UNIVERSIDADE FEDERAL DO PARANÁ

Marcelo Bueno Batista

Determinação do Regulon das Proteínas Fnr1, Fnr2 e Fnr3 em *Herbaspirillum seropedicae* SmR1 em resposta à diminuição nos níveis de oxigênio

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Tese apresentada como requisito parcial para a obtenção do grau de Doutor em Ciências (Bioquímica) pelo Programa de Pós-Graduação em Ciências (Bioquímica), Setor de Ciências Biológicas, Departamento de Bioquímica e Biologia Molecular, da Universidade Federal do Paraná.

Orientadora: Profª Drª Rose Adele Monteiro
Co-orientador: Profº Drº Ray Dixon

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TERMO DE APROVAÇÃO

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Curitiba, 24 de fevereiro de 2015

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Fnr é uma proteína reguladora transcricional amplamente distribuída em organismos procariotos onde evoluiu para conectar as variações das concentrações de oxigênio no ambiente ao aparato transcricional. Estruturalmente, a proteína Fnr apresenta em sua região N-terminal um motivo conservado de quatro cisteínas, importantes para a ligação de um cluster $[4Fe\text{-}4S]^{2+}$, sensível ao oxigênio, e um domínio C-terminal contendo um motivo hélice-volta-hélice de ligação ao DNA. Sob condições limitantes de oxigênio, a ligação do cluster $[4Fe\text{-}4S]^{2+}$ provoca alterações conformacionais que permitem a dimerização e consequente ligação da proteína Fnr ao DNA alvo, para a regulação transcricional de genes essenciais à adaptação aos baixos níveis de oxigênio. A bactéria fixadora de nitrogênio *Herbaspirillum seropedicae* SmR1 apresenta três genes codificando para proteínas reguladoras transcricionais homólogas a Fnr, as quais foram designadas como Fnr1, Fnr2 e Fnr3. A função destas proteínas, na regulação da expressão gênica em resposta à limitação de oxigênio, foi determinado através da análise transcriptômica por RNA-Seq de mutantes nos genes *fnr1, fnr2* e *fnr3* e também através da caracterização fisiológica e bioquímica destes mutantes. Adicionalmente, a ligação das três proteínas Fnr aos seus promotores alvo foi determinada através da técnica de ChIP-Seq. Os resultados obtidos demonstraram que as proteínas Fnr1 e Fnr3 atuam em conjunto na modulação da composição da cadeia respiratória em resposta aos níveis de oxigênio, permitindo uma distribuição adequada do fluxo de elétrons sob condições limitantes de oxigênio e também a modulação da taxa de consumo de oxigênio durante diferentes fases de crescimento de *H. seropedicae*. A importância das proteínas Fnr para regulação da atividade da nitrogenase também foi estudado. As proteínas Fnr1 e Fnr3 parecem ser importantes para a modulação indireta da fixação de nitrogênio e da atividade da proteína NifA durante os diferentes estágios de crescimento. Este mecanismo de regulação permitiu uma eficiente adaptação da bactéria às flutuações nas concentrações de oxigênio. Adicionalmente, um provável mecanismo de regulação de alguns genes alvo de Fnr através da formação de heterodímeros entre Fnr1 e Fnr3 foi identificado. Por fim, os resultados sugerem que a atividade da proteína Fnr2 não é regulada pelos níveis de oxigênio.

Palavras-chave: *Herbaspirillum seropedicae,* Fnr, Regulação Transcricional

ABSTRACT

Fnr is a transcriptional regulatory protein widely distributed in prokaryotes which evolved to connect the changes in the environmental oxygen concentrations to the transcriptional apparatus. Structurally, the Fnr protein has at its N-terminal region a conserved motif of four cysteines that are important for the binding of an [4Fe-4S] $^{2+}$ cluster sensitive to oxygen, and a C-terminal domain containing a helix-turn-helix motif for DNA binding. Under conditions of limited oxygen, binding of the cluster $[4Fe\text{-}4S]^{2+}$ induces conformational changes that allow dimerization and subsequent binding of the Fnr protein to the target DNA for the transcriptional regulation of genes essential for the adaption to low oxygen levels. The nitrogen fixing bacteria *Herbaspirillum seropedicae* SmR1 has three genes coding for transcriptional regulator proteins homologous to the Fnr protein, which were designated as Fnr1, Fnr2 and Fnr3. In this study the role of these proteins in the regulation of gene expression in response to oxygen limitation was studied through RNA-Seq transcriptomic analysis of mutants in the *fnr1*, *fnr2* and *fnr3* genes and also through different physiological and biochemical characterizations of these mutants. Additionally, the binding of the three Fnr proteins to its target promoters was determined by using the ChIP-Seq technique. The results showed that the Fnr1 and Fnr3 proteins act together in the modulation of respiratory chain composition in response to oxygen levels, allowing an appropriate distribution of electron flow under conditions of limited oxygen and also the modulation of oxygen consumption rate during different stages of growth. Thus, the Fnr1 and Fnr3 proteins seem to be important for the indirect modulation of nitrogen fixation and activity of NifA protein during the different stages of growth. These regulatory mechanisms may allow efficient adaptation of the bacteria to fluctuations in oxygen concentrations. In addition, a potential regulation mechanism of some Fnr target genes through formation of heterodimers between Fnr1 and Fnr3 was identified. Finally the activity Fnr2 protein appears not to be regulated by the oxygen levels.

Key-words: *Herbaspirillum seropedicae*, Fnr, Transcriptional Regulation

1 INTRODUÇÃO E OBJETIVOS

O sequenciamento do genoma da bactéria fixadora de nitrogênio *Herbaspirillum seropedicae* SmR1 (PEDROSA et al., 2011) revelou a presença de três genes codificando proteínas reguladoras transcricionais homólogas a Fnr (SPIRO, STEPHEN; GUEST, 1990; SPIRO, 1994), as quais foram designadas como Fnr1 (Locus_tag[1]: Hsero_3197; Ref Seq[2]: YP003776587.1), Fnr2 (Locus_Tag: Hsero_2381; Ref Seq: YP_003775788.1) e Fnr3 (Locus_Tag: Hsero_2538; Ref Seq: YP_003775945.1). Assim como a proteína Fnr de *Escherichia coli*, as proteínas Fnr de *H. seropedicae*, apresentam em seu domínio N-terminal um motivo conservado contendo quatro cisteínas, as quais são importantes para a ligação de um cluster $[4Fe\text{-}4S]^{2+}$, e um domínio C-terminal contendo um motivo hélice-volta-hélice de ligação ao DNA, típicos de proteínas pertencentes à família Crp-Fnr de reguladores transcricionais (revisado em KORNER et al., 2003). Isto sugere que as proteínas Fnr de *H. seropedicae,* assim como a Fnr de *E. coli*, podem atuar no controle global da expressão gênica em resposta à variações nos níveis de oxigênio (O_2).

A respiração aeróbia confere um enorme benefício metabólico para as células, pois a quantidade de energia gerada é muito maior em relação à respiração de aceptores de elétrons alternativos, na ausência de O_2, como nitrato, ou ainda em relação à fermentação (UNDEN; BONGAERTS, 1997). Em seu ambiente às bactérias são frequentemente expostas à diversas variações das condições de sobrevivência, incluindo flutuações nas concentrações de oxigênio, e necessitam adaptar-se a essas mudanças de forma rápida e eficiente para que muitos processos metabólicos fundamentais não sejam prejudicados. Para que essa adaptação seja energeticamente mais eficiente, as bactérias respondem às mudanças a sua volta por meio da modificação da expressão de múltiplos genes. A resposta a estas mudanças frequentemente ocorre por meio de proteínas que se ligam ao DNA, capazes de reconhecer sequências específicas nos promotores de gene alvo para ativar ou reprimir a transcrição gênica (PEREZ; GROISMAN, 2009). O controle do início de transcrição, por proteínas reguladoras de transcrição, é uma etapa fundamental no controle da expressão gênica em resposta às mudanças ambientais.

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[1] Locus Tag: Identificador sistematicamente aplicado a cada gene durante projeto de sequenciamento de um genoma.

[2] Ref Seq: Identificador da base de dados RefSeq do NCBI (*National Center for Biotechnology Information*) . Esta é uma base de dados anotada e curada contendo sequências nucleotídicas (RNA e DNA) e seus produtos.

Deste modo o circuito definido pela conexão entre as proteínas regulatórias e os genes alvo determina o repertório de produtos gênicos que um organismo sintetiza no momento que ele encontra um sinal em particular (PEREZ; GROISMAN, 2009).

H. seropedicae é um bactéria aeróbia, capaz de fixar nitrogênio sob condições de microaerofilia e limitação de nitrogênio fixado. Os mecanismos relacionados à adaptação de *H. seropedicae* aos baixos níveis de oxigênio ainda não são bem compreendidos, porém essa bactéria apresenta um elevado potencial genético de adaptação a baixos níveis de oxigênio devido à presença de uma cadeia respiratória ramificada (PEDROSA et al., 2011). Em *H. seropedicae* SmR1, grande parte das adaptações para sobrevivência aos baixos níveis de oxigênio pode estar ocorrendo através da ação regulatória das proteínas homólogas a Fnr, assim como descrito para a proteína Fnr de *E. coli*, para as proteínas FixK1 e FixK2 de *B. japonicum* e a proteína Anr de *P. putida* KT2440 (CONSTANTINIDOU et al., 2006; UGIDOS et al., 2008; MESA et al., 2008). As proteínas FixK1, FixK2 e Anr são homólogas à Fnr de *E. coli*, fazendo parte da família Crp-Fnr de reguladores transcricionais (KORNER et al., 2003).

Com o advento das novas técnicas de sequenciamento de nova geração (SCHUSTER, 2008; KOBOLDT et al., 2013), o número de genomas sequenciados depositados em bases de dados nos últimos anos têm aumento exponencialmente. Com isso, por meio de análises *in silico*, é possível facilmente identificar outros organismos que apresentam múltiplas cópias de genes codificando para proteínas com as características de uma proteína Fnr (KORNER et al., 2003). Por exemplo, *Burkholderia pseudomalei* 1710b (TUANYOK et al., 2008) e *Herminiimonas arsenicoxydans* (MULLER et al., 2007) apresentam genes codificando para duas proteínas Fnr, enquanto *Cupriavidus metalidurans* CH34 (JANSSEN et al., 2010) e *Ralstonia eutropha* H16 (POHLMANN et al., 2006) codificam três e cinco proteínas do tipo Fnr, respectivamente.

Porém, até o presente, o papel destes múltiplos genes codificando proteínas Fnr encontrados nos diversos genomas ainda não foi determinado, com exceção de *B. japonicum* em que a função de FixK1 e FixK2 na cascata regulatória envolvendo a transmissão do sinal do baixo O_2 via FixL-J já foi estudado (MESA et al., 2008).

