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Biosynthesis of *Salmonella enterica* [NiFe]-hydrogenase-5: Probing the Roles of System-specific Accessory Proteins

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Nonstandard abbreviations

Hyd-1, hydrogenase-1; Hyd-2, hydrogenase-2; Hyd-5, hydrogenase-5; MB, methylene blue; MBH, membrane bound hydrogenase(s); *Rh. leguminosarum*, *Rhizobium leguminosarum*; *R. eutropha*, *Ralstonia eutropha*

Abstract

A subset of bacterial [NiFe]-hydrogenases have been shown to be capable of activating dihydrogen-catalysis under aerobic conditions, however it remains relatively unclear how the assembly and activation of these enzymes is carried out in the presence of air. Acquiring this knowledge is important if a generic method for achieving production of O_2-resistant [NiFe]-hydrogenases within heterologous hosts is to be developed. *Salmonella enterica* serovar Typhimurium synthesizes the [NiFe]-hydrogenase-5 (Hyd-5) enzyme under aerobic conditions. As well as structural genes, the Hyd-5 operon also contains several accessory genes that are predicted to be involved in different stages of biosynthesis of the enzyme. In this work, deletions in the *hydF*, *hydG* and *hydH* genes have been constructed. The *hydF* gene encodes a protein related to *Ralstonia eutropha* HoxO, which is known to interact with the small subunit of a [NiFe]-hydrogenase. HydG is predicted to be a fusion of the *R. eutropha* HoxQ and HoxR proteins, both of which have been implicated in the biosynthesis of an O_2-tolerant hydrogenase, and HydH is a homologue of *R. eutropha* HoxV, which is a scaffold for [NiFe] cofactor assembly. It is shown here that HydG and HydH play essential roles in Hyd-5 biosynthesis. Hyd-5 can be isolated and characterised from a Δ*hydF* strain, indicating that HydF may not play the same vital role as the orthologous HoxO. This study therefore emphasises differences that can be observed when comparing the function of hydrogenase maturases in different biological systems.

Keywords: Biosynthesis; Electrochemistry; Hydrogenase; Iron-sulfur cluster; Metallocenter assembly

Introduction

Photosynthetic microbial water splitting to give sustained bio-H_2 production ($H_2O + h\nu \rightarrow H_2 + ½O_2$) is not currently possible because of inhibitory interactions between O_2 and the natively synthesized H_2-producing enzymes (hydrogenases) of cyanobacteria and algae [1]. However, a mechanistic understanding of how [NiFe]-hydrogenases can catalyse H_2-oxidation in air (defined as "O_2-tolerance") is well developed for periplasmically-oriented, membrane-bound [NiFe]-hydrogenases (MBH) that are produced and functional in O_2, such as *Salmonella enterica* serovar Typhimurium [NiFe]-hydrogenase-5 (Hyd-5) [1-8]. A challenge lies in understanding how such enzymes are assembled, and the ultimate aim of such work is to provide a blueprint of the essential genes required to produce a functional [NiFe]-hydrogenase in the presence of O_2.

The final structure of an O_2-tolerant MBH comprises a "large" subunit (≈60 kDa) containing the [NiFe]-active site, a "small" subunit (≈30 kDa) containing FeS clusters, and an integral membrane cytochrome *b* subunit (Figure 1). In general, assembly of active [NiFe]-hydrogenases is achieved *via* the products of two different operons [8, 9]. The proteins produced from specialised [Ni-Fe] cofactor assembly genes located in a *hyp* operon are essential for building the active site, including the synthesis of the unusual catalytic centre ligands CO and CN [10-12]. A dedicated *hyp* operon is found in every bacterium that produces [NiFe]-hydrogenases [10-12]. In addition, each individual [NiFe]-hydrogenase also requires further, often system-specific, assembly proteins that are often encoded by the same operon as the enzyme itself (Figure 1) [9]. Finally, the twin-arginine transport (Tat) system also plays a crucial role in transporting the correctly folded MBH from the cytoplasm to its final location at the periplasmic side of the cytoplasmic membrane [13].

The most significant progress in the field of MBH maturation and assembly has been provided by studies on *Ralstonia eutropha* (now re-named as *Cupriavidus necator* but herein referred to as

"*R. eutropha"* to clarify links to previous studies) [9, 14-16] and *Escherichia coli* [10-12, 17, 18]. However, it is challenging to examine the impact of O_2 on hydrogenase assembly using these bacterial systems. *R. eutropha* couples H_2 oxidation to O_2 reduction and must therefore be grown under at least microaerobic conditions, while *E. coli* only synthesizes MBH under anaerobic conditions. In light of this, insightful studies into hydrogenase maturation have been performed using *Rhizobium leguminosarum*, a N_2-fixing soil bacterium that can be grown under very low and high O_2 conditions and produces one [NiFe] MBH [19-21]. To complement this previous work, we have used *S. enterica* as a model system to study hydrogenase assembly. *S. enterica* produces three different MBH under different environmental conditions [22]: O_2-tolerant [NiFe]-hydrogenase-1 (Hyd-1) is anaerobically synthesized; O_2-sensitive [NiFe]-hydrogenase-2 (Hyd-2) is anaerobically synthesized; but O_2-tolerant Hyd-5 is normally aerobically synthesized [2, 22, 23]. In addition to the bio-technological importance of this work, there is also medical relevance to probing the assembly of *S. enterica* uptake hydrogenases since H_2 is an essential source of energy during the initial stages of a *Salmonella* bacterial infection [22, 24, 25], and identifying assembly mechanisms can therefore suggest targets for antimicrobial drug development.

