Activation of a [NiFe]-hydrogenase-4 isoenzyme by maturation proteases

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Abstract

Maturation of [NiFe]-hydrogenases often involves specific proteases responsible for cleavage of the catalytic subunits. Escherichia coli Hycl is the protease dedicated to maturation of the Hydrogenase-3 isoenzyme, a component of formate hydrogenlyase-1. In this work, it is demonstrated that a Pectobacterium atrosepticum Hycl homologue, HyfK, is required for hydrogenase-4 activity, a component of formate hydrogenlyase-2, in that bacterium. The P. atrosepticum ΔhyfK mutant phenotype could be rescued by either P. atrosepticum hyfK or E. coli hycl on a plasmid. Conversely, an E. coli Δhycl mutant was complemented by either E. coli hycl or P. atrosepticum hyfK in trans. E. coli is a rare example of a bacterium containing both hydrogenase-3 and hydrogenase-4, however the operon encoding hydrogenase-4 has no maturation protease gene. This work suggests Hycl should be sufficient for maturation of both E. coli formate hydrogenlyases, however no formate hydrogenlyase-2 activity was detected in any E. coli strains tested here.

Keywords:
Escherichia coli; Pectobacterium atrosepticum; formate hydrogenlyase; hydrogenase; maturase; protease.

Abbreviations:
GC, gas chromatography; LB, Lysogeny Broth; LSLB, Low Salt Lysogeny Broth.

Supplementary Material is available with the online version of this article.
E. coli Hyd-3 is a member of the group 4A [NiFe]-hydrogenases [10] and a component of the formate hydrogenlyase-1 (FHL-1) complex [11]. It is encoded within the hycABC-DEFGHI operon that includes the gene for the protease [6, 12]. Interestingly, laboratory strains of E. coli encode two separate group 4A [NiFe]-hydrogenases, each predicted to be part of distinct formate hydrogenlyase complexes. Thus in addition to Hyd-3, the E. coli [NiFe]-hydrogenase-4 isoenzyme (Hyd-4) is encoded by the hyfABCDEFGHIIJ-focB operon [13] and is predicted to be a component of a formate hydrogenlyase-2 (FHL-2) complex [11]. FHL-1 and FHL-2 share the same core architecture, with FHL-2 predicted to contain extra membrane-embedded components [11]. FHL activity is normally maximal under fermentative conditions when the enzyme catalyses the oxidation of carbon dioxide and couples this directly to the reduction of protons to molecular H₂. Thus group 4A [NiFe]-hydrogenases have a physiological role in the evolution of hydrogen gas [11]. Directly demonstrating the enzymatic activity of E. coli FHL-2 or Hyd-4 has proven challenging. Under laboratory conditions, the enzyme appeared to be neither transcribed nor enzymatically active [14–16], although there is some evidence for a physiological role in H₂ metabolism under some specific environmental conditions [17, 18]. In addition, disruption of Hyd-4 genes alone did not affect overall H₂ production by E. coli [19, 20], again indicating that cellular Hyd-4 activity was very low or absent under the conditions tested. It is also clear that the E. coli hyfABCDEFGHIIJ-focB operon does not encode any homologue of HycI (Fig. 1a) nor any other protease [2, 13]. Therefore, while the large subunit of Hyd-4 (HyfG) shares a high degree of sequence identity with HycE including the catalytic triad (Fig. 1b), it must also be considered that the apparent low activity of Hyd-4 may stem from incomplete maturation of the enzyme.

In this work, we set out to test the initial hypotheses that the E. coli hyf operon is not sufficiently expressed, and that HyfG is not correctly processed, such that a hydrogenase-null phenotype is observed. To do this we took a recombineering approach and constructed 15 new strains (Table 1 and Supplementary Material, available in the online version of this article) with alternative promoters and/or φHyfG::hycE fusion alleles at the native hyc locus on the chromosome. None of the new strains displayed any Hyd-4 activity (Table 1). Briefly, a group of E. coli strains with modified hyf transcriptional promoter regions were generated using P1 phage transduction [21] and allelic exchange [22]. All engineering was carried out in single copy on the chromosome, and the strains’ ability to produce H₂ gas under fermentative conditions was assayed by gas chromatography [23]. Initially, an E. coli K-12 strain (MG056G1, Table 1) was constructed based on the MG1655 parent strain [24] but encoding an internal 10-His tag between residues Gly-85 and Ala-86 within the HyfG protein. The rationale here was that a similarly modified version of HycE (Hyd-3) had retained full activity [25] and that the tag would allow
Table 1. Rational engineering of the E. coli hyf operon does not induce H\textsubscript{2} production

