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A H₂-Oxidizing, 1,2,3-Trichlorobenzene-Reducing Multienzyme Complex

Isolated from the Obligately Organohalide-Respiring Bacterium

Dehalococcoides mccartyi Strain CBDB1

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Originality-Significance Statement: This work presents the first detailed description of the Hup uptake hydrogenase in *Dehalococcoides* species and identifies and characterizes a respiratory enzyme complex that exhibits hydrogen-dependent trichlorobenzene-reducing activity. The findings of this study have a great impact on our understanding of the biochemistry and bioenergetics of these strictly anaerobic organohalide-reducing bacterial species.

Summary

Dehalococcoides mccartyi is a small, slow-growing bacterium of the phylum *Chloroflexi* that conserves energy using aliphatic and aromatic organohalides as electron acceptors, and hydrogen as sole electron donor. A recent study identified a protein complex in the membrane of strain CBDB1 comprising a Hup hydrogenase, a complex iron-sulphur molybdoprotein and a reductive dehalogenase (RdhA) that catalyses reduction of 1,2,3,4-tetrachlorobenzene. Using a combination of size-exclusion chromatography, in-gel hydrogenase activity-staining, immunological analysis and mass spectrometry, we identified here a large molecular mass protein complex solubilized from the cytoplasmic membrane of *D. mccartyi* strain CBDB1 that catalysed H₂-dependent reduction of 1,2,3-trichlorobenzene (1,2,3-TCB) to 1,3-DCB. In-gel zymographic staining revealed H₂:benzyl viologen oxidoreductase activity associated with the complex and immunological analysis identified co-elution of CbdbA195, the predicted catalytic subunit of the iron-sulphur molybdoenzyme, the chlorobenzene-specific RdhA, CbrA, and traces of HupL, the catalytic subunit of the Hup hydrogenase. Quantitative reverse transcriptase PCR analyses indicated that the expression of the *hupL* and *cbdbA195* genes was induced by 1,2,3-TCB but not by hydrogen. Together, these data identify and describe a protein-based electron-transfer complex catalysing H₂ oxidation coupled to chlorobenzene reduction.

Introduction

Dehalococcoides mccartyi is a strictly anaerobic, obligately organohalide-respiring bacterium belonging to the *Chloroflexi* phylum (Kube et al., 2005; Löffler et al., 2013). *D. mccartyi* is remarkable for a number of reasons, but primarily because it uses only hydrogen as an electron donor and organohalide compounds as electron acceptor. Moreover, it lacks the ability to synthesize quinones (Schipp et al., 2013), which raises the question how it conserves energy.

Different *D. mccartyi* strains harbour variable numbers of *rdhAB* operons, which encode, respectively, the catalytic and putative membrane anchor subunits of cobalamin (B₁₂)-dependent reductive dehalogenases (Zinder, 2016a). The large number of reductive dehalogenase homologous proteins (RdhAs) is presumed to confer different organohalide acceptor specificities to each strain and in agreement with this presumption, there is limited overlap in the distribution of these enzymes between strains (Hug et al., 2013). For example, the chlorobenzene-reducing *D. mccartyi* strain CBDB1, the subject of the current study, encodes 32 RdhAs (Kube et al., 2005). This large number of *rdhAB* genes is all the more remarkable because these bacteria have small genomes of around 1.4 Mbp (Löffler et al., 2013).

The genome of *D. mccartyi* strain CBDB1 also encodes four different [NiFe]-hydrogenase enzymes (Kube et al., 2005; Hartwig et al., 2015). While the Hyc, Ech and Vhu hydrogenases all are predicted to have their respective active site oriented towards the cytoplasm (Seshadri et al., 2005), only the Hup hydrogenase has its active site on the outer leaflet of the cytoplasmic membrane and is thus appropriately oriented for energy conservation. Hup is also the most abundant of the hydrogenases (Morris et al., 2006), and is classified in the group I [NiFe]-hydrogenases (Vignais and Billoud, 2007). Hup is proposed to be important in H₂-uptake during organohalide respiration (Kube et al., 2005; Mansfeldt et al., 2014; Hartwig et al., 2015; Kublik et al., 2016).

