FEBS Letters 589 (2015) 3141–3147

FEBS Letters

journal homepage: www.FEBSLetters.org

Dissection and engineering of the *Escherichia coli* formate hydrogenlyase complex

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ARTICLE INFO

Article history:
Received 11 August 2015
Revised 26 August 2015
Accepted 28 August 2015
Available online 7 September 2015

Edited by Miguel De la Rosa

Keywords:
Fermentation
Formate hydrogenlyase
[NiFe]-hydrogenase
Mutagenesis
Genetic engineering
Escherichia coli

ABSTRACT

The *Escherichia coli* formate hydrogenlyase (FHL) complex is produced under fermentative conditions and couples formate oxidation to hydrogen production. In this work, the architecture of FHL has been probed by analysing affinity-tagged complexes from various genetic backgrounds. In a successful attempt to stabilize the complex, a strain encoding a fusion between FdhF and HycB has been engineered and characterised. Finally, site-directed mutagenesis of the *hycG* gene was performed, which is predicted to encode a hydrogenase subunit important for regulating sensitivity to oxygen. This work helps to define the core components of FHL and provides solutions to improving the stability of the enzyme.

1. Introduction

Escherichia coli is a Gram negative bacterium that can grow under a broad spectrum of environmental conditions [1]. As a facultative anaerobe, *E. coli* prefers to respire with O_2 as terminal electron acceptor, but in the absence of O_2 the bacterium has the option to switch to anaerobic metabolism. Moreover, in the absence of all exogenous respiratory electron acceptors, including O_2, *E. coli* performs a mixed-acid fermentation. Interestingly, under such anaerobic fermentative conditions with glucose, *E. coli* will produce hydrogen gas and this ability to generate biohydrogen offers the prospect of using *E. coli* as a source of fully renewable H_2. In order to produce H_2, formate is generated from pyruvate under anaerobic conditions and initially secreted from the cell. At a late stage in fermentation the formate is reabsorbed and disproportionated into CO_2 and H_2 by the formate hydrogenlyase (FHL) complex [2]. Thus, formate is the predominant substrate for H_2 production in *E. coli* under fermentative conditions and FHL is the predominant producer of H_2. FHL activity was described in 1932 [3] but, despite the genetics and some biochemistry being reported [4–6], it was only very recently that the intact FHL enzyme was isolated from *E. coli* [7].

The structural genes for FHL include *fdhF*, which encodes a formate dehydrogenase containing pyranopterin guanine dinucleotide and a [4Fe–4S] cluster as cofactors [6], and some of the members of the *hycABCDEFGHI* operon (Fig. 1). Of these, the *hycE* gene encodes a [NiFe]-hydrogenase subunit, while *hycB*, *hycF* and *hycG* are predicted to encode Fe–S proteins [2,8]. The two integral membrane proteins (HycC and HycD) are hypothesised to anchor the catalytic subunits to the cytoplasmic side of the inner membrane (Fig. 1) [2,8].

The [NiFe]-hydrogenase component of FHL, Hyd-3, is of particular interest as it is a nickel-dependent hydrogenase dedicated to H_2 production rather than H_2 oxidation [7]. Hyd-3 comprises a catalytic large subunit, HycE, that contains the Ni–Fe–Co–2CN$^-$ active site cofactor, and an electron transferring small subunit, HycG, that is predicted to contain a single 4Fe–4S cluster similar to the proximal cluster found in standard

Abbreviations: BN-PAGE, blue native polyacrylamide gel electrophoresis; IMAC, immobilized metal affinity chromatography

Author contributions: J.S.M. designed and performed research, analysed data and prepared data for publication. M.C.H. performed research. T.P. performed research, analysed data and wrote the paper. F.S. secured funding for the research, supervised the research, designed the research, analysed data and wrote the paper.

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http://dx.doi.org/10.1016/j.febslet.2015.08.043
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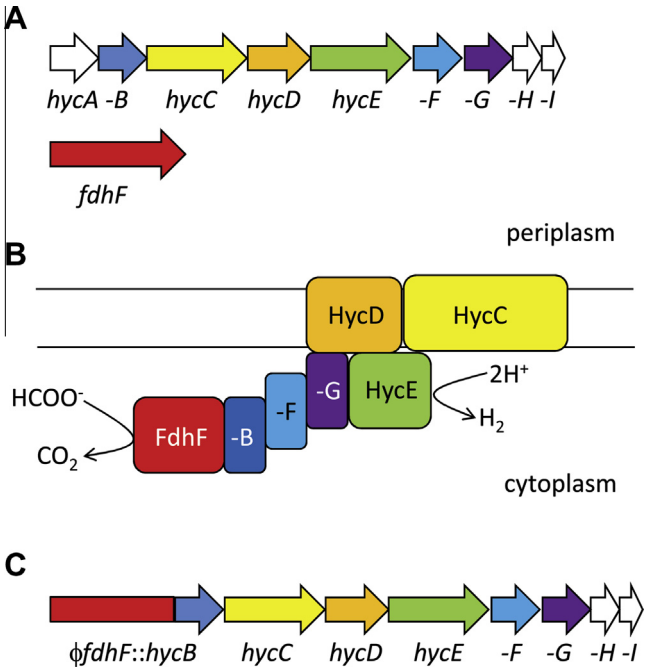

