Fe] hydrogenase genes in *Dehalococcoides ethenogenes*

In *Ralstonia eutropha*, a gram- negative facultative lithoautotrophic bacterium, the genes for both membrane bound and soluble hydrogenases have been well studied at the molecular level (Tran-Betcke et al 1990; Kortluke and Friedrich 1992). The membrane bound group-1 [Ni-Fe] hydrogenase is considered to be a hydrogen-uptake enzyme. Considerable high sequence similarity exists between membrane-bound hydrogen uptake hydrogenases in different organisms (Vignais et al 2001). Therefore, the genome database of *Dehalococcoides ethenogenes* (www.tigr.org) was screened by a blast search for membrane bound group-1 [Ni-Fe] hydrogenase coding regions using the amino acid sequences of the small and the large subunits and sequences of other accessory genes of membrane bound hydrogenase from *Ralstonia eutropha* as input. ORFs coding for membrane bound group-1 [Ni-Fe] hydrogenase were detected in the same contig (6871) of the genome with good similarity (Table. 6.1).

Table. 6.1 Genes in the *Dehalococcoides ethenogenes* genome, which show high similarity to hydrogenase related genes in *Ralstonia eutropha*.

	Gene products of		Similarity[b]	Function in *R. eutropha*
	R. eutropha	*D. ethenogenes*[a] hox or hup operon		
1	HoxK	HupS	$3x10^{-45}$	[Ni-Fe] Hydrogenase small subunit
2	HoxG	HupL	$7x10^{-59}$	[Ni-Fe] Hydrogenase large subunit
3	HoxZ	HupC	Not found	Membrane anchoring cytochrome b
4	HoxM	HupD	$9x10^{-16}$	C-Terminal Peptidase
5	HypA1	HypA1	$9x10^{-16}$	Incorportation of Ni/maturation
6	HypB1	HypB1	$1x10^{-48}$	Nickelin/Ni Insertion
7	HypF1	HypF1	$1x10^{-30}$	CN/CO delivery
8	HypC	HypC	$3x10^{-11}$	Chaperone/maturation
9	HypD	HypD	$7x10^{-81}$	Fe/S protein maturation
10	HypE	HypE	$2x10^{-82}$	Purine derivative binding
11	HoxA	HupA	$3x10^{-13}$	Response regulator
12	HoxJ	HupJ	$7x10^{-30}$	Histidine kinase

[a] All hup and hyp genes of *D. ethenogenes* are located in contig 6871.
[b] Numbers represent E-values of blast searches.
hup: uptake hydrogenase genes, hyp: genes coding for hydrogenase modifying proteins.

6.2.2 [Ni-Fe] Hydrogenase genes in strain CBDB1

Specific PCR primers were designed from the genome data of *Dehalococcoides ethenogenes* (www.tigr.org) by similarity search and multiple alignments of different [Ni-Fe] hydrogenase genes (DCH1 Forward; 5'-GCGGGTTTAGACAAAAGAACCA-3' and DCH3 Reverse; 5'-GTGGTGGAAATGATGAAGGC-3'). Successful amplification of a 3-kb hydrogenase gene locus was achieved from genomic DNA of strain CBDB1 by PCR (Fig. 6.1).

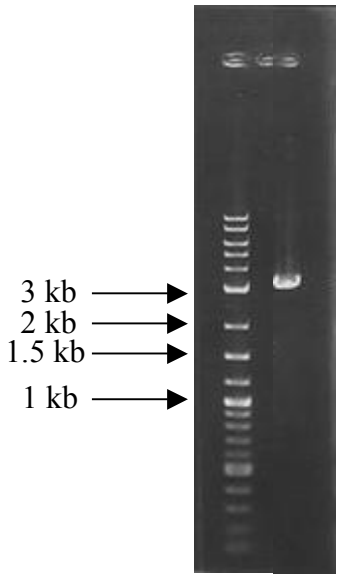


Fig. 6.1 Putative membrane bound [Ni-Fe] hydrogenase gene cluster amplified from genomic DNA of strain CBDB1 by PCR. Lane 1, DNA standard marker (2-log DNA ladder). Lane 2, negative control. Lane 3, DNA-fragment amplified by PCR using primers DCH1 and DCH3.

The PCR amplified DNA-fragment was purified and sequenced using sequencing primers having "GC" at their 3' end. The nucleotide sequence is given as figure 6.2.

The upstream region of hupS was amplified by a PCR using primers hupS1 and hupS2 with genomic DNA as template. The region downstream to hupD was also amplified by a separate PCR using primer pair hupD1 and hupD2 (The primer sequences are given in Material and methods section).

GCG CAGGCACACG ATGCCGAGCA TGAACGGTAA GTTGGCAAGC ACTAGACTGG

1 CCTATAGCGC CGGCGTAGAC TAAAGCACCAT TCTCAAATT**A GTAGG**AAAA**A TG**TTTAATAC

61 TAAACTTACT AGACGGGATT TTGTACAGCT AGCCGCCGGC TCTACCGCCG CCCTCAGTCT

121 GGGTGCTTTG AAGCTCCCCG AATTTGAAAA AATGTTTGCC GAGGCTTTGA AAGAAATCCC

181 GGTTATATGG CTACAGGGTG CCGGCTGCAA GGGATGCACT ATCTCTACTT TCAACGTTGT

241 ATCGCCCACC ATCCAGGATT TACTGCTTAC TTCGGTAGTC CCCGGTACTC ATGTATCCAT

301 GCAGTTCCAC CCCACCATTA TGGCCGCCCA GGGTGACCTG GCTATGAACA CTATTACCGA

361 TACTGCCGCT AAAGGGCTGT TTGTACTGGT AATTGAAGGC TCTGTACTCT TCAAAGGAGG

421 CGGGATTTAC TCCGAAGTCT GCGAAAAAAA AGGCGAAGGA ATCACCCTGC TGGAGCATGT

481 ACTGAATCTG GCTCCCAAGG CACTGGCCAT TGTGGCTGCC GGTACTTGTG CCGCCTTCGG

541 AGGCATCACC GCTGCCGCCC CCAACCCCAC CGGTGCCAAG GCTCTTGAAG AAATTCTTAA

601 AGACCATAAT ATAACTACCC CGGTTGTAAA CCTGCCCGGC TGCCCTCCTC ATCCGGATTG

661 GGTAGTGGGC ACTCTGGCTA CCATACTTAT GAGCGGACTT GACGCACTTG ACCTTGATAA

721 GATGGGCCGC CCCAAGGCTT ATTACGGCAA ACTCCTTCAT GACCAGTGTC CGCGCCGCGG

781 CCACTACGAA ATGGTTCTGT TTGCCACCAA GCTGGGGGGG CCTTACTGCC TGTTCCTGGT

841 CGGCTGTAAA GGCCCGGTAA CCTATTATGA CTGTTCTGAC AGGCTCTGGA ACAATAAAAC

901 CCCTTGGTGC GTAGAAGCTG ATTCCACGTG CATCGGCTGT GCCCACCCCT GTTCCCCGGA

961 TGCCGTTTCT CACATGTTTG AAGCCCCGCC TCTATTCAAT AGTACTGACA AACTGGCTAT

1021 CGGTATTGCC GGCACTGCCG TTGTACTGAC TGCCGGTGTA GCTGCTGTAG AACTTGCTAA

1081 AAAAGCCAAG CGGAATGCCG CGAAAAAGGG TTAGTAGACC GTAATCTTAC CTTT**GGAGAA**

1141 GAAA**ATG**CAG AAGATAGTAA TTGATCCCAT AACGAGGATT GAAGGTCATC TTAAAATCGA

1201 AGCCACCGTA GACGGCGGCG AGGTTAAAGA TGCCAAATGT GTCGGCACTT TGTTCCGCGG

1261 CTTTGAAATA TTTATGAAAG ACCGTGACCC GCGTGATGCT GTTCACATTA CCCAACGCAT

1321 CTGCGGTGTC TGCCCCACCT CTCACGGCAC TACCGCCGCT TTAAATTTGG ACGCTGCCTT

1381 TGGTGTAGCT GACAAGATAC CGAATAACGG GCGGATACTC CGCAACCTTA TTCAGGGTGC

1441 CAACTACATT GCCTCTCATA TAGTCCATTT CTACCATCTG GCCGCCTTGG ATTATGTAGA

1501 TGTAACCGAA GTAGCAGATT ACGACGGCAC TGATCCTGAA CTTTTGAAAG TCAAGGATTT

1561 TATTTCACGG GCTCTGGCTG CCGGGGATAT GTCCATGCTG GCTCCCTTCT ACCCCAGATA

1621 CGAGGGCGAC TACCGTTTGC CCAAGAAGGT CGCCCAAGCC GCAGTCGCCC ATTACGTTAA

1681 AACTTCGAAC ACGCGCCGCC CGGCTCATGA TATGTCCGCC ATTTACAGTG GCCGTCCGCC

1741 CCACAGTGTG GCAGTGGTTG CTGGCGGTGT TACTTTACAC CCCAGCACAG ACAGCACTTC

1801 AAATTCTACG AGTAAGCTGA ATACCCTGTG CAACTTTACT GATAATGTAA ACATACCTGA

1861 CGTTATTGTA GTAGCTGAGT CTTATCCTGA TTACATCGGT ATCGGCGTGG GCTGCGGTAA

1921 CCTGCTCAGC TATGGTGTGT TTGACCTCGA GGCCAGCGGC ACCAATCTGG CTACTCGCCA

1981 GAGGCTGTCC ACTCAGGGCG TGGTTTCCGC ATCTGACCTG GCTCACCGTA CCTTTGACCC

2041 CAGCAAGATC ACCGAATCCA CCAAATACAG CCGGTTCAAG GGTGATATTA CTGAATATTC

2101 CCTGAACGAG GTAATCGAAC CCGAATTTAC CAAGGCTGAC GGCCATTCCT GGCTTAAAGC
2161 TCCCCGCTAC GACGGCACTC CCTCCGAAGT AGGCTCTCTG GCCCGTATGG TAGTCAACTA
2221 CGTATCCGGT GACCTCTTGG TCCAGCAGAT GGCGAATGAT ACTCTGTCCC ATTTCGGTGC
2281 CGGTCCTGCT GCTCTCTTCA GCACTCTCGG CAGACATGTC GCCCGTGCTC CGGAATGCAA
2341 GATTGTGGCT GACGAAATGG TCAAGTGGGT TATGGAACTT AAGATTGGCG AACCGGTATG
2401 TGCCGATTAC GAAATACCCG AAAGTGCCGA AGGCATGGGT CTCTGGGAGG CTCCCCGCGG
2461 TGCTCTTGGC CACTGGATAA AGATTGAAAA CCACAAGATC AGCAACTACC AGTGTGTAGT
2521 TCCTACTACT TGGAACTGTT CACCTAAGGA CGGACAAGGT GTTTACGGTC CGGTTGAGCA
2581 GGCTCTTATC GGAACCAAGG TACGTGATAA CGATAATCCC TTTGAGCTGG TTCGTATTGT
2641 CCGCTCGTTT GACCCGTGTT TGGCCTGTGC CCGTCACCTG ATCTCCCCTA CGGGCAATGA
2701 AATCAGCCGC TTCCGCGTCT ACTAAGT**ATG** TTTTTTAATC CGGGGAGTGA ATCTCCCAAA
2761 CCTATATTGG TCCTGGGTAC AGGCAATATA CTCTTGAGTG ATGAAGGTGC CGGGGTACGC
2821 TGCATAGAGC GGCTATCCCG GTTTCCTGTT CCTGAAGATG TGGAGCTGTA TGACGGCGGC
2881 ACCGCCGCCA TGGATTTACT GGACGTGATA TCCGGGCGTG AAAAAATATT TATACTGGAT
2941 GCGGTTCACG GCGGAGACGA ACCCGGCATG ATATACCGCT TCCGCCCCGA AGATATCAAA
3001 ACCGAGCAGA AAATAGATAT CTCTTTCCAC CAGATGGGGC TGATGGAAAT ACTTAATCTG
3061 GCCAAATACC ATGACGCCCT GCCAAAGGAT ATTATAATCT ACGGTATCCA GCCCGGCAGT
3121 ATGGCACCGG GGTTTGAGCT TACCCCGGCA GTAGACAAGG CTGTAAACCG GGTGGTGGAA
3181 ATGATGAAGG CAGAGCTGGA CATCAACTAG CCCCGGCTAT AAATGCCGGA GTTACAAGGG
3241 CGGGCTTTTA CCGCCTTTTT TTTATTACCC AGATATCC

Fig. 6.2 Nucleotide sequence of the DNA fragment (*hup* gene locus) carrying the putative genes coding for the [Ni-Fe] hydrogenase small, large subunits (hupS and hupL) and an accessory protein (hupD). ORFs 1, 2, and 3 (hupS, L and D) are underlined (amplified by a PCR with primer pairs DCH1 and DCH3). Upstream and downstream regions of the hydrogenase gene cluster amplified by separate reactions are not completely shown.
Putative ribosome-binding site and start codon of each *hup* gene is given in bold fonts. The hypothetical sequences upstream the start codon of hupS for the regions -35 and -10 are shaded in grey and marked with arrows. The putative rho-independent transcriptional terminator (GTTACAAGGGCGGGCTTTTACCGCCTTTTTTTT) downstream the hupD stop codon is shaded in gray.