O objetivo desse estudo foi compreender qual a função das proteínas Fnr na regulação da expressão gênica global em *H. seropedicae.* A presença de três genes *fnr* em *H. seropedicae* permite propor algumas hipóteses quanto as suas funções:

1º : Fnr1, Fnr2 e Fnr3 apresentam diferentes sensibilidades ao oxigênio;

2º : Fnr1, Fnr2 e Fnr3 apresentam diferentes sítios de ligação ao DNA ou diferentes afinidades a determinados promotores alvo;

3º : Uma das proteínas Fnr podem ser necessária para ativação da expressão de outro gene *fnr,* o qual posteriormente transmitiria o sinal de baixo oxigênio;

4º : Uma das proteínas Fnr pode ter adquirido ao longo a evolução uma nova função, atuando em resposta a uma outra molécula sinal que não o oxigênio;

Estas hipóteses apesentam uma ampla e fascinante gama de possibilidades para o estudo do papel destas três proteínas Fnr em *H. seropedicae.* Por tudo isso, neste estudo buscou-se analisar o conjunto de genes alvo de cada proteína Fnr principalmente através do emprego das técnicas de RNA-Seq (WANG et al., 2009; NAGALAKSHMI et al., 2010) e ChIP-Seq (PARK, 2009; LEFRANÇOIS et al., 2010), aliadas ao emprego de outras análises bioquímicas e fisiológicas de mutantes em cada um dos genes *fnr* de *H. seropedicae*.

Além disso, o papel das proteínas Fnr1 e Fnr3 na fixação de nitrogênio e sobre a expressão e atividade da proteína NifA, a principal proteína ativadora transcricional dos genes *nif,* também foram estudados, pois resultados préviamente obtidos relacionaram a atividade de uma versão N-truncada da proteína NifA de *H. seropedicae* com a proteína Fnr em *E. coli.* A proteína NifA N-truncada apresentou uma redução na atividade e ainda foi mais susceptível a degradação quando foi testada na estirpe mutante *fnr*- de *E. coli* (MONTEIRO et al., 2003).

Por fim, este estudo teve como principais objetivos:

- Determinação do conjunto de genes regulados pelas três proteínas Fnr em *H. seropedicae* em resposta à diminuição dos níveis de oxigênio;
- Determinação do papel das proteínas Fnr sob a fixação de nitrogênio e atividade da proteína NifA.

2 REVISÃO DA LITERATURA

2.1 O gênero *Herbaspirillum*

O gênero *Herbaspirillum* foi assim denominado por apresentar bactérias em forma de **espiral** encontradas em associação com plantas **herbáceas** (BALDANI et al., 1986). Atualmente o gênero é composto pelas seguintes espécies[3]: *H. aquaticum* (DOBRITSA et al., 2010), *H. aurantiacum* (CARRO et al., 2012), *H. autotrophicum* (DING; YOKOTA, 2004), *H. canariense* (CARRO et al., 2012), *H. chlorophenolicum* (IM et al., 2004), *H. frisingense* (KIRCHHOF et al., 2001), *H. hiltneri* (ROTHBALLER et al., 2006), *H. huttiense* (DING; YOKOTA, 2004), *H. huttiense subsp. huttiense* (DING; YOKOTA, 2004), *H. huttiense subsp. putei* (DOBRITSA et al., 2010), *H. lusitanum* (VALVERDE et al., 2003), *H. massiliense* (LAGIER et al., 2012), *H. putei* (DING; YOKOTA, 2004), *H. rhizosphaerae* (JUNG et al., 2007), *H. rubrisubalbicans* (BALDANI et al., 1996), *H. seropedicae* (BALDANI et al., 1986) e *H. soli* (CARRO et al., 2012).

Os membros do gênero *Herbaspirillum* foram isolados principalmente do solo, e plantas. Bactérias deste gênero têm sido estudadas principalmente por serem promotores do crescimento vegetal, porém *H. rubrisubalbicans* é uma espécie endofítica patogência para uma variedade específica de cana-de-açucar (revisado em MONTEIRO et al., 2012). Adicionalmente, bactérias deste gênero também já foram isoladas de pacientes com leucemia (ZIGA et al., 2010; CHEN et al., 2011). Outros isolados incluem potenciais patógenos em aneurismas aórticos (MARQUES DA SILVA et al., 2006) bactérias presentes em secreções respiratórias de pacientes com fibrose cística (COENYE et al., 2002; SPILKER et al., 2008) e mais recentemente *H. massiliense* foi isolada a partir de fezes (LAGIER et al., 2012).

2.1.2 *Herbaspirillum seropedicae*

H. seropedicae, uma bactéria gram-negativa, da classe β das proteobactérias, foi o primeiro representante do gênero *Herbaspirillum* descrito. É uma bactéria endofítica, sendo isolada da rizosfera e dos tecidos de plantas importantes economicamente como

[3] A lista de espécies do gênero *Herbaspirillum* foi atualizada de acordo com o website do LSPN, do inglês: "*List of Prokaryotic names with Standing in Nomenclature"* acessado através do link: http://www.bacterio.net/index.html em fevereiro de 2014.

cana-de-açúcar, milho, trigo e arroz (BALDANI et al., 1986). Por ser endofítico, em contraste com diazotrofos da rizosfera que competem com a microflora do solo por fontes de carbono, *H. seropedicae* vive em um ambiente mais uniforme e protegido, o interior dos tecidos da planta, o que pode explicar sua contribuição na fixação biológica do nitrogênio (OLIVARES et al., 1996). Além de converter o nitrogênio atmosférico a amônio, é capaz de produzir promotores do crescimento vegetal como o ácido indolacético, giberilinas e citoquininas (REIS et al., 2000). A inoculação de arroz com *H. seropedicae* leva a um aumento do peso seco das plantas (JAMES et al., 2002). Outros estudos mostraram que a estirpe de *H. seropedicae* (LR15) inoculada em arroz foi capaz de colonizar a superfície das raízes e os tecidos internos, e causar um aumento da biomassa radicular (RONCATO-MACCARI et al., 2003).

O sequenciamento do genoma da estirpe SmR1 de *H. seropedicae* (PEDROSA et al., 2011) juntamente com estudos de interação *Herbaspirillum*-bactéria, revisado em MONTEIRO et al., 2012, revelam o grande potencial de aplicação de bactérias deste gênero como biofertilizante. Os biofertilizantes podem ser definidos como substâncias baseadas em micro-organismos vivos que quando aplicados à semente, superfície da planta ou ao solo, colonizam a rizosfera ou o interior da planta promovendo o seu crescimento. Esta promoção do crescimento pode se dar pelo suprimento de nutrientes primários para a planta hospedeira ou pelo aumento da bio-disponibilidade destes nutrientes (VESSEY, 2003).

2.2 Fixação Biológica de Nitrogênio (FBN)

O nitrogênio é um elemento de grande importância, pois é componente essencial das proteínas, ácidos nucléicos e outras biomoléculas. Depois da água é o principal fator limitante no desenvolvimento e crescimento de plantas. O nitrogênio representa cerca de 2% do peso seco total da planta. As plantas não são capazes de diretamente utilizar o dinitrogênio gasoso - $N_{2(g)}$ - o qual compõe cerca de 80% da atmosfera, por isso absorvem o nitrogênio do solo através de suas raízes principalmente nas formas de amônio ou nitratos. A limitada bio-disponibilidade do nitrogênio e a dependência deste elemento para o crescimento de plantas importantes na agricultura têm levado ao aumento da produção de fertilizantes nitrogenados no mundo (SANTI et al., 2013). O uso destes fertilizantes podem causar diversos danos ambientais, como a emissão de

óxidos nitrosos tóxicos, a eutrofização de lagos e rios e a acidificação do solo (DIXON; KAHN, 2004).

Somente um pequeno grupo de procariotos é capaz de utilizar o dinitrogênio atmosférico, por meio de um processo chamado fixação biológica de nitrogênio (FBN) (YOUNG, 1992). Este processo consiste na conversão no N_2(g) a NH_3, uma forma que pode ser usada pelas plantas. As bactérias responsáveis pela fixação de nitrogênio são chamadas de diazotróficas e possuem um complexo enzimático denominado de nitrogenase, bastante conservado em diazotrofos simbiontes e de vida livre, responsável pela conversão do nitrogênio gasoso em amônia (BURRIS, 1991).

2.2.1 Tipos de interação entre planta-microrganismo para a FBN

Diferentes tipos de associações e/ou interações ocorrem entre bactérias diazotróficas e suas respectivas plantas hospedeiras para fixação biológica de nitrogênio (FBN). A interação mais específica e eficiente para a FBN envolve a formação de nódulos radiculares, principalmente em plantas leguminosas. A formação destes nódulos envolve a interação endossimbiótica dos rizóbios, um grupo de bactérias Gram-negativas pertencentes às Alphaproteobacteria, com leguminosas (família Fabaceae) (revisado em OLDROYD; DOWNIE, 2008). Outro grupo de plantas não-leguminosas, do gênero *Parasponia* (família Cannabaceae), evoluiu independentemente a capacidade de estabelecer uma relação endossimbiótica com os rizóbios (OP DEN CAMP et al., 2012). Ainda, existem um grupo de bactérias Grampositivas da família dos actinomicetos, gênero *Frankia sp*., que podem formar nódulos quando em simbiose com diversas plantas de 8 famílias diferentes, chamadas coletivamente de plantas actinorrízicas (revisado em VERGHESE; MISRA, 2002). Além disso, também foram encontradas cianobactérias fixadoras de nitrogênio (principalmente do gênero *Nostoc sp*.) colonizando diferentes órgãos da planta, seja intracelularmente ou extracelularmente (SANTI et al., 2013).

Em contraste com estas interações simbióticas, alguns diazotrofos, como os do gênero *Azospirillum* spp., *Azoarcus* spp. e *Herbaspirillum* formam interações associativas e/ou endofíticas com as raízes de cereais da família Poaceae. Há relatos práticos no campo, de que grande parte do nitrogênio utilizado por cereais importantes agronomicamente é derivado da FBN, ainda que o nitrogênio fixado nas interações do

tipo associativas ou endofíticas, não seja tão grande quando comparado à quantidade medida em interações endossimbióticas (revisado em SANTI et al., 2013).

Em todas esssas interaçãoes entre a planta e a bactéria, seja do tipo associativa, endofítica ou simbiótica, o benefício esperado para a planta hospedeira seria o nitrogênio fixado provido pelo o parceiro procarioto, o qual recebe em troca uma fonte de carbono altamente reduzida e também alguns outros nutrientes. Ainda, o ambiente formado durante a interação planta-bactéria, pode prover condições apropriadas para proteger o complexo enzimático da nitrogenase da exposição ao oxigênio (SANTI et al., 2013).

As bactérias que colonizam a rizosfera e são capazes que promover algum efeito benéfico promovendoo crescimento de plantas, são chamadas de rizobactérias promotoras do crescimento de plantas (RPCP) (KLOEPPER; BEAUCHAMP, 1992).

As bactérias encontradas em uma associação íntima na superfície externa das raízes são designadas bactérias associativas (ELMERICH, 2007). Já as bactérias endofíticas são definidas como as bactérias extraídas dos tecidos internos das plantas que tiveram sua superfície esterelizada superficialmente, mas deve-se ter uma evidência microscópica da localização interna nos tecidos da planta (HALLMANN et al., 1997; REINHOLD-HUREK; HUREK, 1998).