Fig. 1. Components of the native and engineered FHL system. (A) Genetics of the *E. coli* FHL system. The *hyc* operon is encoded at 61 min on the genome, while *fdhF* is located at 91 min. Genes are colour-coded to match the enzyme cartoon shown in (B). Genes shaded white do not encode structural components of FHL. (B) Cartoon of how FHL is predicted to assemble at the inner membrane. Colour coding matches the genes in panel (A). (C) The engineered *hyc* operon encoding a fusion between FdhF and HycB *via* an HA epitope tag. Native *fdhF* was deleted in this genetic background.

oxygen-sensitive [NiFe]-hydrogenases, rather than the special 4Fe–3S proximal cluster found in oxygen-tolerant hydrogenases [9]. This special cluster is ligated by six cysteine residues [10], which enable the cluster to release two electrons towards the active site when O_2 attacks, thus reducing O_2 to water [11,12].

Using a genetic engineering approach, the isolation of *E. coli* FHL has proven possible [7]. It remains a challenge to understand the molecular architecture of FHL, and to stabilise enzyme activity sufficiently to allow both characterisation and new applications. In this work, further genetic engineering approaches, coupled with mutagenesis, have been used in order to dissect the FHL complex. Using blue native (BN)-PAGE a stable core 131kDa complex of HycB, -E, -F, and -G was identified. In addition, to maintain a fully-assembled FHL complex in vitro, a strain was constructed that would encode a fusion protein between the formate dehydrogenase component FdhF and its predicted partner HycB. The addition of a covalent linker between FdhF and HycB did not adversely affect FHL activity and a stable complex could be isolated from this strain. Finally, a program of targeted mutagenesis was focused on the HycG Fe–S cluster in an attempt to engineer air-stability into the enzyme.

2. Materials and methods

2.1. Construction of bacterial strains

This work was based on *E. coli* K-12 MG1655 [13] and strains constructed and employed in this study are listed in Table 1. Strains carrying gene deletions are based on MG059e1, which carries a *hycE*^{His} allele on the chromosome [7]. Each deletion allele was generated by PCR and assembled in pMAK705 before being moved onto the chromosome by homologous recombination [14]. For site-directed mutagenesis of *hycG*, a ~1.25 kbp fragment of DNA covering the *hycG* gene and ~300 bp of upstream DNA was amplified by PCR and cloned into pMAK705 as an XbaI-KpnI fragment. The positions of the cysteine substitutions were determined by sequence analysis (Supp Fig. S1) and site-specific mutations in *hycG* were introduced by Quikchange (Stratagene) and transferred to the chromosome of MG059e1 (Table 1) [14].

Strain MGe1fZB produces FdhF as an N-terminal fusion protein to HycB joined by a linker sequence containing a hemagglutinin (HA) tag flanked by three glutamines on each side. To construct this strain, ~500 bp of DNA upstream of *hycA* with the 3′ end of the fragment stopping 6 bp away from the *hycA* start codon, was cloned as an XbaI-BamHI fragment into pBluescript. The *fdhF* gene, starting from the ATG start and ending at the penultimate codon was amplified and cloned as a BamHI-EcoRI fragment. Finally, the *hycB* gene lacking its start codon was amplified using a long primer that incorporated the linker and cloned as an EcoRI and HindIII fragment. The complete ϕ*fdhF*^{HA}-*hycB* fusion allele was then subcloned as an XbaI-HindIII fragment into pMAK705 and introduced into MG059e1 [14]. To upregulate expression of ϕ*fdhF*^{HA}-*hycBCDEFGHI*, the synthetic *T5* promoter, *lac* operator and ribosome binding site from pQE60 (Qiagen) was inserted onto the chromosome directly upstream of the fusion. First, the *Eco*RI site present in the *T5* promoter region of pQE60 was removed by site-directed mutagenesis (GAATTC–GAATAC). Next, 102 bp covering the *T5* promoter/operator region from modified pQE60 was amplified and cloned into pBluescript as an *Eco*RI-*Bam*HI fragment. Then ~500 bp of DNA upstream of *hycA* was cloned as a KpnI-EcoRI fragment. Finally, the *fdhF* part of the ϕ*fdhF*^{HA}-*hycB* construct

Table 1
E. coli strains constructed for this study.

Name	Relevant genotype	Source
MG1655	*E. coli* K-12: F$^-$, λ$^-$, *ilvG*-, *rfb*-50, *rph*-1	[13]
MG059e1	As MG1655, *hycE*^{His}	[7]
MGE1dB	As MG059e1, Δ*hycB*	This work
MGE1dC	As MG059e1, Δ*hycC*	This work
MGE1dF	As MG059e1, Δ*hycF*	This work
MGE1dG	As MG059e1, Δ*hycG*	This work
MGE1dZ	As MG059e1, Δ*fdhF*	This work
MACdF	As MG059e1, Δ*hycC*, Δ*fdhF*	This work
MAC47	As MG059e1, *hycG* G47C	This work
MAC120	As MG059e1, *hycG* G120C	This work
MAC131	As MG059e1, *hycG* G131C	This work
MAC12	As MG059e1, *hycG* G47C, G120C	This work
MAC13	As MG059e1, *hycG* G47C, G131C	This work
MAC23	As MG059e1, *hycG* G120C, G131C	This work
MAC123	As MG059e1, *hycG* G47C, G120C, G131C	This work
MGe1fZB	As MG059e1, Δ*hycA*, Δ*fdhF*, ϕ*fdhF*^{HA}::*hycB*	This work
FZBup [pREP4]	As MGe1fZB, P_{T5} ϕ*fdhF*^{HA}::*hycB* [pREP4: Kan^R, *lacI*$^+$]	This work

cloned into the pBluescript construct containing the *hycA* upstream-*T5* promoter assembly. The construct was moved onto pMAK705 and then the chromosome of strain MGe1fZB [14] that had been transformed with pREP4 encoding LacI.