Sequence analysis of the amplified fragment revealed a group of three genes (Fig. 6.2). Searches in the protein and DNA sequence data bases allowed the identification of the three complete ORFs as the large (hupL) and the small (hupS) subunits of [Ni-Fe] hydrogenases and an additional gene coding for a C-terminal peptidase (an accessory gene for [Ni-Fe] hydrogenase maturation, hupD) which follows immediately (2 bp) downstream the hupL stop codon (Fig. 6.2).

The ATG start codon of hupS as well as hupL is immediately (4 nucleotides) preceded by a sequence corresponding to a hypothetical ribosome-binding site (Fig. 6.2). The sequences TAAGTT and TATAGC are located 42 bp upstream the start codon of hupS, separated by 19 bp, constituting most probably the promotor site regions -35 and -10. A predicted 33 bp putative rho-independent transcription terminator (a 3' stretch of T residues and a 'GC' rich interrupted palindrome just upstream of the 3' poly T region) that would be capable of forming a stable stem-loop structure is observed 20 nucleotides downstream of the hupD stop codon (Fig. 6.2).

6.2.3 Expression and organisation of the operon genes

A 3 kb amplicon was obtained by PCR using cDNA transcribed from mRNA of strain CBDB1 (Fig. 6.3). cDNA synthesis by RT-PCR and subsequent amplification using the specific primers (DCHK and DCH3) reveal that the genes encoding HupS, L and D are transcribed to a polycistronic messenger in strain CBDB1. All three genes (hupSLD) are organised in one operon as shown in Fig. 6.4.

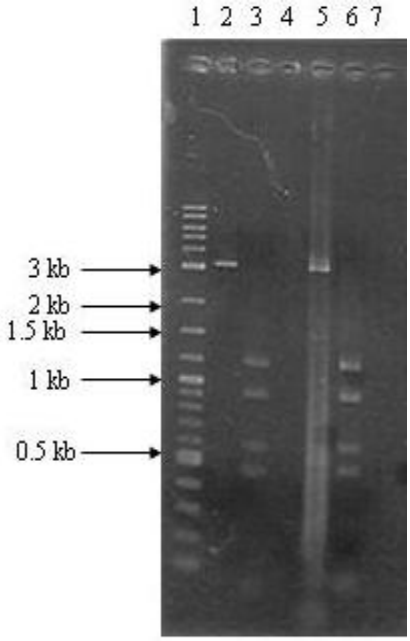


Fig. 6.3 Expression of hupS, L and D of strain CBDB1 indicated by RT-PCR and restriction digestion analysis of PCR and RT-PCR products.
Lane 1, DNA standard marker (2-log DNA ladder). Lane 2, fragment amplified by PCR from genomic DNA. Lane 3, PCR product shown in lane 2 after digestion with BsaA1. Lane 4, negative control reaction for PCR. Lane 5, RT-PCR product. Lane 6, RT-PCR product digested with BsaA1. Lane 7, Negative control for RT-PCR.
Genomic DNA was used as a template in PCR with primer pairs DCH1 and DCH3. mRNA of strain CBDB1 was used in RT-PCR with primer pairs DCHK and DCH3.

To compare the identity and organisation of the genes in the RT-PCR product, a restriction digestion analysis was performed with RT-PCR product as well as with PCR amplified fragment using the restriction endonuclease BsaA1. BsaA1 recognised the same restriction sites in both of the amplified products (Fig. 6.3).

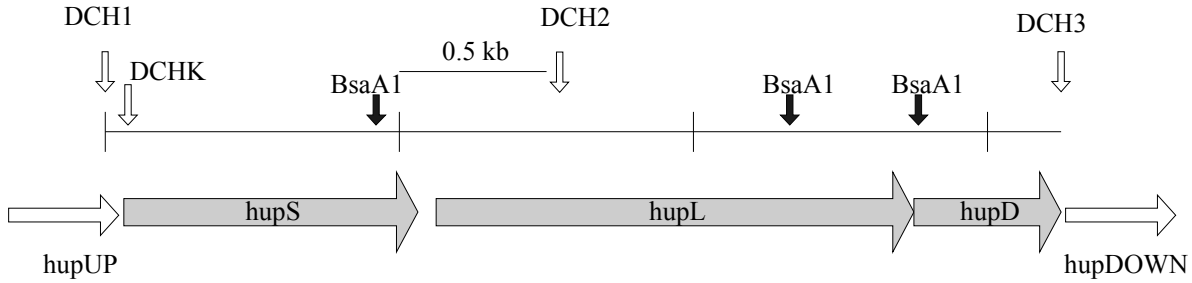


Fig. 6.4 Physical map of analysed putative [Ni-Fe] hydrogenase encoding gene cluster of strain CBDB1. Vertical open arrows, primers used; vertical filled arrows, relevant restriction sites; horizontal arrows, ORFs; horizontal bold arrows, up and down stream sequence of hup locus.

Additionally a nested PCR was performed with RT-PCR product as template using primers DCH2 (an internal forward primer designed from the genome data of *D. ethenogenes*) and DCH3 to amplify a known size of a fragment inside the target gene cluster. A fragment with an expected size of 1600 bp inside the hydrogenase operon was amplified. A fragment of the same size was also amplified in the control experiment in which genomic DNA was used as template (Fig. 6.5).

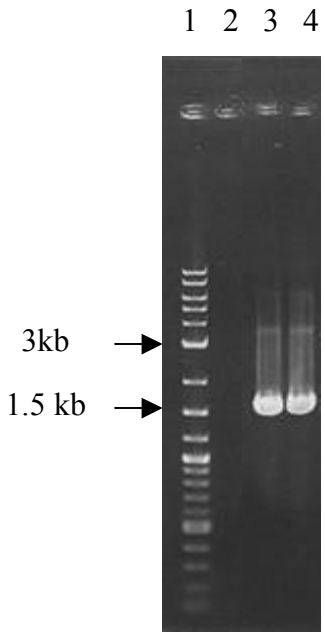


Fig. 6.5 Gene fragments of strain CBDB1 amplified by nested PCR using primers DCH2 and DCH3. Lane 1, DNA standard marker (2-log DNA ladder). Lane 2, negative control without DNA. Lane 3, 1.6-kb fragment amplified from RT-PCR product. Lane 4, 1.6-kb fragment amplified from genomic DNA.

6.2.4 Analysis of the predicted polypeptides

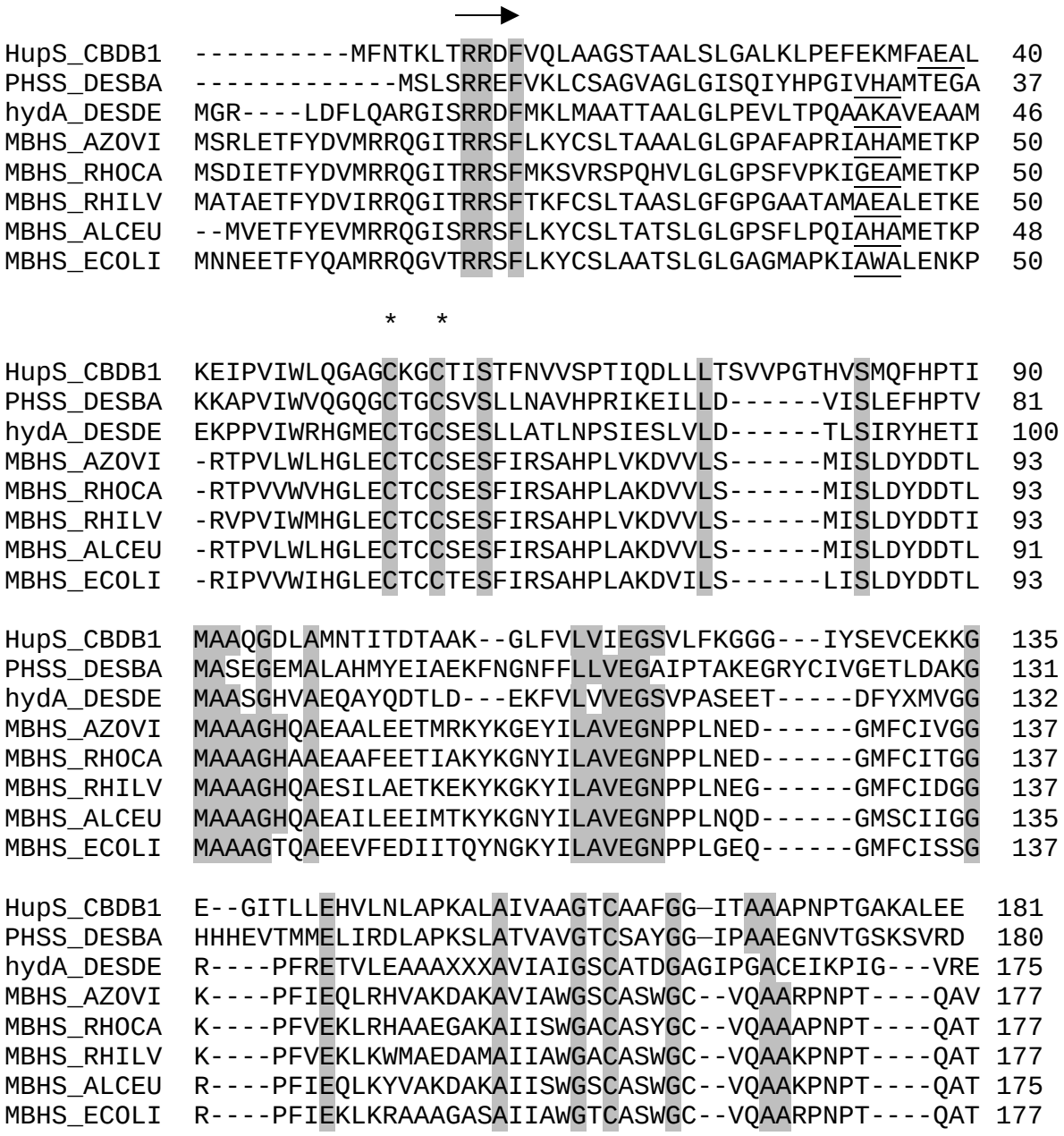
6.2.4.1: ORF1-[Ni-Fe] hydrogenase small subunit (HupS)

ORF 1 from strain CBDB1 is predicted to be a small subunit (HupS) of [Ni-Fe] hydrogenase containing 354 amino acid residues with a calculated molecular mass of 37,258 Da and an isoelectric point of pI = 6.25. HupS shares similarity to many [Ni-Fe] hydrogenase small subunits (Table. 6.2).

