The *Herbaspirillum seropedicae* SmR1 Fnr orthologs controls the cytochrome composition of the electron transport chain

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The transcriptional regulatory protein Fnr, acts as an intracellular redox sensor regulating a wide range of genes in response to changes in oxygen levels. Genome sequencing of *Herbaspirillum seropedicae* SmR1 revealed the presence of three fnr-like genes. In this study we have constructed single, double and triple fnr deletion mutant strains of *H. seropedicae*. Transcriptional profiling in combination with expression data from reporter fusions, together with spectroscopic analysis, demonstrates that the Fnr1 and Fnr3 proteins not only regulate expression of the cbb3-type respiratory oxidase, but also control the cytochrome content and other component complexes required for the cytochrome c-based electron transport pathway. Accordingly, in the absence of the three Fnr paralogs, growth is restricted at low oxygen tensions and nitrogenase activity is impaired. Our results suggest that the *H. seropedicae* Fnr proteins are major players in regulating the composition of the electron transport chain in response to prevailing oxygen concentrations.

*H. seropedicae* is an endophytic diazotroph belonging to the Betaproteobacteria that can fix nitrogen under micro-oxic and nitrogen limiting conditions. It is found in association with different crops such as rice, maize, sugar cane and sorghum1–3. *H. seropedicae* SmR1 is an aerobic bacterium which has a branched respiratory chain comprising different types of terminal oxidases, which potentially could allow the bacteria to exploit respiratory flexibility and survive under microaerobic conditions4. Genome sequencing revealed genes coding for three Fnr-like proteins in *H. seropedicae* SmR14. In many organisms the Fnr protein, which belongs to the CRP-FNR family of transcriptional regulators5, acts as a positive or negative regulator of genes required for the metabolic switch in response to O2 levels6. The Fnr protein of *Escherichia coli* contains an N-terminal sensory domain, which binds an oxygen-labile [4Fe–4S]12+ cluster under oxygen limiting conditions7 and a C-terminal DNA-binding domain, which recognizes a partially palindromic sequence called the Fnr-box or anaerobox, TTGAT-N4-ATCAA5,8.

Various Fnr-related transcriptional regulators of the CRP-FNR family have been reported to be involved in biological nitrogen fixation. The Fnr protein from *Klebsiella pneumoniae* is required to relieve inhibition of NifA activity by its partner regulatory protein NifL under anaerobic conditions8. In *Rhizobium leguminosarum* UPM791 FnrN is responsible for the expression of the high affinity oxidase encoded by fixNOQP that supports growth under microaerobic conditions and is essential for nitrogen fixation10. Similarly the FixK2 protein is also essential for nitrogen fixation in *Bradyrhizobium japonicum* and *Sinorhizobium meliloti*11–12. Representatives of the CRP-FNR family are also known to act negatively in repressing genes related to nitrogen fixation, such as the FixK1 protein from *B. japonicum*12.

As the *H. seropedicae* genome encodes three Fnr-like proteins, we were interested to determine the potential involvement of these three Fnr homologs in nitrogen fixation and in the control of gene expression in response to oxygen limitation. Several representatives of the Betaproteobacteria encode more than one Fnr-like protein in their genome. For example, *Burkholderia pseudomallei* 1710b and *Herminimonas arsenicoxydans* have genes coding for two Fnr-like proteins, whereas *Cupriavidus metallidurans* CH34 and *Ralstonia eutropha* H16 encode...
three and five Fnr-like proteins, respectively. However, to date the functions of these Fnr-like paralogs have not been determined.

In the current study, we sought to attribute function to the three Fnr-like proteins found in *H. seropedicae* and in particular to examine their role in the regulation of electron transport chain composition. We demonstrate that deletion of all three fnr alleles results in a growth phenotype under microaerobic conditions, implying that Fnr proteins may be involved in controlling the expression of respiratory oxidases. By comparing the transcription profiles of the wild-type and triple fnr mutant strains and performing further gene expression and biochemical analyses, we observe that the Fnr proteins not only activate genes required for expression and activity of the high affinity *cbb*$_3$-type oxidase, but also have a major influence on the regulation of the cytochrome *bc*$_1$ complex and on cytochrome *c* biogenesis. This suggests that the *H. seropedicae* Fnr proteins facilitate distribution of the electron flux through cytochrome carriers and the heme-copper oxidase branch of the respiratory chain to increase coupling efficiency under oxygen-limiting conditions.