| E. coli K-12  | Relevant genotype | Source | H\textsubscript{2} production |
|---------------|------------------|--------|-----------------------------|
| MG1655        | F, λ, rph-1      | [24]   | Positive                    |
| MG056G1       | as MG1655, hyfG\textsuperscript{insu} | This Work | Positive                    |
| AF01          | as MG1655, hyfG\textsuperscript{insu}, P\textsubscript{hyfA} | This Work | Positive                    |
| AF02          | as MG1655, hyfG\textsuperscript{insu}, P\textsubscript{hyfA}, ΔhyfC-A:Kan\textsuperscript{r} | This Work | Negative                    |
| AF03          | as MG1655, hyfG\textsuperscript{insu}, P\textsubscript{hyfA}, ΔhyfC-A-I, ΔhyfB:Kan\textsuperscript{r} | This Work | Negative                    |
| AF04          | as MG1655, hyfG\textsuperscript{insu}, P\textsubscript{hyfA}, ΔhyfC-A-I, ΔhyfB | This Work | Negative                    |
| AF06          | as MG1655, hyfG\textsuperscript{insu}, P\textsubscript{hyfA}, ΔhyfC-A-I, ΔhyfB | This Work | Negative                    |
| FTE001        | as MG1655, hyfG\textsuperscript{insu}, P\textsubscript{hyfA}, ΔhyfC-A-I, \textit{ϕhyfG} (nt 1–1569):ΔhyfE (nt 1611–1707) | This Work | Negative                    |
| MG059e1       | as MG1655, hyfE\textsuperscript{insu} | [25]   | Positive                    |
| MGE1dI        | as MG1655, hyfE\textsuperscript{insu}, ΔhyfE | This Work | Negative                    |
| MC4100        | F\textsuperscript{-}, araD139, Δ(araD-fla)169, λ\textsuperscript{-}, e14, ΔflhD5301, Δ(fruK-yeiR)725(fruA255), relA1, rpsL150(Str\textsuperscript{r}), rba22, Δ(fimB-fimE)632(:IS1), deoC1 | [27]   | Positive                    |
| FTD147        | as MC4100, ΔhyfA, ΔhybC, ΔhyfE | [16]   | Negative                    |
| AF05          | as MC4100, ΔhyfA, ΔhybC, ΔhyfE, P\textsubscript{hyfA} | This Work | Negative                    |
| FTE002        | as MC4100, ΔhyfA, ΔhybC, ΔhyfE, P\textsubscript{hyfA}, ϕhyfG (nt 1–1569):ΔhyfE (nt 1611–1707) | This Work | Negative                    |
| FTE003        | as MC4100, ΔhyfA, ΔhybC, ΔhyfE, P\textsubscript{hyfA} | This Work | Negative                    |
| FTE004        | as MC4100, ΔhyfA, ΔhybC, ΔhyfE, P\textsubscript{hyfA}, ϕhyfG (nt 1–1569):ΔhyfE (nt 1611–1707) | This Work | Negative                    |
| FTE005        | as MC4100, ΔhyfA, ΔhybC, ΔhyfE, P\textsubscript{hyfA}, ϕhyfG\textsuperscript{insu} | This Work | Negative                    |
| FTE006        | as MC4100, ΔhyfA, ΔhybC, ΔhyfE, P\textsubscript{hyfA}, ϕhyfG\textsuperscript{insu}, P\textsubscript{hyfG} (nt 1–1569):ΔhyfE (nt 1611–1707) | This Work | Negative                    |
| FTE007        | as MC4100, ΔhyfA, ΔhybC, ΔhyfE, ϕhyfG\textsuperscript{insu}, P\textsubscript{hyfG} (nt 1–1569):ΔhyfE (nt 1611–1707) | This Work | Negative                    |

