Introduction

Biohydrogen is biologically derived molecular hydrogen and is considered a possible replacement for fossil fuels (Benemann 1996). *Escherichia coli* produces hydrogen gas predominantly under fermentative conditions (Sargent 2016) using the formate hydrogenlyase complex that couples formate oxidation to hydrogen evolution (McDowall et al. 2014). One possible approach to engineering additional routes to hydrogen production in *E. coli* is the heterologous expression of non-native enzymes. An attractive proposition for engineering additional hydrogen metabolism into *E. coli* is to assemble functional NADH-dependent hydrogenases (Ghosh et al. 2013; Kelly et al. 2015), which would not compete directly with native sources of reductant for hydrogen production and potentially link the new hydrogenase activity directly into the anaerobic metabolism of the host organism. This is because when *E. coli* is cultured fermentatively a major metabolic challenge for the cell is the recycling of the NADH produced by glycolysis. This process is normally carried out, in the main, by the aldehyde/alcohol dehydrogenase AdhE.

The Knallgas bacterium *Ralstonia eutropha* (also called *Cupriavidus necator*) contains a soluble cytoplasmic enzyme that links NAD+/NADH directly to H₂ biochemistry (Burgdorf et al. 2005a). The *R. eutropha* soluble hydrogenase (SH) is encoded by the *hoxFUYHWI* operon where HoxFU represent the diaphorase half of the enzyme; HoxYH is the [NiFe]-hydrogenase half; HoxI is a sixth subunit of the enzyme; and HoxW is a maturation protease required for final activation of the [NiFe]-hydrogenase catalytic subunit, HoxH (Burgdorf et al. 2005a). Biosynthesis of the Ni–Fe–CO–2CN⁻ cofactor located at the active site of a hydrogenase requires the activity of specific ‘maturase’ proteins (Böck et al. 2006). These include HypA and HypB.

Abstract  Biohydrogen is produced by a number of microbial systems and the commonly used host bacterium *Escherichia coli* naturally produces hydrogen under fermentation conditions. One approach to engineering additional hydrogen production pathways is to introduce non-native hydrogenases into *E. coli*. An attractive candidate is the soluble [NiFe]-hydrogenase from *Ralstonia eutropha*, which has been shown to link NADH/NAD⁺ biochemistry directly to hydrogen metabolism, an activity that *E. coli* does not perform. In this work, three synthetic operons were designed that code for the soluble hydrogenase and two different enzyme maturase systems. Interestingly, using this system, the recombinant soluble hydrogenase was found to be assembled by the native *E. coli* [NiFe]-hydrogenase assembly machinery, and, vice versa, the synthetic maturase operons were able to complement *E. coli* mutants defective in hydrogenase biosynthesis. The heterologously expressed soluble hydrogenase was found to be active and was shown to produce biohydrogen in vivo.

Keywords  Bacterial physiology · Hydrogen metabolism · Synthetic biology · Hydrogenase
(which are involved in nickel processing); the chaperone HypC; and HypeD, HypeE and HypeF, which together build the Fe–CO–2CN− half of the cofactor (Böck et al. 2006). In R. eutropha there are at least two versions of most maturase genes available, together with an extra accessory protein of less clearly defined function, HypX (Burgdorf et al. 2005a; Schifflers et al., 2013). Some reports suggest that the R. eutropha maturases are very specific for the assembly of their cognate [NiFe]-hydrogenases (Burgdorf et al. 2005a; Schiffels et al. 2013) and this is consistent with the hypothesis that different prokaryotic species require their own dedicated maturase machineries (Böck et al. 2006; Lubitz et al. 2014).

In this work, a synthetic biology approach was taken to producing an active R. eutropha soluble hydrogenase in an E. coli host. Synthetic operons encoding the SH and hydrogenase maturases were designed and characterised. An active SH was produced and hydrogen production was recorded.