**Results**

*H. seropedicae* encodes three proteins that share homology with *E.coli* Fnr. The *H. seropedicae* SmR1 genome contains three genes encoding homologs of the Fnr protein, which we designate as fnr1, (Locus Tag: Hsero_3197; Ref seq: YP_003776587.1), fnr2 (Locus Tag: Hsero_2381; Ref Seq: YP_003775788.1) and fnr3 (Locus Tag: Hsero_2538; Ref Seq: YP_003775945.1) The *H. seropedicae* Fnr1, Fnr2 and Fnr3 proteins share 38.4%, 37.5% and 26.9% identity respectively with *E. coli* Fnr. As shown in Figure 1, the Fnr1 and Fnr3 proteins are more similar to each other than to Fnr2. When compared with *E. coli* Fnr, the three Fnr paralogs have characteristic sequence features that are hallmarks of Fnr proteins, including three conserved N-terminal cysteines plus a central cysteine that are thought to co-ordinate the oxygen-labile [4Fe–4S]$^{2+}$ cluster. In addition all three deduced proteins contain the predicted dimerization helix sequence located at the beginning of the C-terminal domain, and a helix-turn-helix DNA binding motif characteristic of members of the CRP-FNR family.

**Figure 1 | Alignment between *H. seropedicae* Fnr1, Fnr2 and Fnr3 proteins and *E. coli* Fnr.** Identical amino acids are indicated by asterisks (* *), high similarity amino acids are indicated by colons (:), and low similarity amino acids by dots (.). Conserved cysteines required for binding of the [4Fe–4S]$^{2+}$ are shown in bold and indicated by thick arrows. The double underlined sequence represents the region of the N-terminal sensory domain that comprises the eight-stranded β-roll. The α-helix required for dimerization is boxed. Highlighted in light-grey is the DNA-binding domain with residues that are important for Fnr-box recognition indicated by thin arrows.
proteins, we carried out a phylogenetic reconstruction and observed that all three are in a clade together with other members of the Fnr group of CRP-FNR superfamily from Betaproteobacteria (Supplementary Fig. S1). This finding is in agreement with the cysteine motif arrangement found in the *H. seropedicae* Fnr proteins, which is characteristic of the Fnr group and clearly divergent from the FnrN and FixK groups commonly represented in the Alphaproteobacteria*. Among the Betaproteobacteria, *H. seropedicae* Fnr1, Fnr2 and Fnr3 branched into a group with the Fnr proteins from *Janthinobacterium sp.* Marseille (Minibacterium massiliensis) and *H. arsenicoxydans*. Within this group *H. seropedicae* Fnr2 is more divergent from Fnr1 and Fnr3 and also from the *J. sp.* Marseille and *H. arsenicoxydans* Fnr proteins.

**Construction of *H. seropedicae* Afnr mutant strains.** To study the role of the three Fnr proteins, single, double and triple fnr deletion mutant derivatives of *H. seropedicae* strain SmR1 were constructed using a sacRB:Km cartridge as described in the Methods section. This strategy allowed the construction of 7 unmarked deletion strains in which all possible combinations of fnr genetic backgounds are available (Supplementary Fig. S2). The promoters and non-coding regions of fnr were retained in these ORF deletions, enabling transcriptomic analysis of fnr mutant strains. Deletion mutants were given the prefix MB, with a number indicating which fnr deletion is present (for example MB1 lacks fnr1, whereas MB13 lacks fnr1 and fnr3 respectively).

**Influence of fnr on growth at low oxygen concentrations.** As Fnr proteins are known to sense oxygen and have an important role as transcriptional regulators during the switch from aerobic to oxygen-limiting conditions, we were interested to determine if deletion of the three *H. seropedicae* fnr genes would influence growth under hypoxic conditions. Accordingly, we compared the growth curves of wild-type strain SmR1 with that of the triple deletion fnr strain MB231, when grown in malate minimal medium with an initial oxygen concentration in the gas phase of 5% and supplemented with 2 or 20 mM ammonium chloride. Oxygen consumption during growth of the cultures was monitored using a gas chromatograph equipped with a molecular sieve column and a TCD detector. Under these conditions, the rate of oxygen depletion in the gas phase was similar for both the wild-type and MB231 strains (Figure 2). However, after 4 hours when the oxygen concentration had decreased to approximately half of the initial concentration, the growth rate of the triple fnr deletion strain was clearly slower than that of the wild-type. Moreover, the optical density (O.D$_{600}$) of the triple mutant strain reached only 0.5 and 0.6 after 10 hours growth in the presence of 2 and 20 mM NH$_4$Cl, respectively, compared with an O.D$_{600}$ of 0.8 reached by the wild type strain in both ammonium chloride concentrations (Supplementary Fig. S3). In contrast we observed no difference in growth rate, when both strains were grown under aerobic conditions with 20.8% oxygen in the gas phase (Supplementary Fig. S4). These results imply that the absence of fnr imposes a growth rate penalty under oxygen-limiting conditions, which may suggest the involvement of at least one of the three Fnr proteins in the regulation of terminal oxidases in response to oxygen, as observed in other bacteria.