A hydrophobicity of HupS determined according to the method of Kyte and Doolittle (1982) using the computer program (http://sosui.proteome.bio.tuat.ac.jp/sosui_submit.html) indicated the presence of a transmembrane helix (KLAIGIAGTAVVLTAGVAAVELA) showing an average hydrophobicity of 0.24.

A typical membrane targeting twin arginine signal sequence motif including (S/T)RRxF (Wu and Mandrand 1993) (Fig. 6.6) is located at residues 8 and 9. Three putative [Fe-S] clusters binding motifs as found in well characterised [Ni-Fe] hydrogenase small subunits from various organisms (Fig. 6.6) were also identified. The secondary structure of the small subunit HupS was estimated by the method of Garnier (Garnier et al 1996). According to that, the subunit would

consist of 34.18 % alpha helical structure, 18.93 % of extended strand and 46.89 % of random coil structure.



```
HupS_CBDB1  ILKDHNITTPVVNLPGCPPHPDWVVGTLATILMSGLD-----ALDLDKMG 226
PHSS_DESBA  FFADEKIEKLLVNVPGCPPHPDWMVGTLVAAWSHVLNPTEHPLPELDDDG 230
hydA_DESDE  LLQKNNIATPVINLPCCPVKPNTLIGTXVYYLTFNAVP------ELDEQA 219
MBHS_AZOVI  PIHKVITDKPIVKVPGCPPIAEVMTGVITYMLTFGKLP------ELDRQG 221
MBHS_RHOCA  PVHKVITDKPIIKVPGCPPIAEVMTGVITYMLTFDRMP------ELDRQG 221
MBHS_RHILV  PIDKVILDKPIIKVPGCPPIAEVMTGVVTFITTFGKLP------ELDRQG 221
MBHS_ALCEU  PVHKVITDKPIIKVPGCPPIAEVMTGVITYMLTFDRIP------ELDRQG 219
MBHS_ECOLI  PIDKVITDKPIIKVPGCPPIPDVMSAIITYMVTFDRLP------DVDRMG 221

                                                    *     *
HupS_CBDB1  RPKAYYGKLLHDQCPRRGHYEMVLFATKLGGP-----YCLFLVGCKGPVT  271
PHSS_DESBA  RPLLFFGDNIHENCPYLDKYDNSEFAETFTKP-----GCKAELGCKGPST  275
hydA_DESDE  RPLIYYGKLLHDNCPRRGQFEAGNYLSDWNDP-AQKDYCLLLMGCKGPKT  268
MBHS_AZOVI  RPKMFYGQRIHDKCYRRPHFDAGQFVEHWDDEGARKGYCLYKVGCKGPTS  271
MBHS_RHOCA  RPAMFYSQRIHDKCYRRPHFDAGQFVEHWDDENARKGYCLYKMGCKGPTT  271
MBHS_RHILV  RPKMFYSQRIHDKCYRRPHFDAGQFVEEWDDEGARKGYCLYKMGCKGPTT  271
MBHS_ALCEU  RPKMFYSQRIHDKCYRRPHFDAGQFVEEWDDESARKGFCLYKMGCKGPTT  269
MBHS_ECOLI  RPLMFYGQRIHDKCYRRAHFDAGEFVQSWDDDAARKGYCLYKMGCKGPTT  271

                                      *   *
HupS_CBDB1  YYDCSDRLWNNKTPWCVEADSTCIGCAHPCSPDAVSHMFEAPPLFNSTDK  321
PHSS_DESBA  YADCAKRRWNNGINWCVEN-AVCIGCVEPDFPDGKSPFYVAE--------  315
hydA_DESDE  YTDCAQVWWNDNANFCINAGSPCSGCSEFGFYGQFSPLYAKQENFSLPGL  318
MBHS_AZOVI  YNACSTVRWNEGTSFPIQAGHGCIGCSEDGFWDKGS-FYERLTTIPQFGI  320
MBHS_RHOCA  YNACSTVPLERRRHFPIQSGHGCIGCSEDGFWDQGS-FYDRLTTIKQFGI  320
MBHS_RHILV  YNACSTVRWNGGVSFPIQSGHGCIGCSEDGFWDNGS-FYDRLTNIHQFGI  320
MBHS_ALCEU  YNACSTTRWNEGTSFPIQSGHGCIGCSEDGFWDKGS-FYDRLTGISQFGV  318
MBHS_ECOLI  YNACSSTRWNDGVSFPIQSGHGCLGCAENGFWDRGS-FYSRVVDIPQMGT  320
```

```
HupS_CDBD1  ----------LAIGIAGTAVVLTAGVAAVELAKKAKRNAAKKG-------  354
PHSS_DESBA  ---------------------------------------------------  315
hydA_DESDE  GQIHADTVGKVVGGATVVGLGAHLIATVASGRLKNNDSEQKKED------  362
MBHS_AZOVI  -EKNADEIGAAVAGGVGAAIAAHAAVTAIKRLQNKGDRP-----------  358
MBHS_RHOCA  -EATADQIGWTATGLVGAAVAAHAAVSVLKRAQKKNEEA-----------  358
MBHS_RHILV  -EANADKVGMTAAGVVGGAIAAHAAVTAVKRLTTKREKADA---------  358
MBHS_ALCEU  -EANADKIGGTASVVVGAAVTAHAAASAIKRASKKNETSGSEH-------  360
MBHS_ECOLI  -HSTADTVGLTALGVVAAAVGVHAVASAVDQRRRHNQQPTETEHQPGNED  369
```

Fig. 6.6 Protein sequence alignment of membrane bound and periplasmic [Ni-Fe] hydrogenase small subunits from various organisms with the HupS of strain CBDB1.
PHSS_DESBA, *Desulfovibrio baculatus* (*Desulfomicrobium baculatus*) (accession number P13063); hydA, *Desulfitobacterium dehalogenans* (accession number AF157641); MBHS_AZOVI, *Azotobacter vinelandii* (accession number P21950); MBHS_RHOCA, *Rhodobacter capsulatus* (*Rhodopseudomonas capsulata*) (accession number P15283); MBHS_RHILV, *Rhizobium leguminosarum* (biovar viciae) (accession number P18637); MBH_ALCEU, *Ralstonia eutropha* (accession number P31892); MBHS_ECOLI, *E. coli* (accession number P19928).
Horizontal arrow marks the twin arginine signal sequence motif, underlined residues at the N-terminus represent the probable site for the signal peptide cleavage, [Fe-S] clusters bridged by cysteine residues are marked with (*) and conserved regions are shaded. Horizontal shaded region at the C-terminus indicate the predicted transmembrane helix.

6.2.4.2: ORF2 [Ni-Fe] hydrogenase large subunit (HupL)

Strain CBDB1 HupL is a polypeptide of 526 amino acids with a mass of 57,523 Da and a pI of 5.95. HupL shows similarity to the large subunits of many [Ni-Fe] hydrogenases. The closest similarity (59 %) was found towards the putative membrane bound [Ni-Fe] hydrogenase large subunit from *Desulfitobacterium dehalogenans* (Smidt et al 1999) (Table. 6.2).

According to the method of Kyte and Doolittle (1982) HupL is predicted to be a hydrophilic polypeptide having an average hydrophobicity of -0.18 (using the computer program http://sosui.proteome.bio.tuat.ac.jp/sosui_submit.html). This indicates that the large subunit has no means of direct attachment with the hydrophobic lipid bilayer.