A further highly abundant protein in *D. mccartyi* strain CBDB1 encoded by the gene locus *cbdbA195* is annotated as a ‘complex iron-sulfur molybdoenzyme’ (CISM; Rothery et al., 2008) and is predicted to be part of a protein complex, termed ‘CISM complex’ (Kublik et al., 2016). Its function remains enigmatic because although the putative catalytic subunit exhibits similarity to the catalytic subunit of respiratory formate dehydrogenases, the lack of the adjacent conserved active-site selenocysteinyl (or cysteinyl) and histidinyll residues that are required for formate dehydrogenase activity, coupled with the fact that *D. mccartyi* strains fail to use formate as an electron donor, indicate that this enzyme has a quite different biochemical function. The gene encoding *CbdbA195* (the major subunit of the CISM complex) is located in a presumptive operon (Fig. 1A) together with a gene encoding an integral membrane subunit (*CbdbA193*) typically found associated with polysulphide reductases or group I [NiFe]-hydrogenases, such as hydrogenase 2 of *Escherichia coli* (Menon et al., 1994; Sargent et al., 1998; Dubini et al., 2002; Pinske et al., 2015). This 403 amino acid- and 10 transmembrane-helix-containing subunit is not predicted to harbour any cofactors but contains a highly conserved glutamate residue in helix 8, which is possibly involved in proton translocation (Zinder, 2016b). Unusually, however, the operon encoding the CISM complex does not encode a presumptive electron-transferring small subunit typically associated with formate dehydrogenases (Kube et al., 2005; Rothery et al., 2008). Instead, the *hup* operon of *D. mccartyi* strain CBDB1 encodes a ferredoxin-like subunit (HupX; Fig. 1A) that might function in this capacity, similarly to what has been proposed for *D. mccartyi* strain 195 (Mansfeldt et al., 2014).

The *hup* operon also encodes a typical iron-sulphur (FeS)-containing hydrogenase small subunit, termed HupS (Hartwig et al. 2015). Interestingly, both the HupS and HupX subunits have their counterparts in hydrogenase 2 of *E. coli*, which are termed HybO and HybA, respectively (Menon et al., 1994; Sargent et al., 1998). Despite the fact that both HupS and HupX are predicted each to have a single transmembrane helix to attach the respective protein

to the membrane, the *hup* operon does not encode a polytopic membrane integral subunit, which is characteristic for these enzymes (Fig. 1A). These features of the *hup* and *cbdbA195-cbdbA193-fdhE* operons have led to the suggestion that the respective gene products might function together in organohalide respiration (Mansfeldt et al., 2014; Kublik et al., 2016; Zinder, 2016b).

The catalytic subunits HupL, CbdbA195 and RdhA all have in common that they are predicted to be located on the outside of the cytoplasmic membrane (Kube et al., 2005). This is because they either bear a Tat (twin-arginine translocation; Palmer and Berks, 2012) signal sequence or HupS of the Hup enzyme carries this sequence and presumably co-translocates HupL through the membrane as a passenger protein (Kube et al., 2005). Recent studies have demonstrated that the HupLS enzyme is actively transported across the membrane in a Tat-dependent manner in the heterologous host *E. coli* (Hartwig et al., 2015), strongly suggesting that this also occurs in *D. mccartyi* strain CBDB1.

With this arrangement of the respiratory chain, it is unclear how this bacterium generates a proton-motive force to conserve energy; however, the recent demonstration that the hydrogenase 2 complex of *E. coli* likely functions as a conformational proton pump (Pinske et al., 2015) suggests that this might be a plausible mechanism also pertinent to *D. mccartyi*.

Before this can be demonstrated, however, it is first important to isolate the catalytically active enzymes involved in H₂-dependent organohalide respiration. In this regard, recent advances were made when an enzyme complex capable of reducing 1,2,3,4-tetrachlorobenzene using reduced methyl viologen (MV^{•+}; $E^{0r} = -446$ mV) as electron donor was identified after blue-native PAGE of extracts derived from *D. mccartyi* strain CBDB1 (Kublik et al., 2016). Proteomic analysis of the complex identified the RdhAs CbrA and CbdbA80, as well as CbdbA195 and HupL, the latter of which was only loosely associated with the complex. In this study we analysed the Hup hydrogenase activity of *D. mccartyi* strain CBDB1 and identified two active, membrane-associated complexes in which it is a

component. Separation of these two complexes by anaerobic size-exclusion chromatography after membrane solubilization identified an enzyme complex capable of catalysing H₂-dependent reduction of 1,2,3-TCB to 1,3-DCB.