**Transcriptional profiling of wild-type and fnr strains using RNA-seq.** The influence of the Fnr proteins on global gene expression under microaerobic conditions was assessed using RNA sequencing. To avoid problems associated with growth rate differences, we grew the wild-type and the fnr ablated strain, MB231 under aerobic conditions (20.8% oxygen) to an optical density of 0.4 and then switched the cultures to microaerobic conditions (initial oxygen concentration of 2%) for 1.5 hours prior to RNA extraction. Comparison of global gene expression patterns revealed that 187 *H. seropedicae* genes were differentially expressed by more than 3-fold, with p values <0.05. In some cases, depending on the genomic context, genes with p-values slightly higher than 0.05 were also considered as being differentially expressed. Of these, 143 were down-regulated in the fnr triple mutant strain, indicating that these genes are activated either directly or indirectly by Fnr under oxygen-limiting conditions. 44 genes were up-regulated in the fnr ablated strain, implying that they are targets for Fnr-mediated repression. A complete listing of differentially expressed genes is provided in Supplementary Dataset 1.

Of the 187 regulated genes, 70 (37.4%) are classified in the cellular process category and 58 genes (31.02%) belong to the metabolism category according to the Clusters of Orthologous Genes (COG) functional classification (Supplementary Fig. S5a). In the cellular process category, 29 genes are related to signal transduction mechanisms, whereas in the metabolism category, 30 genes are related to energy production and conversion (Supplementary Fig. S5b). Most of the genes from the energy and production subcategory, encode important proteins required for synthesis and activity of many of the respiratory electron transport chain components (Table 1 and Supplementary Dataset 1). These findings suggest that the *H. seropedicae* Fnr proteins may facilitate efficient adaptation to the variable oxygen concentrations found in different environments. *H. seropedicae* possesses a branched aerobic respiratory chain comprising four different types of terminal oxidases*. These are represented by the aa$_3$-type (cox) and bb$_3$-type (fix) oxidases in the heme copper oxidase branch and the bd-type and bo$_3$-type oxidases representing the ubiquinol oxidase branch (Figure 3a).

**Figure 2** | Influence of fnr genes on growth during oxygen limitation. The growth of *H. seropedicae* SmR1 (black squares) and MB231 (grey squares) strains were assayed in NFbHP-Malate minimal media supplemented with high ammonium concentration (20 mM NH$_4$Cl) under 5% initial oxygen concentration. The oxygen depletion in the gas phase was monitored for SmR1 (black circles) and MB231 (grey circles). Every two hours 0.5 mL samples from the flask gas phase were analysed by gas chromatography. The data represents the mean of three independent assays performed in duplicate. Error bars indicate standard deviations. In some case these are not visible as they are smaller than the graph points.

Amongst the genes that are potentially activated by Fnr, large changes in transcript abundance were observed in genes required for the biosynthesis and activity of the bb$_3$-type heme-copper oxidase (Table 1). These include the fixN operon encoding the structural components of this oxidase and the maturation genes fixG, fixH, fixL and fixS, which in other bacteria is often organized as an operon*. In contrast, in *H. seropedicae* the fixG, fixH, fixL and fixS genes are dispersed in two distinct operons (Figure 4a), in which fixG and Hsero_3199 (fixH) appear to form an operon with the conserved putative transmembrane protein Hsero_3198 and fixL and fixS apparently form an operon with the heme biosynthesis gene hemN* and Hsero_3206, which encodes a conserved hypothetical protein (Figure 4a). In agreement with the down-regulation observed...
**Table 1** | Differential expression of a subset of genes required for, or implicated in, modulation of the composition of the electron transport chain and nitrate metabolism