Materials and methods

Strain and plasmid generation

Plasmids and bacterial strains used are listed in Tables 1 and 2, respectively. IC011 was constructed by modifying the IC010 strain (Deplanche et al. 2010) using a ΔhybOA allele present on the pMAK705 derivative pRAT58 and the method of Hamilton et al. (1989). HJ001 (as IC011, ΔiscR) and HJ002 (as IC011, ΔiscR, ΔadhE) were assembled by moving the pMAK705-based ΔiscR and ΔadhE alleles constructed by Kelly et al. (2015) sequentially into IC011 by homologous recombination (Hamilton et al. 1989). Synthetic operons were constructed in silico using the OPTIMIZER software (Puigbo et al. 2007). A ribosome binding site and linker (5′-AGGAGGAAAAAAA-3′) was added before each synthetic gene (Kelly et al. 2015) and restriction sites placed at each ends of the gene. The final sequence was then synthesised by Biomatik Corp (USA) and sub-cloned into various expression vectors (Table 1).

35S-methionine radiolabelling

Briefly, E. coli strain K38 [pGP1-2] was transformed with the vectors to be tested. Cultures were grown at 30 °C in M9 minimal medium (Sambrook and Russell 2001), containing all amino acids except methionine and cysteine, before transcription of T7 RNA polymerase was induced by heat shock at 42 °C for 15 min. Rifampicin was then added to inhibit the native RNA polymerase activity and a 1 ml aliquot was spiked with 0.01 μCi of 35S-Methionine. After incubating for 15 min, samples were heated to 105°C, separated by SDS-PAGE, fixed and visualised by autoradiography.

Enzyme assays

In vitro hydrogen oxidation activity was measured spectrophotometrically using the H2-dependent reduction of methyl viologen (MV) in anaerobic cuvettes (Palmer et al. 2010). Strains were grown anaerobically at 37 °C without agitation for 16 h in 500 ml Duran bottles containing LB media supplemented with 0.4% (w/v) fumarate, 0.5% (v/v) glycerol and appropriate antibiotics. Cells were harvested by centrifugation at 2773×g, washed twice in 50 mM Tris.HCl pH 7.8, and the wet weights of cell pellets recorded. Pellets were then suspended in 3 ml 50 mM Tris.HCl pH 7.8 before lysis by French Press (8000 psi) and crude extracts prepared by centrifugation.

For in vivo hydrogen production assays, Hungate tubes containing 5 ml M9 media supplemented with 0.8% (w/v) glucose and 0.2% (w/v) casamino acids were inoculated with 50 μl of pre-culture. Tubes were then inverted and incubated without agitation at 37 °C for 72 h. Headspace hydrogen was quantified using a gas chromatograph (Shimadzu GC-2014). Nitrogen was used as the carrier gas with a flowrate of 25 ml min−1, and the total amount of hydrogen in the headspace (10 ml) was calculated based on a standard curve. Values were normalised by considering the relative optical density of the cultures and the culture volume.

Results

Synthetic hox and hyp operons are expressed in E. coli

To produce the soluble cytoplasmic [NiFe]-hydrogenase from R. eutropha in E. coli a synthetic operon was designed (Fig. 1a). The natural hoxFUHYHWI gene order was maintained, but each synthetic gene was optimised for E. coli codon usage. Next, restriction sites were chosen to bookend each gene (Fig. 1a). The 5811 bp synthetic operon sequence was then synthesised and cloned into pUNI-PROM (AmpR), pSU-PROM (KanR) and pQE-80 (AmpR) expression vectors (Table 1).

To test whether the hoxFUHYHWI genes were being transcribed and translated in E. coli, the K-38[pGP1-2] strain was transformed with the pUNI-SH vector. This allowed T7 polymerase-dependent transcription of the operon and labelling of plasmid-encoded gene products with 35S-methionine. The radiolabelling experiments showed that pUNI-SH encodes at least six polypeptides (Fig. 1b). In order to assign each protein to a specific synthetic gene, a library of in-frame deletion plasmids was prepared.
Following 35S-Met-labelling experiments, it is clear that the catalytic subunits of the SH HoxF and HoxH are correctly and stably synthesised. The remaining four polypeptides are also synthesised but, except for HoxY, appear to migrate in SDS-PAGE at slightly aberrant apparent molecular masses (Fig. 1b). However, the banding pattern on SDS-PAGE matches exactly that of previous work on the native (Burgdorf et al. 2005b) and recombinant (Schiffels et al. 2013) SH enzyme.