Sequence alignment of large subunits of [Ni-Fe] hydrogenases with HupL of strain CBDB1 reveal the presence of two very conserved regions surrounding the two pairs of cysteine ligands of the [Ni-Fe] site near to the N- and C-terminus of the sequence (Fig. 6.7). A conserved histidine residue, which is the endopeptidase cleavage site at the C-terminus of the large subunit, was also found in HupL of strain CBDB1 (Fig. 6.7).

```
HupL_CBDB1  ---------------MQKIVIDPITR-IEGHLKIEATVD-GGEVKDAKCV  33
hydB_DESDE  ---------------MGKVVIDPVTR-IEGHLKVEVEVT-NGVVTDARSI  33
MBHL_RHOCA  -MTTQTPNGFTLDNAGKRIVVDPVTR-IEGHMRCEVNVNDQGIITNAVST  48
MBHL_AZOVI  --MSSLPNASQLDKSGRRIVVDPVTR-IEGHMRCEVNVDASNVITNAVST  47
MBHL_ALCEU  --SAYATQGFNLDDRGRRIVVDPVTR-IEGHMRCEVNVDANNVIRNAVST  47
MBHL_RHILV  -MTIQTPNGFTLDNSGKRIVVDPVTR-IEGHMRVEVNVDENNIIRNAVST  48
MBHL_ECOLI  MSTQYETQGYTINNAGRRLVVDPITR-IEGHMRCEVNINDQNVITNAVSC  49
COOH_RHORU  -------------MSTYTIPVGPLHVALEEPMYFRIEVDGEKVVSVDITA  37
                                      Ni/Fe
HupL_CBDB1  GTLFRGFEIFMKDRDPRDAVHITQRICGVCPTSHGTTAALNLDAAFGVAD  83
hydB_DESDE  GTMFRGLN-AVKSRDPRDATYVTERTCGVCASAHGWASSLCLDDAFG—A  80
MBHL_RHOCA  GTMWRGLEVILKGRDPRDAWAFTERICGVCTGTHALTSVRAVESALG—I  97
MBHL_AZOVI  GTMWRGLEVILKGRDPRDAWAFVERICGVCTGTHALTSVRAVEDALD—I  96
MBHL_ALCEU  GTMWRGLEVILKGRDPRDAWAFVERICGVCTGCHALASVRAVENALD—I  95
MBHL_RHILV  GTMWRGIEVILKNRDPRDAWAFTERICGVCTGTHALTSVRAVENALG—I  96
MBHL_ECOLI  GTMFRGLEIILQGRDPRDAWAFVERICGVCTGVHALASVYAIEDAIG—I  98
COOH_RHORU  GHVHRGIEYLATKRNIYQNIVLTERVCSLCSNSHPQTYCMALESITG—M  85

HupL_CBDB1  KIPNNGRILRNLIQGANYIASHIVHFYHLAALDYVDVTEVADYDGTDPEL  133
hydB_DESDE  KVPAGGRIIRNLITSAMWLHDAALHFYHLSALDFLDITAVAKYQGQDPGL  130
MBHL_RHOCA  TIPDNANSIRNMMQLNLQIHDHIVHFYHLHALDWVNPVNALRADPKATSE  147
MBHL_AZOVI  RIPYNAHLIRNLMDKTLQVHDHIVHFYHLHALDWVNPVNALKADPKATSA  145
MBHL_ALCEU  RIPKNAHLIREIMAKTLQVHDHAVHFYHLHALDWVDVMSALKADPKRTSE  146
MBHL_RHILV  TIPDNANSIRNLMQLALQVHDHVVHFYHLHALDWVDVVSALSADPKATSA  147
MBHL_ECOLI  KVPDNANIIRNIMLATLWCHDHLVHFYQLAGMDWIDVLDALKADPRKTSE  148
COOH_RHORU  VVPPRAQYLRVIADETKRVASHMFNVAILAHIVGFDSL------------  123

HupL_CBDB1  LK--------------VKDFISRALAAGDMSMLAPFYPRYEGD-YRLP-K  167
hydB_DESDE  LR--------------VKEKIGKLIAAG----TAPLTPRYEPDEYCVNDP  162
MBHL_RHOCA  LQQMVSPSHPLSSPGYFRDVQNRLKKFVESGQLGLFKNGYWDNPAYKLPP  197
MBHL_AZOVI  LQQAVSPAHAKSSPGYFRDVQTRLKKFVESGQLGLFSNGYWDNPAYKLPP  195
MBHL_ALCEU  LQQLVSPAHPLSSAGYFRDIQNRLKRFVESGQLGPFMNGYWGSKAYVLPP  196
MBHL_RHILV  LAQSIS-DWPLSSPGYFKDIQTRLKKFVESGQLGPFKNGYWGNASYKLPP  196
MBHL_ECOLI  LAQSLS-SWPKSSPGYFFDVQNRLKKFVEGGQLGIFRNGYWGHPQYKLPP  197
COOH_RHORU  ------------------------------------------------  123

HupL_CBDB1  KVAQAAVAHYVKTSNTRRPAHDMSAIYSGRPPHSVAVV----------A  206
hydB_DESDE  ELVTLAVSHYLKALDMQAKAKKMSALFAGKQPHQSSIV----------V  201
MBHL_RHOCA  EADLMATTHYLEALDLQKEVVKVHTIFGGKNPHPNWLVGGVPCPINVDGV  247
MBHL_AZOVI  EADLMAVAHYLEALDLQKDIVKIHTIFGGKNPHPNYMVGGVACAINLDDV  245
MBHL_ALCEU  EANLMAVTHYLEALDLQKEWVKIHTIFGGKNPHPNYLVGGVPCAINLDGI  246
MBHL_RHILV  EANLMAVAHYLEALDFQKEIVKIHTIFGGKNPHPNWLVGGVPCPINVDGT  246
MBHL_ECOLI  EANLMGFAHYLEALDFQREIVKIHAVFGGKNPHPNWIVGGMPCAINIDES  247
COOH_RHORU  ------FMHVMEAREIMQDTK--EAVFGNRMDIAAMAIG----------  154
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HupL_CBDB1  GGVTLHPSTDSTSNSTSKLNTLCNFTDNVNIPDVIVVAES---YPDYIGI  253
hydB_DESDE  GGVTMLPNIEVVEQYRSLLLEQIDFLENVYLQDVLTFGTGPLLPLAQAGV  251
MBHL_RHOCA  GAVGA-INMERLNLVSSIIDRCTEFTRNVYLPDLKAIGGFYKE—WLYGG  294
MBHL_AZOVI  GAAGAPVNMTSLNFVLERIHEAREFTRNVYLPDVLAVAGIYKD—WLYGG  293
MBHL_ALCEU  GAASAPVNMERLSFVKARIDEIIEFNKNVYVPDVLAIGTLYKQAGWLYGG  296
MBHL_RHILV  GAVGA-INMERLNMVTSIIDQLIEFNDKVYVPDIMAIGSFYKD—WLYGG  293
MBHL_ECOLI  GAVGA-VNMERLNLVQSIITRTADFINNVMIPDALAIGQFNKP-WSEIGT  295
COOH_RHORU  -GVKYDLDKDGRDYFIGQLDKLEPTLRDEIIPLYQTNPSIVDR—TRGIG  201

HupL_CBDB1  GVGCGNLLSYGVFDLEASGTNLATRQRLSTQGVVSASDLAHR-TFDPS--  300
hydB_DESDE  GGGYNNFLSFGGFGLDDE-----KKNFFLPAGVIMDGDLSKVMAVDQS--  294
MBHL_RHOCA  GLSGQSVLSYGDIPENPN—DFSAGQLHLPRGAIINGNLNEVHDVDTTDP  342
MBHL_AZOVI  GLAAHNLLSYGTFTKVPY--DKSSD—LLPAGAIVGGNWDEVLPVDVRDP  340
MBHL_ALCEU  GLAATNVLDYGEYPNVAY--NKSTD—QLPGGAILNGNWDEVFPVDPRDS  337
MBHL_RHILV  GLSGKNVLAYGDVPEHAN—DYSEASLKLPRGAIINGNLAEVFPVDHADP  341
MBHL_ECOLI  GLSDKCVLSYGAFPDIAN—DFGEKSLLMPGGAVINGDFNNVLPVDLVDP  343
COOH_RHORU  VLSAADCVDYGLMGPVAR----------GSGHAYD-------------  226

HupL_CBDB1  -KITESTKYSRFK--GDIT-EYSLNEVIEP---------------EFTK  330
hydB_DESDE  -KVTEGVTYAWYK--DSPKGDHPYDSDTVP---------------DIKR  325
MBHL_RHOCA  EQVQEFVDHSWYDYGEPGMGLHPWDGRTEPKFELGPNLKGTRTNIENIDE  392
MBHL_AZOVI  EEIQEFVSHSWYSYADETKGLHPWDGVTEPKFELGPNTKGSRTHIQEIDE  390
MBHL_ALCEU  QQVQEFVSHSWYKYADESVGLHPWDGVTEPNYVLGANTKGTRTRIEQIDE  387
MBHL_RHILV  EQIQEFVTHSWYKYPDESKGLHPWDGITEPHYELGPNAKGTKTNIEQLDE  391
MBHL_ECOLI  QQVQEFVDHAWYRYPNDQVGRHPFDGITDPWYNPG-DVKGSDTNIQQLNE  392
COOH_RHORU  ----------------------------------------------VRK  229

HupL_CBDB1  ADGHSWLKAPRYDGTPSEVGSLARMVVNYVSGD------LLVQQMANDTL  374
hydB_DESDE  KDAYSFVKAPRYDGKPVEVGNLARMLV------------MQPKPFMDIV  362
MBHL_RHOCA  GAKYSWIKAPRWRGNAMEVGPLAATSSVTRKGH------EDIKNQVEGLL  436
MBHL_AZOVI  AHKYSWIKAPRWRGHAMEVGPLARYIIAYASGR------EYVKEQVDRSL  434
MBHL_ALCEU  SAKYSWIKSPRWRGHAMEVGPLSRYILAYAHARSGNKYAERPKEQLEYSA  437
MBHL_RHILV  GAKYSWIKAPRWRGNAMEVGPLARWVIGYAQNK------AEFKDPVDKVL  435
MBHL_ECOLI  QERYSWIKAPRWRGNAMEVGPLARTLIAYHKGD------AATVESVDRMM  436
COOH_RHORU  QAPYAVYDRLDFEMALGEHG----------------------------  249

HupL_CBDB1  SHFGAGPAA----------LFSTLGRHVARAPECKIVADEMVKWVM  410
hydB_DESDE  AKYSIKPG-----------------VVARHAARAYEAVILAKEMLNWCN  394
MBHL_RHOCA  RDMNLPV----------SALFSTLGRTAARALEAEYCCRLQKHFFD  472
MBHL_AZOVI  AAFNQSTGLNLG-------LKQFLPSTLGRTLARALECELAVDSMLDDWQ  481
MBHL_ALCEU  QMINSAIPKALGLPETQYTLKQLLPSTIGRTLARALESQYCGEMMHSDWH  487
MBHL_RHILV  KDLGLPV----------TALFSTLGRTAARALESQWAGYQMRYFQN  471
MBHL_ECOLI  SALNLPL----------SGIQSTLGRILCRAHEAQWAAGKLQYFFD  472
COOH_RHORU  ------------------------DVWSRAMVRWQEALTSIGLIRQCLR  274
```

```
                                                      DL DL
HupL_CBDB1  ELKIGEP----VCADYEIP------ESAEGMGLWEAPRGALGHWIKIE-N  449
hydB_DESDE  ALEAEIG----KVSEFTIPSIGNPPATGQGVGLTEVPRGALGHWIKIK-D  439
MBHL_RHOCA  KLVTNIKNGDSSTANVEKWDPSTWPKEAKGVGMTEAPRGALGHWVKIK-D  521
MBHL_AZOVI  ALVGNIKAGDRATANVEKWDPSTWPKEAKGVGINEAPRGALGHWIRIK-D  530
MBHL_ALCEU  DLVANIRAGDTATANVDKWDPATWPLQAKGVGTVAAPRGALGHWIRIK-D  536
MBHL_RHILV  KLIANIKAGDSNTAFVDKWKPETWPKEVKGVGFTEAPRGRLAHWIRIK-D  520
MBHL_ECOLI  KLMTNLKNGNLATASTEKWEPATWPTECRGVGFTEAPRGALGHWAAIR-D  521
COOH_RHORU  DMP----------DGPTKAGPVPPIPAGEAVAKTEAPRGELIYYLKTNGT  314

                    DL
HupL_CBDB1  HKISNYQCVVPTTWNCSPKDGQGVYGPVEQALIGTKVRDNDNPFELVRIV  499
hydB_DESDE  HKTENYQMVVPTTWNFSPKDAQGNYGPLEKALIGVPVPDENNPINIVRVV  489
MBHL_RHOCA  GRIENYQCVVPTTWNGSPRDSKGNIGAFEASLLNTKMERPEEPVEILRTL  571
MBHL_AZOVI  GKIENYQAIVPTTWNGTPRDHLGNIGAYEAALLNTRMERPDEPVEILRTL  580
MBHL_ALCEU  GRIENYQCVVPTTWNGSPRDYKGQIGAFEASLMNTPVVNPEQPVEILRTL  586
MBHL_RHILV  GKIDNYQCVVPTTWNGSPRDPTGNIGAFEASLMDTPMSNPTQPLEILRTI  570
MBHL_ECOLI  GKIDLYQCVVPTTWNASPRDPKGQIGAYEAALMNTKMAIPEQPLEILRTL  571
COOH_RHORU  DRPERLKWRVPTYMN---------WDALNVMMAGARIS------DIPLIV  349

                Ni/Fe
HupL_CBDB1  RSFDPCLACARHLISPTGNEISRFRVY  526
hydB_DESDE  RSLNPCLACAIHLIDPQTNEIHKFKIS  516
MBHL_RHOCA  HSFDPCLACSTHVMSAEGAPLTTVKVR  598
MBHL_AZOVI  HSFDPCLACSTHVMSPDGQELTRVKVR  607
MBHL_ALCEU  HSFDPCLACSTHVMSAEGQELTTVKVR  617
MBHL_RHILV  HSFDPCLACSTHVMSPDGQEMARVQVR  597
MBHL_ECOLI  HSFDPCLACSTHVLGDDGSELISVQVR  598
COOH_RHORU  NSIDPCISCTER--------------  359
```

Fig. 6.7 Sequence comparisons of membrane bound [Ni-Fe] hydrogenase large subunits from various organisms with the HupL of strain CBDB1.
hydB, *Desulfitobacterium dehalogenans* (accession number AF157641); MBHL_RHOCA, *Rhodobacter capsulatus* (*Rhodopseudomonas capsulata*) (accession number P15284); MBHL_AZOVI, *Azotobacter vinelandii* (accession number P21949); MBHL_ALCEU, *Ralstonia eutropha* (accession number P31891); MBHL_RHILV, *Rhizobium leguminosarum* (biovar viciae) (accession number P18637); MBHL_ECOLI, *E. coli* (accession number P19927); COOH_RHORU, *Rhodospirillum rubrum* (accession number P31895).
Ni/Fe means that this cysteine is a bridging ligand of both nickel and iron. DL is for residues stabilizing the diatomic ligands. **Histidine** residue at the C-terminus, endopeptidase cleavage site.

6.2.4.3: ORF3: accessory protein for hydrogenase maturation (HupD)

Strain CBDB1 hupD putatively encodes a protein which shows high similarity with the [Ni-Fe] hydrogenase processing protein from *Desulfitobacterium dehalogenans* (Smidt 1999) sharing 43 % of identity and 69 % of similarity. It also shares high identity with HyaD from the *E. coli* hydrogenase 1 (Rossmann et al 1995), HycI from *E. coli* hydrogenase 3 (Theodoratou et al 2000), HupD from *Rhodobacter capsulatus* (Colbeau et al 1993), *Rhizobium leguminosarum* (Hidalgo et al 1992) and *Bradyrhizobium japonicum* (van Soom et al 1993).