| Feature ID | Gene Description | COG<sup>b</sup> | FC<sup>c</sup> | Fnr Box<sup>d</sup> | Sequence<sup>e</sup> | Position |
|------------|-----------------|---------------|----------|------------------|---------------|----------|
| **Position** | | | | | | |
| **Start** | **End** | |
| **ccmC** | ABC-type transport system, permease component protein | O | -3.62 | N | | |
| **dsbC** | thioldisulfide interchange protein | O | -3.22 | Y | | |
| **fnr** | Crp/Fnr family transcription regulator protein | T | -5.51 | Y | | |
| **fnr2** | Crp/Fnr family transcription regulator protein | T | -4.51 | Y | | |
| **fnr3** | Crp/Fnr family transcription regulator protein | T | 0.39<sup>*</sup> | N | | |
| **fixG** | iron-sulfur 4Fe-4S ferredoxin | C | -6.33 | N | | |
| **Hsero_0153** | cytochrome c553 protein | C | -2.37 | N | | |
| **Hsero_1104** | cytochrome c551/c552 transmembrane protein | C | -2.07 | Y | | |
| **Hsero_1490** | 2-polyprenyl-6-methoxyphenol hydroxylase | C; H | -3.27 | N | | |
| **Hsero_4284** | NADH:ubiquinone oxidoreductase protein | C | -1.64 | Y | | |
| **nadH** | NADH dehydrogenase, FAD-subunit cytochrome c reductase | C | -2.43 | Y | | |
| **rubB** | cytochrome Ctype biogenesis transmembrane protein | O | -2.34 | N | | |
| **thiol:disulfide interchange protein** | | | | | | |
| **dsbC** | thioldisulfide interchange protein | C | -2.37 | N | | |
| **ubF** | 2-polyprenyl-3-methyl-6-methoxy-1,4-benzoquinol hydroxylase | H | -2.61 | N | | |
| **cutA** | periplasmic divalent cation tolerance protein | P | -2.12 | N | | |
| **fixN** | cbb3-type cytochrome c oxidase, subunit I | C | -6.33 | Y | | |
| **fixO** | cbb3-type cytochrome c oxidase, subunit II | C | -6.22 | N | | |
| **fixP** | cbb3-type cytochrome c oxidase, subunit III | C | -5.41 | N | | |
| **hemN** | oxygen-independent corynophycinogen III oxidase protein | H | -4.17 | Y | | |
| **Hsero_3206** | conserved hypothetical protein | S | -4.44 | N | | |
| **fixA** | cation transport P type ATPase protein | P | -4.23 | N | | |
| **fixS** | nitrogen fixation protein | P | -6.14 | N | | |
| **narG** | respiratory nitrate reductase alpha chain oxidoreductase | C | -6.30 | N | | |
| **narH** | respiratory nitrate reductase beta subunit protein | C | -5.16 | N | | |
| **narL** | respiratory nitrate reductase transmembrane gamma subunit | C | -4.97 | N | | |
| **narN** | nitrogen fixation protein | C | -5.41 | N | | |
| **maaA** | molybdenum cofactor biosynthesis enzyme A protein | H | -4.00 | N | | |
| **narK1** | nitrate/nitrite transporter protein | P | -6.23 | Y | | |
| **narU** | nitrate/nitrite transporter protein | P | -7.34 | N | | |
| **narX** | nitrate/nitrite sensor histidine kinase | T | -7.11 | Y | | |
| **nar** | nitrate/nitrite response regulator transcription regulator protein | K; T | -6.08 | N | | |
| **petA** | ubiquinoyl-cytochrome C (Iron-sulfur) oxidoreductase | C | -2.78 | Y | | |
| **petB** | cytochrome b subunit transmembrane protein | C | -3.52 | N | | |
| **petC** | cytochrome c1 precursor transmembrane protein | C | -2.48 | N | | |

<sup>a</sup>Genes organized in single transcriptional units are presented first in alphabetical order, followed by predicted operons ordered alphabetically according to the first gene of the operon.

<sup>b</sup>Cluster of Orthologous Genes classification. Letters referring to each specific category are: C, energy production and conversion; H, coenzyme metabolism; K, transcription; O, post-translational modification, protein turnover, chaperone functions; P, Inorganic ion transport and metabolism; T, signal transduction; S, function unknown.

<sup>c</sup>Logarithm of fold change comparing RNA-Seq libraries of H. seropedicae MB231 (feraliberal strain) and SmR1 (wild type) strain.

<sup>d</sup>Presence (Y = yes) or absence (N = no) of putative Fnr-boxes in the promoters of the respective gene. The Fnr boxes were predicted by either PEPPER<sup>1</sup> or Virtual Footprint<sup>2</sup> as described in the Methods section. In some cases manual<sup>14</sup> predictions were also included.

<sup>e</sup>Start and End positions of Fnr boxes related to the predicted translational start site.

<sup>*</sup>Not statistically significant (p-value = 0.74).
in the absence of fnr, both the fixNOP and hemN-Hsero_3206-fixIS putative operons have well conserved Fnr-boxes located at positions −143 and −128 upstream of their respective translational start sites (Table 1). The Fnr-Box in the hemN promoter perfectly matches the consensus TTGAT-N_4-ATCAA, while the fixN promoter Fnr-box, TTGAT-N_4-GTCAA, has only one mismatch (underlined) (Table 1).

Apart from the cbb_3-type heme-copper oxidase, genes encoding the other terminal respiratory oxidases in the H. seropedicae genome were not apparently differentially expressed in response to the presence of Fnr. Although the genome contains two copies of the coxBA operon encoding aa_3-type oxidases, one of these (Locus Tags: Hsero_2311-Hsero_2312) did not appear to be expressed under our experimental conditions. The second coxBA operon (Hsero_4160-Hsero_4161) and its associated coxC (Hsero_4157) and coxG (Hsero_4159) genes do not appear to be Fnr-regulated. This was also the case for the cydAB genes encoding the bd-type oxidase and a bo_3-type oxidase encoded by the cyoABCD operon. However, analysis of transcript abundance suggests that expression of several other components of the respiratory chain are subject to regulation by Fnr. Transcripts mapping to the petABC operon, which encodes ubiquinol-cytochrome c reductase (also known as the cytochrome bc_1 complex or complex III) were down-regulated 7–11 fold in the Fnr mutant compared with the wild-type control (Table 1 and Figure 3b). In addition, significant differential expression was observed for genes encoding cytochrome c oxidase (cbb_3-type oxidase) and the cytochrome bc_1 complex and maturation and expression of c-type cytochromes. Hence, Fnr is likely to influence the flow of electrons through the cytochrome c branch of the pathway in order to optimize energy generation. Additionally, the fnr genes may also control the composition of the quinone pool. Transcripts mapping to ubiF gene which encodes a 2-polyprenyl-3-methyl-6-methoxy-1,4-benzoquinol hydroxylase protein involved in the penultimate step of the ubi-quinone biosynthesis pathway and a gene encoding an alternative

Figure 3 | Fnr regulation of components of the electron transport chain (ETC) in H. seropedicae as determined by transcript profiling. (a) Schematic representation of the probable organization of ETC branches in H. seropedicae based on the genome annotation. (b) Influence of Fnr on differential expression of genes represented in (a). FC indicates fold change.

