Heterologous complementation studies in *Escherichia coli* with the Hyp accessory protein machinery from *Chloroflexi* provide insight into [NiFe]-hydrogenase large subunit recognition by the HypC protein family

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Six Hyp maturation proteins (HypABCDEF) are conserved in micro-organisms that synthesize [NiFe]-hydrogenases (Hyd). Of these, the HypC chaperones interact directly with the apo-form of the catalytically active large subunit of Hyd enzymes and are believed to transfer the Fe(CN)₂CO moiety of the bimetallic cofactor from the Hyp machinery to this large subunit. In *E. coli*, HypC is specifically required for maturation of Hyd-3 while its paralogue, HybG, is specifically required for Hyd-2 maturation; either HypC or HybG can mature Hyd-1. In this study, we demonstrate that the products of the *hypABCDEF* operon from the deeply branching hydrogen-dependent and obligate organohalide-respiring bacterium *Dehalococcoides mccartyi* strain CBDB1 were capable of maturing and assembling active Hyd-1, Hyd-2 and Hyd-3 in an *E. coli* hyp mutant. Maturation of Hyd-1 was less efficient, presumably because HypB of *E. coli* was necessary to restore optimal enzyme activity. In a reciprocal maturation study, the highly O₂-sensitive H₂-uptake HupLS [NiFe]-hydrogenase from *D. mccartyi* CBDB1 was also synthesized in an active form in *E. coli*. Together, these findings indicated that HypC from *D. mccartyi* CBDB1 exhibits promiscuity in its large subunit interaction in *E. coli*. Based on these findings, we generated amino acid variants of *E. coli* HybG capable of partial recovery of Hyd-3-dependent H₂ production in a hypC hybG double null mutant. Together, these findings identify amino acid regions in HypC accessory proteins that specify interaction with the large subunits of hydrogenase and demonstrate functional compatibility of Hyp accessory protein machineries.

INTRODUCTION

[NiFe]-hydrogenases are widespread amongst archaean and bacterial species (Vignais & Billoud, 2007). These enzymes can either oxidize H₂ to generate a chemiosmotic proton gradient via a membrane-based electron transfer chain, as well as provide a source of reducing power, or dissipate accumulated intracellular reductant by reducing protons to produce H₂; some perform both functions under certain physiological conditions (Lubitz et al., 2014; Pinske et al., 2015; Vignais & Billoud, 2007). The active site of all [NiFe]-hydrogenases studied to date has a bimetallic [NiFe]-cofactor that has one carbon monoxide and two cyanide moieties ligated to the iron ion (Lubitz et al., 2014; Böck et al., 2006). These diatomic ligands tune the redox status of the nickel ion to facilitate H₂ activation
enzymes, which synthesize and deliver the cyanide ligands subunit followed by introduction of the nickel ion (Böck et al., 2006; Forzi & Sawers, 2007). Subsequent to successful cofactor insertion, a C-terminal peptide present on the large subunit of most [NiFe]-hydrogenases is cleaved by a hydrogenase-specific protease and further assembly of the enzyme can then be completed (Böck et al., 2006; Pinske & Sawers, 2014).

The Hyp proteins include HypA and the GTPase HypB, which, together with the peptidyl-prolyl cis-trans isomerase SlyD (Zhang et al., 2005), deliver the nickel ion; HypC, which is a small iron- and CO₂-binding protein (Soboh et al., 2013); the FeS cluster protein HypD, which acts as a scaffold for assembly of the Fe(CN)₂CO moiety (Bürstell et al., 2012; Stripp et al., 2013); and the HypE and HypF enzymes, which synthesize and deliver the cyanide ligands (Reissmann et al., 2003). All archaea and bacteria that synthesize [NiFe]-hydrogenases, and that have been characterized so far, have at least one set of the hypABCDEF genes. Some bacteria that synthesize several [NiFe]-hydrogenases possess more than one set of these genes or they have extra copies of genes encoding paralogues of certain Hyp accessory proteins. For example, the betaproteobacterium Ralstonia eutropha (also known as Cupriavidus necator) has more than one set of the hyp genes and each appears to be specific for a particular [NiFe]-hydrogenase synthesized by the bacterium (Wolf et al., 1998). The gammaproteobacterium Escherichia coli also synthesizes multiple hydrogenases but has an extra copy each of the hypC and the hypA genes, which are termed hybG and hybF, respectively, in the genome (Blokesch et al., 2002). HypC is a small protein that interacts directly with the apo-large subunit (Drapal & Böck, 1998; Magalon and Böck, 2000) and is thought to facilitate delivery of the Fe(CN)₂CO moiety into the active site (Böck et al., 2006). In E. coli, the HypC protein is specifically required for maturation of hydrogenase 3 (Hyd-3), which is a component of the H₂-evolving formate hydrogenase (FHL) complex, while its parologue HybG is specifically required for maturation of the H₂-oxidizing Hyd-2 enzyme. Both HypC and HybG can function in the maturation of the O₂-tolerant and H₂-oxidizing Hyd-1 enzyme (Blokesch et al., 2002). As Hyd-4 has not yet been successfully synthesized in an active form (Böck et al., 2006) it is unclear whether HypC or HybG is required for maturation of that enzyme.

The Hyp accessory proteins share a comparatively high amino acid sequence similarity (minimally around 45–50 %) within the bacteria, and even to a certain extent between bacterial and archaeal species, suggesting that their functions are highly conserved (Böck et al., 2006; Vignais & Billoud, 2007). The Hyp proteins from R. eutropha have been heterologously synthesized and are functional in E. coli (Bürstell et al., 2012; Schiiffels et al., 2013; Schiiffels & Selmer, 2015) and have been shown to generate a functional R. eutropha [NiFe]-hydrogenase in the heterologous host. Heterologous expression studies have also been successfully conducted with the hynSL hydrogenase structural genes from the gammaproteobacterium Alteromonas macleodii 'Deep ecotype' and from the photosynthetic gammaproteobacterium Thiocapsa roseopersicina in E. coli (Weyman et al., 2011; Yonemoto et al., 2015). However, no systematic analysis of Hyp protein function between a phylogenetically distantly related bacterium and E. coli has been undertaken. Addressing this question might provide useful information regarding evolutionary conservation of Hyp enzyme function.