Results

Co-regulated expression of the hupXLS-hoxM and cbdbA195-A193-fdhE operons in response to 1,2,3-TCB

The genes encoding the Hup hydrogenase (cbdbA128 through cbdbA131) and the CISM components (cbdbA191 through cbdbA195) are located in distinct operons (Fig. 1A). Analysis of amino acid sequence similarities indicates that the HupL, HupS and HupX components are most similar to HybC, HybO and HybA of [NiFe]-hydrogenase 2 in *E. coli*, and represent, respectively, the large catalytic subunit (36% identity; 52% similarity), the electron-transferring small subunit (36% identity; 53% similarity), and the FeS cluster-containing ferredoxin-like subunit (34% identity; 50% similarity) (Menon et al., 1994; Sargent et al., 1998). HupX also exhibits similarity at the primary structural level to FdoH (40% identity; 58% similarity), the small subunit of the Fdh-O respiratory formate dehydrogenase in *E. coli* (Soboh et al., 2011). The *hoxM* gene encodes a HupL-specific endoprotease, which belongs to a class of hydrogenase-specific endoproteases required for enzyme maturation after cofactor insertion has occurred (Pinske and Sawers, 2016).

The *hup* operon in *D. mccartyi* CBDB1 does not encode a polytopic membrane protein typically encoded in similar operons (Menon et al., 1994); however, such a polytopic membrane protein is encoded within the operon encoding the CISM complex (CbdbA193; Fig. 1A). This polypeptide shares an amino acid sequence identity with HybB of the *E. coli* hydrogenase 2 complex of 28% (52% similarity). Otherwise, CbdbA193 shows a 42% amino acid sequence identity (61% similarity) with NrfD, the polytopic transmembrane subunit of polysulfide reductase from *Caldithrix* (Anantharaman et al., 2016). The other major gene

product encoded within this operon (CbdbA195) shares 40% amino acid identity (55% similarity) over its entire length with formate dehydrogenase N of *E. coli* (Berg et al., 1991).

It is important to stress, however, that the product of *cbdbA195* lacks the key active site residues (U196 and H197) necessary to catalyse formate oxidation (Berg et al., 1991), indicating that the *D. mccartyi* enzyme has a different function. The final gene in the presumptive operon (*cbdbA191*, *fdhE*) encodes a protein with 32% amino acid identity (48% similarity) to FdhE, a formate dehydrogenase-specific chaperone (Lüke et al., 2010).

Transcript levels of both operons were determined after growth in the presence of the chlorinated benzenes 1,2,3-TCB and 1,2,4-TCB, and with and without H₂ gas (Fig. 1B). The results clearly show that *hupL* transcript levels increased 250- to 1500-fold in the presence of 1,2,3-TCB, while those of *cbdbA195* increased 15,000- to 100,000-fold after 24 h compared to the low number of transcript copies per gene copies (see legend to Fig. 1) measured immediately before TCB and/or H₂ addition. A similar result was obtained in the presence of 1,2,4-TCB with the overall transcript levels being slightly lower for both analysed genes (Fig. 1B). Notably, induction of transcription was independent of hydrogen and depended only on the presence of organohalide. These findings indicated that induction of transcription of the *cbdbA195*-*A193*-*fdhE* and *hup* operons was dependent on the presence of the aromatic organohalide but not on the presence of H₂.

Identification of H₂:benzyl viologen oxidoreductase complexes in extracts of D. mccartyi strain CBDB1

Recent studies demonstrated that cell-free extracts of *D. mccartyi* strain CBDB1 show H₂-dependent benzyl viologen oxidoreductase activity (Hartwig et al. 2015). The crude extract derived from cells of *D. mccartyi* strain CBDB1 grown with H₂ and 1,2,3-TCB had a H₂:benzyl viologen oxidoreductase specific activity of 0.68 units mg protein⁻¹ (11.3 nkatal mg protein⁻¹). Gel electrophoretic separation of solubilised protein complexes followed by hydrogenase activity-staining revealed two main active enzyme complexes in *E. coli*,

hydrogenase 1 and 2, which are responsible for H₂-oxidizing activity (Pinske et al., 2012).