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2-polyprenyl-6-methoxyphenol hydroxylase (Hsero_4190) also required for ubiquinone biosynthesis were significantly down regulated in the triple fnr deletion strain (Table 1 and Figure 3). In common with many other Proteobacteria, genes encoding a respiratory nitrate reductase, organized as a narGHJI-moaA operon, are also apparently up-regulated by Fnr in H. seropedicae, as transcripts mapping to this operon decreased 16–100 fold in the triple fnr deletion mutant (Table 1 and Supplementary Dataset 1). In addition, the nitrate/nitrate transporter encoded by narK1U operon and the nitrate-sensing two component regulatory system narXL are also strongly down-regulated in the fnr triple deletion (Table 1 and Supplementary Dataset 1). Although this may suggest the potential to utilise nitrate as a terminal electron acceptor, the function of this respiratory nitrate reductase in H. seropedicae is somewhat enigmatic, as various investigators have failed to demonstrate anoxic growth of this organism in the presence of nitrate14.

Amongst the global changes in transcript abundance, we observed differential expression of two of the three H. seropedicae fnr genes themselves. Whereas, the transcript abundance upstream of fnr3 did not significantly change in the triple deletion mutant, fnr1 and fnr2 were down-regulated 50-fold and 25-fold respectively (Table 1 and Supplementary Dataset 1). This suggests several possibilities that are not mutually exclusive: (a) Fnr3 is required to activate expression of fnr1 and fnr2, (b) Fnr1 and Fnr2 auto activate their respective promoters or (c) a combination of Fnr proteins is required to activate these promoters.

Analysis of Fnr regulation of genes encoding the cbb3-type respiratory oxidase. To confirm the involvement of Fnr in co-regulation of the fixNOP and hemN-Hsero_3206-fixIS operons we constructed fixN::lacZ and hemN::lacZ transcriptional fusions and analysed the expression of β-galactosidase in the various fnr mutant strains.

Figure 4 | Effect of fnr1, fnr2 and fnr3 mutations on expression of the fixNOP and hemN-Hsero_3206-fixIS operons in H. seropedicae. (a) Schematic representation of genomic region encoding the fixNOP and hemN-Hsero_3206-fixIS operons. The genes and predicted functions in the locus are: Hsero_3198, transmembrane protein; Hsero_3199, FixH domain containing protein; fixG, iron-sulfur 4Fe-4S ferredoxin transmembrane protein; fixP, cbb3-type cytochrome c oxidase-subunit III; fixO, cbb3-type cytochrome oxidase-subunit II; fixN, cbb3-type cytochrome c oxidase, subunit I; fixS, nitrogen fixation protein P-type ATPase protein; fixL, cation transport P-type ATPase protein; Hsero_3206, conserved hypothetical protein; hemN, oxygen-independent coproporphyrinogen III oxidase. Black rectangles represent putative FNR-boxes. Genes are not drawn to scale. (b) β-Galactosidase activities of fixN::lacZ and hemN::lacZ fusions incubated for 3 hours under the oxygen concentrations of 2.0% (black bars), 4.0% (dark grey bars), 6.0% (light grey bars) and 20.8% (white bars). CTRL indicates the SmR1 strain carrying the vector plasmid pPW452 (which contains the lacZ gene without a promoter).
compared with the parental strain. Expression of the fixN::lacZ and hemN::lacZ fusions is apparently regulated by oxygen levels in the wild-type background since we observed a reduction in promoter activities upon exposure to increasing oxygen concentrations (Figure 4b). Consistent with the transcriptomics data (Table 1), both operons are apparently subject to regulation by Fnr. Notably, expression from the fixN and hemN promoters was significantly reduced in strains that lack either fnr1 or fnr3, but activity was equivalent to the parental strain in the fnr2 deletion strain MB2 (Figure 4b). This implies that both Fnr1 and Fnr3 are required to activate expression of the fixNOP and hemN-Hsero_3206-fixIS operons and that Fnr2 is not involved in the regulation of expression of the cbb3-type respiratory oxidase. Accordingly, under oxygen-limiting conditions, no growth penalty is observed for the MB2 strain (Supplementary Fig. S7).