2.2. Protein purification

For each strain producing a histidine-tagged protein, two 5 ml aerobic cultures were used to inoculate a 5 l Duran containing LB and 0.4% (w/v) D-glucose. The anaerobic culture was incubated statically at 37 °C for 16 h before the cells were harvested by centrifugation. Isolation of affinity-tagged FHL complexes was as described [7]. Briefly, pellets were suspended in 50 ml B-PER (Invitrogen) cell lysis cocktail supplemented with 50 mM imidazole, a protease inhibitor cocktail (Calbiochem), 10 μg/ml DNase I and 50 μg/ml lysozyme. Suspensions were mixed for 20 min at room temperature before being clarified by centrifugation and the supernatant applied slowly (0.5 ml/min) to a 5 ml immobilized metal affinity chromatography (IMAC) column equilibrated in 20 mM Tris·HCl (pH 7.5), 150 mM NaCl, 50 mM imidazole, 0.02 % (w/v) *n*-dodecyl-β-D-maltoside. Proteins were eluted in the same buffer with a 30 ml linear gradient of 50–1000 mM imidazole.

2.3. Protein analytical methods

Protein samples were separated by SDS–PAGE [15] and, if necessary, transferred to nitrocellulose as described [16]. Proteins were detected using Instant Blue stain (Expedeon), and Western immunoblots were developed using a Qiagen penta-His monoclonal antibody, Invitrogen anti-HA monoclonal antibody, and a Bio-Rad rabbit anti-Mouse HRP-conjugated secondary antibody. Blue native PAGE (BN-PAGE) was performed essentially as described [17] using 4–16% (w/v) acrylamide linear gradient Bis–Tris gels (Novex®, Life Technologies). Tryptic peptide Mass Fingerprinting and data analysis was performed as a service by 'Fingerprints Proteomics Facility', School of Life Sciences, University of Dundee.

2.4. Enzyme assays

Formate-dependent hydrogen evolution in whole cells was assayed using a hydrogen-sensing electrode as previously described [4,18]. Hydrogenase and formate dehydrogenase assays were performed using benzyl viologen as artificial electron acceptor as described [4,19].

3. Results

3.1. The roles of the hyc genes in FHL structure

Incorporation of a deca-histidine affinity/epitope tag at codon 83 of *hycE*, together with a rapid purification protocol, allows isolation of FHL [7]. In this work, the intention was to characterise the purified protein by blue native (BN)-PAGE and to genetically-dissect the complex. First, *E. coli* strain MG059e1 (*hycE*^{His}) was cultured under fermentative conditions and FHL isolated by IMAC in *n*-dodecyl-β-D-maltoside (DDM) detergent. This procedure allowed enrichment of the five cofactor-containing subunits of FHL – FdhF, HycB, HycE, HycF, and HycG – as analysed by SDS–PAGE (Fig. 2). The isolated FHL complex was then analysed by non-denaturing BN-PAGE and was found to migrate as at least three species close to the 200, 132 and 66kDa markers (Fig. 2). The combined mass of all seven subunits at an equimolar stoichiometry would be expected to reach 306kDa. Here, the faster migrating band, located close to the 66kDa marker (Fig. 2), was identified by mass spectrometry as FdhF alone (predicted mass of 79kDa). The slow migrating band migrating around 200kDa was found to contain FdhF, HycB, HycE, HycF and HycG, as well as traces of HycC and HycD, by tryptic peptide mass fingerprinting, while the band migrating ~125kDa was comprised of HycB, HycE, HycF and HycG.

Next, a mutagenic analysis of the FHL structural components was undertaken. In frame, unmarked, deletions of the structural genes (except *hycD*, a deletion strain of which proved not possible to generate) were individually introduced into the MG059e1 (*hycE*^{His}) strains. Each strain was then grown under fermentative conditions and the FHL complexes isolated and analysed by gel electrophoresis (Fig. 2). SDS–PAGE and BN-PAGE showed that the entire FHL complex is destabilized in the absence of HycB, HycF and HycG (Fig. 2), with essentially only the HycE^{His} protein being recovered from these strains (Fig. 2). Interestingly, however, mass spectrometry analysis of the BN-PAGE-treated sample from the Δ*hycG* strain identified not only HycE (70% total sequence coverage, overall score 2357) but also identified two peptide