*E. coli strains were grown under anaerobic fermentative conditions in LB medium supplemented with 0.8 % (w/v) d-glucose at 37 °C for 16h. Production of molecular H\textsubscript{2} in the culture headspace was determined by gas chromatography. Hungate tube headspace gas was injected into a 500 µl loop and separated through a 5A molecular packed column before thermal conductivity detection. A hydrogen standard curve was generated using N\textsubscript{2}:H\textsubscript{2} mixes [23]. LB, Lysogeny Broth.

Further characterization of Hyd-4 at the protein level if the promoter engineering were successful. Next, the MG056G1 strain was further modified to replace the native hyf promoter region with that from the E. coli hyc operon encoding Hyd-3. This new strain (AF01, Table 1) was then extensively modified, first with the genetic removal of hydrogenase-3 activity (resulting in strains AF02 and AF03, Table 1), then by the additional deletion of the gene encoding the hydrogenase-1 catalytic subunit (yielding strains AF04 and AF06, Table 1). Culturing of all of these strains in triplicate 5 ml Lysogeny Broth (LB) supplemented with 0.8 % (w/v) glucose in sealed Hungate tubes for 16h at 37 °C demonstrated that replacement of the hyf promoter region with that of hyc did not result in detectible H\textsubscript{2} production from Hyd-4 (Table 1).

Next, an alternative E. coli K-12 parental strain (based on MC4100 [26, 27]) was tested. The E. coli FTD147 strain (deleted for the genes encoding the catalytic subunits of Hyd-1, -2 and -3 [16]) was modified by replacement of the native hyf promoter with that of the strong T5 promoter from the pQE plasmid series (yielding strain FTE003, Table 1). Growth of this strain under fermentative conditions did not result in any detectible H\textsubscript{2} production from Hyd-4 (Table 1).

Finally, it was considered that potential problems with HyfG processing could be leading to synthesis of an immature, inactive Hyd-4. This hypothesis is based on the fact that the hyc operon encodes no specific maturation protease and the reasonable possibility that HycI might not recognize HyfG as a substrate. In order to test this hypothesis, with the aim of forcing HycI to recognize and activate HyfG, a series of strains were carefully constructed where the C-terminal assembly peptide of HycE was added to the mature sequence of HyfG (Table 1, Fig. 1c). Careful genetic engineering generated a
hyFG:\hyCE fusion sequence that would comprise the first 1569 nucleotides of \textit{hyF} precisely in-frame with \textit{hyC} nucleotides 1611–1707 and retaining the ribosome binding site and initiation codon on the downstream \textit{hyH} gene to mitigate against potential polar effects. The resulting protein sequence is shown in Fig. 1c. This construct was transferred to the chromosome of a number of promoter-engineered strains (note that these all remain \textit{hyC}') using the technique of homologous recombination [22]. No H₂ production from Hyd-4 was detected in any of the engineered large subunit fusion strains (Table 1). Taken altogether, the strain-engineering experiments suggest that additional, unknown, biosynthetic problems are hindering assembly of \textit{E. coli} Hyd-4.

Clearly, making progress in the understanding the biochemistry of Hyd-4-like enzymes requires an alternative model system. Recently, group 4A [NiFe]-hydrogenases from \textit{Pectobacterium atrosepticum} [28], \textit{Trabulsiella guaensis} [29], \textit{Sulfurospirillum multivorans} [30], \textit{Campylobacter concisus} [31] and \textit{Parageobacillus thermoglucosidasius} [32] have been identified as possible candidates for study of this [NiFe]-hydrogenase group. Our sequence analysis suggests that genetic loci encoding each of these 'hyf-type' enzymes contained a \textit{hyfC}-like gene (Fig. 1d). Indeed, bioinformatic analysis of group 4A hydrogenases revealed only four organisms that encode both a Hyd-3 and Hyd-4 orthologue within their respective genomes: \textit{Escherichia coli}, \textit{Shimwellia blattae}; \textit{Hafnia alvei}; and \textit{Koskonia radicincitans} [11, 33]. In every case only one endopeptidase gene is found within the \textit{hyfC}-like operon, and none could be identified within the \textit{hyfC}-like operons (Fig. 1d, organisms linked by the red line). This suggests that a single HyfC-like protease may be sufficient for Hyd-4 biosynthesis, given that a second copy is never conserved.

