FEBS Letters 586 (2012) 536–544

FEBS Letters

journal homepage: www.FEBSLetters.org

Review

# How *Salmonella* oxidises $H_2$ under aerobic conditions

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ARTICLE INFO

*Article history:*
Received 1 July 2011
Revised 22 July 2011
Accepted 28 July 2011
Available online 5 August 2011

Edited by Miguel Teixeira and Ricardo O. Louro

*Keywords:*
*Salmonella*
[NiFe] hydrogenase
Genetic engineering
Protein film electrochemistry
Electron paramagnetic resonance
Oxygen-tolerance

ABSTRACT

***Salmonella enterica* serovar Typhimurium is a Gram negative bacterial pathogen and a common cause of food-borne illness. Molecular hydrogen has been shown to be a key respiratory electron donor during infection and $H_2$ oxidation can be catalysed by three genetically-distinct [NiFe] hydrogenases. Of these, hydrogenases-1 (Hyd-1) and Hyd-2 have well-characterised homologues in *Escherichia coli*. The third, designated Hyd-5 here, is peculiar to *Salmonella* and is expressed under aerobic conditions. In this work, *Salmonella* was genetically modified to enable the isolation and characterisation of Hyd-5. Electrochemical analysis established that Hyd-5 is a $H_2$-oxidising enzyme that functions in very low levels of $H_2$ and sustains this activity in high levels of $O_2$. In addition, electron paramagnetic resonance spectroscopy of the Hyd-5 isoenzyme reveals a complex paramagnetic FeS signal at high potentials which is comparable to that observed for other $O_2$-tolerant respiratory [NiFe] hydrogenases. Taken altogether, Hyd-5 can be classified as an $O_2$-tolerant hydrogenase that confers upon *Salmonella* the ability to use $H_2$ as an electron donor in aerobic respiration.**


## 1. Introduction

*Salmonella enterica* serovar Typhimurium is an enteric, Gram negative bacterium that belongs to a genus that causes disease worldwide. Every year approximately 40 000 cases of salmonellosis are reported in the United States alone [1], while in sub-Saharan Africa infection by a multidrug resistant strain of *Salmonella* is often fatal for immuno-compromised individuals [2]. *Salmonella* is a facultative anaerobe that can grow in a broad range of environmental conditions. The organism is also an intracellular pathogen that must survive an oxidative burst during infection, and negotiate anaerobic and aerobic environments. Molecular hydrogen is produced by the gut flora in animals and can reach concentrations of over 40 μM in some tissues, however only about 14% of this is exhaled [3] with most being oxidised as a microbial energy source. *Salmonella* uses $H_2$ as a respiratory electron donor under both anaerobic and aerobic conditions and genetic inactivation of the enzymes involved results in a loss of virulence [4].

Hydrogenases catalyse the reversible oxidation of $H_2$ and different families are classified based on the cofactor type located at the active site: thus [NiFe], [FeFe], and [Fe] hydrogenases have been identified. The *S. enterica* serovar Typhimurium LT2 (hereafter '*Salmonella*') genome encodes four [NiFe] hydrogenases termed hydrogenase-1 (Hyd-1), -2, -3, and -5. Of these, Hyd-3 forms part of the cytoplasmically-oriented formate hydrogenlyase complex that is responsible for $H_2$ evolution under fermentative conditions [5]. In *Escherichia coli*, which is closely related to *Salmonella*, a second formate hydrogenlyase isoenzyme has been identified and the associated hydrogenase termed Hyd-4 [6]; however *Salmonella* LT2 lacks the genes that would code for a homologous Hyd-4 isoenzyme. Anaerobic respiratory $H_2$ oxidation, or 'uptake', is catalysed by Hyd-1 and Hyd-2 [5], and homologues are present in *E. coli* [7]. The active sites of such respiratory hydrogenases are located at the periplasmic side of the membrane and $H_2$ oxidation is linked to quinone reduction in the membrane.

The *Salmonella* LT2 genome encodes a third respiratory [NiFe] hydrogenase that has no direct homologue in *E. coli*, and so has been termed Hyd-5 here. The Hyd-5 isoenzyme is encoded by the *hydABCDEFGHI* operon (Fig. 1A) where *hydA* encodes the β-subunit and *hydB* encodes the α-subunit of the enzyme. Together, the αβ-dimer forms the minimal core unit of the hydrogenase. The α-subunit harbours the Ni–Fe–CO–2CN cofactor, which is responsible for $H_2$ activation, and the β-subunit contains three iron-sulphur clusters: a proximal [4Fe–4S] cluster closest to the active site, a medial [3Fe–4S] cluster, and a distal [4Fe–4S] cluster [8]. The β-subunit is synthesised as a precursor with an N-terminal twin-arginine signal peptide, which directs the active HydAB

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0014-5793 
doi:10.1016/j.febslet.2011.07.044