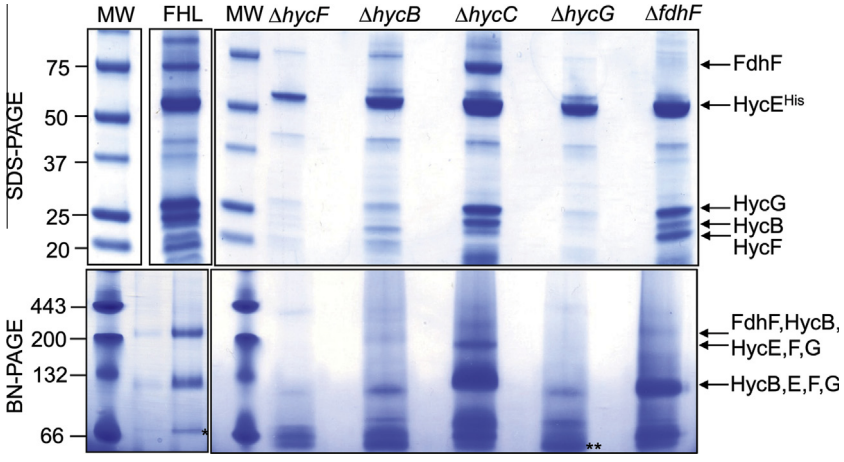

Fig. 2. Genetic dissection and characterisation of the FHL complex. *E. coli* strains MG059e1 (*hycE*^{His}) and derivatives carrying deletions in the structural genes *hycB*, *hycF*, *hycG*, and *fdhF* were grown in rich media under fermentative conditions (0.4% w/v glucose, anaerobically). Detergent-solubilised extracts were separately subjected to IMAC and eluted proteins pooled and concentrated before being separated by SDS–PAGE and BN-PAGE as indicated. The native as-purified complex ('FHL') was then compared to protein recovered from the different genetic backgrounds. Molecular weight markers ('MW') are included and relative molecular masses indicated to the left of the gels. All indicated proteins were identified by tryptic peptide mass fingerprinting. For BN-PAGE, the arrows to the right highlight a 125kDa complex (HycBEFG) and two complexes for FdhF–HycBEFG) that migrate at 180kDa and 220kDa. Also for the BN-PAGE, the band marked * was identified as FdhF only and ** contained peptides from HycE, HycH and HypC.

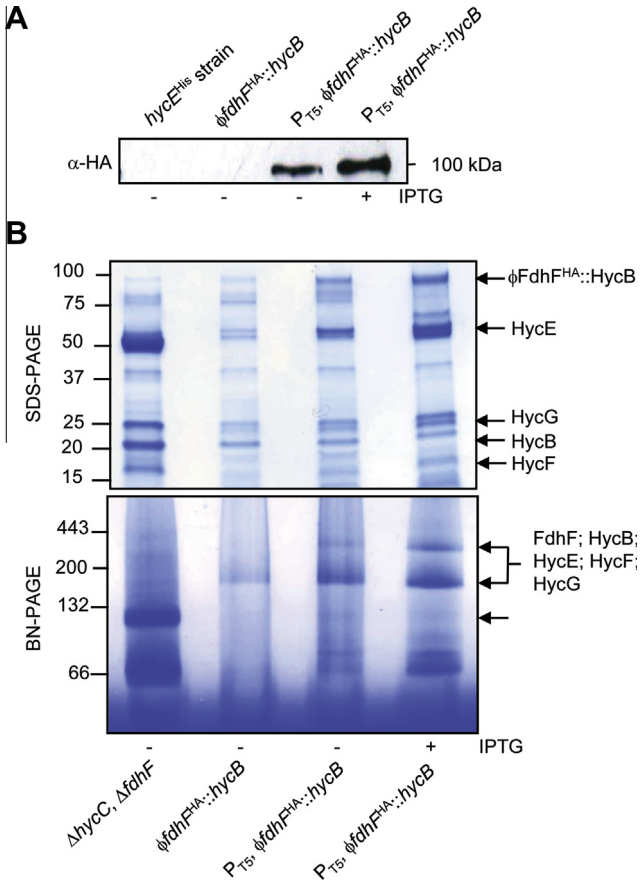

Fig. 3. Characterisation of a strain encoding an FdhF^{HA}–HycB fusion protein. (A) Strains MG059e1 (*hycE*^{His}), MGe1fZB (φ*fdhF*^{HA}::*hycB*) and FZBup [pREP4] (P_{T5} φ*fdhF*^{HA}::*hycB*) were grown in rich media under fermentative conditions. The FZBup [pREP4] strain was also grown in the presence of 1 mM IPTG where indicated. Whole cell samples were then separated by SDS–PAGE and analysed for the presence of the FdhF^{HA}–HycB fusion protein by Western immunoblotting with a monoclonal anti-HA antibody. (B) The strains MACdf (*hycE*^{His}, Δ*hycC*, Δ*fdhF*), MGe1fZB and FZBup [pREP4] were grown under the conditions outlined in (A) but at larger (5 l) scale before detergent-solubilised extracts were analysed by IMAC, SDS–PAGE and BN-PAGE as indicated. The positions of molecular weight markers are noted to the left of the gels and all indicated proteins were identified by tryptic peptide mass fingerprinting. For BN-PAGE, the arrows to the right highlight a 125kDa complex from the Δ*fdhF*Δ*hycC* strain and two complexes for FdhF–HycB fusion that migrate at 180kDa and 220kDa.

masses corresponding to the HycH protein (representing 20% coverage, overall score 114) and one peptide mass corresponding to HypC (representing 24% coverage, overall score 93).