Proteins similar to HupD have been found to be responsible for the C-terminal cleavage of the [Ni-Fe] hydrogenase large subunit during the maturation of the enzyme. Figure 6.8 shows the sequence comparison of HupD from strain CBDB1 with other related proteins.

```
HupD_CBDB1  --MFFNPGSESPKPILVLGTGNILLSDEGAGVRCIERLS-RFPVPEDVEL  47
hydD_DESDE  ---MLQP------KIMVMGVGNVLLSDEGLGVQFLTLLS-QETLPDNVEL  40
HYAD_ECOLI  ---------MSEQRVVVMGLGNLLWADEGFGVRVAERLYAHYHWPEYVEI  41
HUPD_THIRO  --------MRSDPEILVLGIGNLLWADEGFGVRAVEALQRHWVMSSNVQL  42
HUPD_BRAJA  --MPT---SSQDNRILVLGIGNILWADEGFGVRAVEEFHRRYAVPDNVTI  45
HUPD_RHILV  MTIPYPLGPPPAPRILVLGIGNILWADEGFGVRAVEAFHKAYELSDNVTI  50
HUPD_RHOCA  ------MPAFKPERVLVLGIGNVLWADEGFGVRCVERMAETHALPANVRL  44
HOXM_ALCEU  ------------MVVAMGIGNVLWADEGFGVRCIETLQQRYQFAPQVCL  37

HupD_CBDB1  YDGGTAAMDLLDVISGREKIFILDAVHGGDEPGMIYRFRPEDIKTEQ-KI  96
hydD_DESDE  LEGGTAGLELVHLIKEVDYLIIVDAVNAKDEPGAIFRFEPEDIRVMPSQF  90
HYAD_ECOLI  VDGGTQGLNLLGYVESASHLLILDAIDYGLEPGTLRTYAGERIPAYLSAK  91
HUPD_THIRO  LDGGTQGIYLVDRVRKADVLVVFDAVDYGLPPGTMKRVEDDEVPRFLGAK  92
HUPD_BRAJA  LDGGTQGLYLVNYLEEADRLIVFDAIDYGLEPGRLKLVRDDEVPRFTGAK  95
HUPD_RHILV  LDGGTQGLYLVQFVNEHDRLIVFDAIDYGLEPGTMKVVEDDEVPKFTGAK 100
HUPD_RHOCA  LDGGTQGLYLLPFLEEAEALIVFDAVDFGFTPGTLVTMRDDDVPAFMGAK  94
HOXM_ALCEU  VDGGTQGLYLIHHVQAASRLLIFDAIDYGLPPGTLRIIEDEAVPKFLGAK  87

HupD_CBDB1  DISFHQMGLMEILNLAKYHDALPKDIIIYGIQP-GSMAPGFELTPAVDKA  145
hydD_DESDE  EVSFHQVGILEVLAMANILGTAPK-TLIFGIQP-KSLDMGLELTPEIRGV  138
HYAD_ECOLI  KMSLHQNSFSEVLALADIRGHLPAHIALVGLQPAMLDDYGGSLSELAREQ  141
HUPD_THIRO  KMSLHQTGFQEVLALAAMLGDYPKHLLLIGVQPVELDDFGGSLRPQIKER  142
HUPD_BRAJA  KMSLHQTGFQEVISAADLLGRCPKHLVLIGCQPLDLEDWGGPLTPPVRDQ  145
HUPD_RHILV  KMSLHQTGFQEVLSAADFMGHYPERLTLIGCQPLDLEDWGGPLTAPVRGV  150
HUPD_RHOCA  KMSLHQTGFQDVIATAQLMGYCPSRMTLIGCQPVELEDYGGSLRPAVAGQ  144
HOXM_ALCEU  KMSLHQTGFQEVLLLAQLTGQYPQQVVLIGCQPEELEDYGGSLRRVMKAA  137
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HupD_CBDB1  VNRVVEMMKAELD-----------------------------------  158
hydD_DESDE  LPRVKDLVLEEIQHII---------------------------------  154
HYAD_ECOLI  LPAAEQAALAQLAAWGIVPQPANESRC----LNYDCLSMENYEGVRLRQY  187
HUPD_THIRO  ITPAIEMALEYLAQFGVVARRREDSTVDSPHLPHPSLDLIAYESGRPGPE  192
HUPD_BRAJA  IAPSIDLACQVLAEWGVTVSRRSAPLAESERLLANDIDHANYEMR--PA-  193
HUPD_RHILV  IPAAIETAVRVLRSWGVAVTARPEGAAVPP-LLEHDIDFERYERRAEPAA  199
HUPD_RHOCA  IDFAIAEAVRELRAWG-IEVTKGATIS—NDLVDPSLARDAYERGRPSED  191
HOXM_ALCEU  VEDAVEKGADLLRRWGGMPVPRTAELAPAEAVTVPHLALDRYEAERPSPR  187

HupD_CBDB1  ---------------------------- 158
hydD_DESDE  ---------------------------- 154
HYAD_ECOLI  RMTQEEQG-------------------- 195
HUPD_THIRO  IACRIGDERVLTSLSARTVQPGNPHGDRQ 219
HUPD_BRAJA  ---------------------------- 193
HUPD_RHILV  LNC------------------------- 202
HUPD_RHOCA  EACRIGDHRFFPSAAKVRA---------- 210
HOXM_ALCEU  AACRIGDERFMPQDL------------- 202
```

Fig. 6.8 Sequence comparisons of the deduced HupD of strain CBDB1 with other related proteins.
hydD_DESDE, *Desulfitobacterium dehalogenans* (accession number AF157641.2); HYAD_ECOLI, *E. coli* Hydrogenase 1 (accession number P19930); HUPD_THIRO, *Thiocapsa roseopersicina* (accession number Q56362); HUPD_BRAJA, *Bradyrhizobium japonicum* (accession number Q45251); HUPD_RHILV, *Rhizobium leguminosarum* (accession number P27649); MBHL_RHOCA, *Rhodobacter capsulatus* (*Rhodopseudomonas capsulata*) (accession number Q03004); HOXM_ALCEU, *Ralstonia eutropha* (accession number P31909).

Table. 6.2. Identity and similarity percentages between predicted HupS, HupL and HupD polypeptides of strain CBDB1 and respective subunits of other [Ni-Fe] hydrogenases.

Subunit	Organism (Swissprot/NCBI Acc No)	Number of amino acid residues	Identitity (%)	Similarity (%)
[Ni-Fe] hydrogenase small subunit	*Dehalococcoides ethenogenes strain* 195 (Translated homologous region from the genome data of *D. ethenogenes*) (www.tigr.org)	354	86	88
	Desulfomicrobium baculatum (1CC1_S)	283	42	62
	Clostridium acetobutylicum (NP_00098668)	291	42	60
	Archaeoglobus fulgidus DSM (AB89863)	353	41	54
	Desulfovibrio baculatus (P13063)	315	40	59
	Shewanella oneidensis MR-1 (Q8CVD3)	378	40	57
	Desulfovibrio desulfuricans (ZP_00129920)	317	38	57
	Desulfovibrio desulfuricans G20(ZP_00129917)	317	38	56
	Desulfitobacterium dehalogenans (AAF13046)	362	38	55
	Desulfitobacterium hafniense (ZP_00129920)	375	37	56
	Desulfovibrio gigas (HQDVSG)	314	37	53
	Desulfovibrio vulgaris (P21853)	317	36	55
	Aquifex aeolicus VF5 (AAC07047)	349	36	53
	Campylobacter jejuni subsp. *Jejuni* (CAB73521)	379	35	52
	Wolinella succinogenes (P31884)	386	35	50
	Azotobacter vinelandii (P21950)	358	34	54
	Thiocapsa roseopersicina (AAA27409)	360	34	52
	Ralstonia eutropha (P31892)	360	33	54
	Escherichia coli O157:H7 (BAB34551)	372	33	52
	Helicobacter pylori J99 (AAD06157)	384	33	48
[Ni-Fe] hydrogenase large subunit	*Dehalococcoides ethenogenes strain* 195 (Translated homologous region from the genome data of *D. ethenogenes*) (www.tigr.org)	526	90	91
	Desulfitobacterium dehalogenans (AF157641.2)	516	43	59
	Desulfovibrio vulgaris (P21852)	567	39	57
	Desulfovibrio fructosovorans (1FRF_L)	564	39	54
	Desulfovibrio gigas (QDVLG)	551	38	52
	Desulfovibrio baculatus (P13065)	514	38	50
	Desulfovibrio desulfuricans (AAF43138)	543	37	51
	Shewanella oneidensis MR-1 (Q8EF87)	567	35	50
	Methylococcus capsulatus (AAK52318)	597	35	50
	Bradyrhizobium japonicum (P12636)	596	35	49
	Azotobacter vinelandii (P21949)	602	34	50
	Ralstonia eutropha (P31891)	617	33	51
	Rhizobium leguminosarum (P18636)	596	33	50

Subunit	Organism (Swissprot/NCBI Acc No)	Number of amino acid residues	Identitity (%)	Similarity (%)
Accessory protein for hydrogenase maturation (HupD)	*Dehalococcoides ethenogenes strain* 195 (Translated homologous region from the genome data of *D. ethenogenes*) (www.tigr.org)	158	89	91
	Desulfitobacterium dehalogenans (AF157641.2)	154	43	65
	Geobacter metallireducens (ZP_00082076)	172	43	65
	Dechloromonas aromatica RCB (ZP_00203781)	159	41	59
	Escherichia coli CFT073 (AAN82174)	164	39	59
	Escherichia coli K12 (AAC76029)	164	39	59
	Pseudomonas hydrogenovora (BAA13225)	164	38	57
	Desulfovibrio gigas (CAA11501)	165	37	58
	Desulfovibrio desulfuricans G20 (ZP_00129915)	165	36	60
	Azotobacter vinelandii (AAA19501)	207	36	57
	Desulfovibrio fructosovorans (A40591)	164	35	53
	Rhodobacter capsulatus (CAA78798)	210	34	58
	Azotobacter chroococcum (AAA64448)	209	34	57
	Desulfovibrio desulfuricans G20 (ZP_00129915)	173	34	52
	Bradyrhizobium japonicum (Q45251)	193	32	55
	Rhizobium leguminosarum (P27649)	202	33	57
	Ralstonia eutropha (P31909)	202	33	50

Overall, all three subunits of the predicted hydrogenase from strain CBDB1 share high similarity with the amino acid sequences of many periplasmic and membrane bound [Ni-Fe] hydrogenases, while showing no significant sequence similarity to [Fe]-only or to the families of cytoplasmic soluble hydrogenases.

6.3 Discussion

Sequencing of the PCR amplified putative [Ni-Fe] hydrogenase operon from chromosomal DNA of strain CBDB1 showed that the structural genes hupS and hupL were only 30 nucleotides apart and there were only two base pairs separating hupL from the hupD coding for a putative accessory protein (Fig. 6.2). Therefore, co-transcription of the structural genes along with hupD was anticipated. Amplification of all three genes by RT-PCR from mRNA supported this conclusion (Fig. 6.3). This would imply that the co-transcription from a promoter preceding hupS will result in a 3-kb polycistronic hupSLD mRNA (Fig. 6.2).

The absence of a putative rho-independent transcription terminator between hupS and hupL further strengthens the idea that the genes are transcribed as a polycistronic messenger. However, such a characteristic rho-independent transcription terminator was found downstream of hupD (Fig. 6.2). The RT-PCR experiment using primer pairs that were designed to detect co-transcription of the three neighbouring genes resulted in the expected product size demonstrating the operon organisation hupSLD (Fig. 6.4). Strain CBDB1 uses hydrogen as the only electron donor for respiration (Adrian et al 2000 a) and this aspect hampered us to perform experiments to check for the hydrogenase gene expression while strain CBDB1 utilizes an alternative electron donor.