Fnr influences the cytochrome content of H. seropedicae. As the transcriptome analysis implicates Fnr as a regulator of genes involved in cytochrome c biogenesis, we compared the spectral features of wild-type and fnr deletion strains. We noticed that strains lacking the fnr1 gene were deficient in a pink pigment when cultured in liquid media (Figure 5a). To further explore this observation we analysed reduced minus oxidized spectra of protein extracts obtained from SmR1 and the fnr mutant strains (Figure 5b and Supplementary Fig. S8). Spectra of the wild type strain were consistent with the presence of c-type (α-band located around 550 nm) and b-type (α-band shoulder around 560 nm) cytochromes in the protein extract. Similar spectral features were found in strains lacking either fnr2 or fnr3. However, all strains lacking fnr1 appeared to be deficient in cytochrome content, which may account for the observed differences in culture pigmentation. In order to obtain further biochemical support for the spectral features observed, we stained protein extracts from SmR1 and fnr mutant strains for covalently bound heme (Figure 5c and Supplementary Fig. S8). In the wild-type strain SmR1, we detected five bands, which presumably represent c type cytochromes. The protein of approximately 34 KDa (band 1) could represent FixP by comparison with the heme staining profile of the fixN mutant strain, RAM21 (Supplementary Fig. S9) and by analogy with studies on the FixNOQP proteins from other bacteria. Notably, the level of this protein was significantly diminished in strains lacking fnr1, consistent with decreased expression of the fixNOP operon observed in the transcriptome (Table 1 and Figure 3) and lacZ-fusion analysis (Figure 4). The bands 2, 4 and 5 may represent PetC, cytochrome c553 (Hsero_0153), and cytochrome c551/c552 (Hsero_1104), respectively, based on the apparent molecular masses and expression pattern of these proteins, which were identified as being activated by Fnr in the transcriptome analysis (Table 1 and Figure 3). All five c-type cytochromes, including band 4, were absent in strains lacking both fnr1 and fnr3 (MB13 and MB231). This is in agreement with the loss of the cytochrome α-band in the UV-visible difference spectra in strains lacking both the fnr1 and fnr3 genes (Figure 5b and Supplementary Fig. S8). Taken together with the data from transcription profiling, these results suggest that both Fnr1 and Fnr3 are necessary to maintain the level of c type cytochromes in H. seropedicae under microaerobic conditions.

Deletion of the three fnr genes impairs nitrogenase activity and growth on dinitrogen. In the analysis reported so far, strains were
grown in minimal media containing a high concentration of fixed nitrogen, which represses nitrogen fixation in *H. seropedicae*. Since the *cbb*3-type heme-copper oxidase is known to have an important role as a terminal oxidase that supports nitrogen fixation under microaerobic conditions in the *Rhizobacteriaceae* and is subject to regulation by Fnr proteins, we were interested to determine if nitrogen fixation is influenced by the presence of the Fnr paralogs in *H. seropedicae*.

When *fnr* mutant strains were grown under N-deficient conditions (with 0.5 mM sodium glutamate) in semi-solid medium and tested for the ability to reduce acetylene as a measure of nitrogenase activity, no significant differences were observed in comparison with the wild-type strain (Figure 6a). The RAM21 strain (*fixN* mutant) was also not deficient in acetylene reduction when grown under these conditions (Supplementary Fig. S10). However, since semi-solid medium enables bacteria to move towards optimal oxygen concentrations appropriate for growth, we sought a more rigorous method to determine the influence of limiting oxygen on nitrogenase activity in the mutant strains. When the *fixN* mutant strain RAM21 was grown in nitrogen-deficient liquid medium, under conditions of oxygen limitation (initial oxygen concentration of 5% in the gas phase) we observed that acetylene reduction was not severely compromised in comparison with the wild-type (Figure 6b). This suggests that the *cbb*3-type oxidase is not required to support nitrogenase activity in *H. seropedicae* under the oxygen-limiting conditions imposed in this experiment. In contrast, the nitrogenase activity of the MB231 mutant strain was severely impaired compared to the wild-type strain under these conditions (Figure 6b). Given the pleiotropic effects on expression of the electron transport components in the triple *fnr* deletion strain it is likely that the electron flux is insufficient to support nitrogenase activity in the *fnr* deletion strain under oxygen-limiting conditions. In agreement with this result, we observed that diazotrophic growth of the triple *fnr* mutant strain was also compromised in liquid N-free medium (Supplementary Fig. S11).