Three different complexes, which showed H₂:benzyl viologen oxidoreductase activity, were identified in the extract derived from *D. mccartyi* strain CBDB1 (Fig. 2). These complexes were termed A, B and C. Careful excision of these activity bands from the polyacrylamide gel and analysis by mass spectrometry after trypsin digestion identified the major proteins shown in Fig. 2 (a more complete list is in Table S1). Complexes A and B included CbdbA195 and CbdbA193, while complexes B and C identified HupL, as well as both subunits (VhuA and VhuG) of the soluble Vhu hydrogenase. Three polypeptides were found to be common to all three complexes: HupX, the ferredoxin-like FeS subunit encoded in the *hup* operon (Fig. 1A) and two RdhAs, CbrA (CbdbA84) and CbdbA80. Both of these RdhAs have been identified in previous studies as the most abundant reductive dehalogenases in *D. mccartyi* strain CBDB1 (Adrian et al., 2007; Kublik et al., 2016).

Similar hydrogenase-containing enzyme complexes were also identified in *D. mccartyi* strain DCMB5 (Figure S1) and mass spectrometric analysis of the prominent activity bands B and C revealed the same major proteins as identified in extracts of CBDB1 (data not shown). Together, these data reveal that for two different isolates of *D. mccartyi* two enzyme complexes could be identified, both of which exhibited hydrogenase activity. Both complex B and complex C included HupLSX, along with two different RdhAs, CbdbA80 and CbrA, while only the more slowly migrating complex B included CbdbA195 and CbdbA193.

Fractions enriched in complex B exhibit H₂-dependent 1,2,3-TCB-reducing activity.

A crude extract derived from *D. mccartyi* strain CBDB1 was prepared by solubilising cells with digitonin, clarifying the mixture by centrifugation, and then separating the protein complexes by size-exclusion chromatography on a Superdex-200 HR column. Eluted fractions were initially tested for total H₂-oxidizing activity using benzyl viologen as electron acceptor. Fractions containing activity ranged from fractions 4 through 10 with an activity peak in fraction 7 (estimated size of 150 kDa) (Fig. 3A). Analysis of these fractions after

native PAGE and hydrogenase activity staining showed that these activities correlated with complex C activity, which includes HupLS (Fig. 3B).

The activity profile of complex B revealed it eluted earlier than complex C during size exclusion chromatography, indicating a larger molecular mass compared with complex C (Fig. 3B). The peak activity fractions were 3 and 4, and the profile of complex B correlated with a size based on molecular mass standards of between 300 and 500 kDa (data not shown).

A previous study (Jayachandran et al., 2004) had indicated that crude extracts of *D. mccartyi* strain CBDB1 catalyse H₂-dependent 1,2,3-TCB reduction. Each fraction from the size exclusion column was therefore tested for H₂-dependent reduction of 1,2,3-TCB (Fig. 3A, dark grey histogram). It could be shown that this activity correlated well with the elution profile of complex B, with a maximal product concentration of 1,3-DCB (4 µM after 2 h incubation, corresponding to 99.6 pkat mg protein⁻¹) produced in fraction 4. Incubation of the sample in the presence of either air or 100% N₂ failed to result in any 1,3-DCB being detected, indicating that activity was H₂-dependent. Moreover, this activity was reliant on the presence of titanium(III) citrate in the assay buffer, which indicated that strongly reducing conditions were necessary for activity to be observed, similar to what was previously described for whole-cell hydrogenase activity (Jayachandran et al., 2004). The H₂-dependent, 1,2,3-TCB-reducing activity in the crude extract delivered a product concentration of 14.1 µM 1,3-DCB after a 2 h incubation (corresponding to 49 pkat mg protein⁻¹).

We then examined the distribution of the polypeptides CbdbA195, HupL and CbrA using peptide-specific antibodies (Fig. S2). Essentially, the distribution profiles of both CbdbA195 and CbrA correlated with both the H₂-oxidizing activity of complex B and with the H₂-dependent 1,2,3-TCB-reduction activity (Fig. 3C), suggesting that CbdbA195 (+ CbdbA193) and CbrA (CbdbA84) were mainly responsible for the latter activity. It should be noted, however, that traces of HupL could also be detected in fraction 4, although the peak signal of

HupL was in fraction 7 and thus correlated with the peak activity of H₂:benzyl viologen oxidoreductase (Fig. 3B).