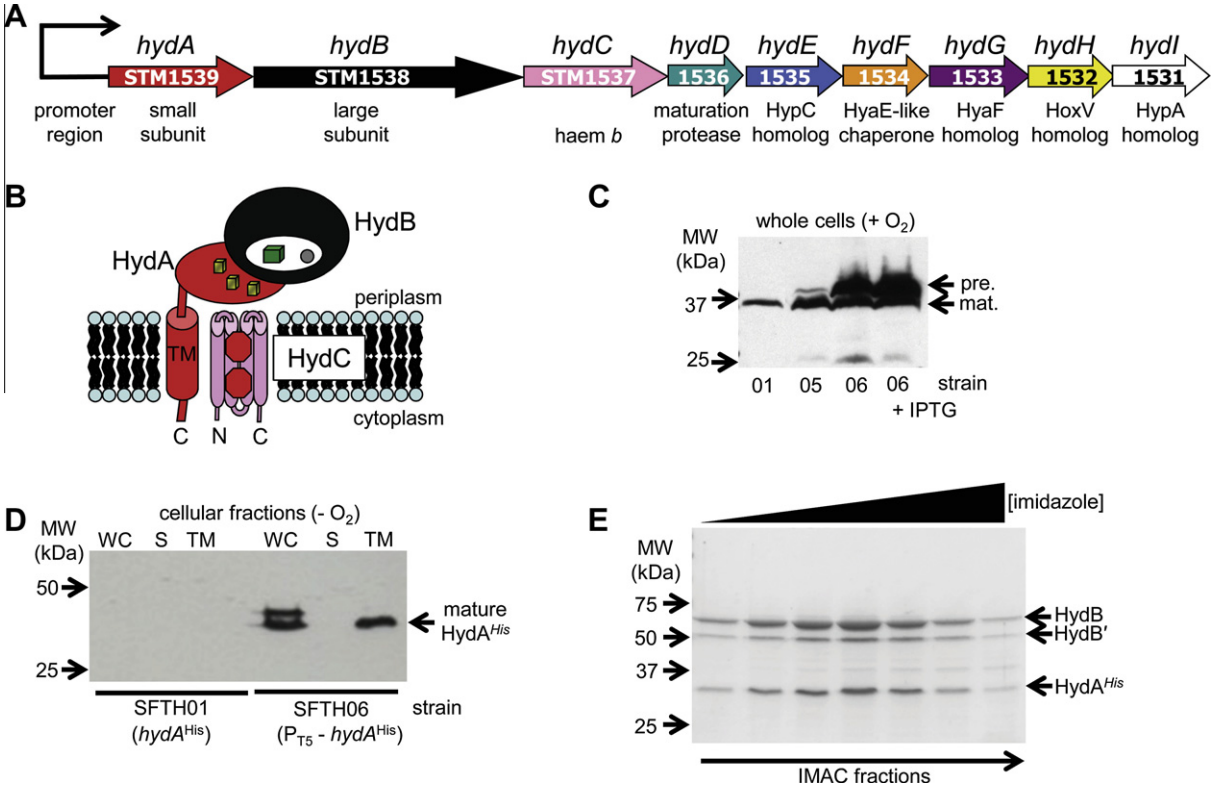


**Fig. 1.** The *Salmonella hyd* operon and isolation of the Hyd-5 enzyme. (A) The *hyd* operon encoding Hyd-5. The promoter region is denoted by the *arrow* and the predicted functions of each gene product are given. (B) A cartoon depicting the predicted structure and topology of Hyd-5 in the *Salmonella* plasma membrane with the enzyme subunits colour-coded as in (A). The C-terminal transmembrane domain ('TM') on HydA is shown and the bound cofactors are indicated by the following symbols: *green cube* = [NiFe] cofactor; *grey circle* = Mg; *yellow cube* = [FeS] cluster; *red octagon* = *b*-type haem. (C) Western immunoblot of whole cells grown aerobically in rich media with 0.5% (v/v) glycerol. Strains SFTH01 ($hydA^{His}$, '01'), SFTH05 ($P_{tat}$-$hydA^{His}$, '05'), and SFTH06 ($P_{T5}$-$hydA^{His}$, '06') were grown to mid-log phase before harvesting. Strain SFTH06 was also grown in presence of 2 mM IPTG ('+ IPTG'). Whole cell samples were separated by SDS-PAGE, blotted and challenged with an anti-pentaHis monoclonal antibody (Qiagen). (D) Strains SFTH01 ($hydA^{His}$) and SFTH06 ($P_{T5}$-$hydA^{His}$) were grown anaerobically in rich media before whole cells were harvested ('WC'), broken by sonication, and soluble ('S') and total membrane proteins ('TM') separated by SDS-PAGE, blotted, and challenged with the anti-His-tag antibody. (E) Protein fractions from the IMAC elution profile separated by SDS-PAGE. The α-subunit (HydB), engineered β-subunit ($HydA^{His}$), and a proteolysed fragment of the α-subunit (HydB'), are indicated.

heterodimer to the periplasm [9]. The roles of the remaining gene products remain to be experimentally tested, though sequence analysis suggests roles in enzyme biosynthesis (Fig. 1A).

The *hyd* operon is maximally expressed under aerobic conditions, suggesting that Hyd-5 may be active under an oxic environment [10]. In vivo studies of *Salmonella* infections in a mouse model showed the *hyd* operon was initially expressed in the liver and spleen, then later in the infection in the ileum [11]. Using microelectrodes, molecular $H_2$ levels of 50 μM (0.06 atm) [12,13] and average molecular oxygen levels of 35 μM (0.03 atm) have been measured in mouse spleen tissue [14]. As a result, it has been postulated that Hyd-5 may be responsible for coupling $H_2$ oxidation to $O_2$ reduction in a respiratory electron transport chain [10]. This is an interesting possibility since most [NiFe] hydrogenases are expressed under anaerobic conditions and are rapidly inactivated by $O_2$. We classify non-standard hydrogenases that can catalyse $H_2$ oxidation in the presence of $O_2$ as "$O_2$-tolerant". The best-characterised $O_2$-tolerant hydrogenases are from the Knallgas bacterium *Ralstonia eutropha* [15,16], the extremophile *Aquifex aeolicus* [17–19] and the facultative anaerobic bacterium *E. coli* [7].

In this paper the isolation and characterisation of *Salmonella* Hyd-5 is reported. Genetic approaches are taken to deliberately up-regulate expression of the *hyd* operon, which allowed an affinity-tagged version of Hyd-5 to be isolated. The purified enzyme comprises a heterodimer of the HydA and HydB subunits. No other co-purifying proteins were observed. Protein film electrochemistry (PFE) demonstrates that isolated Hyd-5 retains $H_2$ oxidation activity, functions in low levels of $H_2$ and sustains that activity in high levels of $O_2$. Electron paramagnetic resonance (EPR) spectroscopy reveals, at high potential, a complex signal in the FeS cluster region that integrates to close to two spins/molecule and is comparable to that observed in other $O_2$-tolerant hydrogenases [7,16,17,19,20]. It is concluded that Hyd-5 is an $O_2$-tolerant hydrogenase and that this property is exploited by *Salmonella* to allow respiratory $H_2$ uptake under aerobic conditions.

## 2. Materials and methods

### 2.1. Bacterial strains

The parental strain used in this study was *S. enterica* serovar Typhimurium LT2. The SFTH01 ($hydA^{His}$) strain was constructed as follows: DNA covering part of the *hydA* gene up to the final codon (Lys-367) was amplified with oligonucleotide primers SalHydA-H1 (5′-GCGCGGTACCGCGCATCATGGGGCTGCGTGCAGGC-3′) and SalHydA-H2 (5′-GCGCAGATCTTTTTTTCTCTTCCGGAGCG TTTTC-3′), digested with *Kpn*I and *Bgl*II and cloned into pFAT210 [21]. Next, DNA covering the initiation codon of *hydB*, including the upstream sequence containing a putative ribosome binding site, was amplified using SalHydA-H3 (5′-GCGCAAGCTTGCTCCGGAAGAGAAAAA ATAATTAT-3′) and SalHydA-H4 (5′-GCGCTCTAGATTCAAGATAGTGG CGACCGCCAGC-3′), and cloned into the new construct as a

*Hind*III-*Xba*I fragment. Finally, the entire *hydA*$^{His}$ allele was excised from pFAT210 by digestion with *Xba*I and *Kpn*I and cloned into similarly digested pMAK705 [22]. This vector was used to move the *hydA*$^{His}$ allele onto the *Salmonella* chromosome by homologous recombination as described [22] to yield strain SFTH01.