**Discussion**

In order to survive in rapidly changing environments and explore diverse habitats, many bacteria adjust the composition of their respiratory chains to cope with fluctuating oxygen concentrations. In many cases this involves regulation of the expression of terminal oxidases in order to optimize energy generation. The global transcriptional regulator Fnr, and its various orthologs, provide a widespread mechanism for sensing oxygen and communicating this to the transcriptional apparatus in order to balance the levels of different terminal oxidases, according to prevailing environmental conditions. Accordingly, we observe that Fnr is required to activate the expression of genes required for the synthesis and activity of the
high-affinity cbu3-type heme copper oxidase in H. seropedicae, as is the case in other Proteobacteria.16,17 To balance respiratory requirements under oxygen-limiting conditions, Fnr and its orthologs commonly participate in negative regulation of the expression of other terminal oxidases, for example, the bd-type and bo3-type oxidases in E. coli and A. vinelandii18,19 and the bo3-type and CI0 oxidases in Pseudomonas putida.20 However, our transcriptomics data indicate that this is not the case in H. seropedicae. We only observe differential expression of the genes encoding the cbu3-type oxidase. Expression of the other terminal oxidases is not apparently affected by absence of the three H. seropedicae Fnr proteins. However, in contrast to other well-studied systems, the Fnr proteins in H. seropedicae appear to have a major influence on the composition of the complete electron transport chain that feeds electrons from NADH, through the ubiquinone pool to the cytochrome bc1 complex and onto the c-type cytochromes that are substrates for the heme-copper oxidases. This is clearly demonstrated by the depletion of c-type cytochromes and the down regulation of genes encoding the various components of this branch of the electron transport chain in the triple fnr mutant. Hence it would appear that the Fnr proteins play a major role in regulating the configuration of the H. seropedicae electron transport chain in order to exploit respiratory flexibility and optimize energy coupling in response to oxygen availability.

The cbu3-type heme copper oxidase is likely to be required for growth at very low (<0.5%) oxygen concentrations20 and can support symbiotic nitrogen fixation at nanomolar levels of dissolved oxygen (reviewed in 20). Nevertheless, the fixN insertion mutant of H. seropedicae RAM21 was competent to support nitrogenase activity under oxygen-limiting conditions implying that the cbu3-type oxidase is not required to support nitrogen fixation in this organism. Perhaps this result is not surprising, given that, to our knowledge, this oxidase is not required for nitrogen fixation in other free-living diazotrophs. By analogy with other nitrogen-fixing bacteria, it is possible that the bd-type oxidase supports nitrogenase activity in H. seropedicae. This oxidase is critical for microaerobic diazotrophy in Klebsiella pneumoniae21, it provides respiratory protection for nitrogenase in Azotobacter vinelandii22,23 and it is utilized as a terminal oxidase to support symbiotic nitrogen fixation in Azorhizobium caulinodans.24 In contrast, the triple fnr deletion strain was compromised with respect to both nitrogenase activity and diazotrophic growth, which presumably reflects the major role played by Fnr in reconfiguring the electron transport chain under oxygen limiting conditions in H. seropedicae.

The results presented here do not provide a rationale for the existence of multiple Fnr proteins in H. seropedicae. Potentially, each ortholog may exhibit differential sensitivity to oxygen, recognize different DNA targets or have different propensities to dimerise under aerobic conditions. The transcript profiling reveals that expression of fnr3 is constitutive, whereas fnr1 and fnr2 expression is apparently positively controlled by one or more of the Fnr orthologs. The fnr1 gene, which is located close to the genes required for the maturation and activity of the cbu3-type oxidase, appears to play a critical role in regulating transcription of the fixNOP and hemV-Hsero_3206-fixIS operons and in controlling the expression of c-type cytochromes. Although, fnr3 also appears to be required to express the cbu3-oxidase, we cannot rule out the possibility that it is required to activate transcription of fnr1. In contrast, fnr2 does not appear to be required, either for expression of this oxidase or c-type cytochromes, under the experimental conditions employed here. Further detailed characterization of the three Fnr paralogs will be necessary in order distinguish their precise roles in gene regulation in H. seropedicae.

Methods

Bacterial strains and plasmids. H. seropedicae and E. coli strains and plasmids used are listed in Supplementary Table S1.

Growth conditions. E. coli strains were grown at 37°C in LB medium.25 H. seropedicae strains were grown at 30°C in NFbHP-Malate medium26 supplemented with NH4Cl or 0.5 mM sodium glutamate. Appropriate antibiotics were used when required. For experiments requiring different oxygen concentrations, the air in the gas phase of Suba Seal® stopped culture flasks was exchanged by injecting argon into the flasks for 30 minutes. To obtain different oxygen levels a given volume of air was injected back into the flask. The oxygen levels in the gas phase were verified by gas chromatography using a molecular sieve column and a TCD detector.

Identification and Analysis of H. seropedicae Fnr Orthologs. The sequences of Fnr1, Fnr2 and Fnr3 from H. seropedicae SmR1 were aligned with E. coli K12 substr. MG1655 Fnr (Ref: NP_415850.1) and cyanobacterium A. vinelandii (Ref: YP_003799173.1), using Muscle software.27 A limited number of sequences were selected, eliminating redundant information, while maintaining representative taxonomic diversity. All sequences selected (Supplementary Table S2) were checked to have the CRP-Fnr superfamily protein signatures, CNMP_Binding_3 PS50042 and HTTR_CRP_2 PS51063, proposed by PROSITE.28 For phylogenetic tree reconstruction, an amino acid alignment was made using Muscle.29 Maximum likelihood (ML) trees were derived using the JTT matrix-based model30 after bootstrapping 1,000 replicates of each original data set using the MEGA 5.05 software.31