Activation of [NiFe]-hydrogenases requires specific maturation subunits for activity (Wolf et al. 1998). In addition, hypC1, hypD1, hypE1 and hypF1 all have roles to play (Böck et al. 2006; Burgdorf et al. 2005a; Schiffels and Selmer 2015). Thus, an 8213 bp synthetic hypA2B2F2C1D1E1X operon was assembled (Fig. 1a) and cloned into pUNI-PROM (AmpR) and pSU-PROM (KanR) (Table 1). Construction of plasmid-borne in-frame deletions and in vivo pulse-labelling with 35S-methionine established that the maturation proteins were being produced in E. coli (Fig. 1c).

Finally, an alternative R. eutropha maturase operon, the 7115 bp hypA1B1F1C1D1E1X version, and derivatives were also constructed (Fig. 1a; Table 1). This synthetic operon was also found to be capable of producing

### Table 1 Plasmids used and constructed in this work

| Plasmid name | Details | Antibiotic resistance | References |
|--------------|---------|-----------------------|------------|
| pUNI-PROM    | As pT7.5 Tabor and Richardson (1985) with tatA promoter and RBS cloned EcoRI–BamHI | Amp | Jack et al. (2004) |
| pSU-PROM     | As pSU40 Bartolome et al. (1991) with tatA promoter and RBS cloned EcoRI–BamHI | Kan | Jack et al. (2004) |
| pUNI-SH      | As pUNI-PROM with synthetic hoxFUYHWI operon cloned BamHI–HindIII | Amp | This work |
| pUNI-A2-X    | As pUNI-PROM with synthetic hypA2B2F2C1D1E1X operon cloned BamHI–HindIII | Amp | This work |
| pSU-A2-X     | As pSU-PROM with synthetic hypA2B2F2C1D1E1X cloned BamHI–HindIII | Kan | This work |
| pUNI-A1-X    | As pUNI-PROM with synthetic hypA1B1F1C1D1E1X cloned BamHI–HindIII | Amp | This work |
| pSU-A1-X     | As pSU-PROM with synthetic hypA1B1F1C1D1E1X cloned BamHI–HindIII | Kan | This work |
| pQE80-SH     | As pQE80 (Qiagen) with synthetic hoxFUYHWI operon cloned BamHI–HindIII | Amp | This work |
| pGP1-2       | Encoding T7 polymerase under a temperature-dependent promoter | Kan | Tabor and Richardson (1985) |

### Table 2 E. coli strains used and constructed in this study

| Strain | Relevant genotype | Antibiotic resistance | Source/references |
|--------|-------------------|-----------------------|-------------------|
| MC4100 | F−, araD139, Δ(argF-lac)U169, ptsF25, deoC1, relA1, flbB5301, rpxL150 | None | Casadaban and Cohen (1979) |
| IC010  | As MC4100, ΔhyaB, ΔhyeE | None | Deplanche et al. (2010) |
| IC011  | As MC4100, ΔhyaB, ΔhybOA, ΔhyeE | None | This work |
| HJ001  | As MC4100, ΔhyaB, ΔhybOA, ΔhyeE, ΔixcR | None | This work |
| HJ002  | As HJ001, ΔadhE | None | This work |
| BEF314 | As MC4100, ΔhypB-E::cam | Cam | Jacobi et al. (1992) |
| BW25113| F−, Δ(araB-D)567, Δ(rhaB-D)568, ΔlacZ4787::rrnB-3, hsdR514, rph-1 | None | Baba et al. (2006) |
| JW2696 | As BW25113, Δhypo::kan | Kan | Baba et al. (2006) |
| JW2697 | As BW25113, ΔhypB::kan | Kan | Baba et al. (2006) |
| JW2698 | As BW25113, Δhypo::kan | Kan | Baba et al. (2006) |
| JW2699 | As BW25113, ΔhypD::kan | Kan | Baba et al. (2006) |
| JW2700 | As BW25113, ΔhypE::kan | Kan | Baba et al. (2006) |
| JW5433 | As BW25113, ΔhypF::kan | Kan | Baba et al. (2006) |
| K38    | HftC, phoA4, pit-10, tonA22, ompF627, relA1, λ+ | None | Lyons and Zinder (1972) |
protein product when examined by $^{35}$S-methionine labelling (Fig. 1d). Many of the encoded proteins can be readily identified; however, HypF1 (predicted to be 40 kDa) is not clear (Fig. 1d). A follow-up SDS-PAGE experiment was designed to generate greater separation in the 40 kDa region (Fig. 1e). In this case, HypB1, HypD1 and HypE1 could be readily identified; however, the synthesis of HypF1 could not be established (Fig. 1e).