The SFTH01 strain was further modified by the addition of non-native transcriptional promoter regions upstream of the *hydA*$^{His}$ gene at the native chromosomal locus. DNA upstream of the native *hydA* promoter region was amplified using oligonucleotides HydX1 and HydX2 (5′-GCGCAAGCTTCTTATCCCCTGGCTGCCATCCGGCG-3′ and 5′-GCGCGAATTCCCCAAACCACTATAGTTAGTGTGGGG-3′), digested with *Hind*III and *Eco*RI and cloned into similarly digested pBluescript. DNA downstream of the *hydA* promoter, covering the initiation codon on *hydA*, was cloned into this construct following amplification using primers HydX5 and HydX6 (5′-GCGCGGAT CCATGCAAACACAAGATACATTTTATC-3′ and 5′-GCGCT CTAGATT CAGGTATAGGCGGGCAGCCTGGC-3′) and digestion with *Bam*HI and *Xba*I. The resultant construct was termed pHydApromswap and is designed to allow the cloning of additional promoter sequences as engineered *Eco*RI-*Bam*HI fragments upstream of *hydA*. Two non-native promoter sequences were chosen. Firstly, the *tatABCD* operon promoter region from *E. coli* K-12, a constitutive promoter [23], was excised from pUNIPROM [24] as an *Eco*RI-*Bam*HI fragment was cloned into pHydApromswap. Secondly, the strong, normally LacI-repressible T5 promoter/lac operator element from the pQE series of overexpression plasmids (Qiagen Inc.) was chosen. In this case it was necessary to remove an *Eco*RI restriction site present in this promoter region. Thus the promoter was amplified with primers T5 forward (5′-GCGCGAATTCAAATCA-TAAAAAATTTATTTGC-3′) and T5 reverse (a very long primer incorporating a base change to remove the *Eco*RI site present in the promoter: 5′-GGCCGGATCCTTTCTCCTCTTTAATGTATTC TGTGTG AAATTGTTATCC-3′) and cloned as an *Eco*RI-*Bam*HI fragment into pHydApromswap. Next, the $P_{tat}$-*hydA* and $P_{T5}$-*hydA* promoter/lac operator constructs were moved separately into pMAK705 as *Xba*I-*Hind*III fragments, and then subsequently onto the chromosome of SFTH01 to give strains SFTH05 ($P_{tat}$-*hydA*$^{His}$) and SFTH06 ($P_{T5}$-*hydA*$^{His}$).

### 2.2. Bacterial growth and protein purification

For small-scale expression tests, bacteria were cultured aerobically overnight in 25 ml volumes at 37 °C in 'low-salt' LB medium (NaCl 5 g/l) supplemented with 0.5% (v/v) glycerol. Small-scale fractionations were performed on cultures grown anaerobically overnight at 37 °C in media not further supplemented. Cells were pelleted by centrifugation, washed twice in 50 mM Tris.HCl pH 7.5, before being suspended (0.1 g cells (wet weight) per ml) in the same buffer. Cells were broken by sonication and separated into soluble protein and total membrane fractions by ultracentrifugation.

For purification of the Hyd-5 enzyme, two methods were devised. First, SFTH06 was cultured in a 5 l Duran bottle in low-salt LB medium anaerobically at 37 °C for 16–17 h. Cells were harvested by centrifugation at 5000×*g* for 40 min at 4 °C and typically yielded a cell pellet of approximately 9 g (wet weight). Following washing in 50 mM Tris HCl pH 7.5, the cell pellet was taken up in 50 ml B-PER solution (Thermo scientific) supplemented with DNase I, lysozyme, a protease inhibitor cocktail (Calbiochem) and 75 mM imidazole. The suspension was agitated for 30 min at ambient temperature before debris was removed by centrifugation at 18 700×*g* for 12 min. The supernatant was subjected to immobilised metal affinity chromatography (IMAC) by loading directly onto a 5 mL HisTrap HP column (GE Healthcare) previously equilibrated with 50 mM Tris.HCl, pH 7.5, 150 mM NaCl, 75 mM imidazole, 0.1% (v/v) Triton X-100. To elute bound protein a linear gradient of 75–500 mM imidazole, in the same buffer, was applied. Fractions containing the Hyd-5 protein were identified by SDS-PAGE and InstantBlue (Expedeon protein solutions) staining. Hyd-5 fractions were pooled and concentrated by centrifugation using a 10 kDa molecular weight cut-off Vivaspin 20 device (Sartorius stedim).

For the second purification method, cell pellets were resuspended in 100 mM Tris–HCl, pH 7.5, supplemented with DNase I, lysozyme and Complete™ EDTA-free protease inhibitor (Roche Molecular Biochemicals). Cells were disrupted by three passages through a French pressure cell at 19 000 psi, unlysed cells were removed by centrifugation at 4000×*g* for 60 min at 4 °C and membranes were isolated from the supernatant by ultra centrifugation at 150 000×*g* for 1.5 h at 4 °C. The membrane pellet was resuspended in 100 mM Tris–HCl, pH 7.5, to a final protein concentration of 10 mg/ml and dispersed by adding Triton X-100 to a final concentration of 3% w/v and gently agitating at 4 °C for 45 min. Insoluble material was removed by ultracentrifugation at 150 000×*g* for 1.5 h at 4 °C before 1 mM dithiothreitol and 40 mM imidazole was added to the cleared supernatant and applied to a 5-ml HisTrap Chelating HP column (GE Healthcare) equilibrated with 20 mM Tris, pH 7.2, 50 mM NaCl, 60 mM imidazole, 1 mM dithiothreitol, 0.02% (w/v) Triton X-100. Bound proteins were eluted by application of a 55 ml linear gradient of 60–1500 mM imidazole in the same buffer. Fractions containing purified Hyd-5 were identified by absorbance at 420 and 280 nm and these were pooled and concentrated to 1 ml by ultrafiltration, washed in 20 ml of column buffer containing no imidazole, re-concentrated and snap-frozen in liquid nitrogen.

### 2.3. Protein film electrochemistry

All electrochemical experiments were performed in a $N_2$-filled anaerobic glove-box (M. Braun ⩽2 ppm $O_2$). A pyrolytic graphite 'edge' (PGE) rotating disk electrode (geometric surface area 0.03 $cm^2$) was used with an electrode rotator (EcoChemie Autolab Rotator) that fitted tightly above a gas-tight, glass, electrochemical cell. A three-electrode configuration was used, with a Pt wire counter electrode and a calomel reference electrode (RE) located in a reference arm containing aqueous 0.10 M NaCl and connected to the working electrode compartment via a Luggin capillary. The temperature of the working compartment was controlled by using a water jacket, whereas the reference electrode remained at ambient temperature, 25 °C. The potential ($E$) was corrected with respect to the standard hydrogen electrode (SHE) after calibrating the reference electrode with a quinhydrone/quinhydrol mixture (Aldrich) at pH 7.0 and pH 4.0 at 25 °C. All potentials are quoted relative to SHE. An Autolab PGSTAT128N electrochemical analyser was used with an electrochemical detection module and NOVA software (EcoChemie). All experiments were carried out in a mixed buffer system [25] prepared using purified water (18.2 M cm, Millipore). The buffer was calibrated to pH 7.4 at 37 °C. Gases were of Premier/Research Grade and supplied by Air Products or BOC. Mass-flow controllers (Smart-Trak, Sierra Instruments) were used to supply precise mixtures of gases to the cell headspace at a constant flow rate. To prepare each film of Hyd-5, the electrode was prepared by sanding with P800 Tufbak Durite sandpaper; enzyme solution (2 μl at ≈5 μM) was then successively applied and withdrawn from the electrode surface over a period of ca. 10 s and the electrode was held under a stream of water to remove non-adsorbed enzyme. In all experiments, the electrode was rotated at a constant rate that was sufficiently high to provide efficient supply of substrate and removal of product from the electrode surface. In electrochemical measurements of hydrogenase activity a steady decrease in current is observed over time and this is ascribed to loss of active enzyme from the electrode surface [26]. We describe this process as "film loss" and the current in Fig. 2 has been "film-loss corrected" by subtracting the rate of activity loss measured at 100% $H_2$.