In this study, we examined whether the Hyp machinery from the strictly anaerobic organohalide-respiring bacterium Dehalococcoides mccartyi strain CBDB1, which is a member of the deeply branching Chloroflexi phylum (Kube et al., 2005), is functional in E. coli. D. mccartyi CBDB1 has a small genome of 1.4 Mbp, yet due to its H₂-dependent lifestyle it has the coding capacity for four [NiFe]-hydrogenases (Kube et al., 2005). The most abundant enzyme, Hyp, exhibits similarity to group I [NiFe]-hydrogenases and is proposed to be important in H₂-uptake during organohalide respiration (Kube et al., 2005; Mansfeldt et al., 2014). D. mccartyi strains have a single copy of each hyp gene in their genome (Kube et al., 2005; Póritz et al., 2013; Seshadri et al., 2005). This suggests that the HypC protein of the bacterium exhibits flexibility with regard to its capacity to facilitate the maturation of different hydrogenase apo-large subunits. We demonstrate in this study that this is indeed the case and show that the Hyp machineries from D. mccartyi CBDB1 and E. coli exhibit reciprocity in their function. Moreover, these findings guided an amino acid-exchange programme with E. coli HybG allowing identification of amino acid motifs that might be important in recognition of the hydrogenase apo-catalytic subunit.

METHODS

Strains and growth conditions. All strains used in this study are listed in Table 1. E. coli strains were routinely grown at 37 °C on LB agar plates or with shaking in LB broth (Miller, 1972). For the analysis of hydrogenase activity, cells were cultivated under anoxic conditions at 37 °C without agitation. E. coli cells were grown either in M9 minimal medium (Miller, 1972) or in the rich medium, TGYEP (1 % w/v peptone from casein, 0.8 % w/v glucose, 0.5 % w/v yeast extract, 100 mM potassium phosphate, pH 6.5 and 0.1 % (v/v) including the trace element solution SLA; Hormann & Arendsen, 1989) as described (Begg et al., 1977). When required, anhydrotetraacycline (AHT) was added at a final concentration of 200 µg ml⁻¹ to induce expression of genes cloned in pASK-IBA3+ derivatives. AHT was added to the cultures when an OD₆₀₀ of 0.3 had been attained. Cells were harvested when cultures had reached an OD₆₀₀ of between 0.8 and 1.2. Where indicated the growth medium was supplemented with 100 µM NiCl₂ and, when required, the antibiotics ampicillin, kanamycin or chloramphenicol were added to a final concentration of 100 µg ml⁻¹, 50 µg ml⁻¹ or 12 µg ml⁻¹, respectively. Cells were harvested anaerobically by centrifugation at 5000 g for 15 min and at 4 °C. Cell pellets were used immediately or stored at −20 °C until use.
<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BEF314</td>
<td>MC4100 ΔhypB-hypE Ω(hyp : : cat pACYC184)</td>
<td>Jacobi et al. (1992)</td>
</tr>
<tr>
<td>DHP-A</td>
<td>MC4100 ΔhypA</td>
<td>Jacobi et al. (1992)</td>
</tr>
<tr>
<td>DHP-B</td>
<td>MC4100 ΔhypB</td>
<td>Jacobi et al. (1992)</td>
</tr>
<tr>
<td>DHP-C</td>
<td>MC4100 ΔhypC</td>
<td>Jacobi et al. (1992)</td>
</tr>
<tr>
<td>DHB-G</td>
<td>MC4100 ΔhypG</td>
<td>Blokesch et al. (2001)</td>
</tr>
<tr>
<td>DHP-D</td>
<td>MC4100 ΔhypD</td>
<td>Jacobi et al. (1992)</td>
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<td>DHP-E</td>
<td>MC4100 ΔhypE</td>
<td>Jacobi et al. (1992)</td>
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<td>DHP-F2</td>
<td>MC4100 ΔhypF</td>
<td>Paschos et al. (2002)</td>
</tr>
<tr>
<td>FTD150</td>
<td>MC4100 ΔhyaB, ΔhybC, ΔhycE, ΔhyfB-R</td>
<td>Redwood et al. (2008)</td>
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<td>JW2958</td>
<td>BW25113 ΔhybG : : KanR</td>
<td>National BioResources Project (NIG, Japan)</td>
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<tr>
<td>MC4100</td>
<td>F', araD139, Δ(argF-lac)U169, λ−, rpsL150, relA1, deoC1, βhfD5301, Δ(fruK-yeiR)725( fruA25), rbsR22, Δ(fmb-fimE)632( : : IS1)</td>
<td>Casadaban (1976)</td>
</tr>
<tr>
<td>SHH228</td>
<td>DHP-C ΔhybG</td>
<td>This study</td>
</tr>
<tr>
<td>SHH229</td>
<td>BEF314 ΔhybG : : KanR</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<td>pCP20</td>
<td>FLP + , λcl857 +, λp3 Rep +, AmpR, CmR</td>
<td>Cherepanov &amp; Wackernagel (1995)</td>
</tr>
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<td>phybGstrept</td>
<td>pASK-IBA3, hybG +, AmpR</td>
<td>Soboh et al. (2014)</td>
</tr>
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<td>pCThybG-HQL</td>
<td>Like phybGstrept but deleted in codons 17, 18 and 19 (encoding amino acids HQL)</td>
<td>This study</td>
</tr>
<tr>
<td>pCThybG + GS</td>
<td>Like phybGstrept but including an insertion of two codons after codon 33 (encoding amino acids GS)</td>
<td>This study</td>
</tr>
<tr>
<td>pCThybG-HQL + GS</td>
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<tr>
<td>pCThybG-CEGN</td>
<td>Like phybGstrept but deleted in codons 34 through 37 (encoding amino acids CEGN)</td>
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<td>pCThybG-Ystop</td>
<td>Like phybGstrept but with the introduction of a stop codon at codon position 77</td>
<td>This study</td>
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<td>pJA36</td>
<td>pBR322, hypBCDE + from E. coli, AmpR</td>
<td>Jacobi et al. (1992)</td>
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<tr>
<td>pJA1021</td>
<td>pACYC181 hypC + CmR</td>
<td>Jacobi et al. (1992)</td>
</tr>
<tr>
<td>pSHH9</td>
<td>pET28a +, hypC + from D. maccarii CBDB1 with C-terminal His-tag, Kan'</td>
<td>This study</td>
</tr>
<tr>
<td>pSHH10</td>
<td>pET28a +, ΔhypD from D. maccarii CBDB1 with N-terminal His-tag, Kan'</td>
<td>This study</td>
</tr>
<tr>
<td>pSHH11</td>
<td>pET28a +, ΔhypE from D. maccarii CBDB1 with N-terminal His-tag, Kan'</td>
<td>This study</td>
</tr>
<tr>
<td>pSHH18</td>
<td>pACYC-Duet-I hypXSLhoxM + (cbdbA128-cbdbA131) from D. maccarii CBDB1, Cm'</td>
<td>This study</td>
</tr>
<tr>
<td>pSHH20</td>
<td>pASK-IBA3 +, hypABFCDE + (cbdbA1395-cbdbA1402) from D. maccarii CBDB1, Amp'</td>
<td>This study</td>
</tr>
<tr>
<td>pSHH23</td>
<td>pASK-IBA3 +, hypFCDE + (cbdbA1399-cbdbA1402) from D. maccarii CBDB1, Amp'</td>
<td>This study</td>
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<tr>
<td>pSHH24</td>
<td>pASK-IBA3 +, hypCD + (cbdbA1400 and cbdbA1401) from D. maccarii CBDB1, Amp'</td>
<td>This study</td>
</tr>
<tr>
<td>pT-hybG-hypDEF</td>
<td>pT7-7, ΔhypD, ΔhybG, ΔhybGstrept, ΔhybF, Amp'</td>
<td>Soboh et al. (2014)</td>
</tr>
</tbody>
</table>
Cultivation of *D. mccartyi* CBDB1. Pure cultures of *D. mccartyi* strain CBDB1 were grown anaerobically in a synthetic, bicarbonate-buffered mineral medium reduced with 1.5 mM Ti(III) citrate (1.5 mM with regard to Ti(III)) in glass serum bottles as described (Adrian *et al.*, 1998). Hydrogen was used as an electron donor and 1,2,3-trichlorobenzene in a 400 mM stock solution in hexadecane (Holliger *et al.*, 1992) as electron acceptor. The carbon sources were 5 mM sodium acetate and CO₂. Cultures were grown at 30 °C until cell numbers reached approximately 5 × 10⁷ – 1 × 10⁹ cells ml⁻¹. The cell numbers were determined microscopically by direct counting on 2 % (w/v) agarose-covered slides using a mixture of one volume of cell suspension and one volume of DAPI solution 5 mg ml⁻¹ (Sigma-Aldrich). Cells were harvested anaerobically by centrifugation at 15 000 g for 30 min and at 4 °C. Cell pellets were used immediately or stored anaerobically at –80 °C until use.