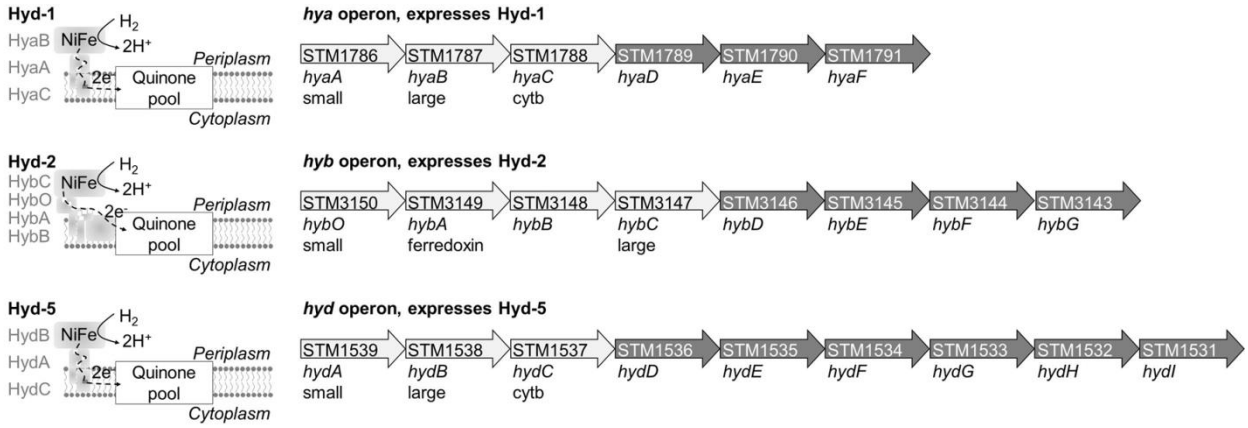

Figure 1. The operons encoding *S. enterica* respiratory [NiFe]-hydrogenases. The grey arrows correspond to genes that encode assembly proteins, while the white arrows represent structural proteins, as depicted to the left of the Figure.

A comparison of the operons encoding each *S. enterica* hydrogenase immediately emphasises the additional number of proteins required to assemble a [NiFe] MBH in air, relative to anaerobically produced enzymes (Figure 1). Six proteins (HydD-I) are synthesized from the *hyd* operon that do not end up in the final hydrogenase enzyme. We have chosen to study the roles of *S. enterica* HydF, HydG and HydH because, based on their sequence, these proteins are predicted to carry out a diverse range of functions, and therefore represent a good basis with which to test how similar the *S. enterica* Hyd-5 proteins are to analogues from *R. eutropha*, *E. coli* and *Rh. leguminosarum*.

S. enterica HydF and HydG are both predicted to play a role in the maturation of the FeS cluster-containing small subunit and belong to the Pfam family 'HyaE' (PF07449) and the 'HupH hydrogenase expression protein, C-terminal conserved region (HupH_C)' family (PF04809), respectively [26]. In particular, HydF is similar to *R. eutropha* HoxO, which has been shown to interact directly with the *R. eutropha* MBH small subunit [15] and is essential for correct assembly of the enzyme [27]. HydF is also similar to HupG from *Rh. Leguminosarum*, which has a role in enzyme assembly under aerobic conditions [19, 28]. HydG is particularly interesting as it probably represents an unusual structure compared to previously studied 'HupH_C' family proteins. The *S. enterica* protein appears to be a fusion of two *R. eutropha* accessory proteins - HoxQ and HoxR – both of which have been implicated in assembly of a correctly O_2-tolerant MBH [14, 15].

S. enterica HydH is predicted to be involved in assembling the [NiFe] cofactor necessary for large subunit maturation. Proteins with homology to *S. enterica* HydH (comparable to *R.*

eutropha HoxV and *Rh. leguminosarum* HupK) seem to be solely required for the assembly of aerobically synthesized hydrogenases and are thought to act as scaffolds for the initial assembly of the cofactor prior to insertion into the apoenzyme.

The proteins we have chosen not to study, *S. enterica* HydD, HydE and HydI are respectively identified as: a maturation protease found in the operons of all [NiFe] MBH and comparable to the structurally characterized protein *E. coli* HybD [29, 30]; a large subunit maturase similar to *E. coli* HypC, *R. eutropha* HoxL [31] and *Rh. leguminosarum* HupF [28], which have well characterised roles in enzyme assembly; and a HypA homolog [2], which is believed to be involved in the final step of incorporating nickel into the metallocentre [32].