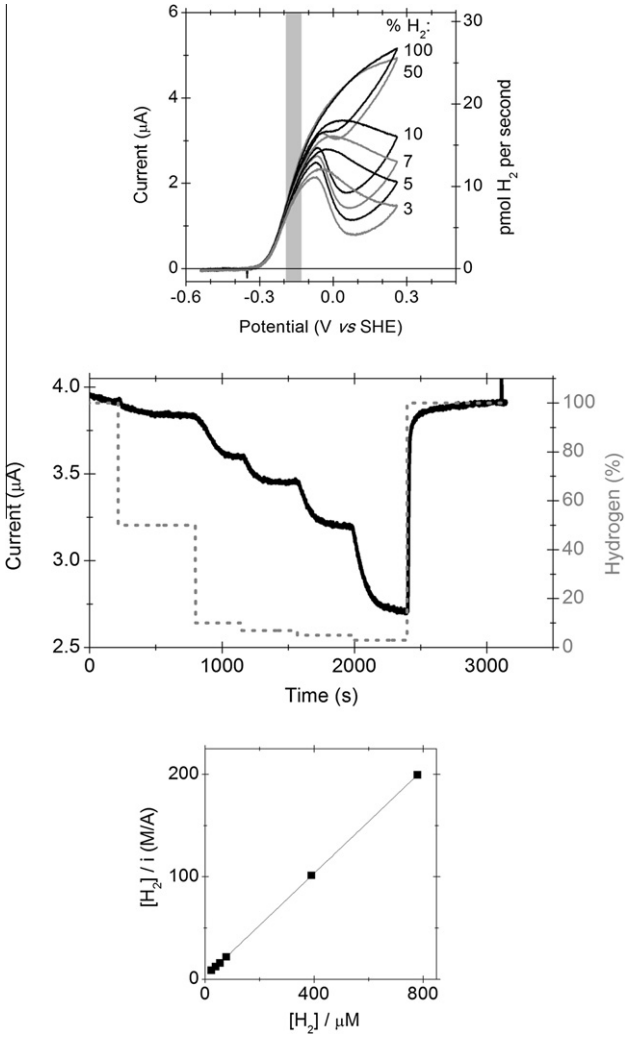


**Fig. 2.** Top panel: $H_2$ oxidation activity as a function of atmospheric $H_2$ concentration and electrode potential. The left hand axis shows current and the right hand axis depicts the equivalent rate of $H_2$ oxidation at the electrode surface. The 2 mV $s^{-1}$ scans were measured at pH 7.4, 37 °C with an electrode rotation of 5000 rpm. $H_2$ in Ar mixtures were made with total gas flow rate of 1000 scc/min and the electrode potential was poised at 0.54 V for 400 s between each scan to ensure complete gas equilibration. The vertical gray bar depicts the range of reducing potentials activity is measured over in the benzyl viologen activity assay. Middle panel: $H_2$ oxidation activity as a function of stepwise changes in $H_2$ concentration at 80 mV. The left hand axis shows current changes (thick black line) and the right hand axis shows changes in gas composition (dotted grey line). Apart from the static electrode potential, all other experimental conditions were the same as in the top panel. Bottom panel: Hanes-Woolf analysis of middle panel data.

### 2.4. $H_2$ oxidation activity assay

A sealed cuvette containing 1 ml of 50 mM oxidised benzyl viologen (Fluka) in pH 7.4 mixed buffer [25] was $H_2$ saturated and placed in a UV/vis spectrometer (Varian Cary 1E) in a water-jacketed sample holder equilibrated to 37 °C. A precise quantity of activated *Salmonella* Hyd-5 was measured in the glove-box using a gas-tight Hamilton syringe. The syringe needle was sealed by pushing it into a rubber bung and the hydrogenase was then transferred in the syringe to the UV–vis spectrometer. The enzyme was injected into the sealed cuvette and the oxidation of $H_2$ was followed by monitoring the reduction of the benzyl viologen at 600 nm. A molar absorbance coefficient of 7.4 $mM^{-1}$ $cm^{-1}$ was used to convert the change in absorbance to the rate of $H_2$ oxidation [27]. A Bradford assay was used to assess the concentration of the *Salmonella* Hyd-5 [28].

### 2.5. Electron paramagnetic resonance spectroscopy

Samples for EPR were prepared using enzyme in a 100 mM Mes, 100 mM NaCl, 10% glycerol pH 6 buffer. This was transferred to a 'titration cell', similar to that first described by Dutton [29] previously purged with Ar. For precise temperature control the cell was water jacketed, and to avoid inward leakage of atmospheric $O_2$ it was kept under positive gas pressure. A stirrer bar ensured constant mixing of the enzyme solution. The working electrode of a combination platinum ring disk electrode (Mettler Toledo, InLab Redox Micro) together with a micro Ag/AgCl reference electrode (WPI, DRIREF-2) was used to measure the potential of the enzyme solution in the cell. Potentials are quoted with respect to SHE using the correction $E_{SHE} = E_{Ag/AgCl} + 195$ mV at 20 °C (calibrated with quinhydrone at pH 4.0 and pH 7.0). Potential-poised samples were obtained by adding sub-μL quantities of 100 mM redox mediators to give a final concentration of 40 μM (methylviologen, benzylviologen, phenazinemethosulfate, indigotetrasulfonate, naphthoquinone and 2-hydroxyl-1,4-naphthoquinone) followed by substoichiometric amounts of potassium ferricyanide and sodium dithionite (both from Sigma–Aldrich). EPR samples were withdrawn by using gas overpressure to force aliquots of approximately 250 μL of enzyme solution through a stainless steel outlet tube into a high precision EPR tube (Wilmad, 714-PQ-7) that had been purged with the gas mixture of the cell. This sample volume was sufficient to fill the resonator length completely. The sample was flash-frozen in an isopropanol-slush over liquid $N_2$.

As-aerobically purified enzyme of concentration 25 μM (determined using a Bradford assay [28]) was titrated to oxidising potentials and EPR samples were taken in the order +143, +265, +385 and +41 ± 5 mV. The remaining enzyme solution was then reduced under $H_2$ for 4.5 h and a sample was taken at 340 ± 5 mV. Finally the cell was flushed with Ar and potassium ferricyanide was added to produce a final enzyme sample at 65 ± 5 mV. All EPR samples were prepared at 20 °C.