Comparing the H₂:benzyl viologen oxidoreductase and H₂-dependent 1,2,3-TCB-reducing activities after anoxic size-exclusion chromatography revealed that approximately 60% and 58.5% of the original activities applied to the column, respectively, were recovered. This indicated that complex B retained significant activity after chromatographic separation.

The specific activity of H₂:benzyl viologen oxidoreductase in fraction 7 was estimated to be 1.4 U mg protein⁻¹ (23.3 nkat mg protein⁻¹; representative of 3 independent experiments), while that of H₂-dependent 1,2,3-TCB-reducing activity of the peak fraction 4 was 0.1 nkat mg protein⁻¹ (6 mU mg protein⁻¹). In comparison, the specific activity of methyl-viologen-dependent 1,2,3-TCB-reducing activity was 0.44 nkat mg protein⁻¹ (26.4 mU mg protein⁻¹) in the crude extract and approximately 1.5 nkat mg protein⁻¹ (90 mU mg protein⁻¹) in fraction 4.

This indicates that the H₂-dependent 1,2,3-TCB-reducing activity represented approximately 6% of the methyl viologen-dependent RdhA activity in this fraction.

Discussion.

In this study we have successfully released the H₂-oxidizing, 1,2,3-TCB-reducing multienzyme complex from the cytoplasmic membrane of *D. mccartyi* strain CBDB1, and separated it from the bulk of the HupLS hydrogenase activity. This finding strongly supports earlier data suggesting that quinones do not play a role in electron transfer within this complex (Jayachandran et al., 2004; Schipp et al., 2013; Kublik et al., 2016). Moreover, the main polytopic transmembrane component of this complex, encoded by *cbdbA193* (Fig. 1A), is not predicted to have a heme *b* cofactor. The *CbdbA193* membrane protein is related at the amino acid sequence level to FdoH, the small subunit of a formate dehydrogenase (Soboh et al., 2011), the NrfD component of polysulfide reductases (Anantharaman et al., 2016) and in particular to the transmembrane subunit HybB of hydrogenase 2 in *E. coli* (Menon et al.,

1994). Like CbdbA193, HybB lacks quinone-binding sites and recent studies (Pinske et al., 2015) strongly suggest that it acts as a conformational proton pump. This would be congruent with a similar function tentatively suggested for CbdbA193 (Kublik et al., 2016; Zinder, 2016b). Further components of this complex (Fig. S3) have homologues in the Hyd-2 enzyme of *E. coli*, in particular HupL, which is similar to the catalytic subunit HybC, as well as HupS, which is homologous to HybO, the electron-transferring and FeS-containing small subunit (Menon et al., 1994; Pinske et al., 2015; Sargent et al., 1998). Finally, HupX (Figs. 1A, S3) is similar to the ferredoxin-like electron transferring protein HybA of the hydrogenase 2 enzyme complex (Dubini et al., 2002). HybA has been shown to be essential for hydrogenase 2-dependent electron transfer to and from menaquinone in the cytoplasmic membrane of *E. coli* (Pinske et al., 2015). This protein also has an important role in mediating reverse electron flow for H₂ production by the hydrogenase 2 complex and, consequently, in the other direction, also for the presumptive electron transport resulting in proton pumping. The lack of identifiable quinones, together with the demonstrated uncoupler-independence of H₂-oxidizing, TCB-reducing activity in *Dehalococcoides* strains (Jayachandran et al., 2004; Nijenhuis and Zinder, 2005) all strongly support the contention that a proton gradient is likely to be generated by a proton-pumping mechanism.

The catalytic subunit of all three enzymes in the H₂-oxidizing complex isolated from the membranes of *D. mccartyi* CBDB1 is predicted to be on the outer leaflet of the cytoplasmic membrane (Kube et al., 2005; Kublik et al., 2016). All 32 RdhA polypeptides, the CbdbA195 protein, as well as the small subunit HupS of the Hup hydrogenase carry Tat signal peptides. While HupS likely co-transport the catalytic subunit HupL via a 'piggy-back' mechanism, as has been demonstrated for oxidoreductases in other organisms (Palmer and Berks, 2012), it remains unclear how HupX is transported across the cytoplasmic membrane. Its functional homologue HybA in *E. coli* bears its own Tat signal peptide (Dubini et al., 2002); however, HupX lacks a signal sequence, but might be co-transported either with HupLS or with