A fragment with an expected size of 1600-bp amplified inside the gene cluster by nested PCR from RT-PCR product using the oligonucleotides DCH2 and DCH3 (Fig. 6.5) additionally proved the organisation of hupS, L and D genes in the hydrogenase coding operon (Fig. 6.4).

6.3.1 Classification

Based on the size of the small (354 aa) and the large subunit (526 aa) and the presence of a conserved histidine residue at the end of second [Ni-Fe] signature of the large subunit (Fig. 6.7), the hydrogenase encoded by hupSL of strain CBDB1 can be classified as a group-1 enzyme (Table. 6.3). Such a conserved histidine residue is found in three of the four classes of hydrogenases (Table. 6.3) except in group-4 membrane bound hydrogen evolving hydrogenases (Fox et al 1996) in which an arginine residue at the position of the conserved histidine is found (Fig. 6.7).

Table. 6.3 Classification of [Ni-Fe] hydrogenases based on their physiological function and length. Hydrogenase of strain CBDB1 falls in the group-1 based on its function, length and its conserved C-terminus (Table obtained from Vignais et al 2001).

Group	Function	Length of polypeptides (aa residues)		C-terminus His-residue of large subunit	Location of Hydrogenase
1	Membrane bound hydrogen uptake hydrogenases	S	291-384		M/P
		L	428-633	+	
2	2a) Cyanobacterial uptake hydrogenases	S	320		C
		L	531	+	
	2b) Hydrogen sensing hydrogenases	S	330-360		C
		L	475-485	+	
3	3a) F_{420} reducing hydrogenases	S	228-259		M/C
		L	398-456	+	
	3b) Bidirectional hyperthermophilic hydrogenases	S	237-266		C
		L	412-429	+	
	3c) Methyl viologen reducing hydrogenases	S	287-308		Unknown
		L	418-474	+	
	3d) Bidirectional NAD linked hydrogenases	S	178-209		C
		L	474-488	+	
4	Membrane bound hydrogen evolving hydrogenases	S	143-255		M
		L	358-569	-	

M, membrane-associated enzymes; C, cytoplasmic soluble enzymes; P, periplasmic enzymes.

The twin arginine signal sequence of HupS from strain CBDB1 (Fig. 6.6) makes clear distinction from soluble cytoplasmic hydrogenases at the genetic level. This signal peptide indicates that the protein can be recognised by a specific protein translocation system known as the TAT translocation pathway by which the correctly folded active dimer can cross the membrane (Wu et al 2000; Berks et al 2000; Voordouw 2000). Such a mechanism has been demonstrated in *E. coli* hydrogenase 1 and hydrogenase 2 (Sargent et al 1998) as well as the membrane bound [Ni-Fe] group-1 hydrogenases of *Wolinella succinogenes* (Gross et al 1999) and *Ralstonia eutropha* (Bernhard et al 2000).

Thus, the presence of the twin arginine signature in HupS of strain CBDB1 is an additional characteristic of this family of group-1 hydrogenases. Therefore, the two hydrogenase subunits of strain CBDB1 can be placed among group-1 membrane bound hydrogen uptake hydrogenases

based on its physiological, chemical and genetic characteristics (Table. 6.3). However, in contrast to all other membrane bound group-1 hydrogenases, the hydrogenase operon of strain CBDB1 lacks a gene homologous to cytochrome b coding gene (Fig. 6.9). The periplasmic [Ni-Fe] hydrogenases of *Desulfovibrio gigas* and *Desulfovibrio fructosovorans* also lack cytochrome b genes and hence they remain in the periplasmic space (Fig. 6.9).

6.3.2 Cytochrome b in membrane bound hydrogenases

In general, operons encoding membrane-bound uptake hydrogenases consist of the two structural genes S and L followed by a gene coding for a transmembrane cytochrome b to anchor the heterodimers to the membrane (hoxZ or hupC) (Fig. 6.9).

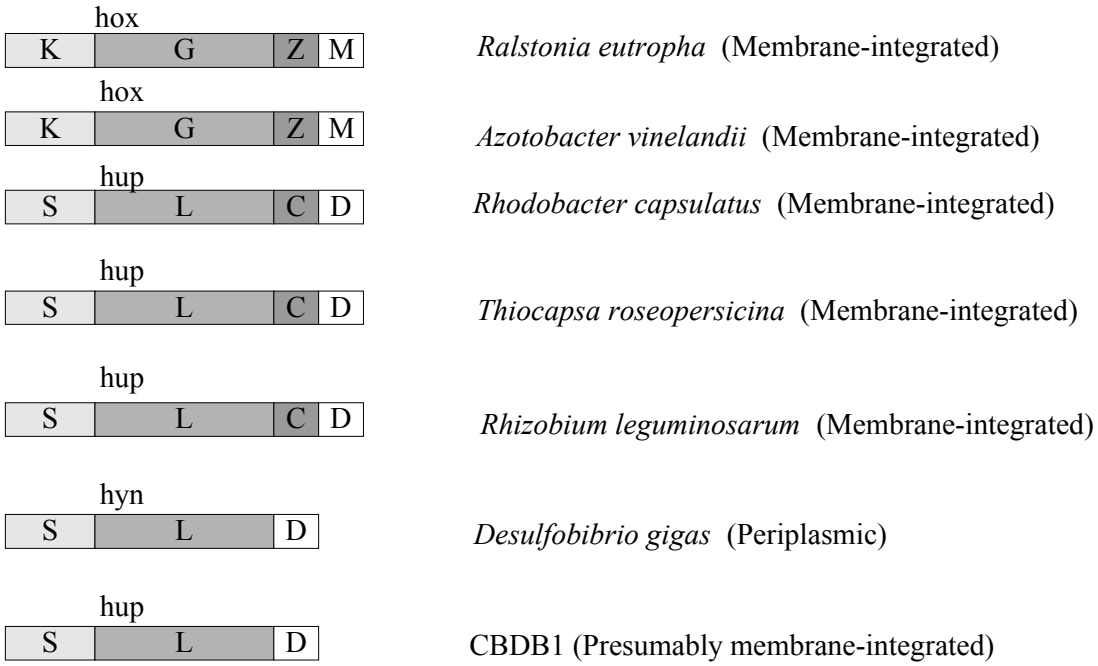

Fig. 6.9 Examples of [Ni-Fe] hydrogenase gene clusters. Functionally homologous genes are similarly shaded. The hydrophobic subunit cytochrome b (Hox Z or Hup C) adjacent to the large subunit (Hox G or Hup L) anchors the catalytic dimer to the membrane and hence is referred to as membrane-integrated. So far hydrogenases lacking this subunit are believed to be located in the periplasm but not membrane bound.

Since cytochrome b gene is not identified in strain CBDB1, the catalytic subunits (HupS and HupL) were screened for transmembrane helical segments that could attach the enzyme to the membrane. The small subunit (HupS) exhibited a strong transmembrane segment at C-terminus (Fig. 6.6) with a hydrophobicity of 0.24. Surprisingly none of the small subunits of described hydrogenases showed similar transmembrane helices with a hydrophobicity close to the value predicted for HupS of strain CBDB1 (Table. 6.4. Only few examples are shown). Whereas, the third subunit (cytochrome b) of the membrane bound hydrogenases show at least three transmembrane segments with high hydrophobicity.

Table. 6.4 Comparison of predicted number of transmembrane helices and average hydrophobicity of HupS from strain CBDB1 with other [Ni-Fe] hydrogenase small subunits (Computer program used for the prediction of transmembrane helices and average hydrophobicity; http://sosui.proteome.bio.tuat.ac.jp/sosui_submit.html).

Organism (Acc no)	Small subunit			Cytochrome b	
	Tm	Hydrophobicity	Reported location	Tm	Hydrophobicity
Ralstonia eutropha (P31892)	-	-0.21	M	5	0.36
Azotobacter vinelandii (P21950)	-	-0.18	M	4	0.28
Azotobacter chroococcum mcd 1 (P18190)	-	-0.36	M	4	0.25
Bradyrhizobium japonicum (P12635)	-	-0.14	M	3	0.34
Rhizobium leguminosarum (P18637)	-	-0.12	M	3	0.30
Rhodobacter capsulatus (P15283)	-	-0.23	M	3	0.15
Wolinella succinogenes (P31884)	-	-0.11	M	5	0.42
Desulfitobacterium dehalogenans (AAF13046)	-	-0.054	M	3	0.11
Desulfovibrio vulgaris (P21853)	-	-0.059	P	No cytochrome b present	
Desulfovibrio baculatus (P13063)	-	-0.055	P	No cytochrome b present	
Strain CBDB1	1	0.24	M	No cytochrome b present	

M, membrane associated enzymes; P, periplasmic enzymes; Tm, number of transmembrane helices.

The high hydrophobicity of HupS which is almost as high as that of cytochrome b of known membrane associated hydrogenases and several folds higher than the small subunits of other hydrogenases suggests the capability of HupS to attach the catalytic dimer HupSL to the cytoplasmic membrane. The higher percent of alpha helices in the secondary structure of HupS from strain CBDB1 (about 35 %) is much higher than those of other hydrogenases (about 20 %, data not shown) also indicates that the deduced protein encoded by hupS is more hydrophobic than the other membrane bound [Ni-Fe] hydrogenase small subunits.

Small subunits of all membrane bound [Ni-Fe] hydrogenases differ from those of the periplasmic enzymes by having a C-terminal hydrophobic extension (Menon et al 1992) showing a slightly higher hydropobicity than the enzymes located in the periplasm (Table. 6.4). Therefore, periplasmic localisation of HupS can consistently be excluded considering the strong hydrophobicity of HupS which is higher than those of membrane bound and periplasmic enzymes (Table. 6.4). The assumption that the enzyme is anchored in the membrane by the alpha helices and high hydrophobicity of HupS is supported by the experimental studies, which showed that hydrogenase activity is detected with the membrane fraction (Chapter 4). This apparently excludes the periplasmic location of hydrogenase activity in this strain.

6.3.3 An open question to hydrogen oxidation coupled to electron transport

The so called respiratory uptake hydrogenases have the capacity to support growth by transferring electrons from hydrogen by cytochrome b which is membrane bound and anchors the complex into an electron transport chain. This couples electron transfer proton translocation across the membrane. Thus the hydrogen oxidation is linked to reduction of various electron acceptors. In strain CBDB1, chlorobenzenes are the terminal electron acceptors and the chlororespiration in strain CBDB1 is a membrane-associated process (Chapter. 5).

While the hydrogenase heterodimer can alone catalyse hydrogenase reactions with artificial electron acceptors, cytochrome b is essential for physiologically relevant redox reactions (Dross et al 1992; Menon et al 1992; Sayavedra-Soto and Arp 1992). The isolated enzyme from *Wolinella succinogenes* containing cytochrome b, catalysed the reduction of the water soluble menaquinone analogue DMN by hydrogen whereas, the other form lacking cytochrome b did not (Dross et al 1992).

Similarly, a mutant of *Wolinella succinogenes* substituted in histidine residues of cytochrome b did not catalyse the reduction of DMN (a soluble analogue of menaquinone) but was still able to catalyse the reduction of benzyl viologen (Gross et al 1998). This indicates that the conserved histidine residues that ligate the heme groups in the membrane anchoring cytochrome b are essential for its physiological activity.

The absence of cytochrome b in strain CBDB1 could indicate that quinones are not involved as electron mediators in the electron transport of strain CBDB1. This is in agreement with the observation that hydrogenase of strain CBDB1 did not reduce DMN (a menaquinone analogue) (Chapter. 4). In addition, a menaquinone specific antagonist HOQNO had no effect on the dechlorination reaction mediated by cell suspensions of strain CBDB1 using hydrogen as electron donor (Chapter. 5). In contrast, all known membrane bound hydrogenase systems use either either menaquinone or ubiquinone as an electron acceptor in the electron transport chain.