Next, the Δ*fdhF*, *hycE*^{His} strain was studied and IMAC performed (Fig. 2). In the absence of FdhF a stable ~125kDa species was identified by BN-PAGE (Fig. 2) and confirmed by mass spec sequence analysis to contain HycB, -E, -F, and -G. In addition, analysis of the complex from a Δ*hycC*, *hycE*^{His} strain, lacking part of the membrane domain, suggested the remainder of the FHL complex appeared assembled in the absence of HycC and could be isolated by IMAC (Fig. 2). The FHL complex lacking HycC migrated at a similar ~125kDa position when analysed by BN-PAGE (Fig. 2), although the larger species containing FdhF was also identified by mass spectrometry analysis for this sample (Fig. 2). Finally, the Δ*fdhF* and Δ*hycC* alleles were combined into a single strain that resulted in purification of HycE^{His} together with HycB, -F and -G (Fig. 3B), although the complex appeared rather unstable during BN-PAGE analysis (Fig. 3B).

Taken altogether, these experiments suggests that the most stable, minimal, core of the FHL complex comprises HycB, -E, -F, and -G, and that this ~125kDa species may be formed in the absence of FdhF and/or HycC.

3.2. Engineering an FdhF–HycB fusion protein within the FHL complex

One challenge to overcome if FHL activity is to be harnessed for biotechnological applications is the loose attachment of the FdhF formate dehydrogenase to the FHL complex. To seek a molecular genetic solution, the *fdhF* gene was first deleted from the MG059e1 (*hycE*^{His}) strain then a construct was assembled that would replace the *hycA* gene (encoding a transcriptional repressor) with a version of *fdhF* fused to *hycB via* an HA epitope tag. The new strain (MGe1fZB) was analysed for production of the φ*fdhF*^{HA}::*hycB* allele by Western immunoblot, however none was detectable (Fig. 3A). The strain yielded only low levels of protein after IMAC (Fig. 3B) and FHL activity in unbroken cells was very low (Fig. 4). In order to address this, a strong T5 promoter (under control of the *lac* operator) was engineered upstream of the φ*fdhF*^{HA}::*hycB* allele. The new strain FzBup (*hycE*^{His}, P_{T5} φ*fdhF*::*hycB*, Δ*hycA*, Δ*fdhF*, pREP4 [Kan^{R}]) was then assayed for whole cell FHL activity (Fig. 4). The FzBup strain demonstrated improved FHL activity, which could be enhanced by growth in the presence of IPTG (Fig. 4). The full-length FdhF^{HA}–HycB fusion protein was identifiable by Western immunoblotting (Fig. 3A), and this led to a concomitant improvement in the ability to isolate active FHL from this strain (Fig. 3B). The fusion complex migrates close to the 200kDa marker when analysed by BN-PAGE (Fig. 3B) and tryptic peptide mass spectrometry showed that FdhF, and the other FHL components, are present in this species. Finally, enzymatic assays with benzyl viologen as electron acceptor demonstrate that both hydrogenase and formate dehydrogenase activities are enhanced in the purified fusion complex (Fig. 4).

These data show that covalent attachment of FdhF to HycB does not adversely affect assembly or physiological activity of the FHL complex, and this may be a key strategy in facilitating further characterisation of the complex.

3.3. Site-directed mutagenesis of the hycG gene

Naturally O_2-tolerant [NiFe]-hydrogenases contain a special cofactor within the small subunit that has a critical role in the mechanism of oxygen tolerance [11,20,21]. Two extra cysteines modify the properties of the proximal Fe–S cluster allowing it to adopt a 4Fe–3S structure capable of delivering two electrons to the active site when oxygen attacks [21]. In standard O_2-sensitive hydrogenases the extra cysteines are replaced by glycine residues and the Hyd-3 small subunit, HycG, contains only one Fe–S cluster that bears the amino acid signatures of the proximal cluster of an O_2-sensitive enzyme (Supp Fig. S1). Here, an attempt was made to retro-fit an O_2-rescue mechanism into FHL. Sequence analysis was used to predict three possible locations for the two supernumerary cysteines present in O_2-tolerant enzymes (Supp Fig. S1) and the MG059e1 (*hycE*^{His}) strain was modified by introducing *hycG* G47C, G120C, and G131C alleles into the chromosome under all possible combinations.

The single substitutions of G47C, G120C and G131C in HycG had no effect on in vivo formate hydrogenlyase activity (Fig. 5A). The three variant enzymes were amenable to purification and behaved similar to the native enzyme when analysed by SDS–PAGE and BN-PAGE (Fig. 5B and D). However, analysis of the three double mutants uncovered some differences. The strain producing the G47C/G120C double variant displayed weak FHL activity in vivo (Fig. 5A) and IMAC directed towards HycE^{His} recovered predominantly the Hyd-3 large subunit (Fig. 5C), which was itself inactive when assayed with benzyl viologen. Similarly, HycG triple variant

G47C/G120C/G131C resulted in low cellular FHL activity (Fig. 5A) and a destabilized complex upon purification (Fig. 5C and D).