CbdbA195 as a passenger protein. The aldehyde oxidoreductase PaoABC of *E. coli* is exported to the periplasm as a heterotrimer via Tat (Lee et al., 2014), suggesting HupX might be a passenger in a HupSLX complex. An alternative possibility is that HupX is co-transported with CbdbA195, as a recent combined transcriptomic and proteomic study performed with *D. mccartyi* strain 195 (formerly *D. ethenogenes*) (Mansfeldt et al., 2014) identified a strong correlation in abundance between Det0112, the homologue of HupX, and CbdbA195, which would be in accord with this proposal. Co-transport of HupX and CbdbA195 would require, however, that both proteins are synthesized at the same time and our RNA analyses at least demonstrate that both the *hupXLS* and *cbdbA195-cbdbA194-fdhE* operons show similar regulation patterns. Notably, expression of both operons is induced when strain CBDB1 is grown with TCBs, but transcription appears to be unaffected by H₂.

By making use of antibodies raised against the catalytic subunits of all three enzymes in the TCB-reducing complex, we show that a tight association between CbdbA195 and CbrA exists and after gel-filtration the distribution of these two proteins correlated strongly with the H₂-oxidizing, 1,2,3-TCB-reducing activity. This is also in good agreement with CbrA being the main reductive dehalogenase involved in reduction of 1,2,3,4-tetrachlorobenzene and 1,2,3-TCB (Adrian et al., 2007). Notably, however, the HupL component appeared to be only very loosely associated with the complex, which substantiates our earlier findings (Kublik et al., 2016). The results presented here indicate that the CISM-CbrA enzymes formed the bulk of the active complex. Nevertheless, because traces of HupL were still associated with the active TCB-reducing complex (see the weak signal in the western blot in Fig. 3C), it is premature to state that CbdbA195 is involved in the H₂-oxidizing activity of the complex.

The generally recognized mechanism of reductive dehalogenase enzymes requires the reduction of Co^{II} species to Co^I (Bommer et al., 2014; Payne et al., 2015; Schubert and Diekert, 2016). Formally, the redox potential of this couple is around $E^{\circ} = -480$ mV for isolated norpseudovitamin B₁₂ when not associated with a protein; however, the standard

redox potential is raised by approximately 100-120 mV when norpseudovitamin B₁₂ is part of the catalytic site of the enzyme, as has been demonstrated for the isolated tetrachloroethene-reducing PceA enzyme (Kräutler et al., 2003; Schumacher et al., 1997; Schubert and Diekert, 2016). However, the two [4Fe-4S] clusters in RdhA have a low midpoint potential of around -440 and -480 mV at slightly alkaline pH (Schumacher et al., 1997; van de Pas et al., 1999), and these presumably deliver the electrons to the Co atom. It is possible that the reduction of these clusters required for catalysis by CbrA is the reason the activity of the complex is dependent on titanium (III) citrate ($E^{\circ} = < -480$ mV at concentrations above 1 mM). Nevertheless, it remains to be determined how the energy conserved by this complex *in vivo* is sufficient to reduce Co^{II} to Co^I and to pump a proton through the complex. Based on the findings presented here, however, we can now attempt to address these questions by incorporating this enzyme complex into proteoliposomes to study the bioenergetic mechanisms underlying energy conservation by this unique enzyme complex.

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Authors' contributions

SH, ND, and AK carried out all experiments with *Dehalococcoides mccartyi*; SH and UL performed the qRT-PCR studies; and DT, MvB and LA carried out mass spectrometric analyses. RGS, SH and LA drafted the manuscript and RGS, LA, MvB, and UL conceived the study. All the data presented in this manuscript are original data and all authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests and have no conflict of interest.

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Figures legends.

Figure 1. Expression of the *hupL* and *cbdbA195* genes is induced in response to trichlorobenzenes.

A. Organisation of the *hup* and *cbdbA915-cbdbA193-fdhE* operons in the genome of *D. mccartyi* strain CBDB1 (Kube et al., 2005). The locus tag of each respective gene is indicated.