Em contraste com os endossimbiontes, não é possível observar a formação de uma estrutura diferenciada, como os nódulos, no processo de colonização de plantas por bactérias associativas ou endofíticas. As bactérias endofíticas invadem os tecidos da planta, porém não colonizam as células internamente. Estas bactérias, como *Herbaspirillum seropedicae* SmR1, colonizam apenas os espaços intercelulares das plantas (MONTEIRO et al., 2012). Os diazotrofos endofíticos podem apresentar uma vantagem sobre os diazotrofos associativos, pois como colonizam o interior das raízes, podem se estabelecer em nichos específicos que provém condições mais apropriadas para uma fixação de nitrogênio mais efetiva e subsequentemente uma melhor transferência deste nitrogênio fixado para a planta hospedeira (REINHOLD-HUREK; HUREK, 1998, 2011).

As rizobactérias diazotróficas foram identificadas em diversos gêneros de proteobactérias, incluindo os gêneros *Acetobacter*, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Burkholderia*, *Enterobacter*, *Herbasprillum*, *Gluconoacetobacter* e *Pseudomonas* (revisado em BALDANI et al., 1986; DÖBEREINER et al., 1993; VESSEY, 2003; SCHMID; HARTMANN, 2007;

RICHARDSON et al., 2009). Entre estes, *Azoarcus* spp., *Herbaspirillum seropedicae* e *Gluconoacetobacter* são reconhecidos como endofíticos. Estes diferem de outras rizobactérias, como *Azospirillum* e *Azotobacter*, no sentido em que apresentam uma associação muito íntima com plantas, não sendo capazes de sobreviver bem no solo (REINHOLD-HUREK; HUREK, 1998). Algumas bactérias endofíticas listadas acima contribuem significativamente para a melhora do crescimento de plantas como milho, arroz e trigo, em condições controladas e/ou em campo (revisado em SANTI et al., 2013).

2.3 Regulação da Fixação Biológica de Nitrogênio em *Herbaspirillum seropedicae*

A regulação da fixação biológica de nitrogênio em *H. seropedicae* têm sido extensivamente estudada (revisado em (PEDROSA et al., 2001, 2011; CHUBATSU et al., 2012). Os genes relacionados a fixação de nitrogênio em *H. seropedicae* estão localizados numa região contígua de aproximadamente 40 Kb, contendo 46 ORFs compreendendo pelo menos sete operons que estão sobe o controle do fator sigma-54 da RNA polimerase e do ativador de transcrição NifA (Figura 2-1).

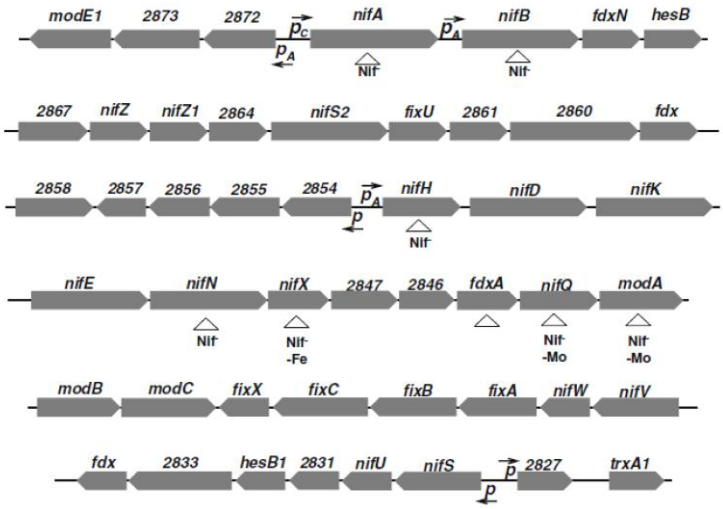

Figura 2-1. Organização estrutural dos genes *nif* em *H. seropedicae* SmR1. As flechas em cinza representam os genes, enquanto que os triângulos brancos representam mutações com fenótipo caracterizado como Nif-, ou deficiente na fixação de nitrogênio. –Mo e –Fe indica um fenótipo Nif- em meio com deficiência de molibdênio e ferro, respectivamente. *p*A e *p*C indicam promotores dependentes de NifA e NtrC, respectivamente. *p* indica um promotor provavelmente regulado por NifA, inferido por análise de sequência. Números indicam ORFs preditas de acordo com o genoma de *H. seropedicae* SmR1. Fonte: Chubatsu et al., 2012.

Em *H. seropediocae,* os genes estruturais para o complexo enzimático da nitrogenase (*nifHDK*), bem como outros genes relacionados com sua atividade e síntese de cofatores, estão localizados em um único operon *nifHDKENXHsero_2847Hsero_2846fdxA* (KLASSEN et al., 1999, 2003) . Este operon é transcrito a partir de um promotor à montante de *nifH* (MACHADO et al., 1996; KLASSEN et al., 1999; SOUZA et al., 2010). Um sítio de ligação para IHF e dois sítios para NifA foram localizados neste promotor (MACHADO et al., 1996). O nível de transcrição de *nifH* é duas vezes maior em relação a *nifD* ou *nifK*, sugerindo atenuação da transcrição no operon (PEDROSA et al., 2001). O gene *nifB* de *H. seropedicae* está localizado a jusate de *nifA* e codifica uma proteína envolvida na síntese do cofator FeMoco da nitrogenase. O promotor do gene *nifB* contém dois sítios de ligação a NifA e um promotor do tipo σ^{54}. Adicionalmente, a expressão de *nifB* foi estimulada por IHF em *E. coli* e experimentos de DNA *band-shift* mostraram que a proteína IHF liga-se ao promotor do gene *nifB* de *H. seropedicae* (REGO et al., 2006).

A expressão dos genes *nif* requer a proteína NifA, a qual é uma proteína da família das EBPs (do inglês: *enhancer binding proteins*) dependentes do fator sigma-54 da RNA polimerase. As proteínas desta família ativam a transcrição da seguinte maneira: a RNA polimerase-σ^{54} liga-se a sequência promotora no DNA formando um complexo fechado que é incapaz de iniciar a transcrição. Para a formação do complexo aberto as proteínas EBP ligam-se a uma sequência à montante do promotor (~150 pares de base). Subsequentemente, uma dobra no DNA, em geral induzida pela proteína IHF, permite que a EBP (NifA) faça contato com a RNA polimerase-σ^{54} ligada ao promotor. A seguir, a hidrólise do ATP pela EBP provoca a isomerização do complexo fechado, a formação do complexo aberto e início da transcrição (WIGNESHWERARAJ et al., 2008).

Em *H. seropedicae* a proteína NifA responde aos níveis de oxigênio e nitrogênio fixado (SOUZA et al., 1999) e sua expressão está sob o controle do sistema Ntr (CHUBATSU et al., 2012). O sistema Ntr controla o metabolismo de nitrogênio de maneira global em bactérias e inclui diversas proteínas que regulam a expressão de genes requeridos para a mobilização de fontes de nitrogênio em condições de limitação de amônio (MERRICK; EDWARDS, 1995). Em *H. seropedicae* esse sistema é composto pela uridiltransferase GlnD, pelas proteínas da família PII GlnB e GlnK, o transportador de amônio AmtB, a glutamina sintetase GlnA, a adeniltransferase GlnE e o sistema de dois componentes NtrB-NtrC (CHUBATSU et al., 2012). Diversos estudos

de caracterização das proteínas do sistema Ntr em *H. seropedicae* (revisado em CHUBATSU et al., 2012), sugerem que este sistema é bastante similar ao descrito para *E. coli* e outros diazotrofos (para revisão ver: NINFA et al., 2001; ARCONDÉGUY et al., 2001). Um esquema da atuação do sistema Ntr na regulação da fixação de nitrogênio em *H. seropedicae* e também na atividade da proteína NifA é apresentado na Figura 2-2.

2.3.1 Regulação da expressão e atividade da proteína NifA

A transcrição do gene *nifA* em *H. seropedicae* é dependente da RNA polimerase σ54 e é ativada por NtrC sob condições limitantes de nitrogênio (SOUZA et al., 1999, 2000). O promotor do gene *nifA* é bastante complexo em *H. seropedicae*, contendo dois sítios par NtrC, três prováveis sítios para NifA e um sítio para ligação de IHF (*integration host factor*) (SOUZA et al., 1991). A expressão de *nifA* é primariamente dependente de NtrC, e enquanto NifA não é essencial para sua própria transcrição, é requerida para sua expressão máxima (WASSEM et al., 2002). Considerando que a atividade da proteína NifA é sensível ao oxigênio, a expressão de *nifA* em condições limitantes de oxigênio é 50% maior em condições limitantes de oxigênio (WASSEM et al., 2002). A proteína IHF exerce duas funções sobre o promotor *nifA*. Ativa a transcrição dependente de NtrC e reprime a transcrição dependente de NifA, prevenindo a super-expressão da proteína NifA (WASSEM et al., 2000, 2002).

A atividade da proteína NifA de *H. seropedicae* é regulada em resposta aos níveis de oxigênio e de nitrogênio fixado, sendo que a NifA é diretamente inativada em resposta ao aumento dos níveis de oxigênio (SOUZA et al., 1999). O mecanismo de regulação da atividade da proteína NifA por oxigênio não é completamente compreendido, porém provavelmente envolve um motivo de quatro cisteínas localizados entre o domínio central e o linker IDL. Souza e colaboradores (1991) propuseram que esse motivo de cisteína seria provavelmente responsável pela ligação de um grupamento metálico, que agiria como um sensor direto de oxigênio (SOUZA et al., 1991). Análises de mutagênese sítio-dirigida mostraram que cada uma das cisteínas do motivo, é essencial para a atividade da proteína. Porém estes resíduos não interferem na capacidade da proteína NifA em ligar o DNA (OLIVEIRA et al., 2009).

Já a regulação da proteína NifA em resposta aos níveis de nitrogênio fixado, em *H. seropedicae*, se dá por meio da interação da proteína PII, GlnK, com o domínio GAF de NifA (OLIVEIRA et al., 2012). Mutantes *glnK*- tiveram a atividade de nitrogenase

bloqueada, muito embora a expressão de *nifA* estivesse ocorrendo a níveis normais. O fenótipo de fixação de nitrogênio foi restaurado quando a estirpe *glnK*- foi complementada com uma plasmídeo expressando GlnK, ou também com a expressão de uma NifA N-truncada (sem o domínio GAF) de *H. seropedicae* (NOINDORF et al., 2011). Em acordo com esses resultados, outros autores já havia demonstrado que na ausência do domíno N-terminal a atividade da proteína NifA não regulada pelos níveis de nitrogênio fixado (SOUZA et al., 1999; MONTEIRO et al., 1999a, 1999b).

Os resultados descritos acima sugerem o seguinte mecanismo para o controle da expressão dos genes *nif* em *H. seropedicae.* Sob condições de excesso de nitrogênio, a proteína GlnB, constitutivamente expressa, está na sua forma não uridirilada, o que permite que NtrB mantenha NtrC na sua forma inativa não fosforilada. Como resultado a expressão do operon *nlmAglnKamtB* é reprimida (Figura 2-2). Por outro lado, quando as concentrações de nitrogênio fixado se tornam limitantes, a expressão de glnK ocorre a partir do promotor *nlmAglnKamtB* , que é NtrC-dependente. GlnK é rapidamente uridililada pela proteína GlnD. Nessas condições, GlnK na sua forma uridirilada interage com o domínio GAF na região N-terminal de NifA, liberando a interação inibitória do doímino GAF com o domíno catalítico de NifA, provocando a ativação da proteína NifA e consequente ativação dos genes *nif* (Figura 2-2). Reciprocamente, quando os níveis de nitrogênio fixado aumentam, GlnK é rapidamente desuridirilada, formando um complexo GlnK-NifA inativo. Sendo assim, o complexo GlnK-UMP-NifA é ativo, enquanto o complexo GlnK-NifA inibi a atividade de NifA. Esse mecanismo deve permitir uma rápida modulação da expressão dos genes *nif* em *H. seropedicae* em resposta à disponibilidade de amônio extracelular (CHUBATSU et al., 2012).