A true test of O_2-tolernace requires an electrochemical assay, however Hyd-3 has proven particularly unstable when attached to an electrode [7]. Instead, and alternative assay was devised here that would determine the stability of Hyd-3 activity during exposure to air. The assay measures benzyl viologen-dependent hydrogenase activity of purified enzymes over time (Fig. 5E). Samples of *E. coli* Hyd-1 and *Salmonella enterica* Hyd-5, known to be O_2-tolerant [22,23], were used as controls. The enzymes were left exposed to air at room temperature and aliquots were withdrawn and assayed over 6 h (Fig. 5E). The hydrogenase activity in the Hyd-1 and Hyd-5 samples did not degrade over the six-hour timescale of this experiment (Fig. 5E). Conversely, an aliquot of isolated Hyd-3 lost 60% of its activity over the same time (Fig. 5E). The HycG G131C variant initially maintained its activity level over the first hour, but then collapsed to levels observed for native Hyd-3 (Fig. 5E).

These data suggest that FHL is partly amenable to mutagenesis, and that HycG in particular can withstand incorporation of cysteine side-chains. Analysis of the HycG G131C variant also gives an indication that it may be possible to overcome some issues of stability displayed by the FHL complex.

4. Discussion

4.1. The core complex of FHL

The inherent instability of the *E. coli* FHL complex is long-known, especially the loose attachment of FdhF to the Hyd-3 component [24]. Although some FdhF-containing FHL species could be identified here, particularly in the native enzyme and the Δ*hycC* mutant (Fig. 2), the mutagenic analysis, coupled with purification and BN-PAGE analysis, suggest that the most stable core complex of FHL comprises HycB, HycE, HycF and HycG. Together, these four proteins have a predicted mass of 131 174 Da, which is a close mass match to the species found to migrate close to the 132kDa marker in BN-PAGE, and also shown to contain the HycBEFG proteins by mass spectrometry. Potentially, a strain lacking FdhF and the membrane domain (HycCD) could provide a homogeneous, core complex suitable for crystallographic studies. Unfortunately, the Δ*fdhF*, Δ*hycC* strain characterised here suggests the HycBEFG complex may not be further stabilised in this genetic background (Fig. 3B).

The *hycB* gene encodes an Fe–S cluster binding protein that is predicted to form the direct partner subunit for formate dehydrogenase (FdhF) in FHL (Fig. 1). BN-PAGE showed that FdhF was not present in the absence of HycB but that HycF and HycG could be co-purified with $HycE^{His}$, albeit in reduced amounts (Fig. 2). The *hycF* gene also encodes an Fe-S protein, this time with homology to the Nqo9/NuoI/TYKY protein of Complex I [7]. BN-PAGE showed that the FHL complex was largely destabilized in the absence of HycF (Fig. 2). The third Fe–S protein in FHL is encoded by the *hycG* gene, which encodes the putative small subunit of Hyd-3 and as such is predicted to form the direct partner subunit for HycE in FHL (Fig. 1). $HycE^{His}$ was essentially the only FHL component that could be recovered from the mutant strain (Fig. 2) and BN-PAGE confirmed that the FHL complex was destabilized (Fig. 2). Thus, removal of any of the members of the core HycBEFG 131kDa complex has a destabilizing effect on the enzyme.

Interestingly, tryptic peptide mass fingerprinting of the BN-PAGE-analysed sample from the Δ*hycG* background identified peptides attributable to HycH (residues 5–12 and 79–98) and HypC (residues 44–65). While the interaction of HypC with precursor HycE is well known [25], the role of HycH is poorly understood. The amount of HycH co-purifying with $HycE^{His}$ in this experiment is likely to be far from equimolar, since a clear HycH polypeptide is not obvious by SDS–PAGE (Fig. 2), however this work gives the first indication that HycH may interact with HycE and/or HypC, in particular in the absence of the Hyd-3 small subunit HycG. Future research will concentrate on this potential new role for HycH, an accessory protein with homologues encoded within the formate hydrogenlyase operons of several bacterial species.

4.2. Engineering structural stability into FHL

Early sequence analysis predicted that HycB could the direct partner subunit for FdhF within FHL [5,24]. In an attempt to engineer stability into the FHL complex the FdhF formate dehydrogenase was genetically fused to the HycB protein. This approach resulted in an active FHL enzyme (Fig. 4) and the complete loss of the ~131kDa HycBEFG complex as observed by BN-PAGE (Fig. 3B). Instead, the physical attachment of FdhF to HycB led to an increased abundance of two electrophoretic species around 200kDa (one at ~180kDa and another at ~220kDa) that were both shown to contain FdhF, HycB, HycE, HycF and HycG by mass spectrometry (Fig. 3B). The predicted mass of a complete complex of the soluble proteins would be 210 547 Da, and a band migrating