We describe how a series of *S. enterica* strains, carrying separate deletions in each of the *hydF*, *hydG* and *hydH* genes, have been generated to produce an affinity-tagged version of Hyd-5 from its native chromosomal locus, but under control of the T5 promoter [23]. These engineered *S. enterica* strains enable the synthesis of enzyme from aerobically or anaerobically grown bacteria. The comparison of cellular and periplasmic H_2 oxidation activity from anaerobically grown cells was used to indicate how assembly is impacted by the different deletions, even in the absence of O_2. The reactivity of purified Hyd-5 enzyme was studied using protein film electrochemistry, a technique which provides direct control of the voltage used to push/pull electrons in/out of the enzyme and quantitative analysis of the sensitivity of the enzyme to inhibition by O_2 [33].

Materials and methods

Strain construction

S. enterica strains studied in this work are summarized in Table 1. To generate strain Δ*hydF*, a 500 bp DNA fragment upstream of the *hydF* gene was amplified using primer pair HydFupXba

(5’ GCGCTCTAGAGCAACTGATGCCTGCGGTCTATCTCG-3’) and HydFupBam (5’-GCGCGGATCCGATAGTCATTTTTTTAAATGCTCCGG-3’), and a 500 bp DNA fragment downstream of the *hydF* gene amplified using primer pair HydFdownBam (5’-GCGCGGATCCACGCAATAGAATGAAAGGTAACACACG-3’) and HydFdownHind (5’-GCGCAAGCTTGCGGCAATTCTGGCGTAATCGGCG). To generate Δ*hydG*, amplification of 500 bp DNA fragments up- and downstream of the *hydG* gene were amplified using primers pairs HydGupXba (5’- GCGCTCTAGACCTTAGCAGGCGCATGGCTGCTGAC-3’) and HydGupBam (5’- GCGCGGATCCATGATTCACGTGTGTTACCTTTCATTC-3’), and HydGdownBam (5’-GCGCGGATCCATCGATGAAGGTAATAGTTCGTGC-3’) and HydGdownHind (5’- GCGCAAGCTTCGATAGCCAGAGGCGAGACATCCAGG-3’) respectively. Upstream DNA fragments were digested with XbaI and BamHI and downstream fragments digested with BamHI and HindIII, and both ligated into the similarly digested pBluescript II KS^+. To produce strain Δ*hydH*, 500 bp upstream of the *hydH* gene was amplified using primer pair HydHupHind (5’-GCGCAAGCTTCGTAAAACAGCCGG-3’) and HydHupBam (5’-GCGCGGATCCCACGAACTATTACC-3’), digested with HindIII and BamHI, and ligated into similarly digested pBluescript II KS^+. The 500 bp downstream region was amplified using primer pair HydHdownBam (5’- GCGCGGATCCTGAAGGAGCTGGCC-3’) and HydHdownXba (5’- GCGCTCTAGACAGTTTAGGGATACC-3’), digested with BamHI and XbaI and ligated into the pBluescript II KS^+ construct digested with the same enzymes. All pBluescript KS II^+ inserts were digested with XbaI and HindII and ligated into similarly digested pMAK705 [34] to provide pMAK_Δ*hydG*, pMAK_Δ*hydF* and pMAK_Δ*hydH*. All gene deletions were generated on the LB03 chromosome by allelic exchange, following the pMAK homologous recombination method previously described [34] to

yield strains LB04 (Δ*hydG*), LB05 (Δ*hydF*) and LB06 (Δ*hydH*). Each mutant strain encodes a modified HydA protein that bears a hexa-histidine tag at its C-terminus and does not possess a transmembrane helix on the small subunit [23].

Table 1. Strains constructed in this work. The LB0… labels are included as in-house cataloguing references.

Strain	Relevant Genotype	Source
Parental (LB03)	*S. enterica* LT2a, P^{hydA}_{T5}, $hydA^{\Delta TM\text{-}His}$	[23]
Δ*hydF* (LB05)	as LB03, Δ*hydF*	this work
Δ*hydG* (LB04)	as LB03, Δ*hydG*	this work
Δ*hydH* (LB06)	as LB03, Δ*hydH*	this work
Δ*tat* (LB03T)	as LB03, Δ*tatABC*::$Apra^R$	this work

The genetically modified LB03 strain was also modified to generate a *tatABC* deletion mutant, Δ*tat* . In this case, the lambda red method was used to replace the *tatABC* genes with an apramycin resistance cassette on the *S. enterica* LB03 chromosome. Further confirmation of a non-functional Tat pathway was provided from tests that showed that the Δ*tat* strain was unable to grow on agar containing 2% (w/v) SDS [35] (not shown).

Whole cell H_2 oxidation assay

Each *S. enterica* strain was cultured anaerobically in low salt (5 g/L NaCl) Luria Broth (LB) and the Δ*tat* culture was also supplemented with 50 μg/mL apramycin. Cells were harvested, washed and 100 mg of cells re-suspended in 200 μL of 50 mM Tris.HCl. Hydrogen oxidising activity was measured in cuvettes containing H_2-saturated buffer and the artificial electron acceptor benzyl viologen [36]. The reaction was started by the addition of intact cells and H_2 dependent benzyl viologen reduction was measured at an absorbance of 600 nm.

Periplasmic fraction H_2 oxidation assay

Parental, Δ*hydG*, Δ*hydF* and Δ*hydH S. enterica* strains were cultured anaerobically in low salt LB. The cells were harvested, periplasms isolated by EDTA/lysozyme/sucrose treatment and then subjected to rocket immunoelectrophoresis. Periplasm samples were electrophoresed into agar containing Hyd-5 specific antibody and incubated under H_2 saturated conditions with BV and Tetrazolium Red at 37 °C, as described previously [23, 37].