Strain construction. *E. coli* strains were constructed using P1kc-mediated phage transduction (Miller, 1972) to introduce the respective defined deletion mutation from the appropriate donor strain obtained from the Keio collection (Baba *et al.*, 2006). When required, the plasmid pCP20 was used to remove the kanamycin antibiotic resistance cassette as described (Cherepanov & Wackernagel, 1995).

Plasmid construction and site-directed mutagenesis. Genomic DNA from anaerobically grown *D. mccartyi* strain CBDB1 was isolated using a Nucleospin Tissue kit (Macherey-Nagel) according to the manufacturer’s instructions. High-fidelity PCR amplification of genomic DNA was performed using Velocity DNA polymerase (Bioline). The sequences of all primers used in PCRs are listed in Table S1, available in the online Supplementary Material.

To construct the plasmids pSHH20, pSHH23 and pSHH24 (Table 1) the AHT-inducible expression vector pASK-IBA3+ (IBA-Life Sciences) was used. The pACYC Duel-1 vector (Novagen) was used for the generation of plasmid pSHH18, and the pET-28a(+) vector (Novagen) was used to make the plasmids pSHH9, pSHH10 and pSHH11.

The entire hypABCDFE gene cluster encoding the Hyp accessory proteins from *D. mccartyi* CBDB1 was amplified by PCR using the primer pair #1 and #2 (Table S1); the primer pair #3 and #4 was used to amplify the hypXLshoxM gene cluster (Kube *et al.*, 2005); hypX is equivalent to *cbdaB131*. The primers were designed containing restriction sites corresponding to restriction sites of the expression vectors used for cloning. The BamHI/PstI-digested fragments and vectors were assembled using the T4 DNA Ligase protocol (Thermo Fisher Scientific).

To construct pSHH9, pSHH10 and pSHH11 the individual hyp genes were amplified by PCR and the resulting DNA products were ligated into the appropriate XbaI/XhoI-digested (hypC) or NdeI/HindIII-digested (hypD, hypE) vector. To clone the hypFCD gene cluster and the hypCD cluster, with a C-terminal Strep-tag on HypC in each case, the genes were amplified as two fragments and after digestion assembled in the respective expression vector. For hypCD/DEF the primer pairs #13 and #14 (fragment A; BamHI/Smal) and #15 and #16 (fragment B; Smal/PstI) were used. For hypCD the primer pairs #14 and #17 (fragment A; XbaI/Smal) and #8 and #15 (fragment B; Smal/HindIII) were used. The authenticity of all cloned DNA fragments was validated by DNA sequencing.

Site-directed mutagenesis of the hypG gene in plasmid phygStrep (Table 1) was carried out using the QuikChange site-directed mutagenesis strategy (Agilent Technologies). The oligonucleotide primers used for mutagenesis experiments are listed in Table S1.

All plasmids were transformed into the appropriate *E. coli* strains as described (Chung *et al.*, 1989).

Preparation of crude cell extracts. Cell paste from *E. coli* WT MC4100, or mutants carrying recombinant plasmids, was suspended at a ratio of 1 g (wet weight) per 3 ml in 50 mM MOPS pH 7 including 5 μg DNase ml⁻¹ (Sigma-Aldrich) and 0.2 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich). Cells were disrupted aerobiocally by sonication (30 W power for 5 min with 0.5 s pulses) using a Sonopuls HD 3100 sonifier (Bandelin Electronic). Unbroken cells and cell debris were removed by centrifugation for 30 min at 15 000 g and 4 °C. The resultant supernatant was termed the crude extract and, unless otherwise stated, was used for all studies reported herein. Subcellular fractionation of the crude extract to deliver soluble cytoplasmic and membrane fractions was performed by ultra-centrifugation at 120 000 g for 1 h as described (Sawers *et al.*, 1985).

*D. mccartyi* CBDB1 cells were disrupted in an anaerobic chamber (Coy; 95 % N₂; 5 % H₂ atmosphere). The cell pellet was suspended in 500 μl of anaerobic 50 mM MOPS pH 7 including 5 μg ml⁻¹ DNase, 0.2 mM phenylmethylsulfonyl fluoride and 2 mM sodium dithionite. The cell suspension was incubated with 1.5 % (w/v) digitonin for 45 min with gentle agitation followed by sonication (30 W power for 2 × 30 s with 0.5 s pulses). Unbroken cells and cell debris were removed by centrifugation for 30 min at 15 000 g at 4 °C. The resultant supernatant (crude extract) was used immediately or was stored at –20 °C until use.

Determination of protein concentration was performed using the method of Lowry *et al.* (1951).