Considering the above observation with strain CBDB1, it is assumed that the strong hydrophobic C-terminus of HupS could bind to the membrane but that the complex does not transports electrons to a quinone pool contrary to other membrane bound hydrogen uptake hydrogenases.

Thus, membrane bound hydrogenase of strain CBDB1 has a topology of subunits which has never been observed in any of the membrane bound hydrogenases described yet (Figs. 6.4, 6.9 and 6.10).

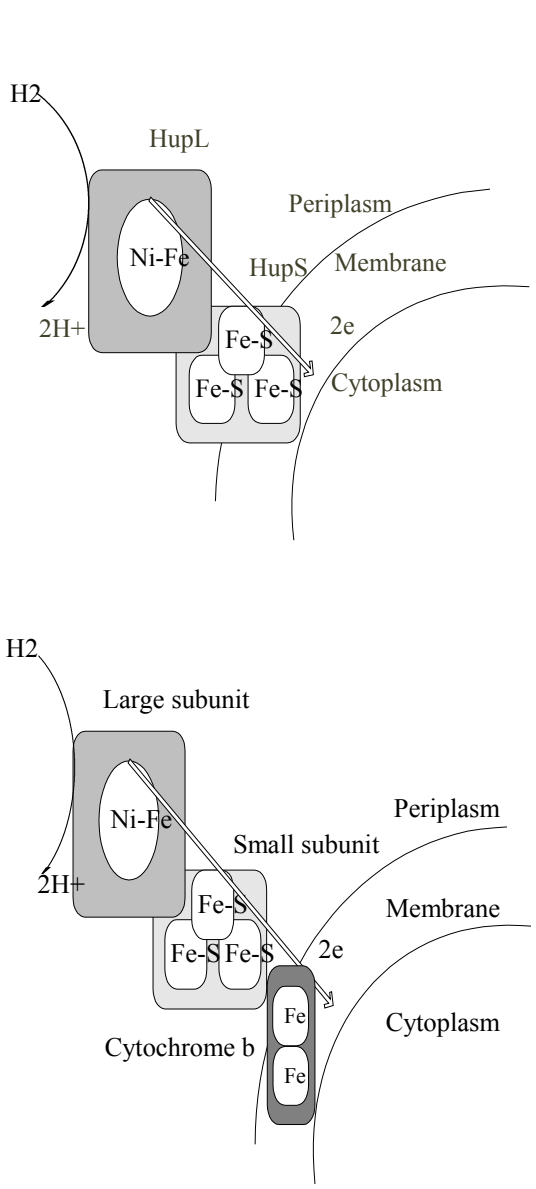

Fig. 6.10 Hypothetical topology of the catalytic subunits in [Ni-Fe] hydrogenase of strain CBDB1 (A) and the other so far described membrane bound hydrogenases (B). The hydrophobic subunit (cytochrome b) in so far described hydrogenases anchors the catalytic heterodimer in the membrane. This protein is replaced by a hydrophobic helix of HupS of the hydrogenase in strain CBDB1.

This new type of a membrane bound group-1 [Ni-Fe] hydrogenase without cytochrome b in a chlorobenzene respiring bacterium lends itself to further studies for new insights into electron transport and energy conservation of anaerobic dehalorespiration processes.

7. Hydrogen oxidation coupled to chlorobenzene reduction: An energy conserving dehalorespiratory system in *Dehalococcoides* species strain CBDB1

Abstract

An energy conserving mechanism is operative in the dehalorespiring *Dehalococcoides* species strain CBDB1. According to that, ATP synthesis is coupled to the proton motive force that is generated upon H_2 oxidation by hydrogenase and concomitant electron transport in the membrane resulting in a reduction of chlorobenzene. The theoretical maximal ATP/e ratio $(nATP/n_e)_{max}$, and H^+/e ratio for chlorobenzene reduction by hydrogen were calculated and compared with the ATP yield estimated from the experimental molar growth yield of strain CBDB1 growing by HCB reduction with hydrogen. Based on the results mentioned in the earlier chapters, probable reasons for the low growth yield of strain CBDB1 are suggested.

The ATP-synthase inhibitor DCCD inhibited the dechlorination reaction in cell suspensions with hydrogen as electron donor indicating that a proton motive force builds up during the dechlorination reaction. The latter effect was partially relieved by the addition of the protonophore TCS. This suggests that the release of the proton motive force allows the reductive dechlorination to continue.

7.1 Introduction

Electron transport phosphorylation is employed in most forms of life to conserve energy. According to this process, a thermodynamically favorable transfer of electrons from an electron donor to a suitable terminal acceptor via membrane bound redox components generates a proton motive force (pmf) across the membrane (Mitchell 1961). This pmf is used by ATP-synthase to couple proton transfer back across the membrane to the synthesis of ATP. NADH (E_o' NAD^+/NADH = -320 mV) obtained from substrate oxidation is used as an electron donor by most of the higher forms of life to reduce molecular oxygen (E_o' O_2/H_2O = +818 mV) in mitochondria, a multicomplex aerobic electron transport respiratory chain.

In contrast, microorganisms use various electron donors and acceptors. In case of strain CBDB1, hydrogen is used as electron donor to reduce chlorobenzenes (terminal electron acceptors) (Adrian et al 2000 a, Jayachandran et al 2003). Therefore, an energy conserving electron transport from hydrogen to chlorobenzene employing electron transport phosphorylation is expected. The electrochemical potential difference $\Delta E_o'$ between H_2 (E_o' of H^+/H_2 couple = -414 mV) and the chlorobenzene couples (for eg, E_o' of HCB/PeCB +478 mV) is in the same order of magnitude as for the oxidation of NADH with O_2 reduction in aerobic systems. However, a low growth yield of strain CBDB1 utilising HCB (2.1±0.24 g of cell protein/mol of Cl^- released) prompted us to focus on biochemical studies on chlorobenzene respiration by strain CBDB1 in order to derive possible information on energy conservation and probable reasons for the low growth yield of strain CBDB1.

7.2 Results

7.2.1 Effect of DCCD on reductive dechlorination

DCCD, an inhibitor of ATPase activity blocks the proton channel of this enzyme and prevents influx of protons into the cell (White 1995). In the presence of 0.5 mM DCCD, the reductive dechlorination reaction by intact cells with hydrogen as electron donor was almost completely inhibited (Fig. 7.1). The same concentration of DCCD did not have any effect on either hydrogenase or dehalogenase activity.

The inhibitory effect of DCCD was partially released upon the addition of 1 μM protonophore TCS (Fig. 7.1).

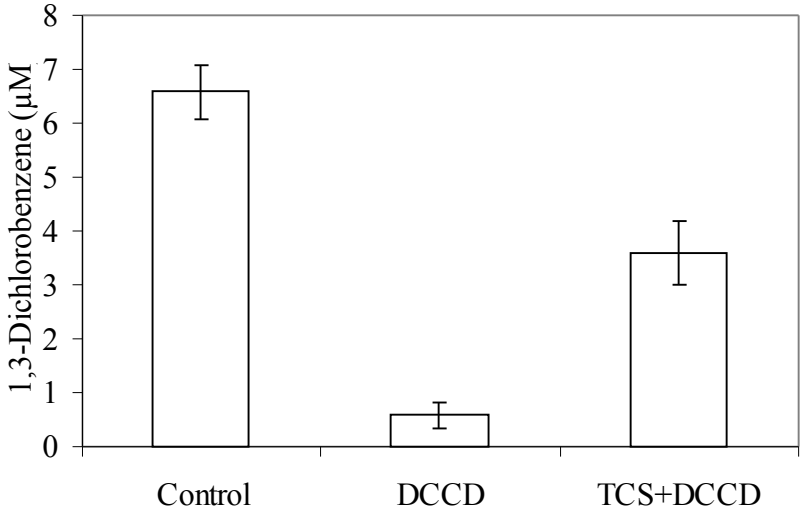

Fig. 7.1. Effect of DCCD and TCS on reductive dechlorination of 1,2,3-TCB by cell suspensions of strain CBDB1 in the presence of a nominal concentration of 2 mM H_2 as electron donor. The dechlorination reaction was carried out as described in Materials and methods.

7.2.2 Theoretical ATP/e and H^+/e ratios

The theoretical maximum ATP/e ratio $(nATP/n_e)_{max}$, can be calculated from the cellular phosphorylation potential ($\Delta G_p'$) of a cell and the change in standard redox potential difference ($\Delta E_o'$) of the energy providing reaction.

The maximum number of moles ATP, which can be generated by converting a mole of reactant to product is given by the relationship,

$$n_{ATP} = \frac{\Delta G'_o(redox\ react)}{\Delta G'_p} = -n\,F\,\frac{\Delta E'_o(redox\ react)}{\Delta G'_p}$$

because $\Delta G_o' = -n\ F\ \Delta E_o'$

where, F is the Faraday constant (96,485 J/mol V), n is the number of participating electrons.
Dividing by the number of electrons involved results in the maximal ATP ratio.

$$(n_{ATP}/n_e)_{max} = \frac{\Delta G_o'(redox\ react)}{n\ \Delta G'_p} = -F\ \frac{\Delta E_o'(redox\ react)}{\Delta G'_p}$$

From HCB, the different reductive dechlorination steps of the various chlorobenzenes are accompanied by free energy changes of -146 to -171 kJ/mol. For calculations, an average value of $\Delta G_o' = -160$ kJ/mol is used.

The change in free energy of a reaction depends on the concentration of the reactants. The free energy changes for the phosphorylation reaction of ADP to ATP with inorganic phosphate (P_i) ADP+ $P_i \leftrightarrow$ ATP is given by the equation,

$$\Delta G' = \Delta G_p' = \Delta G_o' + RT\ \ln\left(\frac{[ATP]}{[ADP][p_i]}\right)$$

Where,

$\Delta G'$ is the change in free energy; $\Delta G_o'$ is the change in free energy under standard conditions at pH 7.

$\Delta G_o'$ is known to be 32 kJ/mol (Kröger et al 2002). The general gas constant R has a value of 8.314×10^{-3} kJ/mol K and at 25°C the absolute temperature T has a value of 298.15 K.

Based on the findings that in *W. succinogenes* the concentration of ATP is equal that of ADP, Kröger et al (2002) calculated $\Delta G_p'$ for this organism to be between 44 to 50 kJ/mol ATP assuming P_i concentrations of 10 and 1 mM.

The low cell numbers of *Dehalococcoides* species strain CBDB1 do not allow to perform similar measurements with this organism. Assuming that the ratio of [ATP]/[ADP] may have a value between 0.1 and 10 and the P_i concentration may vary between 10 and 1 mM as assumed by Kröger et al (2002), $\Delta G_p'$ will have values between 37.5 to 54 kJ/mol ATP.

For estimating the maximal ATP/e ratio, an average value of $\Delta G_p' = 46$ kJ/mol is assumed.

$$(n_{ATP}/n_e)_{max} = \frac{\Delta G_o'(redox\ react)}{n\ \Delta G'_p} = \frac{160\ kJ/mol}{2 \times 46\ kJ/mol} = 1.74$$

Assuming 3 protons are translocated across the membrane per molecule of ATP synthesised (Brune et al 1987), the ATP/e ratio of 1.74 results in a maximum H^+/e ratio of 5.22. This means that the H^+/e ratio of chlorobenzenes (HCB through TCBs) respiration by strain CBDB1 would be either 4 or 5 as the H^+/e ratio is assumed to be an integer number. In theory, the potential difference between hydrogen (E_o' H^+/H_2 = -414 mV) and HCB (a mean E_o' +425.7 mV for several chlorobenzene couples starting dechlorination with HCB) (Dolfing and Harrision 1992) is sufficient to account for the synthesis of 2-3 ATP/Cl^- (two electrons are needed for a Cl^- removal) via a chemiosmotic mechanism.