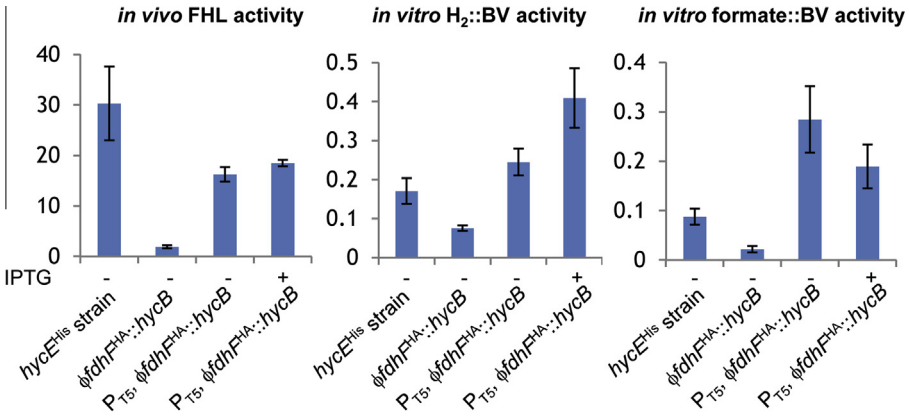

Fig. 4. $FdhF^{HA}$–HycB retains enzymatic activity. Strains MG059e1 (*hycE*^{His}), MGe1fZB (*φfdhF*^{HA}::*hycB*) and FZBup [pREP4] (P_{T5} *φfdhF*^{HA}::*hycB*) were grown in rich media under fermentative conditions. The FZBup [pREP4] strain as also grown in the presence of 1 mM IPTG where indicated. Intact cells were then assays for formate hydrogenlyase activity (left panel) where the units of activity were micromole H_2 produced *per* minute *per* gram cells. Proteins were then purified by IMAC, concentrated and assayed for hydrogenase activity (middle panel) or formate dehydrogenase activity (right panel) using benzyl viologen (BV) as electron acceptor. Specific activities are given as micromole BV reduced per minute per milligram protein. Bars represent standard error of the mean.

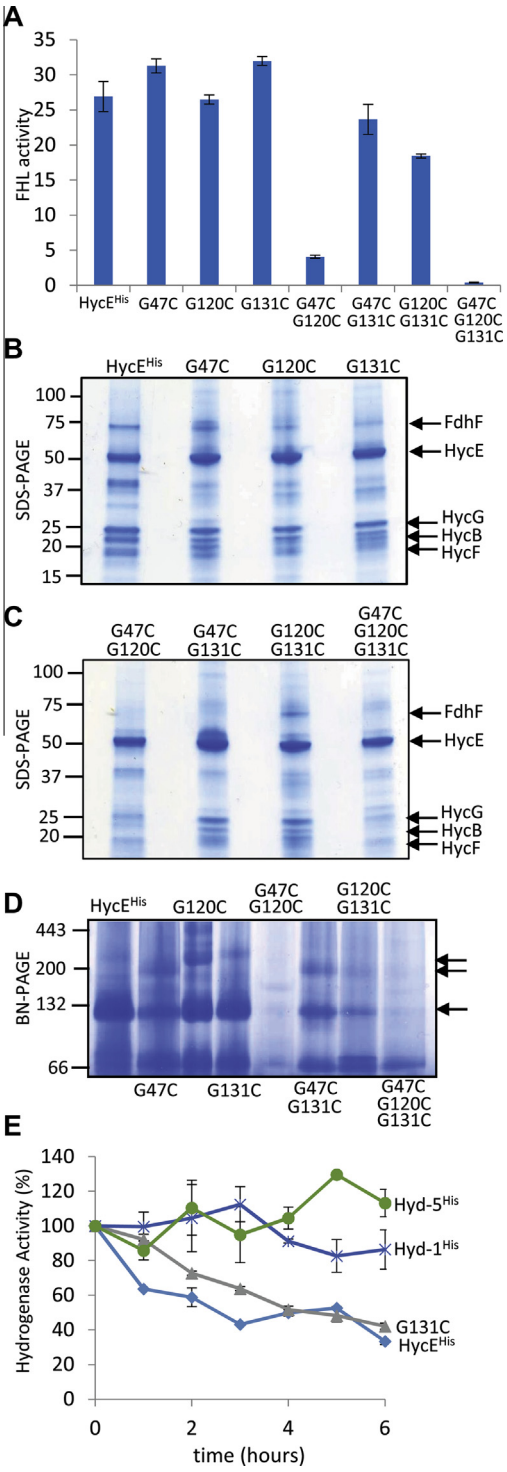

Fig. 5. Site-directed mutagenesis of *hycG*. Strain MG059e1 (*hycE*^{His}) and its derivatives carrying mutations in the *hycG* gene encoding G47C, G120C and G131C substitutions (and combinations) were grown in rich media under fermentative conditions. (A) Intact cells were assayed for FHL activity where the units of activity were micromole H_2 produced *per* minute *per* gram cells. Large scale fermentative cultures were prepared before FHL and its variants were isolated by IMAC, pooled and analysed by SDS–PAGE (B and C), where the arrows indicate proteins identified by tryptic peptide mass spectrometry, and BN-PAGE (D). For BN-PAGE, the arrows to the right highlight a 125kDa stable complex and the larger minor complexes that migrate at 180kDa and 220kDa. (E) Aliquots of purified FHL together with the HycG G131C derivative and *E. coli* Hyd-1 and *S. enterica* Hyd-5 were incubated exposed to air at 25 °C before being periodically assayed for H_2-linked BV reductase activity. Hydrogenase activity was plotted as a percentage of the total starting activity at time-point zero. Error bars represent standard error of the mean.