B. Quantitative RT-PCR analysis of the *hupL* and *cbdbA195* gene transcripts after incubation

of cells with the indicated respective trichlorobenzenes as electron acceptors (see Supplementary Materials for details of the Experimental procedures) is shown. Note that the relative increase in transcripts per gene copy within 24 h is shown. Due to the preceding starvation in the absence of TCBs and hydrogen, the initial transcript levels were, respectively, 4×10^{-3} and 3×10^{-5} transcripts/gene copies for *hupL* and *cbdbA915* and increased to 0.1 and 7 transcripts per gene copy during the incubation. The dark grey bars indicate incubation with H_2 and the light grey bars indicate incubation with N_2 in the gas phase. The error bars represent the standard deviation from duplicate cultures, each analysed in triplicate by RT-qPCR.

Figure 2. Identification of H_2 :benzyl viologen oxidoreductase complexes after in-gel activity staining.

Active hydrogen-oxidizing enzyme complexes were separated anaerobically by native PAGE (7.5% w/v polyacrylamide) and the gel was subsequently incubated in 50 mM MOPS buffer pH 7 including benzyl viologen and triphenyltetrazolium chloride, and under an atmosphere of H_2 gas (Ballantine and Boxer, 1985; Pinske et al., 2012). The active enzyme complexes (labelled as A, B, C on the left of the gel) identified in the extract of *D. mccartyi* strain CBDB1 are indicated. The hydrogenase complexes in extracts of *E. coli* are shown on the right side of each gel as a control: Hyd-1, hydrogenase 1; Hyd-2, hydrogenase 2; Hyd-3, hydrogenase 3; Fdh-O/N indicates a hydrogenase side-activity of formate dehydrogenases O and N (Soboh et al., 2011). Approximately 5 μ g of crude extract from *D. mccartyi* strain CBDB1 and 1 μ g of *E. coli* crude extract were applied to the gel as a control. The CbdbA195, CbdbA193 and [NiFe]-hydrogenase-related polypeptides identified by mass spectrometric analysis of isolated gel fragments are shown on the left side of the figure. The number of polypeptides identified is indicated in parentheses. A more complete list of identified polypeptides is given in Table S1.

Figure 3. Identification of a H₂:1,2,3-TCB oxidoreductase enzyme complex in solubilized membrane samples derived from *D. mccartyi* strain CBDB1.

Protein complexes present in crude extract (~ 1 mg protein) treated with digitonin were separated by size-exclusion chromatography (see Supplementary Material for details of Experimental procedures) and aliquots of each elution fraction were analysed for H₂:benzyl viologen oxidoreductase activity (white histograms in part A), H₂: 1,2,3-TCB oxidoreductase activity (dark grey histograms in panel A), in-gel hydrogenase activity (panel B) or by western blotting (panel C). Panel A. The total elution volume is given below the graphic, the fraction numbers analysed are given as F1, F2 etc. and the dotted line represents the absorption profile measured at 280 nm. The total respective enzyme activity in each fraction is shown as a percentage of the original total activity applied to each column run. Activities are shown with standard deviation for a representative chromatographic separation and are derived from the average of two independent measurements. Note that the measurement of H₂:benzyl viologen oxidoreductase activity in fractions 1 through 4 was carried out once. 100% activities: H₂:benzyl viologen oxidoreductase was 0.68 units mg protein⁻¹ (11.3 nkat mg protein⁻¹: nkat is equivalent to nmoles of substrate converted per second); and H₂:1,2,3-TCB oxidoreductase was 14.1 ± 2.1 μM 1,3-DCB (corresponding to 49 pkat mg protein⁻¹) produced after a 2 h incubation at room temperature. Panel B. Aliquots (5 μl) from the indicated fraction were separated anaerobically by native PAGE and stained for hydrogenase activity. CE, indicates crude extract (5 μg protein), and the arrows on the right of the gel show the migration position of the respective active B and C complexes. Panel C. Shown is a western blot in which aliquots of fractions (10 μl) were separated by denaturing SDS-PAGE using 10% w/v polyacrylamide gels. After transfer to a nitrocellulose membrane, the polypeptides attached to the nitrocellulose membrane were identified using a mixture of affinity-purified antibodies directed against CbdbA195 (1:8000), HupL (1:500) and CbrA

(1:8000). The molecular mass markers are shown on the left as kDa and the migration position of the respective polypeptides is indicated on the right. CE, indicates crude extract.