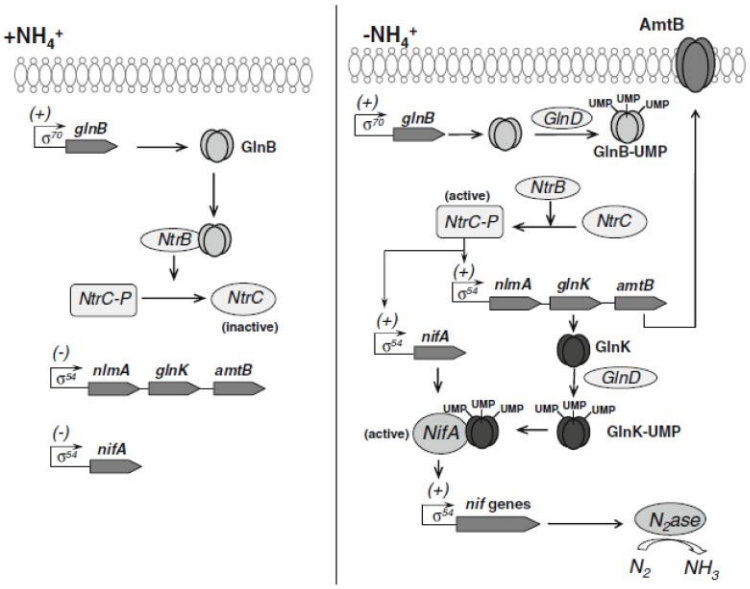

Figura 2-2. Regulação da fixação de nitrogênio pelo sistema Ntr em resposta aos níveis de amônio em *H. seropedicae* SmR1. GlnB é constitutivamente expressa a partir de um promotor σ70. Em altos níveis de nitrogênio fixado, o aumento na concentração da glutamina induz a desudirilação da GlnB pela proteína GlnD. Nestas condições, NtrB desfosforila NtrC, inativando-a (painel esquerdo). Sob baixos níveis de nitrogênio, NtrB fosforila NtrC, a qual ativa a transcrição de genes alvo, como *nifA* e o operon *nlmAglnKamtB.* Nestas condições, GlnB e GlnK estão totalmente uridiriladas por meio da atividade da proteína GlnD. A proteína GlnK uridirilada, ativa aproteína NifA, a qual, por sua vez, ativa a transcrição de outros genes nif e consequentemente permite a biossíntese do complexo enzimático da nitrogenase em sua forma ativa (painel direito). **Fonte: Chubatsu et al., 2012.**

2.4 A família Crp-Fnr dos reguladores transcricionais

Os membros da família Crp/Fnr dos reguladores transcricionais apresentam basicamente dois domínios: um domínio N-terminal sensório capaz de ligar-se ou monitorar pequenas moléculas efetoras (O_2, NO_3^-, NO, CO AMPc, 2-oxoglutarato, etc) e um domínio C-terminal do tipo hélice-volta-hélice de ligação ao DNA (revisado em KORNER et al., 2003). Os dois principais, e melhor caracterizados, membros dessa família são as proteínas Crp e Fnr. Historicamente a proteína Crp, foi o primeiro fator de transcrição a ser purificado (ZUBAY et al., 1970; EMMER et al., 1970) e ter sua estrutura cristalográfica resolvida (MCKAY; STEITZ, 1981). A proteína Crp é um regulador transcricional implicado no controle da repressão catabólica (STÜLKE; HILLEN, 1999). Na presença de glucose, os níveis de AMP cíclico em *E. coli* diminuem e assim não ocorre a ativação da proteína Crp e a consequente expressão de genes implicados na utilização de outros açúcares. A proteína Crp é ativada quando AMP cíclico liga-se ao seu domínio N-terminal. A ligação do AMPc leva a uma alteração conformacional na proteína, que permite sua dimerização e ligação ao DNA.

A proteína Crp atua como um regulador de transcrição global controlando mais de 200 genes em *E. coli* (ZHENG et al., 2004; SHIMADA et al., 2011). Em contraste, a proteína Fnr, é um regulador transcricional envolvido na regulação da expressão gênica em resposta ao oxigênio (SPIRO; GUEST, 1990; GREEN et al., 2001). Quando os níveis de oxigênio se tornam limitantes, há a ligação de um grupamento $[4Fe\text{-}S]^{2+}$ no domínio N-terminal da Fnr, o que provoca alterações conformacionais que levam a sua dimerização e consequente ligação ao DNA (LAZAZZERA, B. A. et al., 1996). O grupamento $[4Fe\text{-}S]^{2+}$ da proteína Fnr de *E. coli* é ancorado na região N-terminal através de 4 resíduos de cisteína, Cys20-Cys23-Cys29-Cys122 (SHARROCKS et al., 1990). O domínio de ligação ao DNA da proteína Fnr, localizado na região C-terminal, tem alta similaridade com o domínio de ligação ao DNA presente na proteína Crp (SPIRO et al., 1990).

A primeira classificação da família Crp/Fnr dividiu as proteínas desta família em três grupos baseados nas informações acerca de seus domínios funcionais: i) um grupo representado principalmente por Fnr e FixK; ii) um grupo contendo a proteína Crp e iii) um grupo contendo a principalmente as proteínas CysR e NtcA (FISCHER, 1994). Mais tarde com o aumento do número de proteínas da família Crp/Fnr nas bases de dados, uma análise filogenética mais compreensiva classificou 365 membros da família em 21 grupos (KORNER et al., 2003). Outro estudo focado nas proteínas Fnr, FixK e DNR de Alpharoteobacteria, revelou algumas características co-evolutivas dos reguladores transcricionais da família Crp/Fnr em relação aos seus respectivos sítios de ligação ao DNA (DUFOUR et al., 2010).

Recentemente MATSUI e colaboradores (2013) propuseram uma maior e mais compreensiva classificação dos membros da família Crp/Fnr, apresentando também uma proposta sobre a origem de seus integrantes. Estes autores fizeram uma busca por homólogos das proteínas Crp/Fnr nos genomas plasmidiais e cromossomais de 1969 espécies com genoma depositado no NCBI em junho de 2012, sendo 1845 espécies de bactérias e 124 espécies de árqueas. Nesta análise os autores classificaram 1455 proteínas anotadas como homólogas as proteínas da famíla Crp/Fnr, em 12 diferentes grupos, e também fizeram uma proposta sobre como a diversidade funcional dos membros da família Crp/Fnr surgiu (MATSUI et al., 2013). Apesar de a classificação filogenética apresentada por KORNER e colaboradores (2003) ter utilizado apenas 365 proteínas, esta sugeriu pela primeira vez uma classificação filogenética, bastante útil para a descrição de novos membros da família Crp/Fnr que têm surgido à medida que novos genomas vão sendo sequenciados e disponibilizados nos bancos de dados.

A classificação filogenética proposta por Korner e colaboradores, consiste em 21 grupos taxonômicos na família Crp/Fnr. Dentre estes grupos, as proteínas dos grupos Fnr, FnrN e FixK, exercem sua atividade regulatória por meio do monitoramento direto ou indireto do sinal de baixo oxigênio intracelular. As proteínas dos grupos Fnr e FnrN, possuem um motivo de cisteínas conservadas no domínio N-terminal para ligação do cluster $[4\text{-Fe-}4S]^{2+}$ permitindo o monitoramento direto dos níveis de O_2. A única diferença entre estes grupos é o espaçamento do motivo de cisteínas. No grupo Fnr tal motivo apresenta a configuração $Cys\text{-}X_2\text{-}Cys\text{-}X_5\text{-}Cys\text{-}X_n\text{-}Cys_{(central)}$, enquanto que no grupo FnrN a configuração $Cys\text{-}X_2\text{-}Cys\text{-}X_7\text{-}Cys\text{-}X_n\text{-}Cys_{(central)}$ é observada (KORNER et al., 2003). Já as proteínas do grupo FixK, sendo a proteína FixK2 de *Bradyrhizobium japonicum* a principal representante, não apresentam estas cisteínas conservadas e recebem o sinal de baixo O_2 através do sistema de dois componentes FixLJ (NELLEN-ANTHAMATTEN et al., 1998).

2.4.1 Função da proteína Fnr de *E.coli* e outras ortólogas da família Crp/Fnr

A proteína Fnr de *E. coli* é a melhor caracterizada de seu grupo (dentro da família Crp/Fnr) tanto bioquímica, quanto funcionalmente. Caracterizações bioquímicas permitiram a completa elucidação do processo de montagem e degradação do cluster $[4Fe\text{-}4S]^{2+}$ (CRACK et al., 2008) e também como a protease ClpXP (METTERT; KILEY, 2005) influencia na dinâmica ou no controle dos níveis de Fnr em *E. coli* em resposta aos níveis de oxigênio, permitindo até mesmo construção de modelos matemáticos que descrevem esse processo (TOLLA; SAVAGEAU, 2010, 2011).

Até o momento, ainda não há uma estrutura para a proteína Fnr, mas devido a sua similaridade de sequência a mesma pode ser modelada sobre a estrutura da proteína Crp de *E. coli* (SCHULTZ et al., 1991). Recentemente, a estrutura da proteína FixK2 de *B. japonicum* ligada ao DNA foi descrita (BONNET et al., 2013). Tal estrutura só pôde ser obtida quando a cisteína da posição 183 (C183) foi mutada por uma serina e uma cauda de histidinas foi adicionada a região C-terminal, o que sugeriu que a oxidação do resíduo Cys-183 poderia estar envolvida na diminuição da afinidade de ligação ao DNA. Essa suspeita foi confirmada através da análise da estrutura fina da proteína ligada ao DNA. Os autores discutem que a conversão do grupamento tiol do resíduo Cys-183 a um grupamento $-SO_2^-$ ou SO_3^- poderia causar não somente um impedimento estérico da interação proteína-DNA, mas também repulsão eletrostática

com o grupo fosfato negativamente carregado da cadeia do DNA (BONNET et al., 2013). Adicionalemente, o resíduo Cys-183, presente próximo ao domínio de ligação ao DNA, havia sido previamente implicado num mecanismo de sensibilidade de FixK2 a agentes oxidantes e espécies reativas de oxigênio (MESA et al., 2009). Sendo assim, pode-se concluir que FixK2 também é sensível ao status redox celular (MESA et al., 2009; BONNET et al., 2013).