Continuous-wave EPR experiments were performed using an X-band (9–10 GHz) Bruker EMX spectrometer (Bruker BioSpin GmbH, Germany) with an X-band Super High Sensitivity Probehead (Bruker) equipped with a low-temperature helium flow cryostat (Oxford Instruments CF935). For determining *g* values, the magnetic field was calibrated at room temperature with an external 2,2-diphenyl-1-picrylhydrazyl standard (*g* value 2.0036). A background spectrum was recorded under identical conditions and subtracted from the EPR spectrum of the enzyme sample. Data analysis was performed using the program *EasySpin* [30]. Spin quantification was carried out using a calibration line obtained with copper perchlorate samples (20, 50, 100, 150, and 200 μM $CuSO_4$ in 2 M $NaClO_4$ (aq) adjusted to pH 1.22 with HCl) measured under non-saturating conditions.

## 3. Results

### 3.1. Isolation of Salmonella Hyd-5

The *hydABCDEFGHI* operon (Fig. 1A) is predicted to encode a membrane-bound uptake [NiFe] hydrogenase (Hyd-5) where the core HydAB heterodimer would be anchored to the periplasmic face of the cytoplasmic membrane by a single transmembrane domain located at the C-terminus of HydA (Fig. 1B) [5]. To isolate active enzyme, and avoid bottlenecks in cofactor biosynthesis or protein targeting that might be encountered upon overexpression, it was decided to affinity-tag the enzyme at its native chromosomal locus. Recombinant DNA technology was used to incorporate a hexa-Histidine tag at the extreme C-terminus of HydA and Western analysis of the $HydA^{His}$ strain (SFTH01) revealed low

levels of mature HydA under aerobic, but not anaerobic conditions (Fig. 1C and D). Unfortunately, attempts to purify Hyd-5 from the SFTH01 strain were hampered by the naturally very low level of expression of the *hyd* operon.

In order to up-regulate expression of the *hyd* operon, again in situ at its native chromosomal locus, the STFH01 (*hydA*$^{His}$) strain was further modified. The constitutive *tat* promoter ($P_{tat}$) from *E. coli* [23] and the synthetic, very strong, promoter from the pQE70 overexpression vector ($P_{T5}$) were chosen. Production of HydA$^{His}$ in the SFTH05 ($P_{tat}$-*hydA*$^{His}$) and SFTH06 ($P_{T5}$-*hydA*$^{His}$) strains was analysed in whole cells by Western immunoblotting (Fig. 1C and D). The SFTH05 ($P_{tat}$-*hydA*$^{His}$) strain produces increased levels of mature HydA$^{His}$, as well as detectable levels of the HydA$^{His}$ precursor that would contain its N-terminal signal peptide (Fig. 1C). The SFTH06 ($P_{T5}$-*hydA*$^{His}$) strain produces the highest levels of both precursor and mature HydA$^{His}$ (Fig. 1C). Addition of exogenous IPTG made little difference to the production of HydA$^{His}$, suggesting that the strong T5 promoter is already de-repressed in *Salmonella* (Fig. 1C). Indeed, inspection of the *Salmonella* LT2 genome suggests it does not encode a close homologue of LacI, which would be required for efficient repression of transcription from this engineered promoter.

Clearly, the SFTH06 ($P_{T5}$-*hydA*$^{His}$) strain produces high levels of HydA$^{His}$ (Fig. 1C). The domination of the precursor form, however, suggests either that cofactor levels are not high enough to meet demand (especially under aerobic conditions), or that the Tat translocase is saturated. In order to naturally boost [NiFe] cofactor levels, which by analogy with *E. coli* should be maximally produced under anoxic conditions [7,31], the SFTH06 ($P_{T5}$-*hydA*$^{His}$) strain was instead cultured under anaerobic conditions (Fig. 1D). Fractionation of the SFTH06 ($P_{T5}$-*hydA*$^{His}$) strain into soluble protein and total membrane fractions revealed that the HydA$^{His}$ precursor was unstable and rapidly degraded during the fractionation process (Fig. 1D). Conversely, the mature HydA$^{His}$ protein remained stable and tightly associated with the membranes (Fig. 1D).

The SFTH06 ($P_{T5}$-*hydA*$^{His}$) strain was chosen as the most suitable host for isolation of Hyd-5. The strain was cultured anaerobically into late stationary phase. Two Hyd-5 purification protocols were then devised: a 'whole cell protocol' involving rapid cell breakage with a chemical cocktail, and a 'membrane protocol' involving an initial purification of cell membranes. Both methods gave good yields of active Hyd-5 enzyme. For example, from 9 g (wet weight) of cells 1.4 mg of protein was obtained using the 'whole cell protocol'. The sample contained only HydA and HydB (Fig. 1E), including a small amount of a truncated form of HydB that had apparently been proteolytically clipped towards its C-terminus (Fig. 1E).

### 3.2. Purified Hyd-5 is enzymatically active and shows tolerance to oxygen

Protein film electrochemistry (PFE) is a technique that has revolutionised studies of hydrogenases [32]. In PFE a small quantity of protein is adsorbed directly onto an electrode and, if electron transfers and catalytic activities are fully maintained in the adsorbed state, a great deal of mechanistic information can be obtained [32]. The catalytic activity of the enzyme responds directly to the applied electrode potential and is monitored through the electrical current recorded in the presence of substrate, which in turn relates directly to the turnover frequency of the enzyme. The electrode with enzyme attached is typically held in a sealed electrochemical cell, through which precise mixtures of gases can be passed. The electrode is then rotated at sufficiently high speed in approximately 5 ml of buffer solution to ensure that the reaction rates measured are not diffusion-limited [32]. Human body conditions of blood pH (7.4) and temperature 37 °C were used for the electrochemical experiments on *Salmonella* Hyd-5.

Fig. 2 shows the results of electrochemical experiments designed to measure the effect of $H_2$ partial pressure on the activity of *Salmonella* Hyd-5. The plots in the top panel are cyclic voltammograms; the electrochemical potential of an electrode coated with *Salmonella* Hyd-5 is raised from −0.54 up to +0.26 V and then back down to −0.54 V at a rate of 2 mV $s^1$ and the resulting current is recorded as a function of potential. Hydrogenase-catalysed $H_2$ oxidation generates a positive electrical current, directly proportional to the rate of $H_2$ oxidation at the electrode surface. In the top panel of Fig. 2 the left *y*-axis denotes the electrical current and the right *y*-axis denotes the equivalent rate of $H_2$ oxidation at the electrode surface. In the benzyl viologen $H_2$ oxidation assay under the same conditions (pH 7.4, 37 °C) a turnover rate of 213 ± 45 $s^1$ was measured when oxidised benzyl viologen was used as the electron acceptor. The activity assay measures $H_2$ oxidation in the potential range of −0.19 to −0.13 V as depicted by the vertical gray bar in the top panel of Fig. 2.

