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

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A B S T R A C T

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.
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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
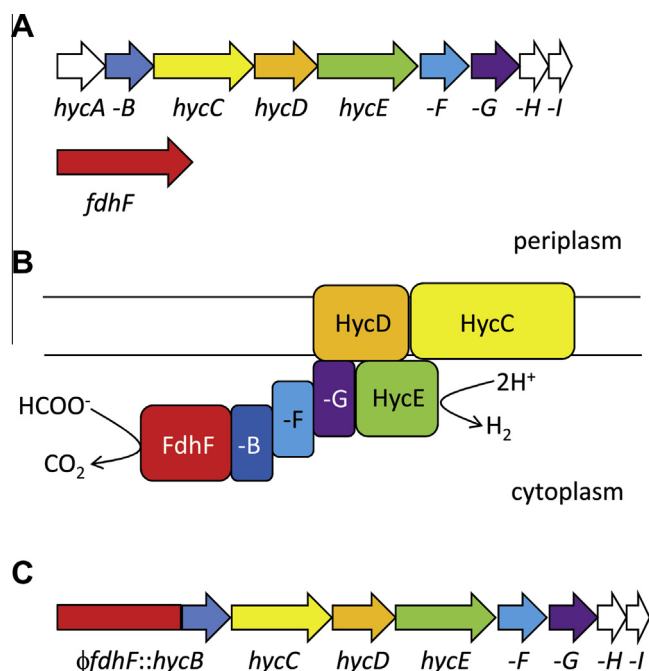
Fig. 1. Components of the native and engineered FHL system. (A) Genetics of the E. coli FHL system. The hyc operon is encoded at 61 min on the genome, while fdhF is located at 91 min. Genes are colour-coded to match the enzyme cartoon shown in (B). Genes shaded white do not encode structural components 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.

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 φfdhF^{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 EcoRI 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 EcoRI-BamHI fragment. Then ~500 bp of DNA upstream of hycA was cloned as a KpnI-EcoRI fragment. Finally, the fdhF part of the φfdhF^{HA}-hycB construct

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

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, ΔhycB	This work
MGE1dC	As MG059e1, ΔhycC	This work
MGE1dF	As MG059e1, ΔhycF	This work
MGE1dG	As MG059e1, ΔhycG	This work
MGE1dZ	As MG059e1, ΔfdhF	This work
MACdF	As MG059e1, ΔhycC, ΔfdhF	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, ΔhycA, ΔfdhF, φfdhF^{HA}::hycB	This work
FZBup [pREP4]	As MGe1fZB, P$_{T5}$ φfdhF^{HA}::hycB [pREP4: Kan^R, lacI$^+$]	This work