Western immunoblotting analysis

S. enterica strains were grown anaerobically in low salt LB. The cells were harvested, washed, boiled in Laemmli sample buffer and the resultant proteins were separated by SDS-PAGE using a 10% or 14% (w/v) polyacrylamide gel. Proteins were transferred onto nitrocellulose membrane and challenged with either an antibody specific to the Hyd-5 large subunit for HydB identification (10% gel) used at a dilution of 1:500, or an antibody specific to the his-tag (Qiagen pentahis antibody) for HydA identification (14% gel) used at a dilution of 1:4000. An HRP-conjugated anti-rabbit IgG secondary antibody (Bio-Rad) was used at a dilution of 1:10,000.

Bacterial growth and protein purification

For aerobic growth, cells were cultured for 16 h in 500 mL of low salt LB in a baffled 2 L conical flask at 37 °C and with rotation at 180 rpm. For anaerobic growth, cells were cultured without shaking for 16 h at 37 °C in a Duran bottle filled to the top containing 6 L of a media mixture of low salt LB and 60 mL of 50% v/v glycerol (Sigma Aldrich). The cells were harvested by centrifugation at 6000 *g* for 30 min at 2 °C, and the cell pellets were stored at -80

°C. The Hyd-5 purification procedure was as previously published [23]. Total protein yields were estimated by multiplying the volume of protein solution collected at the end of purification by the protein concentration, as determined using a Bradford assay.

Methylene blue H_2 oxidation assay

All assays were performed in an anaerobic glove box (Faircrest, O_2 < 2 ppm) filled with nitrogen. An in-house built LED spectrophotometer (University of York) was placed on a magnetic stirrer plate. A 3 mL cuvette containing a small magnetic flea was filled with 2 mL of H_2 saturated 25 μM methylene blue in pH 6 'mixed buffer' solution (15 mM each of TAPS (N-tris(Hydroxymethyl)methyl-3-aminopropanesulfonic acid sodium-potassium salt) (Sigma Aldrich), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Sigma Aldrich), MES (2-(N-morpholino)ethanesulfonic acid) (Sigma Aldrich), and CHES (N-Cyclohexyl-2-aminoethanesulfonic acid) (AMRESCO) and 100 mM NaCl, pH adjusted using HCl or NaOH). A light blocking cap with a small injection hole was placed over the cuvette. Pre-H_2 saturated enzyme was then transferred into the cuvette using a syringe and the reduction of the methylene blue solution was monitored at 626 nm. Absorbances were converted to concentration using the pre-measured molar extinction coefficient of 28,000 M^{-1} cm^{-1}.

Protein film electrochemistry

All electrochemistry was performed in an anaerobic glove box (Faircrest, O_2 < 2 ppm) filled with nitrogen. A three electrode set-up was used in an all-glass cell (built in-house by University of York glassblower), and gas flow through the equipment was controlled using Sierra SmartTrack 50 mass flow controllers [38]. A graphite working electrode 'tip' (constructed by Mechanical

Workshop in Department of Chemistry at University of York) was attached to an OrigaTrod Rotator. The graphite electrode tip was abraded using sandpaper of grade P1200 and 3 μL of enzyme was pipetted directly on to the graphite surface. The rotator and tip were then slotted into the glass electrochemical cell and electrode rotation was controlled using the OrigaBox software. Along with the graphite working electrode, the counter electrode (platinum wire) was also placed within the main electrochemical cell compartment containing 2 mL of mixed-buffer. This compartment was surrounded by a water jacket which was fed by a thermostated water bath set to 37 °C. The main body of the electrochemical cell was connected to the saturated calomel electrode reference electrode side arm via a Luggin capillary. The reference electrode side arm was filled with 100 mM NaCl solution and kept at ambient temperature [38]. Electrochemical measurements were performed using a Compactstat potentiostat (Ivium Technologies) and data was recorded using the Iviumsoft software. All potentials quoted have been converted to the Standard Hydrogen Electrode using the conversion factor E (V *vs* SHE) = E (V *vs* SCE) + 0.241 V [39].

Results

A minor role for HydF in enzymatic activity of Hyd-5

First, the parental strain (LB03) and the Δ*hydF* strain (LB05) were cultured anaerobically. As shown in Figure 2A, Δ*hydF* cells were found to have the same level of H_2 oxidising activity (using BV as electron acceptor) as the parental strain. Next, the periplasmic proteins were isolated and rocket immunoelectrophoresis used to compare the relative amounts of enzyme present in the two strains (Figure 2B). In rocket immunoelectrophoresis, the size of the precipitin arc is used as an estimate of the amount of immunoreactive material present, while the activity

stain can only give an indication if the enzyme is active (the stain is non-quantitative). Analysis of the periplasmic fractions showed unchanged levels of Hyd-5 between the parental and Δ*hydF* strains, confirming that the deletion had not impacted on the synthesis, assembly or transport of the enzyme (Figure 2B). Western immunoblotting confirmed that Hyd-5 was behaving similarly in the parental and Δ*hydF* strain (Figure 3).