\textit{P. atrosepticum} SCR1043 is a γ-Proteobacterium that contains an active FHL-2 and Hyd-4 encoded by a \textit{hyf} operon (Fig. 1a), but no FHL-1 or Hyd-3 isoenzyme [28]. Unlike the \textit{E. coli} \textit{hyf} operon, the \textit{P. atrosepticum} SCR1043 \textit{hyf} operon encodes HyfK (HyfK\textsubscript{\textit{pa}} Fig. 1a), which shares 74% overall sequence identity with \textit{E. coli} HyfC (HyfC\textsubscript{\textit{ec}}). Due to this sequence similarity it was considered that these endopeptidases could be tested for their ability to activate either the Hyd-3-type and the Hyd-4-type hydrogenase. To begin, both \textit{hyfC}\textsubscript{\textit{ec}} and \textit{hyfK}\textsubscript{\textit{pa}} genes were cloned separately in to pQE80L (Amp\textsuperscript{R}) expression vectors using standard PCR and molecular cloning techniques. Both plasmids, and a vector control, were used to transform the \textit{E. coli} Δ\textit{hyfC} strain MGE1dI (Table 1). \textit{E. coli} MGE1dI is based on MG059e1 (as MG1655, \textit{hyfE}\textsubscript{\textit{ec}} [25]) but carries an unmarked in-frame deletion in \textit{hyfC}. The transformed \textit{E. coli} strains were grown in triplicate 5 ml LB 0.2% (w/v) formate, with or without addition of 1 mM IPTG, anaerobically in sealed Hungate tubes, for 24 h at 37°C, before GC analysis of the culture headspace. The MGE1dI (Δ\textit{hyC}l) strain of \textit{E. coli}, containing empty vector control, displayed no physiological FHL-1 activity and did not evolve H₂ gas under fermentative conditions (Fig. 2a). However, incorporation of either \textit{hyfC}\textsubscript{\textit{ec}} or \textit{hyfK}\textsubscript{\textit{pa}} in the \textit{E. coli} Δ\textit{hyfC}l strain rescued H₂ production (Fig. 2a). These data demonstrate that the \textit{hyfK}\textsubscript{\textit{pa}} gene product can facilitate the maturation of the \textit{E. coli} Hyd-3 enzyme.

To study the role of maturation proteases in the activation of Hyd-4/FHL-2, \textit{P. atrosepticum} SCR1043, which contains