The synthetic maturase operons are functional and can activate E. coli [NiFe]-hydrogenases

To test functionality of the maturase operons, genetic complementation of E. coli mutant strains compromised in [NiFe] cofactor biosynthesis was undertaken. The E. coli BEF314 strain ($\Delta hypBCDE$) is devoid of hydrogenase activity (Jacobi et al. 1992). When BEF314 ($\Delta hypB-E$) was transformed with plasmids encoding HypA1-X and HypA2-X, fermentative hydrogen production was restored to native levels (Fig. 2a), indicating that the E. coli FHL had been activated.

Next, individual mutants in each of the hypA-E and hypF genes were examined (Fig. 2b), beginning with hypA. As previously reported (Jacobi et al. 1992), the hypA mutant retains some hydrogenase activity (Fig. 2b). Indeed, under the growth conditions and assay type chosen, here the hypA mutant was able to accumulate hydrogen to the same level as the control strains (Fig. 2b).

HypB is a nickel-binding NTPase (Böck et al. 2006), and fermentative H$_2$ production by the E. coli hypB mutant is low (Fig. 2b). This phenotype can be rescued by production of either HypA1-X or HypA2-X (Fig. 2b), and this demonstrates that both HypB1 and HypB2 are functional.

Next, a $\Delta hypC$ strain was tested. HypC is a small protein that acts as a bridge between the initial [NiFe] cofactor assembly proteins and the empty apoenzyme (Böck et al. 2006). In the hydrogen accumulation assay employed here, the $\Delta hypC$ mutant is able to demonstrate clear hydrogen production activity (Fig. 2b). Nevertheless, co-expression of plasmids encoding HypA1-X and HypA2-X returned hydrogen evolution to native levels (Fig. 2b), suggesting that R. eutropha HypC1 can substitute for E. coli HypC.

The operons encoding HypA1-X and HypA2-X can also rescue the hydrogenase-null phenotype of a hypD mutant (Fig. 2b). However, the complementation of E. coli hypE and hypF mutants, which encode two proteins that interact extensively (Stripp et al. 2015), is less compelling (Fig. 2b). The R. eutropha HypE1 protein (which is identically encoded in both hypA1-X and hypA2-X plasmids) is able to complement a $\Delta hypE$ mutant, although hydrogenase activity is clearly reduced (Fig. 2b). However, in the absence of endogenous E. coli hypF, it seems that R. eutropha hypF1 is completely inactive in this assay (Fig. 2b).

Production of an active SH in E. coli

To explore the activity of the SH, an E. coli host strain (HJ002) was prepared that lacked key native hydrogenase genes (Table 2) and also carried an $\Delta iscR$ allele, which can be beneficial for heterologous production of metalloenzymes (Kelly et al. 2015), and a $\Delta adhE$ allele, with a view to increasing the availability of the NADH substrate under fermentative conditions (Ghosh et al. 2013).

The HJ002 ($\Delta hyaB$, $\Delta hybOA$, $\Delta hycE$, $\Delta iscR$, $\Delta adhE$) strain was transformed with a plasmid encoding hypA2-X operon and then co-transformed with pQE80-SH or pUNI-SH. The pQE80-SH vector, when uninduced, resulted in a comparable level of MV-linked hydrogenase activity to that of the pUNI-SH (Fig. 3a). Induction with progressively higher concentrations of IPTG correlated with a corresponding increase in hydrogenase activity (Fig. 3a). This assay demonstrates that the [NiFe]-hydrogenase half of the SH has been assembled and activated.