Therefore, the corresponding values of ATP/e ratio were calculated for the various H^+/e ratios ranging from 1 to 5. According to that, 1.66, 1.33, 1, 0.66 and 0.33 of ATP/e were obtained for the values corresponding to H^+/e ratio of 5 through 1. To screen out the unlikely ratio, the corresponding percentages relative to the theoretical maximum ratio are compared to those of mitochondrial aerobic respiration with NADH. The ATP/e ratio of mitochondrial respiration is well established and the corresponding percentages were 50 and 57 % of the theoretical maximum (Hinkle et al 1991).

Table. 7.1 Theoretical ATP/e and H^+/e ratios of HCB respiration by strain CBDB1.

e^- donor	e^- acceptor	$\Delta G_o'$ (kJ/mol)	ATP/e ratio		(Assumed/ Maximum) x100	H^+/e ratio	
			Maximum[a]	Assumed		Maximum	Assumed
H_2	Chlorobenzenes	160	1.74	1.66	96	5.22	5
			1.74	1.33	76	5.22	4
	(HCB to TCBs)		1.74	1	57	5.22	3
			1.74	0.66	38	5.22	2
			1.74	0.33	19	5.22	1

[a] $(nATP/n_e)_{max}$; calculated with $\Delta G_p'$ = 46 kJ/mol ATP and the given value of $\Delta G_o'$.

In chlorobenzene respiration by strain CBDB1, the higher (1.74) and the lower (0.33) ATP/e ratio do amount to 96 and 19 % of the theoretical maximum. The assumed ATP/e ratio of 1 was found to be more likely, since it has the value (57 %) as like the mitochondrial system. The ATP gain relative to the theoretical maximum of bacterial respiration is not expected to exceed that of mitochondria by 20 % or more (Kröger et al 2002). Such a theoretical calculations (0.33 ATP/e,

and the assumed ratio of 1 for H^+/e) were carried out for the fumarate respiration by *W. succinogenes* using formate as the electron donor. The theoretical values were verified experimentally yielding about 18 g cells/mol of ATP (Mell et al 1982).

During HCB respiration by strain CBDB1 using hydrogen as electron donor and acetate as carbon source, a total cell protein yield of at most 2.1±0.24 g of cell protein/mol of Cl^- released was calculated (Jayachandran et al 2003). Assuming that the protein content accounts for 50 % of the dry cell weight (Mackiewicz and Wiegel 1998) a maximum of 4.2 g of dry cell mass/mol of Cl^- can be expected. Assuming a molar growth yield (Y_{ATP}) of about 7 g under these conditions (Loubiere and Lindely 1991) as assumed by Miller at al (1997 b) for PCE respiring *Desufurospirillum multivorans*, only about 0.6 mol of ATP per mol of Cl^- released would be generated. This is almost equal to the ATP/e ratio of 0.33 assuming H^+/e of 1 (Table 7.1). This low value of ATP estimated could mean that, in addition to protein synthesis, energy is required for some other purposes in chlorobenzene respiration by strain CBDB1. It might be that reverse electron transport is required which has been anticipated to operative in PCE respiring *Desufurospirillum multivorans* (Miller et al 1997 b). However, the experiment with TCS in Chapter 5 excludes that strain CBDB1 does require reverse electron transport for chlorobenzene reduction *in vivo*.

7.3 Discussion

Inhibition of the dechlorination reaction in cell suspensions with H_2 as electron donor in the presence of an inhibitor of ATP-synthase DCCD suggests that a proton motive force builds up during reductive dechlorination (Fig. 7.1). The inhibitory effect of DCCD was partially released by the addition of the protonophore TCS, probably because of the collapse of the proton motive force up across the membrane and allowing further reductive dechlorination.

The apparent extra-cytoplasmic location of respiratory enzymes (Chapter 4 and 5) and the effect of DCCD on reductive dechlorination by strain CBDB1 suggest a chemiosmotic model where, oxidation of hydrogen by membrane bound hydrogenase leads to proton translocation across the membrane and the formation of a pmf. The electron donor used by strain CBDB1 additionally suggests that, the energy is conserved only by a chemiosmotic process since fermentative growth is unknown with H_2. Hence, exergonic transfer of reducing equivalents from the electron donor (H_2) to terminal electron acceptor (chlorobenzenes) is coupled to the formation of an electrochemical proton gradient across the membrane. This energy can then be used by ATP-synthase to synthesise ATP.

Considering the results obtained in this dissertation, the probable reasons for the low growth yield of strain CBDB1 would be the following:

Molecular analysis of membrane bound group-1 [Ni-Fe] hydrogenase gene locus of strain CBDB1 (Chapter 6) showed that the operon lacks a gene coding for cytochrome b. As discussed in chapter 6, the absence of cytochrome b in strain CBDB1 possibly excludes the involvement of a quinone in the electron transport chain. Our biochemical experiments with HOQNO and DMN in Chapter 5 are additional supportive for the exclusion of a quinone in the electron transport chain of strain CBDB1. The experiment with protonophore clearly excluded the involvement of reverse electron transport in the reductive dechlorination of chlorobenzenes in strain CBDB1 (Chapter 5). Therefore, it is possible that, due to the lack of mediators in this respiratory system, a major part of the energy is released as heat.

Probably the respiration system in strain CBDB1 is not much evolved like in mitochondria or any other bacteria. The simplicity of the system suggests that it might be a precursor of the more developed respiratory systems in other bacteria and in mitochondria.

8. Summary The thesis describes physiological properties of *Dehalococcoides* species strain DBDB1 and enzymes involved in dehalorespiration. Growth of strain CBDB1 with HCB and PeCB provided an efficient system for the cultivation with highly chlorinated benzenes as electron acceptors. The key enzymes, hydrogenase and dehalogenase activities, were membrane associated with catalytic sites oriented towards the outside. Hydrogenase activity was also detected in the cytoplasm. Hydrogenase of strain CBDB1 was found to be highly sensitive to oxygen. Reductive dehalogenase activity detected in cells of strain CBDB1 pregrown with different chlorobenzene congeners as electron acceptors indicated that the different electron acceptors might induce different reductive dehalogenases. Dehalogenase activity of whole cells detected with artificial electron donors indicated that a redox potential of ≤-360 mV is needed for the chlorobenzenes reduction. However, steric effects also influenced dehalogenase activity because a higher reaction rate of dehalogenase activity was measured with methyl viologen (E_o'= -450 mV) compared to ethyl viologen (E_o'= -480 mV). Quinones seem not to be physiological electron mediators in the electron transport of strain CBDB1, because HONOQ, an inhibitor of quinone dependent redox reactions, did not inhibit the reductive dechlorination reaction by intact cells with hydrogen as electron donor. Dechlorination by intact cells with hydrogen as electron donor in the presence of a protonophore, TCS, revealed that strain CBDB1 does not require reverse electron transport. 1,2,3,4-TeCB strongly inhibited the dechlorination by whole cells with hydrogen as electron donor. The precise mechanism of inhibition by 1,2,3,4-TeCB is unknown. However, 1,2,3,4-TeCB is believed to interfere somehow with a step in the electron transport of strain CBDB1 without inhibiting hydrogenase or dehalogenase activity. The putative gene cluster consisting of structural genes hupS and hupL coding for a membrane bound group-1 [Ni-Fe] hydrogenase was amplified and sequenced. The gene cluster also contained hupD, a gene encoding an accessory protein for hydrogenase maturation. Amplification of all three genes by RT-PCR from mRNA confirms that the investigated gene cluster is transcribed as a polycistronic messenger. The amplified operon lacks a gene coding for cytochrome b that is found in all other membrane bound [Ni-Fe] hydrogenases known so far, but is not present in soluble hydrogenases. However, based on the hydrogenase activity in the membrane fraction, a unique hydrophobic segment found in the small subunit (HupS) of the hydrogenase could be responsible for attaching the complex to the membrane. In this aspect, the hydrogenase operon of strain CBDB1 differs from all other membrane bound hydrogenase operons described so far.

9. Zusammenfassung

Die Dissertation beschreibt die physiologischen und enzymatischen Fähigkeiten von *Dehalococcoides* sp Stamm CBDB1. Wachstum des Stammes CBDB1 auf Grundlage von Dehalorespiration mit HCB und PeCB konnte gezeigt werden. Diese Ergebnisse stellen ein neues System für die Kultivierung von Stamm CBDB1 mit hochchlorierten Benzolen als Elektronenakzeptoren zur Verfügung.

Hydrogenase und Dehalogenase sind die Schlüsselenzyme bei der Dehalorespiration. Beide Enzyme sind membrangebunden mit den katalytischen Zentren nach außen. Hydrogenaseaktivität konnte auch im Cytoplasma gemessen werden. Die Hydrogenase von Stamm CBDB1 ist sehr empfindlich gegenüber Sauerstoff, so verloren die Zellen sofort ihre Enzymaktivität, wenn sie der Luft ausgesetzt waren.

Die Aktivität der reduktiven Dehalogenasen in Kulturen von Stamm CBDB1 angezüchet auf verschiedenen Chlorbenzolen weisen darauf hin, dass verschiedene Elektronenakzeptoren unter Umständen unterschiedliche reduktive Dehalogenasen induzieren. Die Dehalogenaseaktivität gemessen an ganzen Zellen mit artifiziellen Elektrondonoren lassen vermuten, dass ein Redoxpotential von $\leq$-360 mV für die Reduktion von Chlorbenzolen benötigt wird. Auch sterische Effekte haben einem Einfluss auf die Dehalogenaseaktivität. So war die Dehalogenaseaktivität, die mit Methylviologen (E_0'=-450 mV) gemessen wurde, höher als die mit Ethylviologen (E_0'=-480 mV).

Chinone scheinen als physiologische Elektronenmediatoren in der Transportkette von Stamm CBDB1 auszuscheiden, weil durch HONOQ, einen Inhibitor von Chinon abhängigen Redoxreaktionen, die Dechlorierung in intakten Zellen mit Wasserstoff als Elektronendonor nicht gehemmt werden konnte. Bei Dechlorierungsversuchen mit intakten Zellen und Wasserstoff als Elektronendonor in Gegenwart des Protonophors TCS stellte sich heraus, dass Stamm CBDB1 nicht auf einen reversen Elektronentransport angewiesen ist.

1,2,3,4-TeCB inhibierte die Dechlorierung in Zellkulturen mit Wasserstoff als Elektronendonor. Der genaue Mechanismus der Inhibition ist unbekannt. Es wird vermutet, dass 1,2,3,4-TeCB den Elektronentransport unterbricht, ohne mit der Hydrogenase oder Dehalogenase zu interagieren.
Ein putatives Gencluster, bestehend aus den Strukturgenen hupS und hupL, die membrangebundene Gruppe 1 [Ni-Fe] Hydrogenasen codieren, wurde aus Stamm CBDB1 amplifiziert und sequenziert. Das Cluster enthielt außerdem hupD, ein Gen, das ein akzessorisches Protein für die Reifung der Hydrogenase codiert. Die Amplifizierung der drei Gene aus mRNA über RT-PCR bestätigte, dass das Gencluster als polycistronischer Messenger transkribiert wird. Dem amplifizierten Operon fehlte ein Gen, das für Cytochrom b codiert. Dieses Gen existiert in allen bisher bekannten membrangebundenen [Ni-Fe] Hydrogenasen, nicht aber in löslichen Hydrogenasen.

Die Hydrogenaseaktivität wurde in der Membranfraktion gemessen. Ein einzigartiges hydrophobes Segment in der kleinen Untereinheit (HupS) der Hydrogenase könnte für das Anhaften an der Membran verantwortlich sein. An diesem Punkt unterscheidet sich das Hydrogenaseoperon von Stamm CBDB1 von den bisher in der Literatur beschriebenen.

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11. List of tables

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