Phylogenetic Analysis. Amino acid sequence retrieval was performed by using a BLASTP search32 against the nonredundant NCBI database. The proteins used as queries were Fnr1, Fnr2 and Fnr3 from H. seropedicae (Ref: TP_003775887.1, YP_003775788.1 and YP_003775945.1, respectively) FixK1 and FixK2 from B. japonicum (Ref: NP_772701.1 and NP_769397.1, respectively), Fnr from E.coli K12 substr. MG1655 (Ref: NP_415850.1) and CydR from A. vinelandii (Ref: Seq: YP_003799173.1). A BLASTP search33 against the nonredundant NCBI database was performed for the orthologs identified in this study.

Construction of H. seropedicae SmR1 fnr deletion and fixN insertional mutant strains. An allelic exchange strategy was used to generate derivatives of H. seropedicae SmR1 for orthologs deletions (Supplementary Fig. S2) and a tetracycline resistance cassette insertion into fixN (Supplementary Fig. S12). The primers used in this work are shown in Supplementary Table S3. For construction of the allele exchange plasmids for fnr mutation the upstream and downstream regions of fnr1, fnr2 and fnr3 were amplified by PCR. These fragments were then ligated to generate fnr deletions, which were cloned into HindIII and BamHI sites of the suicide plasmid pSUP202, to generate pMB1B1, pMB2B2 and pMBBD3 (Supplementary Table S1).

The npt-sacB-sacR cartridge (from pMH1701) was then inserted into BamHI site. We generated three suicide plasmids: pMBBD1 for the 27 bp deletion of fnr1, pMBBD2 for the 276 bp deletion of fnr2 and pMBBD3 for the 267 bp deletion of fnr3 (Supplementary Table S1).

Conjugation was performed between E. coli S171 containing the plasmid of interest and H. seropedicae recipient strains. Conjugation was performed on NFbHP-Malate/LA (3 : 1) agar by mixing recipient and donor strains in two proportions (50/1 and 10/1). Transconjugants were selected on NFbHP-Malate agar supplemented with 30 µg/mL cloramphenicol and antibiotics. One mutant strain resulting from a tetracycline overgrowth was grown overnight in liquid NFbHP-Malate plus 20 mM NH4Cl without antibiotics at 30°C. After incubation 250 µL of the culture were plated on NFbHP-Malate agar supplemented with 20 mM NH4Cl, 5% sucrose, 5 µg/mL nalidixic acid and 80 µg/mL streptomycin. Sucrose is toxic to bacteria that express the sacB gene, therefore only strains that lost the sacB-Rm® cassette by a second homologous recombination event could grow under these conditions. The mutant strains were analysed by PCR using primers (Supplementary Table S3) external to fnr1, fnr2 and fnr3. To construct double and triple H. seropedicae mutant strains the process described above was repeated using different allelic exchange plasmids.

RNA isolation and RNAseq library construction. For total RNA extraction, we grew H. seropedicae SmR1 (wild-type) and MB231 strains (triple fnr mutant) under aerobic conditions to an optical density of 0.4 (cultures were shaken at 120 rpm in air) and then the cultures were switched to microaerobic conditions (initial oxygen concentration 2%) for 1.5 hours. After collection of the cells by centrifugation, the RNA was isolated using a Qiagen RiboPur® extraction kit (Ambion) and subjected to microarray analysis. To test if the RNA was of high quality to proceed to RNA-seq, the RNA was subjected to RNA integrity number (RIN) analysis, an indicator of RNA damage and integrity. We found that the RIN of the RNA was not below 7 and therefore the RNA was considered to be of good quality for RNA-seq. RNA was sequenced and aligned with the Genomic H. seropedicae genome (NC_014323) as reference using the SOLiD 4 platform.

Read Mapping, Differential Expression Analysis and Fnr Binding Site Prediction. The reads were mapped against the H. seropedicae SmR1 genome (NC_014323) as reference using the SOLiD 4 platform.
reference using the CLC Genomics Workbench package. Read counts table was exported into the RobiNA software and both normalization and statistical correction were performed. Genes with fold change lower than three were excluded from the analysis. The initial oxygen concentration in the gas phase as described. Nitrogenase activity is nitrogen-fixation (nif) gene expression in Klebsiella pneumoniae. J. Bacteriol. 183, 1385–1393 (2001).

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**Author contributions**

M.B.R. conceived the work, designed and carried out experiments, analyzed the data and wrote the paper; M.Z.T.S. and H.F. carried out the construction and sequencing of RNA-seq library; R.W., M.B.R.S. and F.P. conceived the work and supervised the study; E.M.S., R.D. and R.A.M. conceived the work, supervised the study, designed experiments, analyzed the data and wrote the paper. All authors approved the final manuscript.

**Additional information**

**Supplementary information** accompanies this paper at http://www.nature.com/scientificreports

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