Next, the ability of the SH to produce H$_2$ in vivo was assessed. The HJ002 ($\Delta hyaB$, $\Delta hybOA$, $\Delta hycE$, $\Delta iscR$, $\Delta adhE$) strain was transformed with either pQE80-SH or pUNI-SH together with either a control plasmid (pSUPROM) or a plasmid encoding hypA1-X or hypA2-X. Hydrogen gas in the headspace was determined by gas chromatography in a single end-point assay (Fig. 3b). The data show that there is H$_2$ production from strain HJ002 carrying pQE80-SH, even without inclusion of the synthetic maturases (Fig. 3b). The native E. coli maturases are clearly able to assemble an active SH (Fig. 3b), thus partly corroborating the reciprocal experiment where the R. eutropha maturases were able to activate E. coli hydrogenase activity (Fig. 2). Furthermore, despite the IPTG-induced pQE80-SH strain having a similar level of
MV-linked hydrogenase activity to the pUNI-SH strain (Fig. 3a), the ability to evolve H₂ in vivo was greatly improved by using the pQE80-SH system (Fig. 3b). During the course of this work, two further studies reported the reconstitution of SH activity in an E. coli host (Ghosh et al. 2013; Schiffels et al. 2013). In both cases, the addition of 100 µM iron salts to the growth medium was required for maximal activation of the hydrogenase (Ghosh et al. 2013; Schiffels et al. 2013). Thus, the synthetic operons under investigation here were also tested in growth media containing 100 µM FeCl₃ (Fig. 3c). In this case, maximum levels of H₂ evolution were recorded at 250 nmol H₂ ml⁻¹ OD⁻¹ (Fig. 3c) compared to 12 nmol H₂ ml⁻¹ OD⁻¹ without iron supplementation (Fig. 3b).

Discussion

Production of an active R. eutropha SH in E. coli

The R. eutropha soluble [NiFe]-hydrogenase enzyme is an attractive system to biotechnologists for several reasons. The SH enzyme is not membrane-bound, like so many other hydrogenases, and this lends the possibility to generate ‘cell factories’ producing vast quantities of active enzyme in the cell cytoplasm. In addition, the ability to link hydrogen oxidation to NAD(P)H cofactor regeneration means this enzyme could have several industrial applications working together with other NAD(P)H-dependent biochemical reactions. Finally, the natural oxygen tolerance of this enzyme (Burgdorf et al. 2005a) may give it an advantage as a possible H₂ producer over the fragile O₂-sensitive [FeFe]-hydrogenase class.

Recent advances have been reported in the production of the SH in E. coli B strains and E. coli K-12 strains. Both (Schiffels et al. 2013) and Ghosh et al. (2013) worked with the native R. eutropha sequences, rather than synthetic sequences as described here, and had differing overall aims to their studies.

Schiffels et al. (2013) aimed to develop an overexpression system to allow purification of the recombinant enzyme. To this end, a plasmid-based system was constructed using the native R. eutropha genes for the SH and associated maturases to allow high-level production and purification of an active SH in E. coli BL21(DE3), a normally hydrogenase deficient strain (Pinske et al. 2011). In this case, increasing extracellular nickel salts (with levels above 1 µM being inhibitory), or providing an extra nickel transporter, proved useful in increasing yield of active enzyme for purification (Schiffels et al. 2013).

Interestingly, Schiffels et al. (2013) found that production of an active SH was dependent on co-expression with the R. eutropha hypA2, hypB2, hypC1, hypD1, hypE1, and hypF2 genes. This is in contrast to the data described here, where the E. coli maturases could active the SH (Fig. 3).

One difference between the two studies could be that the SH expression levels induced by Schiffels et al. (2013) were likely orders of magnitude greater than achieved here. Alternatively, however, it should be noted that the BL21(DE3) strain used by Schiffels et al. (2013) may well be defective in the global regulator FNR, which is necessary for full expression of nickel uptake and hypBCDE in E. coli (Pinske et al. 2011).