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{\textit{P. atrosepticum} HyfK can activate \textit{E. coli} Hyd-3, and \textit{E. coli} HyfC can activate \textit{P. atrosepticum} Hyd-4. (a) \textit{E. coli} strains MC4100 (FHL-1\textsuperscript{+}) and MGE1dI (Δ\textit{hyfC}) were transformed separately with plasmids harbouring \textit{hyfC}\textsubscript{\textit{ec}}, \textit{hyfK}\textsubscript{\textit{pa}} or a vector control (pQE80). Strains were grown anaerobically in LB medium supplemented with 0.2% (w/v) formate, ampicillin and 1 mM IPTG where indicated (+) for 24 h at 37°C. (b) \textit{P. atrosepticum} strains PH002 (Δ\textit{hyfC}, FHL-2\textsuperscript{−}) and PH006 (Δ\textit{hyfK}, Δ\textit{hyfC}) were transformed with plasmids containing \textit{hyfC}\textsubscript{\textit{pa}}, \textit{hyfK}\textsubscript{\textit{ec}} or pQE80. Strains were grown anaerobically in low-salt LB (LSLB) medium supplemented with ampicillin and 1 mM IPTG where indicated (+) for 48 h at 24°C. (c) \textit{E. coli} strains MGE1dI (Δ\textit{hyfC}), FTE003 (Δ\textit{hyfA}, Δ\textit{hyfB}, Δ\textit{hyfC}, Δ\textit{hyfE}, Δ\textit{hyfF}, Δ\textit{hyfG}, Δ\textit{hyfH}, Δ\textit{hyfI}\textsubscript{\textit{ec}}) and FTE004 (Δ\textit{hyfA}, Δ\textit{hyfB}, Δ\textit{hyfC}, Δ\textit{hyfE}, Δ\textit{hyfF}, Δ\textit{hyfG}, Δ\textit{hyfH}, Δ\textit{hyfI}\textsubscript{\textit{pa}}, Δ\textit{hyfJ}, Δ\textit{hyfK}\textsubscript{\textit{pa}}) were transformed with plasmids containing \textit{hyfC}\textsubscript{\textit{ec}}, \textit{hyfK}\textsubscript{\textit{pa}} or a vector control (pQE80). Strains were grown anaerobically in LB medium supplemented with 0.8% (w/v) glucose, ampicillin and 1 mM IPTG for 16 h at 37°C. In all cases, H₂ headspace samples were extracted and analysed by gas chromatography (Shimadzu GC2014 using a 5A molecular packed column with thermal conductivity detection). Data was normalized by OD\textsubscript{600} and culture volume. Error bars represent sD (n=3).}
\end{figure}
active FHL-2, was studied [28]. First, a genetic approach was taken to assess the role of hyfK in hydrogen production. A *P. atrosepticum* double-mutant strain was constructed, using an allele exchange protocol [28], which carried both ΔhybC and ΔhyfK in-frame deletions (PH006, Table 2 and Supplementary Material). The ΔhybC deletion removes all Hyd-2 activity leaving Hyd-4 as the only active hydrogenase in the bacterium [28]. Next, the *P. atrosepticum* ΔhybCΔhyfK double-mutant (PH006), together with the *P. atrosepticum* PH002 parent strain (ΔhybC only), were separately transformed with the pQE80 plasmids containing either hycI*Ec* or hyfK*Pa* or the empty vector as a control. The transformed *P. atrosepticum* strains were then grown in triplicate 5 ml low salt LB cultures (5 g l⁻¹ NaCl as opposed to the commonly used 10 g l⁻¹), with or without addition of 1 mM IPTG, in sealed Hungate tubes, fermentatively for 48 h at 24 °C, before GC analysis of the headspace gases. The *P. atrosepticum* PH002 parent strain (ΔhybC) was able to generate H₂ gas under all conditions (Fig. 2b). However, the ΔhybCΔhyfK double-mutant was incapable of producing any H₂ gas in this experiment when carrying an empty vector (Fig. 2b). This shows the hyfK*Pa* protease gene is essential for FHL-2 and Hyd-4 activity in *P. atrosepticum* SCR11043. Moreover, the *P. atrosepticum* ΔhybCΔhyfKΔhybCΔhyfK double-mutant strain was clearly rescued for H₂ production by inclusion of either hycI*Ec* or hyfK*Pa* (Fig. 2b). These data demonstrate that Hyd-4 isoenzymes do require a maturation step for successful biosynthesis and they also suggest that, in the rare cases where an organism has the capability to produce both FHL-1 and FHL-2, that a single copy of hycI should be sufficient for this task.

This compatibility of HycI*Ec* and HyfK*Pa* for activation of either *E. coli* Hyd-3 or *P. atrosepticum* Hyd-4 points strongly towards the idea that *E. coli* HycI should be capable of maturation of the endogenous Hyd-4 found in *E. coli*. In one final attempt to observe Hyd-4 activity in *E. coli*, the hycI*Ec* and hyfK*Pa* encoding plasmids, and a vector control, were each used to transform the *E. coli* FTE003 and FTE004 strains encoding the HyfG::HycE fusion proteins (Table 1). All strains were grown in triplicate 5 ml LB 0.8% (w/v) glucose cultures, with addition of 1 mM IPTG, in sealed Hungate tubes, for 16 h at 37 °C, before GC analysis. Hydrogen production was only detected in the control strains (Fig. 2c), demonstrating that cellular levels of a maturation protease is not the sole factor limiting Hyd-4 activity in *E. coli*.