PAGE and activity-staining. Unless otherwise specified non-denaturing PAGE was performed under oxidic conditions for the analysis of *E. coli* extracts. When crude extracts from *D. mccartyi* were analysed, non-denaturing PAGE was performed under anoxic conditions in a Coy chamber under an atmosphere of 95 % N₂; 5 % H₂. Separating gels included 0.1 % (w/v) Triton X-100 as described (Ballantine & Boxer, 1985). The crude extracts were incubated with a final concentration of 4 % (w/v) Triton X-100 prior to application (usually 30–60 μg of protein) to the gel, which included 7.5 % (w/v) polyacrylamide. Hydrogenase activity-staining was performed in 50 mM MOPS buffer pH 7.0, as described (Pinske *et al.*, 2012), and included 0.5 mM benzyl viologen (BV) and 1 mM 2,3,5-triphenyltetrazolium chloride (TTC). Gels were incubated at RT under an atmosphere of 100 % hydrogen gas (Pinske *et al.*, 2012). Alternatively, to determine the activity specifically of *E. coli* Hyd-1, staining was done in a 100 % hydrogen atmosphere using 0.3 mM phenazine methosulfate (PMS) as mediator and 0.2 mM nitro blue tetrazolium (NBT) as electron acceptor (Pinske *et al.*, 2012). Experiments were repeated several times always with the same results.

Enzyme activity assays. Determination of total hydrogenase enzyme activity as H₂-dependent reduction of BV was performed according to (Ballantine & Boxer, 1985) except that the buffer used was 50 mM MOPS, pH 7.0. The wavelength used was 578 nm and an extinction coefficient of 8300 M⁻¹ cm⁻¹ was assumed for reduced BV. One unit of enzyme activity corresponded to the reduction of 1 μmol of substrate min⁻¹. Enzyme assays were performed in triplicate using three biological replicates.

All studies with *E. coli* strains containing heterologously expressed genes encoding the Hyp hydrogenase of *D. mccartyi* were carried out anaerobically in a Coy chamber under an atmosphere of 95 % N₂; 5 % H₂.

Mass spectrometric analysis. SDS gel slices were desiccated, dehydrated and proteolytically cleaved overnight at 37 °C using trypsin (Promega) (Jehmlich *et al.*, 2008). Extracted peptides were desalted using ZipTip-μC18 material (Merck Millipore). Peptides were resuspended in 0.1 % formic acid before LC–MS/MS analysis. Mass spectrometry was performed using an Orbitrap Fusion (Thermo Fisher Scientific) coupled to a TriVersa NanoMate (Advion). Five
microlitres of the peptide solution was separated using a Dionex Ultimate 3000 nano-LC system (Dionex/Thermo Fisher Scientific).

Raw LC-MS/MS data were searched using a SEQUEST HT algorithm in Proteome Discoverer v1.4 against a *D. mccartyi* CBDB1 database (containing 1454 protein-coding sequences, downloaded from Uniprot 10/2014) or an *E. coli* K12 database (containing 4305 protein-coding sequences, HupS- / HupL- / HupX- sequences were manually added). The common Repository of Adventitious Proteins database (cRAP) (http://www.thegpm.org/crap/index.html) was utilized to eliminate the most frequently encountered contaminations. Searches were performed with carbamidomethyl on cysteine as fixed modification and oxidation on methionine as variable modification. Enzyme specificity was selected to trypsin with up to two missed cleavages allowed using 10 p.p.m. precursor mass tolerance and 0.1 Da fragment mass tolerance. Peptides were considered as identified with a false discovery rate <1% (FDR, Percolator). A complete list of the peptide and protein identifications is available (Tables S3 and S4).

Amino acid sequence comparisons were made using the BLASTP (basic local alignment search tool) algorithm (BLAST; http://www.ncbi.nlm.nih.gov).

### RESULTS

#### The Hyp machinery from *Dehalococcoides mccartyi* is functional in *E. coli*

The genetic organization of the hyp operon in *D. mccartyi* strain CBDB1 is depicted in Fig. 1. The hypABFCDE genes are collinear on the chromosome of *D. mccartyi*, which is slightly different from the situation with *E. coli* where the hypABCDE genes form an operon, while hypF is located separately on the chromosome (Lutz *et al.*, 1991; Schlessg

#### Table 2. Amino acid similarities of hydrogenase accessory protein between *D. mccartyi* CBDB1 and *E. coli*

<table>
<thead>
<tr>
<th><em>D. mccartyi</em> CBDB1 protein</th>
<th>Length (amino acids)*</th>
<th><em>E. coli</em> orthologue % amino acid similarity (identity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HypA</td>
<td>119</td>
<td>52 (28)</td>
</tr>
<tr>
<td>HypB</td>
<td>218</td>
<td>69 (47)</td>
</tr>
<tr>
<td>HypC</td>
<td>71</td>
<td>Hyc 59 (32); HybG 60 (30)</td>
</tr>
<tr>
<td>HypD</td>
<td>364</td>
<td>61 (41)</td>
</tr>
<tr>
<td>HypE</td>
<td>335</td>
<td>63 (47)</td>
</tr>
<tr>
<td>HypF</td>
<td>763</td>
<td>53 (39)</td>
</tr>
<tr>
<td>HupX</td>
<td>267</td>
<td>HybA 50 (34)</td>
</tr>
<tr>
<td>HupS</td>
<td>354</td>
<td>HyaA 52 (33); HyaO 53 (36)</td>
</tr>
<tr>
<td>HupL</td>
<td>526</td>
<td>HyaB 51 (33); HyaC 52 (36)</td>
</tr>
<tr>
<td>HoxM</td>
<td>164</td>
<td>HybD 59 (38)</td>
</tr>
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</table>

*Number of amino acids in *D. mccartyi* orthologue.
Fig. 2. The hyp genes from *D. mccartyi* are functional in *E. coli*. Aliquots of crude extracts (30 μg of protein) derived from the indicated strains after anaerobic growth were separated by non-denaturing PAGE (7.5 % w/v polyacrylamide) and subsequently stained for hydrogenase enzyme activity using BV and TTC. The enzymes responsible for the respective activity bands are shown on the right of the gel images. The gel in (a) shows complementation of mutants lacking HypD, HypE, HypF or the complete set of HypB, C, D and E proteins, plus HybG (strain SHH229), while (b) shows complementation of mutants lacking the HypA, HypB, HypC or HypC + HybG (strain SHH228) proteins. In (c), complementation of strain DHB-G with the indicated plasmids is shown. As a control, DHP-C (ΔhypC) transformed with pSHH20 is shown. All strains were grown to the stationary phase. In the interest of clarity, the strains and their mutant alleles are shown above each lane. The lanes are

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(a)

(b)

(c)
Comparison of the amino acid sequences of the core Hyp proteins HypC, D, E and F from *D. mccartyi* with their *E. coli* counterparts indicates that they share an overall amino acid sequence identity of 30–47 %, with a similarity of 53–63 % (Table 2). The nickel-insertion protein HypA from both bacteria shares only 28 % identity (52 % similarity), while the HypB orthologues have 47 % amino acid identity (Table 2). These levels of amino acid identity are also in the range observed when comparing the HypC, D, E and F proteins from either *D. mccartyi* or *E. coli* with their counterparts from the thermophilic bacterium *Aquifex aeolicus* and the thermophilic archaea, *Thermococcus kodakarenis*, *Methanocaldococcus jannaschii* and *Methanopyrus kandleri* (Table S2).