at around this mass (and containing FdhF) can also be observed in the native preparation (Fig. 2). This suggests that the ~220kDa species represents the most stable form, possibly complete, of the cytoplasmic domain of FHL (Fig. 3B). It is notable, however, that an intermediate-sized species with a mass ~180kDa is also present in the FdhF–HycB fusion preparation (Fig. 3B). Indeed, a complex of similar electrophoretic mobility is also observed in the protein purified from the Δ*hycC* mutant (Fig. 2) and from some of the *hycG* mutants (Fig. 5D). This possibly indicates that the FdhF–HycB fusion complex is only partially stabilised and that a degree of heterogeneity still exists in this preparation. Nevertheless, it is clear that linking FdhF to HycB could successfully reconstitute the soluble domain of FHL and result in an active enzyme both in vivo and in vitro (Fig. 4). Engineering peptide linkers between weakly interacting proteins has been explored for adding stability and functionality to several biological systems [26,27]. Here, the HA tag (itself comprised of nine amino acid residues) flanked by six glutamines proved to be an effective linker that clearly does not interfere with cofactor loading pathways. Although the exact region of contact between FdhF and HycB is not known, it seems likely that artificially maintaining FdhF in close proximity to HycB is sufficient to maintain FHL activity.

4.3. Engineering air-stability into FHL

Accurate, quantitative and sensitive electrochemical experiments have allowed the classification of [NiFe]-hydrogenases as either 'standard' (sensitive to O_2 attack) or 'O_2-tolerant' (being able to recover and re-activate from O_2 attack) [9]. The molecular basis of hydrogenase O_2-tolerance has been determined as primarily conferred by a special property of the proximal (and medial) Fe–S clusters within the hydrogenase small subunits [20,21]. A six-cysteine coordination shell allows a stable 4Fe–3S cluster to form, which can change conformation and release two electrons into the [NiFe] active site of the large subunit to reduce O_2 to water [12]. The *E. coli* FHL Hyd-3 enzyme is a standard hydrogenase and is predicted to bind a single 4Fe–4S cluster coordinated by four cysteines within HycG. Here, extra cysteine residues were engineered around the predicted proximal cluster and the variant enzymes purified (Fig. 5). The behaviour of *E. coli* Hyd-3 in electrochemical experiments is inconsistent [7], thus as an alternative an "air-stability" assay was devised here based on a Hyd-3 activity with benzyl viologen as artificial electron acceptor (Fig. 5). While the HycG sequence is rather divergent from most other NiFe-hydrogenase small subunits, the G47C substitution was chosen to generate a CXCC motif [9]. In O_2-tolerant hydrogenases the sixth cysteine is located within a WGCV motif that is often WGGV in standard hydrogenases (Supp Fig. S1). Sequence analysis suggested both HycG G120 and G131 should be tested as possible candidates for the sixth cysteine. Although none of the Hyd-3 variants was dramatically increased in their ability to maintain H_2-oxidation activity in air, the HycG G131C variant was more stable than the native enzyme over the first hour of exposure (Fig. 5E). Similar experiments with a Hyd-3-like enzyme from *Klebsiella oxytoca* concentrated on the HycG G47, G50, G113 and G120 residues [28]. In that work, Huang et al. [28] observed a slight increase in the air-stability of a purified G50C G120C double variant *K. oxytoca* Hyd-3, however a G131C variant was not tested to allow a direct comparison here. Engineering of the *K. oxytoca* HycE subunit, the Hyd-3 catalytic subunit, has also been reported [29]. In this case, substitution of HycE G300, predicted to be located within a gas channel leading to and from the active site, with either glutamic acid or methionine lead to increased air-stability of *K. oxytoca* Hyd-3 [29].

Although this work concentrated solely on the Hyd-3 component of FHL, engineering of O_2-tolerance into the entire

FHL complex will also require engineering of the formate dehydrogenase component. The molybdenum-containing FdhF protein is stable to exposure to air in the absence of substrate, but in the presence of formate the enzyme is irreversibly inactivated by O_2 [30].

4.4. Concluding remarks

E. coli FHL is a complex membrane-bound enzyme with the potential to be harnessed for biotechnological applications – either as a biohydrogen producing or as a CO_2 fixing enzyme. This genetic analysis, combined with a rapid purification protocol, established the core, stable enzyme as part of the soluble domain comprising HycBEFG. Concentrating on this core enzyme may be the key to making structural breakthroughs with this fragile complex. In the meantime, synthetic biology approaches, such as engineering gene fusions, can produce a fully functional enzyme under non-native conditions, as has been shown here. Finally, the attempts to retro-fit an Fe–S chemistry-based O_2-tolerance system to FHL were only partly successful. It may be difficult to engineer complete O_2-tolerance into FHL by modification of the HycG subunit alone. This is because HycG already differs considerably from other [NiFe]-hydrogenase small subunits, most notably since it only contains one Fe–S cluster rather than the more usual three [12].

Acknowledgements

This work was funded by the Biological and Biotechnological Sciences Research Council (Grants BB/H001190/1 and BB/I02008X/1 to F.S.). Dr Constanze Pinske is thanked for useful discussions and advice. M.C.H. was a visiting Masters student from Linköpings Universitet, Sweden. T.P. is Royal Society-Wolfson Research Merit Award holder.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.08.043.

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