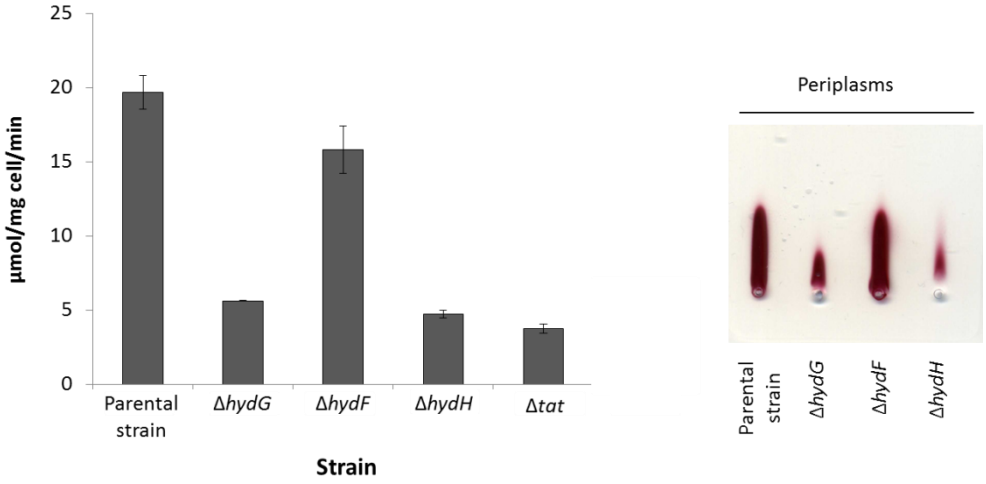

Figure 2. Hydrogen oxidising activities of (A) anaerobically grown whole cells and (B) periplasmic fractions of anaerobically grown parental *S. enterica* and *hyd* deletion strains. **(A)** Hydrogen dependent benzyl viologen reduction activity of whole cells at room temperature. **(B)** Periplasm samples were electrophoresed into agar containing Hyd-5 specific antibody and then incubated under H_2 saturated conditions with benzyl viologen and tetrazolium red at 37°C.

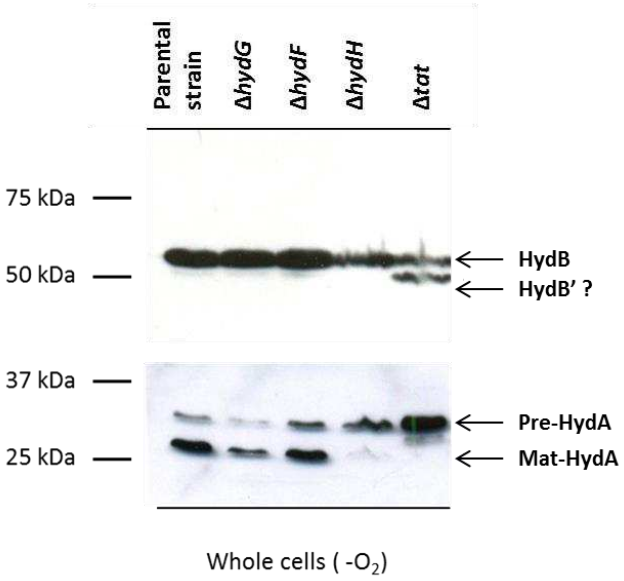

Figure 3. Western analysis of the large and small subunits of Hyd-5 from parental *S. enterica* and different deletion strains grown under anaerobic conditions. A faster migrating band for the large subunit in the Δ*tat* strain is labelled "HydB' ?" since it is close in apparent motility to a proteolysed fragment of HydB observed after purification [2].

Following protein purification, the final amount of Hyd-5 isolated from the Δ*hydF* strain was comparable to that from the parental strain after either aerobic or anaerobic growth (approx. 3 mg protein from 26 g wet cell weight, or approx. 2 mg protein from 8 g wet cell weight, respectively). The SDS-PAGE gels shown in Figure S1 also confirm that the composition of the purified Hyd-5 was unaffected by the deletion of *hydF*.

The activity of the purified enzyme was first assessed using methylene blue (MB) H_2 oxidation assays. As summarised in Table 2, the MB dye assay turnover rates (k_{cat}) are the same order of magnitude for Hyd-5 regardless of whether it was assembled by a *S. enterica* strain which possessed or lacked the *hydF* gene. It is notable, however, that the enzyme produced under aerobic conditions in the absence of HydF exhibited less than half the activity of the native enzyme (Table 2). This effect was reversed when the enzymes were prepared from anaerobically-grown cells (Table 2). This observation is returned to later.

Table 2. Methylene blue H_2 oxidation assay data for Hyd-5 purified from the parental strain (LB03) and the Δ*hydF* mutant (LB05) grown under either aerobic or anaerobic conditions. The turnover number is the average calculated from three repeat experiments and the confidence limits reflect the standard error.