This work presents the first demonstration of cross-species complementation by hydrogenase maturation endopeptidases, highlighting the close evolutionary relationship between group 4 [NiFe]-hydrogenases and demonstrating the critical importance of the HycI-type protease in the biosynthesis of these enzymes. These data are in line with studies showing a endopeptidase for a group 1D hydrogenase was able to activate a different group 1D enzyme within the same organism (*Salmonella enterica*) [34], and one endopeptidase was able to activate both a group 3B hydrogenase and group 4D hydrogenase within the same organism (*Thermococcus kodakarensis*) [35].

Though the proteolytic maturation schedule for [NiFe]-hydrogenases is now dogma, there are known and emerging variations on the canonical pathway for large subunit biosynthesis. Protopelolytic processing is not required for all [NiFe]-hydrogenases, such as examples of the H₂-sensing, Ech- and CODH-linked hydrogenases [36–39]. Indeed, recent genetic engineering work showed that removal of the C-terminal assembly peptide from the membrane bound hydrogenase (MBH) in *Cupriavidus necator* (*Ralstonia eutropha*)) did not disrupt cofactor insertion and resulted in no loss of hydrogenase-specific activity [40]. Given that in *S. enterica* a maturation protease was found to retain the ability to recognize and bind to a large subunit completely lacking the maturation peptide [34], perhaps it should be considered that the maturation protease has a role in hydrogenase biosynthesis beyond the simple cleavage of the C-terminal extension. This could certainly be tested in the *C. necator* system [40] by deleting the gene encoding the processing protease (HoxM [41]) in the strain already lacking the hydrogenase assembly peptide and observing any changes to hydrogenase activity.

It is becoming increasingly clear that the C-terminal assembly peptide may not be the key recognition motif for the protease [34, 42]. Early work showed that swapping of the *E. coli* HycE (Hyd-3) assembly peptide for that of HybC (Hyd-2) led to a ‘dead-end’ fusion protein that could not be processed by any maturation protease tested [43]. While more recently, swapping the HybC (Hyd-2) assembly peptide for that of HyaB (Hyd-1) did not lead to any changes in the requirement for the Hyd-2-specific protease (HybC) for maturation [42, 44].

In conclusion, this work has demonstrated that group 4 [NiFe]-hydrogenases require a functional hycI-like accessory gene for correct biosynthesis. A model bacterium (*P. atrosepticum* SCR11043), which contains an active Hyd-4 and FHL-2 as the only formate hydrogenlyase activity, required the presence of the native hyfK gene product for maturation. The *E. coli* hycI gene could substitute for *P. atrosepticum* hyfK if supplied on a plasmid, providing an explanation of why it is that in rare examples of organisms

### Table 2. Mutagenesis of the *P. atrosepticum* hyf operon

| *P. atrosepticum* strain | Relevant genotype | Source | H₂ production |
|--------------------------|------------------|--------|---------------|
| SCR11043                 | wild-type        | [48]   | Positive      |
| PH002                    | as SCR11043, ΔhybC |        | Positive      |
| PH006                    | as SCR11043, ΔhybCΔhyfK | This Work | Negative      |

*P. atrosepticum* strains were grown under anaerobic fermentative conditions in low salt (LS) LB medium supplemented with 0.8% (w/v) d-glucose at 24 °C for 48 h. Production of molecular H₂ in the culture headspace was determined by GC [28]. GC, Gas Chromatography; LB, Lysogeny Broth; LSLB, Low Salt Lysogeny Broth.
that contain both an FHL-1 and an FHL-2 only one copy of a hycl-like gene is conserved.

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Author contributions
AJF performed research, analysed data, prepared figures for publication, and wrote the paper. GB and TP performed research. SJC and FS supervised the research. FS conceived the study and wrote the paper.

Conflicts of interest
The authors declare that there are no conflicts to declare.

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