*E. coli* strain BEF314 carries a chromosomal deletion of the hypBCDE genes (Jacobi et al., 1992), and after anaerobic growth in rich medium with glucose as an additional carbon source, the hydrogenase enzyme profile was compared with that from the isogenic WT strain MC4100 after separation of the crude extracts in non-denaturing PAGE (Fig. 2a). The crude extract derived from MC4100 (WT) revealed the multiple isoforms characteristic of Hyd-1 and cannot be replaced completely by its counterpart from *D. mccartyi* CBDB1 (Fig. 2a, lane 2). The hydrogenase activity profile of strain SHH229 transformed with pSHH20 shown in Fig. 2(a), lane 3, revealed an essentially identical hydrogenase activity band profile to that observed for BEF314 transformed with pSHH20. This result indicates that HypC from *D. mccartyi* was capable of delivering the [NiFe]-cofactor to the apocatalytic subunits HypB and HypC and HypE of Hyd-2 and of Hyd-3, respectively.

Table 3. Specific total hydrogenase enzyme activity of heterologously complemented *E. coli* hyp gene mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>No plasmid</th>
<th>Specific activity (μmol H₂ × min⁻¹ × mg⁻¹) *</th>
<th>+ pSHH20 (hypABFCDE)</th>
<th>+ pSHH23 (hypFCDE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC4100</td>
<td>n.d.</td>
<td>0.62 ± 0.07</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>BEF314 (ΔhypB-E)</td>
<td>&lt;0.01</td>
<td>0.44 ± 0.17</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>DHP-A (ΔhypA)</td>
<td>0.35 ± 0.09</td>
<td>0.81 ± 0.23</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>DHP-B (ΔhypB)</td>
<td>0.08 ± 0.03</td>
<td>0.61 ± 0.2</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>DHP-C (ΔhypC)</td>
<td>0.06 ± 0.02</td>
<td>0.44 ± 0.13</td>
<td>0.15 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>DHB-G (ΔhypG)</td>
<td>1.3 ± 0.37</td>
<td>1.32 ± 0.2</td>
<td>1.68 ± 0.62</td>
<td></td>
</tr>
<tr>
<td>SHH228 (ΔhypCΔhypB)</td>
<td>&lt;0.01</td>
<td>0.7 ± 0.3</td>
<td>0.14 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>DHP-D (ΔhypD)</td>
<td>&lt;0.01</td>
<td>0.42 ± 0.13</td>
<td>0.19 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>DHP-E (ΔhypE)</td>
<td>&lt;0.01</td>
<td>0.37 ± 0.08</td>
<td>0.22 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>DHP-F2 (ΔhypF)</td>
<td>&lt;0.01</td>
<td>0.41 ± 0.09</td>
<td>0.15 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

*The mean and SEM of at least five independent measurements are shown.*
revealed that approximately 70% of the total enzyme activity compared to WT could be restored by introduction of the hyp genes from D. mccartyi CBDB1 (Table 3).

**D. mccartyi** HypB limits *E. coli* Hyd-1 maturation

In order to identify which of the five Hyp proteins (HypB through F) from *D. mccartyi* CBDB1 failed to function efficiently after heterologous synthesis in *E. coli*, plasmid pSHH20, including the complete hypABFCDE operon from *D. mccartyi* CBDB1, was introduced into a series of *E. coli* mutants lacking individual hyp genes (Jacobi et al., 1992; Table 1). Crude extracts derived from each strain, without plasmid and after transformation with pSHH20, were analysed by non-denaturing PAGE after fermentative growth to stationary phase (Pinske et al., 2012; Richard et al., 1999) and subsequent hydrogenase activity-staining. The results shown in Fig. 2(a, b) reveal that, with the exception of the hypA mutant (DHP-A), all other *E. coli* hyp mutants lacked Hyd-derived hydrogenase enzyme activity. This was also reflected in the findings of the quantitative analysis of total hydrogenase enzyme activity in these crude extracts (Table 3).

Introduction of pSHH20 restored Hyd-1 and Hyd-2 activity to DHP-C (∆hypC), DHP-D (∆hypD), DHP-E (∆hypE) and DHP-F2 (∆hypF), indicating that each of the corresponding hyp gene products from *D. mccartyi* was synthesized and functional in *E. coli*. Plasmid pSHH20 also restored Hyd-1 and Hyd-2 enzyme activities to strain SHH228, which carries deletions in both hypC and hybG, indicating that HypC from *D. mccartyi* can functionally replace both HypC and HybG of *E. coli* (see Fig. 2b, lane 8). This result is also reflected in the quantitative determination of total hydrogenase activity (Table 3).

Notably, mutant DHP-A (∆hypA) lacked only the more slowly migrating isoforms of Hyd-2 enzyme activity (Fig. 2b, lane 1), as well as Hyd-3-dependent H₂ production (data not shown). Introduction of pSHH20 restored the slowly migrating isoforms of Hyd-2, which have been shown to comprise minimally the heterotrimer of HybOAC (Jack et al., 2004; Pinske et al., 2015). Strain DHP-B (∆hypB), on the other hand, failed to show restoration of active Hyd-1 after transformation with pSHH20, despite Hyd-2 activity being restored (Fig. 2b, lane 4). This result indicates that although HypB from *D. mccartyi* CBDB1 can restore active Hyd-2, it lacks a key function to allow effective maturation of Hyd-1. As a control, and at the same time to demonstrate that Hyd-1 neverthless could be matured in this strain, growth was performed in the presence of 100 μM NiCl₂, which can phenotypically complement a hypB allele (Maier et al., 1993; Waugh & Boxer, 1986). Growth with increased Ni²⁺ ion concentrations indeed restored Hyd-1 enzyme activity in strain BEF314/ pSHH20, as well as when the strain was transformed with pSHH23 in which only hypFCDE were present (Fig. 3). These results confirm