No que diz respeito à definição do regulon da proteína Fnr, diversos estudos utilizando abordagens do tipo gene por gene, e também em escala genômica utilizando-se técnicas como microarranjos, RNA-Seq, ChIP-on-chip e ChIP-Seq já foram realizados e ajudaram a elucidar o papel da proteína Fnr de *E. coli* como um regulador global de transcrição em resposta a diminuição dos níveis de oxigênio (SALMON et al., 2003; KANG et al., 2005; CONSTANTINIDOU et al., 2006; GRAINGER et al., 2007; MYERS et al., 2013).

O primeiro estudo em escala global para avaliar o efeito de Fnr e do oxigênio na regulação da expressão gênica foi realizado por Salmon e colaboradores (2003). Estes autores identificaram que cerca de 1/3 do genoma de *E. coli*, ou mais precisamente 1445 genes de um total de 4290, foram diferencialmente expressos em resposta à diminuição dos níveis de oxigênio. Destes1445 genes, 1256 genes eram direta ou indiretamente regulados por Fnr (SALMON et al., 2003). Mais tarde, Kang e colaboradores (2005), em um estudo semelhante, encontraram um conjunto de 962 genes regulados em resposta a Fnr e oxigênio em *E. coli* (KANG et al., 2005). Uma comparação destes dois primeiros estudos revelou que somente 334 genes eram comuns aos dois conjuntos de dados. Ainda assim, 123 genes apareceram como sendo regulados em direções opostas, revelando que somente 211 genes mostraram respostas regulatórias semelhantes nos dois estudos, ou seja, apenas cerca de 10% de todos os 2073 genes com regulação diferencial (CONSTANTINIDOU et al., 2006).

Em 2006 Constantinidou e colaboradores realizaram um novo estudo sobre o papel de Fnr na adaptação de *E. coli* aos baixos níveis de oxigênio na presença de nitrato e nitrito, visando expandir o conhecimento dos mecanismos de co-regulação envolvendo Fnr e o sistema de dois componentes composto por NarXL e NarQP (CONSTANTINIDOU et al., 2006). Neste estudo, além de análises trancriptômicas por microarranjo de mutantes *fnr*, *narXL* e *narP*, resultados de ChIP-on-chip de uma proteína Fnr-3xFlag também foram obtidos. Em resumo, os autores estabeleceram que 103 operons são regulados por Fnr, sendo 68 ativados e 35 reprimidos. Dos 103

operons, 24 foram também co-regulados por NarXL e Fnr, 12 co-regulados por NarQP e Fnr e 7 co-regulados por NarXL, NarQP e Fnr (CONSTANTINIDOU et al., 2006). Grainger e colaboradores (2007), também realizaram estudos de ChIP-on-chip utilizando uma proteína Fnr-3xFlag (GRAINGER et al., 2007). Neste estudo muitos sítios novos de ligação para Fnr foram identificados, mas considerando que as condições de cultivo empregadas por Grainger e colaboradores foram muito diferentes das condições utilizadas nas três outras análises já discutidas, os dados não podem ser diretamente comparados (MYERS et al., 2013).

Em 2013, um estudo bastante complexo re-analisou a distribuição dos sítios de ligação da proteína Fnr no genoma de *E. coli* (MYERS et al., 2013). Neste estudo, Myers e colaboradores, identificaram todos os sítios prováveis de ligação para Fnr utilizando ferramentas de bioinformática e também novos dados de ChIP-on-chip e ChIP-Seq da proteína Fnr. A influência de 3 proteínas NAP ("*nucleoid associated proteins*"), como HN-S, IHF e Fis, sobre a ocupação dos sítios preditos de ligação para Fnr no genoma foi avaliado. Em resumo, 187 sítios prováveis de ligação a Fnr foram identificados, através de análises de bioinformática, no genoma de *E. coli*. Porém, surpreendentemente menos da metade (63 de 187 sítios) tiveram correspondência com um pico de ligação ao DNA na análise do ChIP-Seq. Ainda que os autores tenham considerado a possibilidade de alguns sítios de ligação a Fnr terem sequências flanqueadoras que desfavoreçam a ligação de Fnr, eles sabiam através da revisão da literatura que proteínas NAP poderiam afetar a ligação de outros reguladores de transcrição ao DNA (BROWNING et al., 2010; RIMSKY; TRAVERS, 2011). Para avaliar se as proteínas HN-S, IHF e Fis poderiam afetar a ligação de Fnr a alguns desses sítios "silenciados", uma análise de ChIP-on-chip das proteínas HN-S e IHF, nas mesmas condições das realizadas para a proteína Fnr, juntamente com uma análise de ChIP-Seq publicamente disponível para a proteína Fis (KAHRAMANOGLOU et al., 2011) foi analisada. Aproximadamente todos os sítios silenciados para a ligação da proteína Fnr (111 de 124 sítios) estavam enriquecidos nas análises de imunoprecipitação (ChIP) das proteínas HN-S, IHF ou Fis (MYERS et al., 2013).

Ainda neste último estudo citado os autores encontraram 122 operons diferencialmente expressos em resposta ao oxigênio, porém apenas 63 apresentaram um sítio de ligação para Fnr. Destes 32 operons foram diretamente ativados por Fnr, 21 diretamente reprimidos e outros 10 tiveram a ativação por Fnr inibida pela atividade repressora da proteína Fur. Sendo assim fazendo uma compilação de todos os estudos

de transcriptoma já citados (a saber: SALMON et al., 2003; KANG et al., 2005; CONSTANTINIDOU et al., 2006; GRAINGER et al., 2007), MYERS e colaboradores construíram um modelo para a rede regulatória envolvendo a proteína Fnr em *E. coli* apresentado na Figura 2-3 (MYERS et al., 2013).

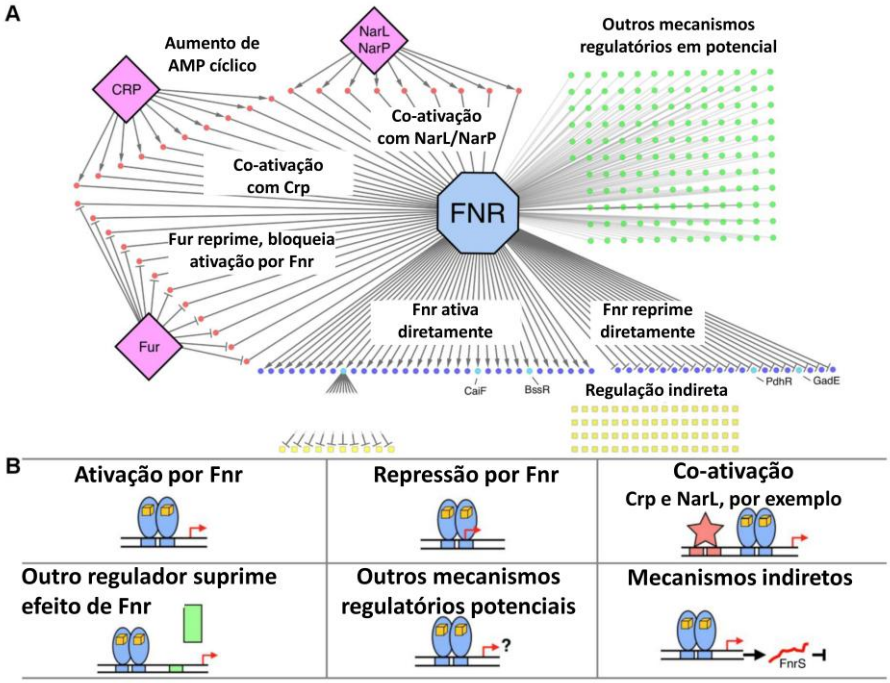

Figura 2-3. Representação esquemática da rede regulatória e das categorias de regulação da proteína Fnr. A - Esquema da rede regulatória da proteína Fnr. A Fnr é mostrada como um octógono azul, enquanto outros reguladores transcricionais (Crp, Fur e Narl) são representados como diamantes cor-de-rosa. Círculos representam os operons com um pico de ligação a Fnr revelado por ChIP-Seq. Círculos em azul escuro, representam operons diretamente dependente de Fnr para expressão, enquanto círculos em azul claro representam alguns promotores que apresentam regulação dependente do RNA pequeno FnrS. Quadrados amarelos representam operons regulados pelos fatores de transcrição CaiF, BssR, PdhR e GadE) que presumidamente controlam a expressão gênica de maneira indireta são representados por quadrados amarelos. Círculos em vermelho representam operons conhecidos por serem co-regulados por Fnr e outro regulador enquanto que os círculos em verde representam outros mecanismos em potencial. B – Cada caixa representa uma categoria de regulação identificada pelo estudo realizado por Myers e colaboradores 2013. Nas categorias 1 e 2 (caixas de cima da esquerda e do meio respectivamente) apresentam ativação ou repressão direta por Fnr (figuras ovais em azul), respectivamente. A categoria 3 (caixa de cima da direita) apresenta a co-ativação com outros fatores de transcrição (por exempo: Crp, NarL ou NarP – estrela vermelha), já a categoria 4 (caixa de baixo à esquerda) mostra que a repressão por um fator de transcrição (por exemplo: Fur – retângulo verde) previne a regulação por Fnr. Na categoria 5 (caixa de baixo ao centro) representa operons com mecanismos de regulação por Fnr ainda não caracterizados, já a categoria 6 (caixa de baixo à direita) mostra a regulação indireta via outros reguladores, como por exempo o pequeno RNA regulatório FnrS (linha vermelha). **Fonte: Adaptado de Myers et al., 2013.**

Além de análises em escala genômica realizadas para a proteína Fnr de *E. coli*, outra proteína de interesse da família Crp/Fnr que se tem bastante informação a respeito de seu regulon é a proteína FixK2 de *B. japonicum.* Análise de microarranjos de DNA

de estirpes mutantes nos genes *fixJ*, *fixK1* e *fixK2*, em comparação com a estirpe selvagem em condições de vida-livre sob condições de microaerofilia (0,5% de O_2) ou em condições simbióticas no caso do mutante *fixK2*, foi utilizado para se obter um entendimento da cascata regulatória envolvendo as proteínas FixLJ, FixK1 e FixK2 (MESA et al., 2008). Neste estudo ficou evidente que o regulon da proteína FixK2 é bem maior do que o das proteína FixJ e FixK1, confirmando o papel central de FixK2 na transmissão do sinal de baixo O_2 nesta cascata regulatória. Um resumo desta cascata revelada por esses autores é apresentado na Figura 2-4.