The results show that *Salmonella* Hyd-5 is not a $H_2$ producer as there is no negative current at low potential (Fig. 2, top panel). Hydrogen oxidation activity commences at potentials more positive than −0.28 V and this "overpotential" requirement does not vary with $H_2$ level. The $O_2$-tolerant membrane bound [NiFe] hydrogenases from *R. eutropha* [15,33], *E. coli* [7], and *A. aeolicus* [17] have also been defined as uni-directional, $H_2$-uptake only enzymes with small overpotentials. At potentials more positive than −80 mV a decrease in activity is observed in the cyclic voltammograms of Fig. 2. This is because the Ni in the active site is oxidised from Ni(II) to a catalytically-inactive Ni(III) oxidation state. The inactivation is fully reversible, as the potential is decreased from +0.26 to −0.08 V the current increases. A useful parameter for comparing the re-activation potentials of [NiFe] hydrogenases is "$E_{switch}$", measured by finding the potential of the minima in a derivative plot of the current/potential data [25]. All $O_2$-tolerant hydrogenases characterised by electrochemistry are found to have positive values of $E_{switch}$ and at pH 6.0, 30 °C, 100% $H_2$ the $E_{switch}$ potential of *Salmonella* Hyd-5 is +0.13 V: for comparison *E. coli* Hyd-1 shows $E_{switch}$ at +0.21 V (data not shown). The experiments shown in Fig. 2 permit assessment of *Salmonella* Hyd-5's Michaelis constant for $H_2$, $K_M(H_2)$. The middle panel shows how $H_2$ oxidation activity (current) changes as a function of $H_2$ concentration at a fixed potential of 80 mV, a potential sufficiently negative to avoid complications due to oxidative inactivation. The corresponding Hanes-Woolf analysis (bottom panel) yields a value for $K_M$ ($H_2$) of 9 μM. This is also within the low range measured for other $O_2$-tolerant hydrogenases [7,15].

The capacity of *Salmonella* Hyd-5 to sustain $H_2$ oxidation activity in the presence of physiologically relevant levels of $O_2$ is established by the results shown in Fig. 3. The top panel shows how the $O_2$ tolerance of the enzyme varies as a function of electrochemical potential in similar levels of $H_2$ (5%) and $O_2$ (3%) to those of the mouse spleen [12–14]. The positive current measured under a gas atmosphere of 3% $O_2$/5% $H_2$ is attenuated compared to the voltammogram measured in $O_2$-free conditions. There are two reasons for the attenuation in oxidation (positive) current: firstly, and most importantly, $O_2$ has an inhibitory effect on *Salmonella* Hyd-5 and therefore the rate of enzyme catalysis decreases under aerobic conditions; secondly $O_2$ reduction at the graphite electrode surface results in negative currents at potentials lower than 0 V which offsets the positive current from $H_2$ oxidation (see 3% $O_2$ data). Comparing the 3% $O_2$ voltammograms in the presence and absence of $H_2$ allows the potential window for $H_2$ oxidation in the presence of $O_2$ to be clearly resolved.

The inhibition of *Salmonella* Hyd-5 by $O_2$ is potential dependent and at potentials more positive than 0 V $H_2$ oxidation is completely shut down in the presence of $O_2$. This is because $O_2$-derived species bind to the Ni(III) oxidation state rendering the enzyme catalytically

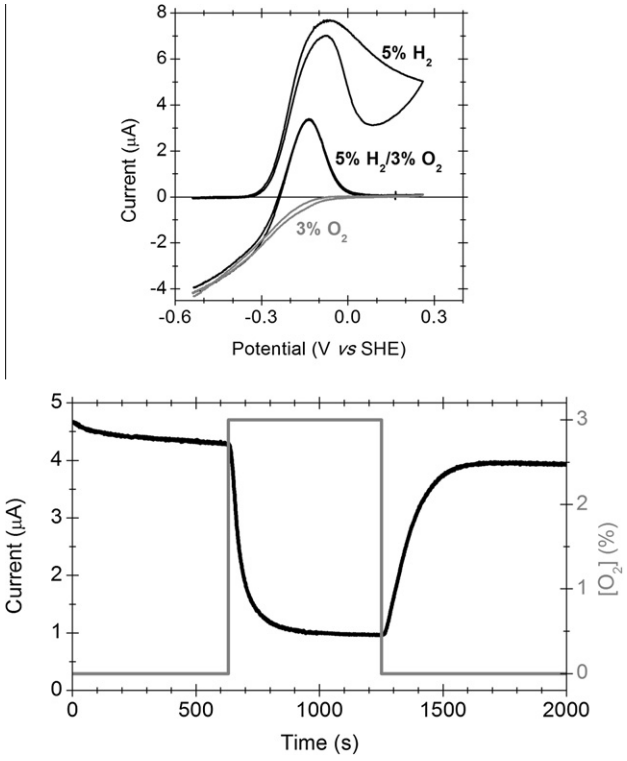


**Fig. 3.** Top panel: Cyclic voltammograms demonstrating $H_2$ oxidation in the presence of $O_2$ by *Salmonella* Hyd-5. Consecutive cyclic voltammograms measured at 2 mV $s^1$, pH 7.4 and 37 °C under gas atmospheres of (1) 5% $H_2$; (2) 5% $H_2$, 3% $O_2$; and (3) 3% $O_2$. The electrode potential was poised at −0.54 V for 400 s between each scan to ensure complete gas equilibration. Bottom panel: Demonstration that Hyd-5 recovers spontaneously, at −80 mV, when $O_2$ is removed. The electrode potential was poised at −80 mV and the current was recorded (left hand axis, thick black line) as the $O_2$ level was pulsed from 0% to 3% (right hand axis, grey line) while the $H_2$ level was kept constant at 5%. Both experiments recorded with electrode rotation of 5000 rpm and Ar as carrier gas with total gas flow rate 1000 scc/min.

inactive. When the electrode potential is decreased the oxidative inactivation is reversed and an increase in $H_2$ oxidation current is seen between 0 and −0.15 V in the top panel of Fig. 3. A potential of −80 mV is thus sufficiently reducing to drive spontaneous re-activation of the $O_2$-inactivated *Salmonella* Hyd-5 states and this is explored in the bottom panel of Fig. 3. Upon removal of $O_2$ from the gas mixture the $H_2$ oxidation current rapidly recovers to the level we would expect if we extrapolated a linear rate of film loss. Fully reversible and rapid recovery from $O_2$ inhibition suggests *Salmonella* Hyd-5 predominantly forms the "ready"/Ni-B Ni(III)-hydroxide inactive state upon reaction with $O_2$. The states generated when *Salmonella* Hyd-5 reacts with $O_2$ were further probed using EPR.