	H_2 oxidation rate (s^{-1})	
	Aerobic growth	Anaerobic growth
parental	7.2 ± 0.05	3.3 ± 0.2
Δ*hydF*	4.0 ± 0.6	6.7 ± 0.1

In order to make a more detailed assessment of the Hyd-5 activity from the different preparations, protein film electrochemistry was employed. When Hyd-5 was adsorbed onto a pyrolytic graphite electrode the electrocatalytic profile of hydrogenase reactivity was the same for enzyme from either a parental (LB03) or Δ*hydF* strain, and this was independent of whether cells were grown under aerobic or anaerobic conditions. The data is shown in Figure 4, which summarises the results of cyclic voltammetry experiments where the voltage of the graphite electrode is increased from -0.55 V to +0.24 V and then 'cycled' back to -0.55 V at 20 mVs^{-1}. Hydrogen oxidation activity is measured as a positive electrical current and, as has been previously observed for *S. enterica* Hyd-5, the enzyme is entirely biased towards H_2 uptake under the conditions of pH 6.0 and 3% H_2, with negligible net reduction (negative) current visible in the black scans of Figure 4. The only effect of O_2 on all the enzyme samples is to inhibit H_2 oxidation at potentials more positive than -0.1 V *vs* SHE. This inhibition corresponds to a drop in the electrical current and this occurs because the enzyme forms an inactive "Ni-B" state in which the active site is oxidised from Ni(II) to Ni(III) [2, 23].

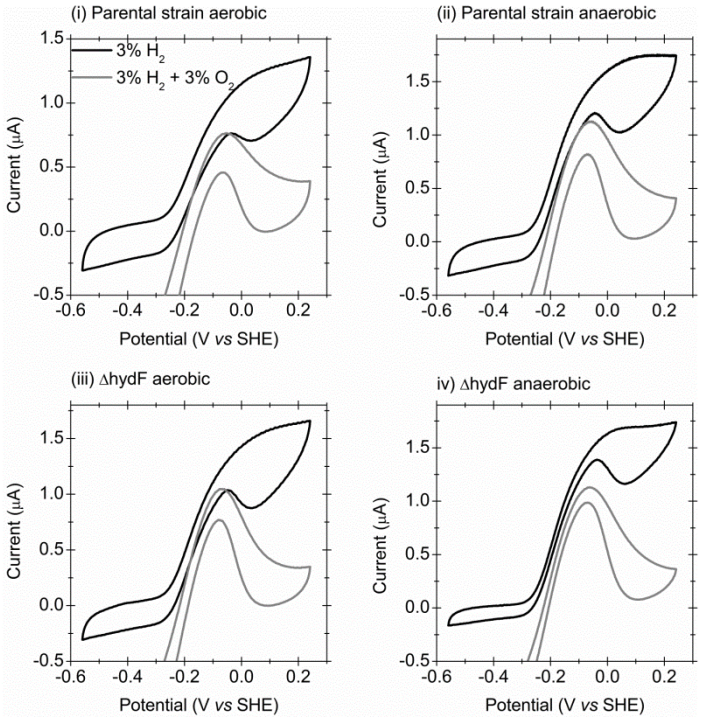

Figure 4. Cyclic voltammetry experiments (20 mV s^{-1}) of Hyd-5 purified from a (top) parental or (bottom) Δ*hydF* strain in the presence of 3% H_2 (black line) and a 3% H_2 and 3% O_2 gas mixture (gray line) either (left) aerobic or (right) anaerobic cell culturing. Other experimental conditions: gas flowing through the glassware at a total rate of 100 scc min^{-1} with N_2 as carrier gas, pH 6.0, 37 °C, electrode rotation rate 2000 rpm.

The formation of the Ni-B state under O_2–free conditions was explored in the slow (0.2 mV s^{-1}) cyclic voltammogram experiments shown in Figure S2. The inset data, showing the first derivative of the current-potential data collected during the reductive (decreasing potential) sweep confirms that the potential of most rapid reactivation, often characterized as "E_{switch}", is effectively unchanged in all cases, indicating that the reactivity of the active site is not impacted in a *hydF* free strain.

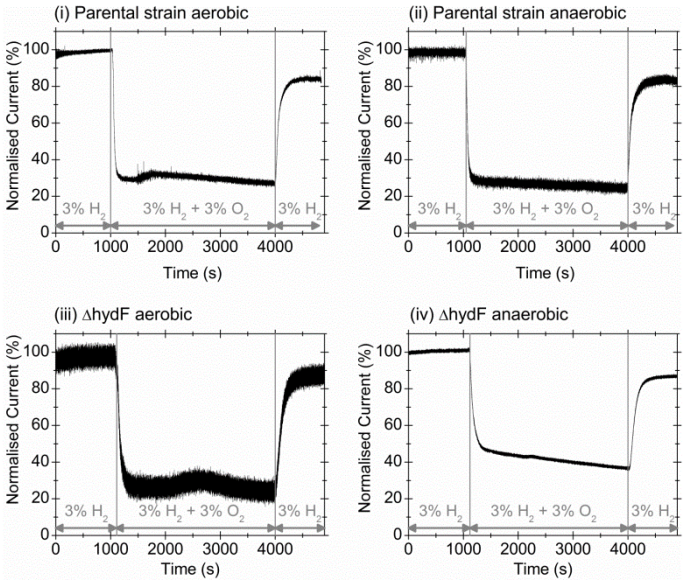

Figure 5. Poised potential experiments at +21 mV *vs* SHE to assess the O_2 tolerance of Hyd-5 isolated from parental (top) and Δ*hydF* (bottom) *S. enterica* strains grown under either aerobic (left) or anaerobic (right) conditions. The different gas phases of the experiment are denoted by the double-headed arrows. The current has been normalised relative to the initial enzyme activity to facilitate comparison between the experiments. Other experimental conditions: gas flowing through the glassware at a total rate of 100 scc min^{-1} with N_2 as carrier gas, pH 6.0, 37 °C, electrode rotation rate 2500 rpm