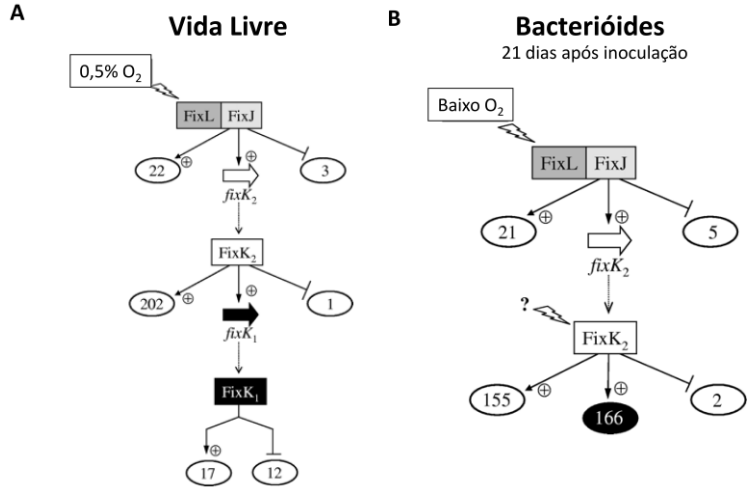

Figura 2-4. Esquema da cascata regulatória desencadeada pelo sinal de baixo O_2 em *B. japonicum*. A - Esquema da cascata regulatória das bactérias em vida livre. Sob condições de microaerofilia (0,5% de O_2) a proteína FixL fosforila FixJ, que por sua vez atua na regulação de 26 genes incluindo *fixK2*. FixK2 expande a cascata regulando uma série de outros genes, incluindo *fixk1*. FixK1, por sua vez atua na regulação de mais alguns genes somente. O regulon da proteína FixK1 foi detectado somente no modo de vida livre. B – Esquema da cascata regulatória nos bacterióides. Semelhante ao descrito em A, o sinal de baixo O_2 é transmitido a FixK2 via o sistema FixLJ. Um grupo de 166 genes ativados por FixK2 (indicado em letras brancas e em fundo preto) não é ao mesmo tempo dependente de FixJ, sugerindo a existência de um mecanismo adicional para a transmissão do sinal de baixo O_2. Isso indica que além da relação hieráquica de transmissão do sinal de baixo O_2 via o sistema FixLJ-FixK2, a proteína FixK2 deve ser capaz de monitorar diretamente o estado redox intracelular através de um mecanismo não conhecido[4] (representado pelo ponto de interrogação seguido pelo desenho de um raio). **Fonte: Adaptado de Mesa et al., 2008.**

FixK1 em *B. japonicum* parece atuar tanto como um ativador, quanto como um repressor de transcrição, tendo em vista que 17 genes tiveram ativação dependente de FixK1 enquanto 12 genes foram reprimidos por essa proteína. Ainda FixK1 parece não

[4] Como apresentado no ínicio do item 2.4.1, o resíduo Cys-183 influencia a atividade de FixK2 (MESA et al., 2009; BONNET et al., 2013). Este resíduo de aminoácido está envolvido no monitoramento direto do status redox intracelular. Tal característica da proteína FixK2, ainda não conhecida no trabalho de Mesa e colobarodores (2008) pode ser o mecanismo não conhecido citado na figura 2-4.

ser importante para o processo de formação dos nódulos e adaptação das bactérias à formação dos nódulos. Porém FixK2, atua principalmente como um ativador na regulação de diversos genes requeridos para adaptação ao modo de vida simbiótico, sendo essencial para a fixação de nitrogênio (NELLEN-ANTHAMATTEN et al., 1998; SCIOTTI et al., 2003; MESA et al., 2008).

2.5 Potencial adaptativo das bactérias às flutuações nas concentrações de oxigênio

A respiração é um processo fundamental para todos os organismos vivos. Neste processo os elétrons provenientes da oxidação de fontes de carbono reduzidas, (estocados na forma de NADH) são transferidos de forma sequencial por uma série de carreadores proteicos ligados ou associados à membrana, a cadeia transportadora de elétrons, até o oxigênio, um aceptor de elétrons com alto potencial redox. A energia livre liberada durante esse processo é usada para guiar a translocação de prótons através da membrana, o que leva à geração de um gradiente eletroquímico que pode ser usado para a síntese do ATP (RICHARDSON, 2000; BUENO et al., 2012). Em bactérias, ao contrário do sistema respiratório de organismos eucarióticos superiores, a cadeia respiratória é ramificada. Durante a exposição a condições limitantes de oxigênio, duas adaptações podem ocorrer para manutenção da síntese de ATP: (i) síntese de oxidases terminais alternativas de alta afinidade, capazes de manter a respiração em baixas concentrações de O_2 ou (ii) utilização de outros substratos como aceptores finais de elétrons, como nitrato, nitrito, óxido nítrico, óxido nitroso, dimetil-sulfóxido (DMSO), trimetilamina N-óxido (TMAO), sulfato, sulfito e fumarato (RICHARDSON, 2000).

Essa flexibilidade respiratória apresentada pelos microorganismos, permitem que estes colonizem muitos habitats sob condições de microaerofilia ou até mesmo sob anoxia. (revisado em RICHARDSON, 2000; PRICE; DRIESSEN, 2010). A mitocôndria de mamíferos, em contrapartida, não apresenta a mesma flexibilidade respiratória. Existe alguma diversidade ao nível das redutases, ou do ramo de entrada dos elétrons na cadeia transportadora, porém no ramo oxidativo há apenas uma oxidase terminal, onde a oxidase do tipo aa_3 é a única que reduz O_2 a H_2O (RICHARDSON, 2000).

A flexibilidade respiratória bacteriana requer uma estratégia que possa assegurar que a célula procariótica seja capaz de sustentar seu metabolismo em um ambiente com mudanças nas tensões de oxigênio. Para isso, as bactérias precisam de sensores dos níveis de O_2. Dois modos de monitoramento do O_2 são: (i) através da proteína receptora

de membrana FixL, uma proteína quinase sensora presente em rizóbios, que contém um grupamento heme (revisado em RODGERS; LUKAT-RODGERS, 2005; GREEN et al., 2009) ou (ii) através de um grupamento $[4Fe\text{-}4S]^{2+}$ presente na proteína Fnr (revisado em CRACK et al., 2008; GREEN et al., 2009; FLEISCHHACKER; KILEY, 2011).

2.6 Análise Transcriptômica

Os organismos precisam estar constantemente adaptando-se às variações das condições ambientais para garantir seu crescimento e desenvolvimento. Para isso, precisam regular a expressão de seus genes em reposta a diferentes sinais ambientais. Grande parte desta regulação acontece ao nível do controle transcricional, por isso a análise do transcriptoma pode revelar os mecanismos que levam um organismo a mudar sua expressão gênica visando adaptar-se a condições específicas. O transcriptoma pode ser definido como o conjunto completo de RNAs mensageiros (mRNA) e RNAs não codificadores (ncRNA) transcritos por uma célula ou organismo sob uma determinada condição em um determinado tempo de seu ciclo de vida. Ao longo dos anos, a pesquisa na área transcriptômica evoluiu da simples detecção de mRNAs transcritos através da técnica de northern blot (ALWINE et al., 1977) até técnicas de larga escala para caracterização do perfil de expressão de genes, utilizando das técnicas de microarranjos (SCHENA et al., 1995) e mais recentemente por técnicas de sequencimento de DNA de nova geração (revisado em NAGALAKSHMI et al., 2001; MOROZOVA et al., 2009).

As primeiras técnicas para análise transcriptômica eram baseadas no conhecimento prévio de genes candidatos. A primeira técnica utilizada foi o northern blot (ALWINE et al., 1977) em que basicamente, o RNA era primeiramente separado eletroforéticamente em gel de agarose, transferido para uma membrana de nitrocelulose ou nylon e então hibridizado com uma sonda de DNA marcada usualmente com ^{32}P. Esta técnica apresenta um baixo rendimento, requer a utilização de grandes quantidades de RNA, o uso de sondas em geral radioativas e ainda está limitada pela abundância e estabilidade do RNA a ser detectado.

O desenvolvimento da técnica de RT-PCR quantitativa em tempo real (BECKER-ANDRÉ; HAHLBROCK, 1989; NOONAN et al., 1990) facilitou a detecção de transcritos, aumentou o rendimento experimental e reduziu a quantidade de RNA requerido.

Outra técnica para a análise transcriptômica desenvolvida na década de 90, foi a técnica dos microarranjos que superou as demais técnicas até então conhecidas, pois permitiu a caracterização simultânea dos níveis de expressão de milhares de transcritos (SCHENA et al., 1995). Apesar deste avanço no número de transcritos que podem ser detectados, as técnicas de microarranjo requerem o conhecimento prévio do genoma e não permitem a detecção de novos transcritos.

Uma alternativa para a análise transcriptômica é a abordagem baseada no sequenciamento de DNA. Uma grande vantagem desta técnica em relação ao microarranjo é a habilidade de se determinar a identidade e também mais recentemente a abundância direta do transcrito detectado. Técnicas de análises transcriptômica baseadas em sequenciamento começaram através do sequenciamento de bibliotecas de cDNA (SEKI et al., 2002) pelo método de Sanger (SANGER et al., 1977). Porém devido o alto custo do sequenciamento de Sanger e o laborioso processo de clonagem utilizando-se hospedeiros bacterianos, este método denominado *Sanger Full-Length cDNA sequencing* (SEKI et al., 2002) passou a ser considerado ineficiente para a análise do transcriptoma total. Após o surgimento do *Sanger Full-Length cDNA sequencing*, outras técnicas baseadas no sequenciamento de Sanger surgiram, como o sequenciamento de ESTs (*Expression Sequence Tags*) (BOGUSKI, 1995) e o SAGE (*Serial Analysis of Gene Expression*) (VELCULESCU et al., 1995).

Em 2005, a introdução da primeira técnica de sequenciamento de nova geração (454/Roche) (MARGULIES et al., 2005) proporcionou uma revolução não somente na genômica, mas também na transcriptômica, pois permitiu uma grande evolução nas técnicas de análise transcriptômica baseada em sequenciamento. Na técnica de sequenciamento de RNA (RNA-Seq) (Figura 2-5), um DNA complementar (cDNA) gerado a partir de um RNA de interesse, é total ou parcialmente seqüenciado através das técnicas de sequenciamento de nova geração. O uso do RNA-Seq para a análise do transcriptoma possibilita a obtenção de uma grande quantidade de dados com resolução de uma única base, e pode ainda auxiliar na re-anotação de genomas, na descoberta e detecção de RNAs não codificadores e também na determinação dos sítios de início e fim de transcrição (MOROZOVA et al., 2009; NAGALAKSHMI et al., 2010).

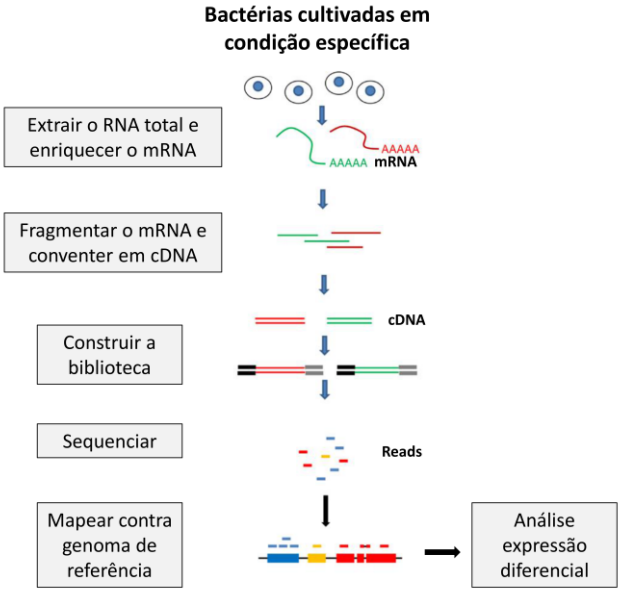

Figura 2-5. Diagrama esquemático representando um protocolo típico para o RNA-Seq. Basicamente o RNA total é purificado das bactérias cultivadas em uma condição específica. O rRNA é removido através de um método de escolha para o enriquecimento do mRNA. O mRNA é fragmentado a uma gama de fragmentos de distribuição uniforme e então convertidos em cDNA para o preparo da biblioteca para o sequenciamento. Após o sequenciamento programas de bioinformática são utilizados para o mapeamento das sequências obtidas contra um genoma de referência. A expressão diferencial dos genes entre as condições aplicadas pode ser analisada com um método de escolha. **Fonte: Adaptado de ZENG; MORTAZAVI, 2012.**

2.7 Estudos de Interação proteína-DNA através de ChIP-Seq

Para o estudo da regulação da expressão gênica é importante que se tenha uma idéia de quais genes estão sendo regulados diretamente por um determinado regulador de transcrição. A busca de um motivo de ligação para uma proteína especifica na região promotora de genes de interesse, através de ferramentas de bioinformática, é uma abordagem bastante útil para se obter uma sugestão dos genes que são diretamente regulados por um determinado fator de transcrição, muito embora essas predições *in silico,* requeiram validações experimentais *in vivo* (TOMPA et al., 2005). Ainda, a presença de uma sequência consenso de ligação para uma proteína, não está sempre relacionado com a ligação da proteína àquela sequência. Da mesma forma que motivos de ligação que se assemelham perfeitamente ao consenso não são ligados pela proteína experimentalmente, motivos poucos conservados podem ser ligados sob condições ambientais específicas (MARTONE et al., 2003; BORNEMAN et al., 2007; MYERS et al., 2013).