![Fig. 3. High nickel concentration restores hydrogenase 1 activity to an *E. coli* hyp operon mutant with a plasmid expressing the hypABFCDE genes from *D. mccartyi*. Non-denaturing PAGE analysis of crude extracts derived from the strain indicated was performed exactly as described in the legend to Fig. 2, with the exception that the gel was stained specifically to detect hydrogenase 1 using PMS and NBT redox dyes. The strains cultivated in the presence of 100 μM NiCl₂ are indicated. The migration position of hydrogenase 1 (Hyd-1) is shown. Whether the plasmids carried hyp genes derived from *D. mccartyi* or *E. coli* is indicated below the figure.](http://mic.microbiologyresearch.org)
that the limitation in Hyd-1 activity was indeed the HypA metallochaperone-enhancer function provided by HypB (Watanabe et al., 2015). While BEF314 alone failed to synthesize active Hyd-1, even in the presence of 100 μM NiCl₂, the recovery of Hyd-1 after transformation with pSHH23 confirmed that sufficient levels of functional HypC, D, E and HypF proteins from D. mccartyi were synthesized in the heterologous host to deliver active Hyd-1 (Fig. 3). Notably, the level of Hyd-1 activity restored after growth of BEF314 carrying a plasmid encoding the E. coli HypC-DEF proteins was similar to that when transformed with pSHH23. Finally, transformation of BEF314 with plasmids encoding E. coli HypA and HypB obviated the requirement for nickel supplementation to reveal Hyd-1 enzyme activity (Fig. 3).

The complete hyp operon from D. mccartyi is required for optimal Hyd activity in E. coli

Introduction of a plasmid (pSHH23) encoding only the HypC, D, E and F accessory proteins from D. mccartyi CBDB1 into the E. coli strains carrying deletions in individual hyp genes failed to restore WT levels of active [NiFe]-hydrogenases (Fig. 2). Quantitative measurement of total hydrogenase enzyme activity in crude extracts of the mutant strains transformed with pSSH23 revealed that only approximately 25–30 % of the WT enzyme activity was recovered after transformation with the plasmid (Table 3). The exception was strain DHB-G (ΔhybG), which showed approximately double the enzyme activity observed with the WT MC4100, even in the absence of a plasmid. This reflects the increased Hyd-3 enzyme activity observed in this strain (see Fig. 2c) and presumably results from increased channelling of [NiFe]-cofactor precursor toward Hyd-3 synthesis (Blokesch et al., 2001). Under the growth conditions used to generate the data in Fig. 2(c), Hyd-3 usually constitutes roughly 80 % of the total hydrogenase enzyme activity in WT cells (Pinske et al., 2011). In strain DHB-G, the activity of Hyd-3 was highly abundant, while that of Hyd-2 was absent and that of Hyd-1 was strongly reduced (Fig. 2c, lane 2). Addition of the complete hyp operon on pSSH20, or the partial operon of hypFCDE on pSSH23, from D. mccartyi did not affect Hyd-3 activity, while Hyd-1 activity increased substantially after addition of these plasmids (Fig. 2c, compare lanes 2, 3 and 4). Hyd-2 enzyme activity was only restored by addition of pSSH20 to the mutant (Fig. 2c, lane 4). As a control, strain DHP-C carrying pSSH20 failed to synthesize high levels of Hyd-3 (Fig. 2c, lane 1). It was noted that plasmids encoding the individual Hyp proteins from D. mccartyi CBDB1 were unable to recover active Hyd-1 and Hyd-2 enzyme activities in the corresponding E. coli deletion strains (Fig. 2 and data not shown). Together, the findings of all these experiments indicate that the complete Hyp machinery from...
*D. mccartyi* can functionally replace the *E. coli* Hyp machinery and assemble catalytically active Hyd-2 and Hyd-3 of *E. coli*. However, the individual Hyp components from *D. mccartyi* CBDB1 appear not to be effective in replacing the corresponding Hyp accessory protein of the host.

**Activity of the hydrogen-uptake hydrogenase HupLSX from *D. mccartyi***

Anoxically cultivated *D. mccartyi* CBDB1 cells (approximately 0.1 g wet weight) were used to make a crude extract. An aliquot of this crude extract (approximately 5–10 μg of protein) was subjected to non-denaturing PAGE and the gel was subsequently stained for hydrogenase enzyme activity. Two strongly staining, and rapidly migrating, hydrogenase activity bands were detected (Fig. 4a). These activity bands migrated significantly further in the gel compared with the activity corresponding to Hyd-1 from *E. coli*. After excision from the gel, the polypeptides in the gel fragments were identified by mass spectrometry to include the HupL catalytic subunit as well as the HupS and HupX electron-transferring subunits of the hydrogen-oxidizing [NiFe]-hydrogenase Hup from *D. mccartyi* CBDB1 (Kube et al., 2005; Table S3). The specific hydrogenase enzyme activity determined for the crude extract from *D. mccartyi* was 1.3 ± 0.05 units mg\(^{-1}\). It was noted that the enzyme activity was highly sensitive to O\(_2\), in agreement with previous observations (Jayachandran et al., 2004).

**Catalytically active HupLS in *E. coli* requires host HypF for maturation**

The HupL and HupS polypeptides are encoded within a putative operon comprising the genes *hupX-hupS-hupL-hoxM* (*cdlbA131-cdlbA128*) (Fig. 1). Originally annotated as *cdlbA131*, this gene has now been renamed *hupX* because its product is a predicted additional electron-
transferring subunit with 50 % amino acid sequence similarity (40 % identity) with HybC, and HoxM is a predicted hydrogenase-specific subunit recognition. Fig. 5(a) shows an amino acid alignment of HypC and HybG of E. coli with HypC of D. maccarthyi. While E. coli HypC is 90 amino acids in length (89 amino acids after removal of the formyl-Met residue; Böck et al., 2006), HybG is 82 amino acids long and HypC from D. maccarthyi CBDB1 is only 71 amino acids in length. Overall, the proteins share approximately 60 % amino acid similarity; however, the alignment identifies three main regions with comparatively variable amino acid sequences (Fig. 5a). The first variable region, V1, lies between amino acid positions 10 and 18 (E. coli HypC numbering); the second, V2, is between amino acid positions 34 and 43; the third region, V3, is at the C-terminus starting from approximately amino acid position 66. Therefore, key amino acid residues in these regions were chosen for a mutagenesis study in which five amino acid variants of HybG were constructed (Fig. 5b). Three of these variants, HybG lacking amino acids 17, 18 and 19 (pCThybG-HQL), or including two additional amino acids (pCThybG+GS) at positions 34 and 35, as well as a construct combining both sets of amino acid exchanges (pCThybG-HQL+GS) delivered HybG derivatives that were more similar to E. coli HypC in these regions (see Fig. 5a). One further construct (pCThybG-CEGN) lacked a four amino acid insertion in the V2 region that is found mainly in enterobacterial HypC proteins. Finally, the fifth variant (pCThybG-YStop) had a truncated C-terminus, similar to that found in HypC from D. maccarthyi, in which a stop codon was introduced at codon 77 in hybG (Fig. 5, a, b). The plasmids encoding these variants, along with the plasmid encoding native HybG carrying a C-terminal Strep-tag (Fig. 5b) and pJA1021, encoding hypC (Jacobi et al., 1992), were introduced into strain SHH228 (ΔhycD ΔhybG), and after growth in rich medium to the stationary phase, whole cells were assayed for hydrogenase activity associated with the plasmid. Heterologously synthesized HupLS migrated slightly more slowly than the enzyme activity identified in a crude extract derived from D. maccarthyi CBDB1 (Fig. 4b, compare lanes 4 and 7).