To quantify the O_2 inhibition, the experiments shown in Figure 5 were conducted. Deleting the *hydF* gene and preparing enzyme from that genetic background was not seen to have any significant impact on the O_2 tolerance of the Hyd-5 enzyme. This is a particularly informative experiment in terms of enzyme maturation because O_2 tolerance arises from the correct assembly of the iron-sulfur relay in the small subunit. Together the electrochemical experiments therefore confirm that fully competent Hyd-5 is extracted from the Δ*hydF* strain, regardless of O_2-level in the growth conditions.

Returning to the MB H_2 oxidation assay data in Table 2, we can rule out the possibility that increased O_2 sensitivity gives rise to decreased Hyd-5 activity from aerobically cultured Δ*hydF* relative to anaerobically grown cells. We therefore cannot infer anything significant from the differences in turnover frequencies measured in the dye assay.

HydG is essential for aerobic and anaerobic Hyd-5 assembly

Deletion of the gene *hydG* had a negative effect on anaerobic whole cell and periplasmic H_2 oxidation (Figure 2). Comparing with the Δ*tat* mutant as a control, which would fail to transport Hyd-5 to the periplasm and so any activity represents only basal activities [8], the periplasmic levels of Hyd-5 are reduced in a Δ*hydG* mutant (Figure 2). This correlates with protein purification experiments, where the yield of purified Hyd-5 from anaerobic Δ*hydG* cells was approximately half that from parental LB03 cultures. The isolated enzyme was completely inactive in the methylene blue (MB) H_2 oxidation assay, regardless of whether it was purified from anaerobically or aerobically grown cells. Similarly, electrochemical measurements conducted on the isolated enzyme also showed very low levels of catalytic current. SDS-PAGE gels of Hyd-5 purified from Δ*hydG* cells grown in high O_2 conditions clearly show misassembled enzyme (Figure S1), where only the precursor of the small subunit is isolated clearly in an immature form that is unable to interact correctly with the large subunit (Figure S1). It is harder to understand the inactivity of enzyme from anaerobically grown Δ*hydG* since Western immunoblots showed the Hyd-5 profile in the Δ*hydG* background to be similar to the parental strain where the small subunit appears processed relative to the Tat mutant control (Figure 3) and SDS-PAGE analysis of small amount purifiable enzyme available from anaerobic cells also shows the isolated Hyd-5 enzyme to contain the same protein components as enzyme from the parental strain.

HydH is essential for aerobic and anaerobic Hyd-5 assembly

In-frame deletion of *hydH* greatly impacts whole cell H_2 uptake in anaerobically cultured *S. enterica*, causing a drop in hydrogenase-catalysed H_2-oxidation and concomitant benzyl viologen reduction (Figure 2). The significant drop in H_2 oxidising activity of the Δ*hydH* whole cells is accompanied by reduced levels of active Hyd-5 in the periplasms of cells grown under O_2-limited conditions (Figure 2).

Western immunoblotting (Figure 3) shows that the predominant form of the Hyd-5 small subunit in Δ*hydH* is the higher weight precursor form of HydA (Pre-HydA), not the fully processed 'mature' HydA (Mat-HydA). Similarly, only the precursor form of HydA was observed from the Δ*tat* mutant (Figure 3). Together these results suggest that in the absence of HydH, the Hyd-5 enzyme is not correctly assembled.

Protein purification and SDS-PAGE analysis of Hyd-5 isolated from Δ*hydH* grown under aerobic and anaerobic conditions provides evidence to further support the hypothesis that HydH plays an essential role in hydrogenase maturation. Regardless of the O_2 level in the growth media, following Ni immobilized metal affinity chromatography Hyd-5 protein yields were very low from the Δ*hydH* strain, decreased by approx. one third and approx. half compared to the parental strain under anaerobic and aerobic growth conditions, respectively. After anaerobic culturing the Hyd-5 isolated from the Δ*hydH* background displayed fragmented large and small subunits in the SDS-PAGE gel (Figure S1). In the HydA region, a very faint band corresponding to the molecular weight of pre-HydA$^{\Delta TMHIS}$ is visible as well as a possible degradation product and a protein band corresponding to the molecular weight of mature HydA$^{\Delta TMHIS}$. As with the Western blot analysis, this is again comparable with results for the Δ*tat* strain, which also produces Hyd-5 enzyme exhibiting various forms of the small subunit. The predominance of

incorrectly processed small subunit over all other Hyd-5 proteins is even greater in the SDS-PAGE analysis of Hyd-5 enzyme isolated from aerobically grown Δ*hydH* (Figure S1).
As would be expected for incompletely processed Hyd-5, in H_2 oxidation dye assays of enzyme purified from Δ*hydH*, tens of minutes elapsed before MB reduction was clearly detected. Because diffusion of H_2 from the cuvette would be significant over these time periods, a quantitative measure of the turnover rate could therefore not be made. Electrochemical measurements of enzymatic reactivity were also inconclusive because the catalytic currents were so low, again implying inactive enzyme.