A caracterização global da ligação de um fator de transcrição em um genoma requer a execução de múltiplos experimentos em variadas condições o que faz esses estudos bastante laboriosos (LEFRANÇOIS et al., 2010). Em *Saccharomyces cerevisiae,* por exemplo, a análise direta da ligação de um fator de transcrição nos promotores de genes alvo, foi feita inicialmente usando-se a técnica de footprinting de DNAse (AXELROD; MAJORS, 1989) e/ou PCR quantitativa a partir de DNA imunoprecipitado com um fator de transcrição (ORLANDO et al., 1997; KUO; ALLIS, 1999). Estes métodos permitem a análise de poucos promotores por vez, e partem de genes candidatos previamente conhecidos. Isto torna a identificação de novos promotores alvo bastante difícil (LEFRANÇOIS et al., 2010). O advento da técnica de microarranjos de DNA permitiu um grande avanço nestes estudos de interação proteína-DNA em escala genômica, pois possibilitou o surgimento da técnica chamada ChIP-chip. Esta técnica é baseada na imunoprecipitação do fator de transcrição com o DNA de interesse, seguida da hibridização em um chip de microarranjos de DNA (HORAK; SNYDER, 2002).

Os estudos de interação proteína-DNA em escala genômica também se beneficiaram das novas tecnologias de sequenciamento em larga escala com o surgimento do ChIP-Sequencing ou ChIP-Seq (JOHNSON et al., 2007; ROBERTSON et al., 2008; PARK, 2009; LEFRANÇOIS et al., 2010; BRDLIK et al., 2014). No ChIP-Seq, ao invés de hibridizar o DNA imunoprecipitado em um chip de microarranjo, uma biblioteca de fragmentos de DNA imunoprecipitados é sequenciada por uma técnica de sequenciamento em larga escala. A aumentada sensibilidade e o reduzido ruído do ChIP-Seq está fazendo com que a técnica baseada em microarranjos (ChIP-chip) seja substituída nos estudos de análise de interação proteína-DNA em escala genômica (LEFRANÇOIS et al., 2010). Adicionalmente, proteínas marcadas com um epítopo exógeno específicio podem ser utilizadas para a imunoprecipitação (por exemplo: c-Myc, His-Tag ou 3xFlag) (revisado em TERPE, 2003), contornando-se a necessidade da geração de anticorpos específicos para cada fator de transcrição. Além disso, a marcação com um epítopo comercial traz a praticidade da utilização de anticorpos disponíveis comercialmente, a possibilidade de marcação de múltiplas proteínas e ainda um baixo nível de imunoprecipitação inespecífica resultando na diminuição do ruído (LEFRANÇOIS et al., 2010).

Um esquema da técnica de ChIP-Seq é apresentado na Figura 2-6. O primeiro passo é a imunoprecipitação da cromatina (ChIP) que envolve o tratamento das células

vivas com formaldeído para que ocorra a formação de ligação cruzadas ou *crosslinkings* entre DNA e proteína no momento do tratamento. O formaldeído atua no acoplamento de aminas primárias próximas e é um agente de *crosslinking* ideal para ChIP-Seq devido ao seu curto tempo de reação e alta permeabilidade pelas membranas biológicas. Além disso, a maioria dos *crosslinkings* proteína-DNA mediados pela adição de formaldeído *in vivo*, são devido a interações fisiológicas genuínas (SOLOMON; VARSHAVSKY, 1985; SOLOMON et al., 1988). Depois do *crosslinking* com formaldeído, os complexos proteína-DNA são liberados das células e fracionados para gerar fragmentos de um tamanho entre 200-800 bp. O DNA fragmentado é então incubado com anticorpos específicos para a proteína de interesse para capturar um conjunto de sequências de DNA ligadas à proteína. Finalmente as sequências de DNA são recuperadas e sequenciadas em sequenciador de nova geração (LEFRANÇOIS et al., 2010; BRDLIK et al., 2014).

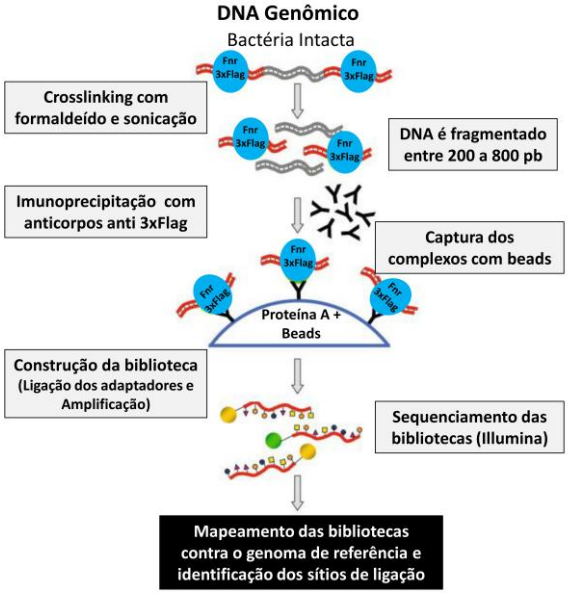

Figura 2-6. Diagrama esquemático representando o protocolo para o ChIP-Seq. Os complexo proteína-DNA são submetidos ao crosslinking com formaldeído. Após a lise das células, o DNA é fragmentado e imunoprecipitado com anticorpos específicos. Em seguida ocorre a reversão do crosslinking e os fragmentos de DNA são preparados para o sequenciamento de nova geração. Os sequências obtidas são então mapeados contra o genoma de referência. **Fonte: Adaptado de BRDLIK et al., 2014).**

3. RESULTADOS

Os resultados obtidos durante o desenvolvimento desta tese foram compilados em três manuscritos, apresentados nas sessões a seguir:

- 3.1 - **The *Herbaspirillum seropedicae* SmR1 Fnr orthologs controls the cytochrome composition of the electron transport chain**;
  Publicado na revista Scientific Reports em setembro de 2013. O formato submetido é apresentado na seção 3.1.

- 3.2 – **Enhanced oxygen consumption in *Herbaspirillum seropedicae fnr* mutants leads to increased NifA mediated transcriptional activation**;
  Este manuscrtio foi previamente submetido para publicação na revista Research in Microbiology em setembro de 2013. No formato anteriormente submetido o manuscrito foi rejeitado. Diante disso, seguindo as sugestões dos referees, mais experimentos foram realizados e o manuscrito foi submetido novamente para publicação na revista BMC Microbiology em dezembro de 2014. O formato atual é apresentado na seção 3.2.

- 3.3 - **ChIP-Seq and RNA-Seq analysis unravel the role of three Fnr proteins in the adaptation of *Herbaspirillum seropedicae* SmR1 to low oxygen levels**;
  Manuscrito em formato de *draft* ainda não submetido.

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

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3.1.1 Abstract

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 cbb_3-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.

3.1.2 Introduction

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 sorghum (BALDANI et al., 1986; OLIVARES et al., 1996; JAMES; OLIVARES, 1998). *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 conditions (PEDROSA et al., 2011). Genome sequencing revealed genes coding for three Fnr-like proteins in *H. seropedicae* SmR1 (PEDROSA et al., 2011). In many organisms the Fnr protein, which belongs to the CRP-FNR family of transcriptional regulators (KORNER et al., 2003), acts as a positive or negative regulator of genes required for the metabolic switch in response to O_2 levels (KILEY; BEINERT, 1998). The Fnr protein of *Escherichia coli* contains an N-terminal sensory domain, which binds an oxygen-labile $[4Fe–4S]^{2+}$ cluster under oxygen limiting conditions (LAZAZZERA, B. A. et al., 1996) and a C-terminal DNA-binding domain, which recognizes a partially palindromic

sequence called the Fnr-box or anaerobox, TTGAT-N_4-ATCAA (SCOTT et al., 2003; KORNER et al., 2003).

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 conditions (GRABBE et al., 2001). In *Rhizobium leguminosarium* 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 fixation (GUTIÉRREZ et al., 1997). Similarly the $FixK_2$ protein is also essential for nitrogen fixation in *Bradyrhizobium japonicum* and *Sinorhizobium meliloti* (NELLEN-ANTHAMATTEN et al., 1998; BOBIK et al., 2006; MESA et al., 2008). Representatives of the CRP-FNR family are also known to act negatively in repressing genes related to nitrogen fixation, such as the $FixK_1$ protein from *B. japonicum* (MESA et al., 2008).

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 pseudomalei* 1710b and *Herminiimonas arsenicoxydans* have genes coding for two Fnr-like proteins, whereas *Cupriavidus metalidurans* 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.

3.1.3 Results

3.1.3.1 *H. seropedicae* encodes three proteins that share homology with *E.coli* Fnr

The *H. seropedicae* SmR1 genome (PEDROSA et al., 2011) 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%, 26.9% and 37.5% identity respectively with *E. coli* Fnr. As shown in Figure 3.1-1, 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 (KORNER et al., 2003). 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.

A phylogenetic affiliation for the CRP-FNR superfamily of transcriptional regulators has been proposed (KORNER et al., 2003; MESA et al., 2006). Proteins from the Fnr and FnrN groups sense oxygen directly via an $[4Fe–4S]^{2+}$ cluster, whereas the FixK members, which lack the conserved cysteine residues required for ligating the cluster, either sense oxygen indirectly, or simply relay the O_2 signal, via the two-component regulatory system FixL-FixJ (NELLEN-ANTHAMATTEN et al., 1998). In order to classify the *H. seropedicae* Fnr 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. S3.1-1). 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 (KORNER et al., 2003). 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.

3.1.3.2 Construction of *H. seropedicae Δfnr* 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 backgrounds are available (Supplementary Fig. S3.1-2). 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).

3.1.3.3 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 3.1- 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_4Cl, respectively, compared with an $O.D_{600}$ of 0.8 reached

by the wild type strain in both ammonium chloride concentrations (Supplementary Fig. S3.1-3). 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. S3.1-4). 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.