Amino acid exchanges in E. coli HybG that enable partial restoration of Hyd-3 activity to a hypC hybG double null mutant

The promiscuity of D. maccarthyi HypC in being capable of heterologously ‘maturing’ the Hyd-1, -2 and -3 provides the basis for identifying key primary sequence features within the HypC family that might be important in large subunit recognition. Fig. 5(a) shows an amino acid alignment of HypC and HybG of E. coli with HypC of D. maccarthyi. While E. coli HypC is 90 amino acids in length (89 amino acids after removal of the formyl-Met residue; Böck et al., 2006), HybG is 82 amino acids long and HypC from D. maccarthyi CBDB1 is only 71 amino acids in length. Overall, the proteins share approximately 60 % amino acid similarity; however, the alignment identifies three main regions with comparatively variable amino acid sequences (Fig. 5a). The first variable region, V1, lies between amino acid positions 10 and 18 (E. coli HypC numbering); the second, V2, is between amino acid positions 34 and 43; the third region, V3, is at the C-terminus starting from approximately amino acid position 66. Therefore, key amino acid residues in these regions were chosen for a mutagenesis study in which five amino acid variants of HybG were constructed (Fig. 5b). Three of these variants, HybG lacking amino acids 17, 18 and 19 (pCThybG-HQL), or including two additional amino acids (pCThybG+GS) at positions 34 and 35, as well as a construct combining both sets of amino acid exchanges (pCThybG-HQL+GS) delivered HybG derivatives that were more similar to E. coli HypC in these regions (see Fig. 5a). One further construct (pCThybG-CEGN) lacked a four amino acid insertion in the V2 region that is found mainly in enterobacterial HypC proteins. Finally, the fifth variant (pCThybG-YStop) had a truncated C-terminus, similar to that found in HypC from D. maccarthyi, in which a stop codon was introduced at codon 77 in hybG (Fig. 5a, b). The plasmids encoding these variants, along with the plasmid encoding native HybG carrying a C-terminal Strep-tag (Fig. 5b) and pJA1021, encoding hypC (Jacobi et al., 1992), were introduced into strain SHH228 (ΔhycD ΔhybG), and after growth in rich medium to the stationary phase, whole cells were assayed.
for FHL activity and crude extracts were prepared for hydrogenase activity staining after native-PAGE.

While extracts of MC4100 (WT) revealed activity bands corresponding to Hyd-1, Hyd-2 and Hyd-3 (Fig. 6a), the negative control SHH228 (ΔhypC ΔhybG) revealed only a weak activity band due to the hydrogenase activity associated with Fdh-N/O enzymes (Soboh et al., 2011). Introduction of plasmid pJAI021 encoding HypC from E. coli restored the activity band due to Hyd-3, and this also correlated with recovery of H2 gas production, which attained levels of 70% of the activity observed for MC4100 (Table 4). Notably, pJAI021 failed to restore Hyd-1 synthesis (Fig. 6). Analysis of strain SHH228 transformed with phybGstrep revealed that both Hyd-2 and weak Hyd-1 activities were restored by the HybGstrep protein (Fig. 6a, b). Surprisingly, FHL enzyme activity of this strain was approximately 3% of the level observed for MC4100 (Table 4); introduction of a HybG variant lacking a C-terminal Strep-tag failed to show any FHL activity (data not shown). Removal of the amino acid residues HQL from region V1 in HybG or introduction of GS residues in region V2 did not have a major effect on either Hyd-1 or Hyd-2 activity, and the pattern of bands looked like that of HybGstrep (Fig. 6 and data not shown). Notably, however, a very low level of FHL activity was detected for the strain transformed with phybG-HQL which was similar to that observed for the strain transformed with phybGstrep (Table 4). Combining the two sets of amino acid exchanges (phybG-HQL+GS) abolished Hyd-1 activity and resulted in a significant reduction of Hyd-2 activity (Fig. 6). Notably, however, H2 production was increased and attained levels of 15% of the FHL activity of MC4100.

Deletion of the CEGN motif from region V2 (Fig. 5a, b) essentially abolished all Hyd enzyme activity (Fig. 6a). Finally, truncation of the C-terminus of HybG resulted in increased Hyd-1 and Hyd-2 enzyme activities compared with the same strain transformed with phybGstrep encoding HybGstrep (Fig. 6). FHL activity attained a value similar to that of SHH228 transformed with phybGstrep (Table 4).

### DISCUSSION

In this study, we present a systematic analysis of the ability of the Hyp protein machinery from a member of the deeply branching Chloroflexi to function in the gammaproteobacterium E. coli. The Hyp machinery from D. mccartyi CBDB1 could substitute for the core HypCDEF accessory proteins of E. coli, resulting in the maturation, to differing degrees, of all three E. coli [NiFe]-hydrogenases. In a reciprocal experiment, the HupLS hydrogenase from D. mccartyi CBDB1 could be shown to deliver an active enzyme in E. coli and this was shown to be dependent on the Hyp machinery, as demonstrated by an absence of activity in a hypF mutant background. A comparison of the ability of the complete hyp operon and individual hyp genes from D. mccartyi to complement either single E. coli hyp mutations or the hypB-E deletion mutation in strain BEF314 (Jacobi et al., 1992) demonstrated that effective complementation only occurred when all of the Hyp proteins from D. mccartyi were present. This finding suggests that the Hyp proteins from D. mccartyi preferentially function with each other. Due to the differences in primary structure between the Hyp counterparts from the two bacteria (Table 2) it is perhaps not surprising that individual Hyp proteins from D. mccartyi were unable to function efficiently with the host’s Hyp proteins.