### 3.3. The EPR signature of Hyd-5

The numerous redox-active [NiFe] and FeS clusters present in hydrogenases has meant that these proteins have been amenable to characterisation by advanced spectroscopic techniques for many years [34,35]. The EPR measurements of potential-controlled samples of *Salmonella* Hyd-5 are shown in Fig. 4. A $[3Fe4S]^+$ signal ($g_{x,y,z}$ = 2.02, 2.01, and 2.00) is visible in the sample of enzyme poised at +41 mV. This signal is assigned to the medial FeS cluster. At more positive potentials the intensity and complexity of signals in this region of the spectra increases. An absolute spin count in the FeS region of the +385 mV spectrum yields 1.6 ± 0.2 spins per molecule which indicates the presence of two $S = 1/2$ species, the $[3Fe4S]^+$ cluster and a "high-potential" (HP) centre. This has previously been observed in EPR spectra of the $O_2$-tolerant hydrogenases *E. coli* Hyd-1 [7], *A. aeolicus* Hase 1 [17–19] and *R. eutropha* membrane-bound hydrogenase (MBH) [16,20], and can be considered a spectroscopic "fingerprint" of $O_2$-tolerance. Most recently Pandelia et al. assigned the HP signal as arising from the proximal [4Fe4S] cluster forming a 3+ oxidation state [19].

In the EPR spectrum of the sample poised at −340 mV broad peaks are visible with *g* values 2.05, 1.98, 1.92, and 1.87. The area of FeS signals in the low potential spectrum is approximately half that at high potential (the ratio is 0.7:1.6). Double integration for the −65 mV enzyme sample also yields 0.7 spins/molecule showing that the EPR active species were fully reduced at this potential. A similar value was reported for *R. eutropha* MBH [16]. The low potential spectra are similar to those reported for *A. aeolicus* Hase 1 [19] and *R. eutropha* MBH [16,20] and have been attributed as arising from complex magnetic interaction between all three reduced FeS clusters.

EPR studies have shown that reaction of the [NiFe] active site with $O_2$ results in at least two inactive Ni(III) paramagnetic forms known as Ni-A (also referred to as the "unready" state) and Ni-B ("ready") [34,35]. The ratio of these forms in any one sample depends upon both the specific enzyme under investigation and the experimental conditions (especially the availability of electrons) under which $O_2$ attacks [33,36]. The Ni-A state is thought to include Ni(III) bound to a product of partial reduction of $O_2$ (a coordinated peroxide has been assigned as one of the options) [8,37] and this form of the hydrogenase can be only slowly reductively re-activated in the presence of $H_2$ [36]. Conversely, the Ni-B state contains a bridging hydroxo ligand [8,35], which is the product of complete reduction of $O_2$, and is rapidly reactivated upon reduction [25]. Both Ni-A and -B signals are seen in EPR spectra of as-isolated *Salmonella* Hyd-5 at high potential (Fig. 4) and this is the same as was observed for as-isolated samples of *E. coli* Hyd-1 [7]. The Hyd-5 signals can be simulated to give typical *g*-values of $g_{x,y,z}$ = 2.31, 2.24 and 2.01 for Ni-A and $g_{x,y,z}$ = 2.31, 2.16 and 2.01 for Ni-B (obtained from 80 K measurements where FeS signals are broadened beyond detection).

## 4. Discussion

*Salmonella* and *E. coli* are closely related bacteria. They are members of the *Enterobacteriaceae*, are γ-Proteobacteria and facultative anaerobes. Both also have flexible respiratory capabilities, being able to use a broad spectrum of electron donors and acceptors for respiration. Hydrogen is a commonly used respiratory electron donor for many prokaryotes [38], and *Salmonella* and *E. coli* both express multiple uptake hydrogenases. Where *Salmonella* differs from *E. coli*, however, is that it contains an extra respiratory [NiFe] hydrogenase (Hyd-5), for which transcription is induced under aerobic conditions. Here, we set out to isolate and characterise the Hyd-5 isoenzyme.

Although the core Hyd-5 enzyme itself comprises only two proteins (HydA and HydB), these are encoded within a large nine-cistron operon *hydABCDEFGHI*. Most of the genes encoded by this operon are predicted to be involved in building the [NiFe] cofactor within the α-subunit (HydD, E, H, and I) or in coordinating assembly and export via interactions with the β-subunit (HydF, G). In order to maintain the correct ratios of these accessory proteins during production of Hyd-5 it was decided to up-regulate the *hyd* operon at its native locus on the *Salmonella* chromosome. This approach proved successful and the addition of a hexa-Histidine tag onto the C-terminus of the β-subunit HydA doubled as an affinity tag for facile protein purification and as an epitope tag allowing detection of the HydA protein by Western analysis. Here it was shown that HydA is produced at a low level under aerobic conditions, and that its production is normally repressed under

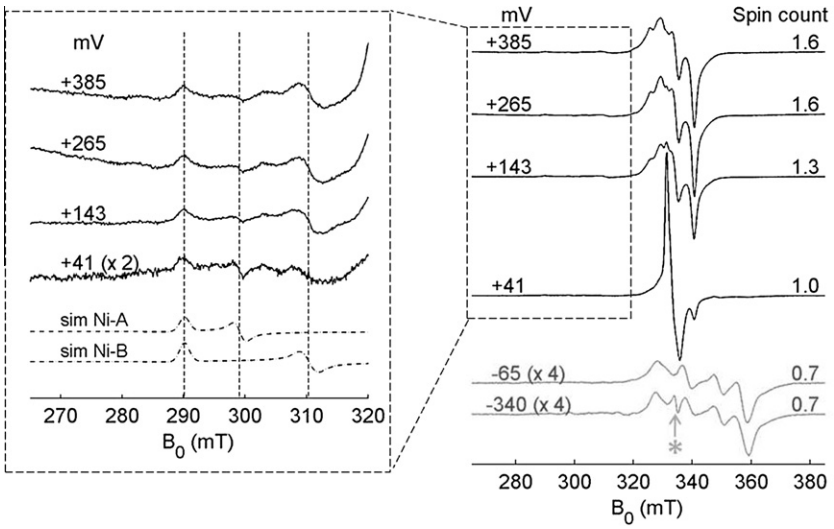


**Fig. 4.** EPR spectroscopy of *Salmonella* Hyd-5. Right: Samples poised at different potentials as shown in mV. The number of spins per molecule of enzyme is indicated by the spin count (error ±0.2). Black spectra are from "as-isolated" potential-poised enzyme samples, gray spectra result from enzyme samples which have been $H_2$ reduced. *g* values: $[3Fe4S]^+$ (+41 mV) $g_{x,y,z}$ = 2.02, 2.01, 2.00; reduced FeS cluster(s) (−340 mV) *g* = 2.05, 1.98, 1.92, and 1.87. Arrow with asterisk indicates reduced viologen mediator (*g* = 2.00). Left: Magnification of the Ni region of selected spectra with simulations of Ni-A and Ni-B (dotted lines). Vertical lines indicate the positions of Ni-A/Ni-B $g_x$ (2.31), Ni-A $g_y$ (2.24) and Ni-B $g_y$ (2.16). Measurement conditions for all spectra: temperature 15 K, microwave power 2 mW, microwave frequency 9.374 GHz, modulation amplitude 1 mT.

anaerobic conditions, thus providing new biochemical data that complement recent gene-expression analyses [10]. Presumably, Hyd-5 activity is deemed superfluous by the cell when the high-level production of Hyd-1 and Hyd-2 is induced under anoxic conditions.