The work of Ghosh et al. (2013) had a similar aim to that described here—integration of an NADH-dependent

Fig. 2 The synthetic maturase operons can complement E. coli hyp mutants. a E. coli strain BEF314 (∆hypBCDE) was transformed with a pSU-PROM vector control (−) or vectors encoding the hypA1-X synthetic operon (pSU1-X) or synthetic hypA2-X operon (pSU2-X). b The Keio collection version of single E. coli deletion mutants in each of hypA, hypB, hypC, hypD, hypE and hypF were transformed with either empty vector (−) or pUNI-PROM-based vectors encoding the hypA1-X synthetic operon (A1-X) or synthetic hypA2-X operon (A2-X). In all cases, strains were grown fermentatively in M9 minimal media supplemented with 0.8% (w/v) glucose before gas chromatography was used to quantify any H₂ accumulated in the headspace after 48 h of incubation at 37 °C. The positive control (+) is the E. coli strain MC4100. Error bars represent SEM (n = 3)
[NiFe]-hydrogenase into E. coli metabolism. Ghosh et al. (2013) cloned the native SH genes behind a strong promoter and demonstrated very high (2 mol H₂ per mole glucose) hydrogen gas production levels in an E. coli K-12 strain that had been deleted for native hydrogenase genes. Interestingly, in this case, the SH was able to substitute for the main alcohol dehydrogenase (AdhE) and restore growth to an adhE mutant under fermentative conditions (Ghosh et al. 2013). Moreover, this growth effect was dependent on supplying 25 µM nickel salts to the medium (Ghosh et al. 2013). The synthetic system described in this work was unable to restore fermentative growth to an E. coli adhE mutant on minimal media, and even the native E. coli K-12 control strains were unable to reach 2 mol H₂ per mole glucose in this experimental setup. Clearly, expression levels must be very different between the Ghosh et al. (2013) experiments and those described in this work.

The work presented here used E. coli K-12 as a host strain and took an alternative synthetic approach to the design of the expression system. All gene sequences were designed by back-translation from protein products and arranged in synthetic operons under the control of single promoters. The modular design of the operons allows facile removal and replacement of each coding sequence, which could allow for future directed evolution or further biochemical studies. This system was found to successfully produce each individual subunit of the SH, and an active [NiFe]-hydrogenase was assembled that was capable of H₂ evolution in vivo. In this system, higher level expression from the T5 promoter (pQE-80-derived) was needed to induce the highest hydrogen production levels.

On the role of maturases in the biosynthesis of [NiFe]-hydrogenases

One relatively surprising result reported here is that active R. eutropha SH could be assayed in E. coli in the absence of its dedicated accessory proteins, thus demonstrating that, in this system, the E. coli maturases could recognise and activate HoxH. This is interesting because
the latter stages of cofactor insertion require some degree of protein–protein interactions, in particular when a HypC-type protein makes direct contact with the catalytic subunit. In the case of *E. coli* [NiFe]-hydrogenase-3, the HypC protein forms a stable complex with HycE until the cofactor loading is complete (Drapel and Böck 1998). *E. coli* has a second HypC homologue, HybG that can substitute when HypC is missing (Blokesch et al. 2001), and it seems also that these proteins can contribute to maturation of the SH. Indeed, the HypC protein from *Dehalococcoides mccartyi* was also recently shown to be able to activate the *E. coli* [NiFe]-hydrogenases (Hartwig et al. 2015).

It was pleasing that the converse experiment also upheld the hypothesis of cross-talk between the different maturation systems. There, the *E. coli* hydrogenases could be activated by the synthetic maturase operons designed here. This was very important in showing that the maturases were functional. The exception was *R. eutropha* HypF1, which is an unusually truncated version of the protein, which was found to be inactive. HypF2, which is normally co-produced with the SH itself, more closely resembles the native *E. coli* protein (Wolf et al. 1998). Note, however, that production of HypF1 was also not clear in the *E. coli* 35S-Met-labeling experiment (Fig. 1) and taken altogether these data show that the *hpaI*-X operon may not be producing an active HypF1 protein. Overall, however, these complementation experiments suggest some previously unsuspected promiscuity may exist between cofactor insertion proteins from different biological systems.

### Concluding remarks

In this work, a synthetic biology approach has been taken to engineering additional hydrogenase biochemistry into *E. coli*. A functional *R. eutropha* SH was produced and, somewhat surprisingly, could be activated by the native maturases found in the host strain. Future work will concentrate on optimising this synthetic system and engineering further non-native hydrogenases.

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### Compliance with ethical standards

**Conflict of interest** The authors declare no conflicts of interest.
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