The exception appears to be the GTPase HypB from E. coli, which was required to ensure efficient maturation of a functional Hyd-1 enzyme in the absence of high nickel ion concentration. While HypB from D. mccartyi CBDB1 was capable of maturing Hyd-2 and Hyd-3, it was not capable of generating WT levels of active Hyd-1 enzyme. HypB is involved in the latter stages of [NiFe]-hydrogenase maturation where it functions together with HypA and the peptidyl-prolyl cis/trans isomerase SlyD to deliver the nickel ion to the active site (Maier et al., 1993; Waugh & Boxer, 1986; Zhang et al., 2005). A recent structural study indicates that HypA proteins likely deliver nickel directly to the hydrogenase large subunit, as exemplified for T. kodakarensis (Watanabe et al., 2015); however,
**T. kodakarensis** lacks a classical HypB protein and instead has a functional homologue (Sasaki et al., 2013) that has an ‘enhancer’-like role in facilitating delivery of nickel to the active site by the HypA protein. The data presented here nevertheless suggest that the HypB protein might recognize specific amino acid residues on the apo-large subunits of hydrogenases during maturation, and that *D. mccartyi* CBDB1 HypB lacks the ability to interact effectively with HyaB, the large subunit of Hyd-1.

**Amino acid motifs in HypC family members with a role in hydrogenase large subunit recognition**

HypC-type chaperones have been shown to interact directly with unprocessed large subunits, or large subunit homologues, in a number of bacterial systems (Albareda et al., 2014; Drapal & Böck, 1998; Jones et al., 2004; Winter et al., 2005) and HypC has been proposed to deliver the completed Fe(CN)$_2$CO cofactor directly into the active site cavity of the catalytic subunit of Hyd-3 (Magalon & Böck, 2000; Blokesch et al., 2001; Böck et al., 2006). This suggests a highly specific interaction between HypC and HycE. The demonstration that the HypC orthologue from *D. mccartyi* CBDB1 yielded active Hyd-2 and Hyd-3 enzymes in *E. coli* guided mutagenesis studies, which identified amino acid motifs in HybG that are important for recognition of HybC and which appear to discriminate against recognition of HycE, the large subunit of Hyd-3. When two short amino acid stretches (the HQL amino acid residues of variable region V1, which are absent from *E. coli* HypC, and the GS residues of V2, which are present in *E. coli* HypC; see Fig. 5a) were exchanged in HybG to mimic the situation in the HypC protein, this resulted in a reduction of Hyd-1 and Hyd-2 activities but at the same time recovery of some H$_2$ production, signifying Hyd-3 activity. While this FHL activity was only approximately 15% of the WT, it nevertheless reveals the importance of these two sets of amino acid residues in directing the interaction with the apo-large subunits. Based on the recently published crystal structure of the HypC–HypD complex isolated from *T. kodakarensis* (Watanabe et al., 2012) both of these regions are not involved in HypD interactions. Surprisingly, HybG carrying a C-terminal Strep-tag also caused a very low but measurable level of H$_2$ production. This finding suggests that extending the C-terminus of HybG with the 8 amino acid Strep-tag might allow low-level maturation of HycE, the catalytic subunit of Hyd-3.

Notably, HypC from *D. mccartyi* CBDB1 lacks an insert of four amino acids (CDEN in HypC; CEGN in HybG), within V2, from amino acids 36–39. These amino acids are also absent in the HypC from *T. kodakarensis* but are predicted to extend a loop between beta-sheets 3 and 4, which might influence the position of a relatively large contact surface extending from amino acid positions 10 to 30 formed by three beta-strands (Watanabe et al., 2012). Removal of these four amino acid residues prevented HybG from functioning in maturing any of the hydrogenases in *E. coli*. This suggests that these residues have an important function in HybG/HypC of *E. coli*. Nevertheless, these residues are not essential in all HypC family members because the majority of HypC orthologues lack this motif.

Finally, truncation of the C-terminus of HybG appeared to have a positive effect on Hyd-1 and Hyd-2 maturation, because both of these activities increased in the mutant strain transformed with a plasmid encoding this HybG variant. No effect on H$_2$ production was observed, suggesting that the C-terminal helix of HypC proteins is also involved in discriminating hydrogenase large subunits. Notably, *E. coli* HypC has a much longer C-terminal helix than HybG, and this possibly influences its ability to interact with HycE and perhaps prevents strong interaction with HybC, the Hyd-2 catalytic subunit. In a recent study involving HupF, a HypC paralogue in *Rhizobium leguminosarum*, the extended C-terminal helix could be shown to stabilize the hydrogenase large subunit HupL upon exposure to oxygen during aerobic growth of the bacterium (Albareda et al., 2012). Truncation of the helix severely affected the O$_2$-stability of hydrogenase maturation.

**Heterologous synthesis of active HupLS from *D. mccartyi***

Our studies also revealed that HupLS from *D. mccartyi* could be synthesized in an active form in the heterologous host *E. coli* and the data demonstrated that HypF from the host was required to complete [NiFe]-cofactor insertion into the large subunit. Comparison of the electrophoretic mobility and subcellular localization of the HupLS enzyme indicated, however, that it was found in the soluble fraction, suggesting either that the Tat signal sequence located on the small subunit HupS failed to be recognized or cleaved by the *E. coli* Tat machinery or that the predicted C-terminal transmembrane helix in HupS was insufficient to maintain the protein associated with the outer leaflet of the cytoplasmic membrane; unusually, the *hupSLX* operon of *D. mccartyi* does not encode a membrane-anchor subunit equivalent to HybB of *E. coli* (Dubini et al., 2002; Kube et al., 2005). Active soluble Hyd-2 of *E. coli* can be detected in a *tat* mutant (Sargent et al., 1998b), indicating that membrane localization is not essential for enzyme activity to be observed with artificial violeno redox dyes.

Together, the data presented in this study strongly suggest that the maturation of [NiFe]-hydrogenases by the Hyp proteins is evolutionarily highly conserved and points to an ancient origin of this cofactor biosynthetic machinery. This is further supported by the demonstration that a [NiFe]-hydrogenase from *Pyrococcus furiosus* can be functionally matured in *E. coli* (Sun et al., 2010). Due to the fact that, up until now, *D. mccartyi* strains were genetically intractable, the findings presented here indicate that *E. coli* could be used as a suitable recombinant host to study key aspects of the organism’s H$_2$ metabolism.
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