### 4.1. What makes a [NiFe] hydrogenase $O_2$-tolerant?

Production of Hyd-5 under aerobic conditions immediately suggests that this isoenzyme must be able to maintain $H_2$ oxidation activity in the presence of $O_2$. To test this directly the core Hyd-5 αβ-dimer was isolated and characterised by PFE. The data show conclusively that Hyd-5 can oxidise $H_2$ in the presence of oxygen. Moreover, since "wiring" Hyd-5 to an electrode is similar in principle to the enzyme passing electrons from the active site to electron acceptors in the cytoplasmic membrane, these in vitro experiments give a good indication that the enzyme can operate as a $H_2$ oxidiser in air in the living cell.

The $O_2$-tolerance characteristics of Hyd-5 were reinforced by EPR analysis, which revealed "typical" characteristics of $O_2$-tolerant [NiFe] hydrogenases including a high potential multiline $[3Fe–4S]^+$ signal. Although the EPR experiments show the as-isolated enzyme contains a mixture of Ni-A and Ni-B (also observed for *E. coli* Hyd-1 [7]) only the Ni-B/Ready state is formed in electrochemical experiments when activated enzyme is exposed to $O_2/H_2$ mixtures. This, taken together with the characteristic low $K_M$ ($H_2$), lack of $H_2$ production activity and small overpotential for the onset of $H_2$ oxidation means that *Salmonella* Hyd-5 has the same properties as uni-directional $O_2$-tolerant respiratory [NiFe] hydrogenases such as Hyd-1 from *E. coli* [7]. We have previously proposed [15] that $O_2$-tolerant [NiFe] hydrogenases retain $H_2$ oxidation activity in $O_2$ because they are able to rapidly reduce $O_2$ to $H_2O$ at positive potentials; the Fe–S relay architecture appears to be crucial in facilitating this rescue by reverse electron transfer [20]. The electrochemical and spectroscopic characteristics of *Salmonella* Hyd-5 reinforce our model [15] for the mechanistic origin of $O_2$ tolerance.

The parallels between the electrocatalytic function and EPR structural data of *Salmonella* Hyd-5 and *E. coli* Hyd-1 agrees with the high sequence homology between these enzymes. The *Salmonella* Hyd-5 α-subunit (HydB or STM1538) shares 67% identity and 80% similarity with the *E. coli* Hyd-1 α-subunit (HyaB). However, in addition to Hyd-5, *Salmonella* is also capable of expressing a hydrogenase with even greater amino acid sequence identity with *E. coli* Hyd-1. There is 92% overall sequence identity, and 98% similarity, between the α-subunits of the *bona fide Salmonella* Hyd-1 (STM1787) and *E. coli* Hyd-1 (HyaB), suggesting that both *Salmonella* Hyd-1 and Hyd-5 should be highly $O_2$-tolerant hydrogenases. However, *Salmonella* Hyd-1 is optimally expressed under anoxic conditions and this suggests that the ability of bacteria to oxidise $H_2$ in air is not just dependent on the final structures of the catalytic subunits of the hydrogenase. For a bacterium to sustain respiratory $H_2$ oxidation under aerobic conditions it must not only produce an enzyme that is tolerant to the presence of $O_2$, it must also be capable of synthesising the necessary catalytic cofactors under aerobic conditions. Therefore a careful distinction should be made between the definition of an $O_2$-tolerant hydrogenase and that of an $O_2$-tolerant hydrogenase operon.

### 4.2. What makes a bacterium capable of expressing an $O_2$-tolerant $H_2$ oxidising enzyme?

What are the differences in the maturation pathways used to build *Salmonella* Hyd-1 and *Salmonella* Hyd-5? Hyd-1 is encoded by the *hyaABCDEF* operon and homologues of HyaC (HydC), HyaD (HydD), HyaE (HydF) and HyaF (HydG) are all encoded by the *hydABCDEFGHI* operon encoding Hyd-5 (Fig. 1A). The extra genes present in the *hyd* operon, and missing from the *hya* operon, encode homologues of HypA (HydI) and HypC (HydE), which are key accessory proteins required for [NiFe] cofactor assembly into the α-subunit. By analogy with the *E. coli* system, the *hypABCDE* operon, which is essential for [NiFe] cofactor assembly, would be maximally expressed under anaerobic conditions [39]. Under aerobic conditions, however, expression of the *hypABCDE* operon would be repressed, which would drastically deplete the amount of [NiFe] cofactor available to any aerobically-expressed hydrogenases. To counter this problem the *hyd* operon therefore encodes additional HypA and HypC homologues. Despite this, assembling such an oxygen-sensitive cofactor under oxic growth conditions remains a challenge. One important final addition to the *hyd* operon is the *hydH* gene, which encodes a homologue of the *R. eutropha* HoxV

protein. In aerobic *Ralstonia* species HoxV acts as a scaffold protein protecting the immature cofactor from oxygen during hydrogenase biosynthesis, and interacts with a HypC homologue (termed HoxL in that system) to facilitate cofactor insertion into the α-subunit [40]. Thus the *Salmonella hyd* operon produces a specialist Hyd-1 homologue, Hyd-5, and has possibly evolved to encode a dedicated cofactor insertion machinery guaranteeing a supply of [NiFe] cofactor to the enzyme under aerobic conditions.

A capability for $O_2$ tolerant $H_2$ oxidation is of great advantage to a bacterium. It has been suggested that in vivo Hyd-5 oxidises $H_2$ with $O_2$ as the terminal electron acceptor, enabling *Salmonella* to conserve energy in the form of ATP production [4,10,11,41]. The aerobic function of Hyd-5 may also contribute to the ability of *Salmonella* to counteract the animal host's immune system [4,10,11,41]. *Salmonella* is an intracellular pathogen and it will probably invade phagocytes in the liver and spleen. Hydrogen oxidation would provide the bacterium with a source of reducing power that is needed by the pathogens oxidative stress-combating enzymes to reduce reactive oxygen species such as peroxide, which is produced by phagocytes during the oxidative burst to destroy invading bacteria [4,10,11,41]. Of course, beyond their role in *Salmonella* infection, the catalytic action of $H_2$-uptake [NiFe] hydrogenases enables a huge range of prokaryotes to utilise molecular hydrogen as a respiratory energy source.

## Acknowledgements

We thank Elena Nomerotskaia for invaluable technical assistance and we are grateful to Dr. Luet L. Wong for use of UV–visible spectroscopic facilities. This research was supported by Biological and Biotechnological Sciences Research Council (BBSRC) Grants BB/H003878/1 and BB/H001190/1, and Engineering and Physical Sciences Research Council Grant EP/D044855D/1 supporting the Oxford Centre for Advanced Electron Spin Resonance (CAESR). A.P. thanks Merton College, Oxford for a Junior Research Fellowship and L.B. thanks BBSRC for a targeted priority studentship (BB/G016690/1).

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