Discussion

On the role of HydF

In studies of the *E. coli* protein for which the Pfam HyaE group is named [13, 40, 41], and the homologous assembly proteins from *R. eutropha*, HoxO [15, 27], and *Rh. leguminosarum*, HupG [20], it has been determined that the function of these proteins is to assist in the maturation of the FeS-containing protein subunit of the final hydrogenase enzyme complex. The specific role is thought to involve binding of the HyaE-homologue to the Tat signal peptide of the hydrogenase small subunit that is synthesized from the same operon [13, 15], perhaps explaining the presence of highly conserved negatively charged residues (Figure S3) [41]. A *Rh. leguminosarum* Δ*hupG* study showed that the higher the oxygen concentration the more important the function of HupG in hydrogenase maturation [20]. Thus, it is possible to rationalise why the deletion of *hyaE* had an insignificant effect on the assembly of the anaerobically synthesized enzyme *E. coli* Hyd-1 [40], but deletion of *hoxO* had a completely deleterious effect on the maturation of the aerobically synthesized enzyme *R. eutropha* MBH [15, 27]. Our results are therefore surprising,

since we show that HydF is redundant in the biosynthesis of *S. enterica* Hyd-5, regardless of O_2 level. We prove that the resultant enzyme is fully active, and that maturation of the proximal [4Fe3S] cluster, which is unique to O_2-tolerant [NiFe]-MBH, has not been negatively impacted by deletion of *hydF*. By conducting protein production experiments under aerobic conditions we know that the hydrogenase-5 assembly cannot simply co-opt the hydrogenase-1 or hydrogenase-2 biosynthetic machinery in order to compensate for the lack of HydF. Further studies are required to understand the interaction of HydF with the Tat signalling peptide in *Salmonella* but it is hopeful to note that not all of the genes present on the *hyd* operon are necessary for synthesis of competent Hyd-5 enzyme.

On the role of HydG

The sequence of HydG resembles that of the C-terminus of *E. coli* HyaF, *R. eutropha* HoxQ and the group's namesake, *Rh. leguminosarum* HupH (Figure S4). However, unlike these previously studied proteins, HydG is part of the subgroup of HupH_C architectures which also contain a C-terminal rubredoxin domain (Figure S4). Overall, *S. enterica* HydG can therefore be thought of as analogous to a fusion of both *R. eutropha* HoxQ and the *R. eutropha* rubredoxin hydrogenase assembly protein HoxR (Figure S5). Based on studies on *R. eutropha* HoxQ [15], HoxR [14] and *Rh. leguminosarum* HupH [20] we would expect HydG to interact with the small subunit of Hyd-5, performing an important role in FeS cluster insertion into HydA. This is consistent with our results, since we show that HydG is essential for the production of active Hyd-5 under both low and high O_2 conditions. Further study of this protein is suggested by our work since the precise role of HydG is obviously more complex given that aerobic growth of the relevant deletion strain yields an incomplete, large subunit-free, hydrogenase product, but processing of both the large

and small subunits occurs anaerobically despite low enzyme activity resulting. It is also highly likely that anaerobic expression induces the *hyaABCDEF* operon, and that HyaF can partially compensate for the loss of HydG.

On the role of HydH

HydH is critical to the assembly, and therefore H_2-oxidising activity, of *S. enterica* Hyd-5 under both aerobic and anaerobic growth regimes. The gene product of *hydH* is predicted to be a homolog of *R. eutropha* HoxV and *Rh. leguminosarum* HupK (see Figure S6 for alignment). *R. eutropha* HoxV is also an essential hydrogenase assembly protein, with a specific role in assembling a $Fe(CN)_2CO$ active site precursor that is mediated by Cys-52 and Cys-366, residues, which are conserved in *S. enterica* HydH (Figure S6) [31]. Similarly, HupK from *Rh. leguminosarum* is essential for hydrogenase activity, playing a role in active site assembly [19]. This correlates with the SDS-PAGE results described here (Figure S1), which show two weak bands at different weights for the active site protein from Hyd-5 purified from anaerobically grown *S. enterica* Δ*hydH*, and a complete loss of all large subunit from aerobically grown *S. enterica* Δ*hydH*.

Conclusion

In this study the *S. enterica hydF* gene has been identified as essentially redundant for Hyd-5 assembly under the experimental conditions tested. This is a helpful result because it shows that heterologous production of an O_2-tolerant [NiFe]-hydrogenase under aerobic conditions may not require transfer of all the genetic information contained within a particular hydrogenase operon, simplifying the possible design of a synthetic biology solar-H_2 microbe. Conversely, the data

surrounding *S. enterica* HydG, a natural fusion carrying a C-terminal HoxR domain, suggest this protein, along with the HoxV homologue HydH, would be critical to include when designing systems requiring [NiFe]-hydrogenase activity, and inhibition of such assembly proteins would offer a route to disabling Hyd-5 assembly in pathogenic strains of *Salmonella*.

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