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Figure 1

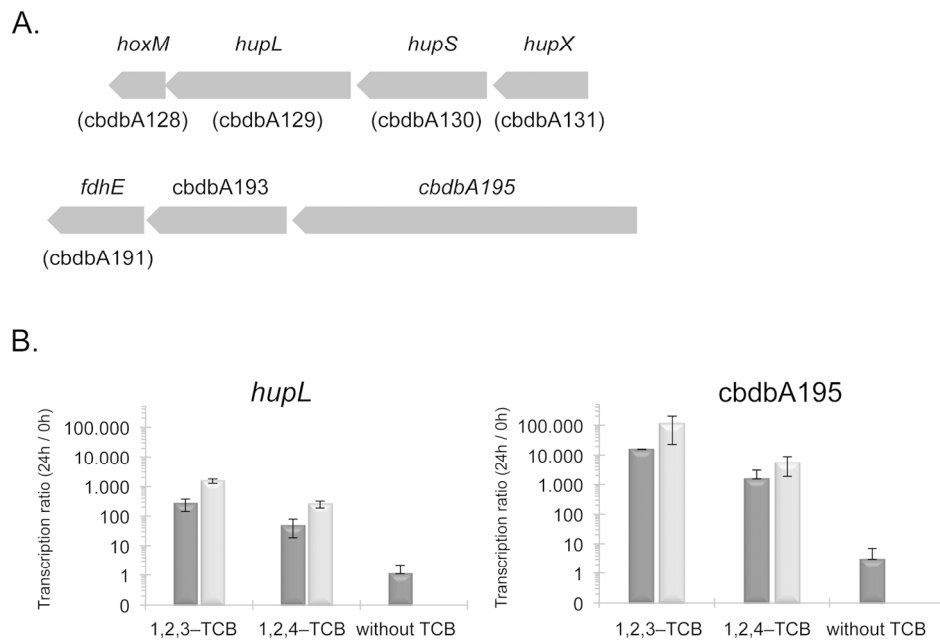


Figure 1

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Accept

Figure 2

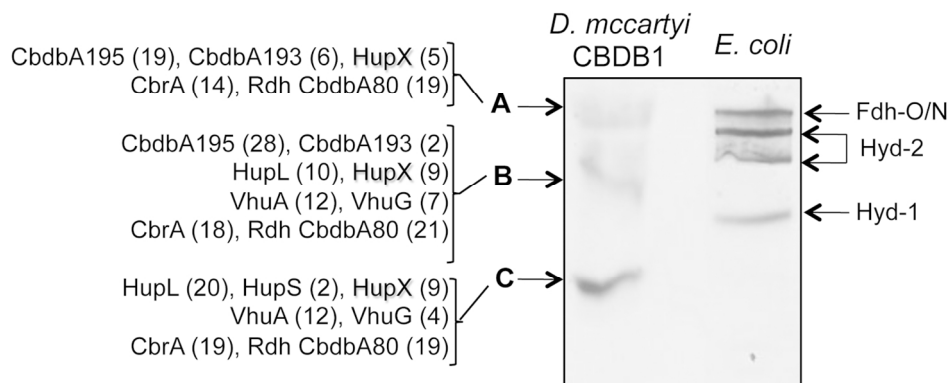


Figure 2

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Figure 3

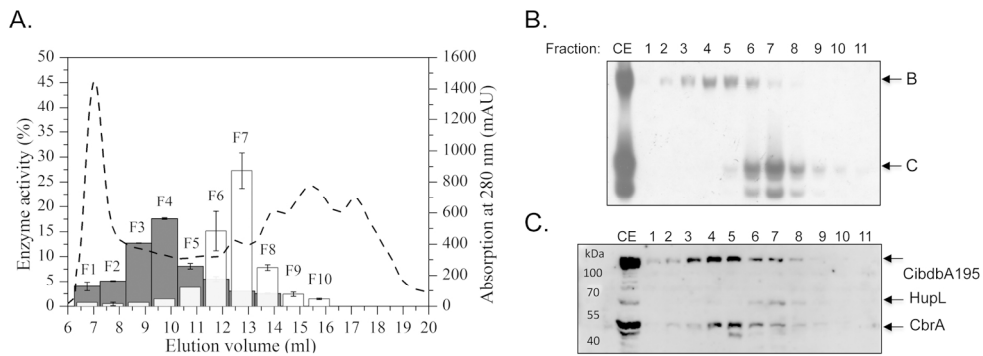


Figure 3

160x69mm (300 x 300 DPI)

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