3.1.3.4 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 3.1-1.

Of the 187 regulated genes, 70 (37.43%) 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 (TATUSOV et al., 2003) (Supplementary Fig. S3.1-5a). 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. S3.1-5b). 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 3.1-1 and Supplementary Dataset 3.1-1). 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 (PEDROSA et al., 2011). These are represented by the aa_3-type *(*cox*)* and cbb_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 3.1-3a). 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 cbb_3-type heme-copper oxidase (Table 3.1-1). These include the *fixNOP* operon encoding the structural components of this oxidase and the maturation genes *fixG, fixH, fixI* and *fixS,* which in other bacteria is often organized as an operon (KOCH et al., 2000; COSSEAU; BATUT, 2004). In contrast, in *H. seropedicae* the *fixG, fixH, fixI* and *fixS* genes are dispersed in two distinct operons (Figure 3.1-4a), in which *fixG* and Hsero_3199 (*fixH*) appear to form an operon with the conserved putative transmembrane protein Hsero_3198 and *fixI and fixS* apparently form an operon with the heme biosynthesis gene *hemN* (LIEB et al., 1998) and *Hsero_3206,* which encodes a conserved hypothetical protein (Figure 3.1-4a). In agreement with the down-regulation observed 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 3.1-1). 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 3.1-1).

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 3.1-1 and Figure 3.1-3b). In addition, significant differential expression was observed for genes encoding cytochrome c_{551}/c_{552} (Hsero_1104) and cytochrome c_{553} (Hsero_0153) a c_4 type cytochrome, that potentially acts as an electron donor to the cbb_3-type heme-copper oxidase (CHANG et al., 2010) (Table 3.1-1 and Figure 3.1-3b). In addition to the apparent influence of *fnr* on the expression of *c*-type cytochromes (Table 3.1-1 and Figure 3.1-3), the Fnr protein(s) also appear to activate genes required for cytochrome *c* biogenesis. The *H. seropedicae* genome encodes system II-like machinery for cytochrome *c* maturation (Supplementary Fig. S3.1-6), including homologs of the ResB (CcsB) and ResC (CcsA) proteins, which are proposed to function in the handling of heme and its ligation to apocytochrome, and the DsbD (CcdA) and ResA (CcsX) proteins that function in the reduction of the disulphide bond in the CXXCH heme binding site (GODDARD et al., 2010; SIMON; HEDERSTEDT, 2011). Transcript profiling revealed that genes encoding all four components were significantly down-regulated in the triple *fnr* deletion strain (Table 3.1-1). The *dsbD* gene is apparently located in an operon downstream of *cutA*, which is also implicated in cytochrome *c* biogenesis (CROOKE; COLE, 1995; YANG et al., 1996). Transcripts mapping to the *ndh* gene, which encodes the non-coupling NADH dehydrogenase II enzyme, increased in the triple *fnr* mutant (Table 3.1-1), indicating that expression of this enzyme is repressed by Fnr as observed in other bacteria (SPIRO et al., 1989; MENG et al., 1997). Perhaps to compensate for this, the expression of Hsero_4284, encoding the energy-conserving NADH dehydrogenase I is apparently activated 3-fold by Fnr (Table 3.1-1). Overall, in comparing the global expression pattern of genes involved in electron transport, it would appear that Fnr activates expression of genes involved in the heme-copper oxidase branch of the aerobic respiratory chain (Table 3.1-1 and Figure 3.1-3). This involves not only regulation of the expression of the cbb_3-type oxidase, but also 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 ubiquinone biosynthesis pathway and a gene encoding an alternative 2-polyprenyl-6-

methoxyphenol hydroxylase (Hsero_4190) also required for ubiquinone biosynthesis were significantly down regulated in the triple *fnr* deletion strain (Table 3.1-1 and Figure 3.1-3). 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 3.1-1 and Supplementary Dataset 3.1-1). In addition, the nitrate/nitrate transporter encoded by $narK_1U$ operon and the nitrate-sensing two component regulatory system *narXL* are also strongly down-regulated in the *fnr* triple deletion (Table 3.1-1 and Supplementary Dataset 3.1-1). 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 nitrate (BALDANI et al., 1986; PEDROSA et al., 2011).

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 3.1-1 and Supplementary Dataset 3.1-1). 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.

3.1.3.5 Analysis of Fnr regulation of genes encoding the cbb_3-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 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 3.1-4b). Consistent with the transcriptomics data (Table 3.1-1), 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 *fnr*3, but activity was equivalent to the parental strain in the *fnr*2 deletion strain MB2 (Figure 3.1-4b) . 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 cbb_3-type respiratory oxidase. Accordingly, under oxygen-limiting conditions, no growth penalty is observed for the MB2 strain (Supplementary Fig. S3.1-7).

3.1.3.6 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 3.1-5a). To further explore this observation we analysed reduced minus oxidized spectra of protein extracts obtained from SmR1 and the *fnr* deletion strains (Figure 3.1-5b and Supplementary Fig. S3.1-8). 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 3.1-5c and Supplementary Fig. S3.1-8). 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. S3.1-9) and by analogy with studies on the FixNOQP proteins from other bacteria (ZUFFEREY et al., 1996; KOCH et al., 1998). 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 3.1-1 and Figure 3.1-3) and *lacZ*-fusion analysis (Figure 3.1-4). The bands 2, 4 and 5 may represent PetC, cytochrome c_{553} (Hsero_0153), and cytochrome c_{551}/c_{552} (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 3.1-1 and Figure 3.1-3). 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 3.1-5b and Supplementary Fig. S3.1-8). 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.

3.1.3.7 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* (BATUT et al., 1989; PREISIG et al., 1993; MANDON et al., 1994) 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 3.1-6a). The RAM21 strain (*fixN* mutant) was also not deficient in acetylene reduction when grown under these conditions (Supplementary Fig. S3.1-10). 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 3.1-6b). 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 3.1-6b). 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. S3.1-11).

3.1.4 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 cbb_3-type heme copper oxidase in *H. seropedicae,* as is the case in other Proteobacteria (BATUT; BOISTARD, 1994; COSSEAU; BATUT, 2004). 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 bo_3-type oxidases in *E. coli* and *A. vinelandii* (COTTER et al., 1990; WU et al., 2000) and the bo_3–type and CIO oxidases in *Pseudomonas putida* (UGIDOS et al., 2008). However, our transcriptomics data indicate that this is not the case in *H. seropedicae*. We only observe differential expression of the genes encoding the cbb_3-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 bc_1 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 cbb_3-type heme copper oxidase is likely to be required for growth at very low (<0.5%) oxygen concentrations (KAMINSKI et al., 1996) and can support symbiotic nitrogen fixation at nanomolar levels of dissolved oxygen (reviewed in MARCHAL; VANDERLEYDEN, 2000). Nevertheless, the *fixN* insertion mutant of *H. seropedicae* RAM21 was competent to support nitrogenase activity under oxygen-limiting conditions implying that the cbb_3-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 pneumoniae* (HILL et al., 1990), it provides respiratory protection for nitrogenase in *Azotobacter vinelandii* (KELLY et al., 1990; POOLE; HILL, 1997) and it is utilized as a terminal oxidase to support symbiotic nitrogen fixation in *Azorhizobium caulinodans* (KAMINSKI et al., 1996). 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 cbb_3-type oxidase, appears to play a critical role in regulating transcription of the *fixNOP* and *hemN-Hsero_3206-fixIS* operons and in controlling the expression of *c*-type cytochromes. Although, *fnr3* also appears to be required to express the cbb_3-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.*

3.1.5 Methods

3.1.5.1 Bacterial strains and plasmids

H. seropedicae and *E. coli* strains and plasmids used are listed in Supplementary Table S3.1-1.

3.1.5.2 Growth conditions

E. coli strains were grown at 37°C in LB medium (SAMBROOK et al., 1989). *H. seropedicae* strains were grown at 30°C in NFbHP-Malate medium (KLASSEN et al., 1997) supplemented with NH_4Cl 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® stoppered 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.

3.1.5.3 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 seq: NP_415850.1) using Muscle software (EDGAR, 2004). The presence of conserved domains in the Fnr homologs was investigated by submitting sequence search to the Pfam database (http://pfam.sanger.ac.uk/) (FINN et al., 2010).

3.1.5.4 Phylogenetic Analysis

Amino acid sequence retrieval was performed by using a BLASTP search (ALTSCHUL et al., 1997) against the nonredundant NCBI database. The proteins used

as queries were Fnr1, Fnr2 and Fnr3 from *H. seropedicae*, (Ref seq: YP_003776587.1, YP_003775788.1 and YP_003775945.1, respectively) FixK1 and FixK2 from *B. japonicum* (Ref seq: NP_772701.1 and NP_769397.1, respectively), Fnr from *E.coli* K12 substr. MG1655 (Ref seq: NP_415850.1) and CydR from *A. vinelandii* (Ref seq: YP_002799173.1). A limited number of sequences were selected, eliminating redundant information, while maintaining representative taxonomic diversity. All sequences selected (Supplementary Table S3.1-2) were checked to have the CRP-FNR superfamily protein signatures, CNMP_Binding_3 PS50042 and HTH_CRP_2 PS51063, proposed by PROSITE (SIGRIST et al., 2010). For phylogenetic tree reconstruction, an amino acid alignment was made using Muscle (EDGAR, 2004). Maximum likelihood (ML) trees were derived using the JTT matrix-based model (JONES et al., 1992) after bootstrapping 1,000 replicates of each original data set (DOPAZO, 1994) using the MEGA 5.05 software (TAMURA et al., 2011).

3.1.5.5 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 containing *fnr* orthologs deletions (Supplementary Fig. S3.1-2) and a tetracycline resistance cassette insertion into *fixN* (Supplementary Fig. S3.1-12). The primers used in this work are show in Supplementary Table S3.1-3. 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 pMBB1D, pMBB2D and pMBB3D (Supplementary Table S3.1-1). The *nptI-sacB-sacR* cartridge (from pMH1701) was then inserted into BamHI site. We generated three suicide plasmids: pMBB1DS for the 279 bp deletion of *fnr1*, pMBB2DS for the 276 bp deletion of *fnr2* and pMBB3DS for the 267 bp deletion of *fnr3* (Supplementary Table S3.1-1).

Conjugation was performed between *E. coli* S17.1 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 20 mM NH_4Cl and antibiotics. One mutant strain resulting from single cross-over was

grown overnight in liquid NFbHP-Malate plus 20 mM NH_4Cl without antibiotics at 30°C. After incubation 250 µL of the culture were plated on NFbHP-Malate agar, supplemented with 20mM NH_4Cl, 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 *sacRB-Km*^{R} cassette by a second homologous recombination event could grow under these conditions. The mutant strains were analysed by PCR using primers (Supplementary Table S3.1-3) external to *fnr1*, *fnr2* and *fnr3*. To construct double and triple *H. seropedicae* mutant strains the process described above was repeated using different allele exchange plasmids.

For *fixN* mutagenesis, the plasmid pHS17058H11 (Genopar Consortium) (PEDROSA et al., 2011) containing the *fixN* gene was subject to a transposition reaction using the EZ-Tn5 <TET-1> Insertion Kit (Epicentre). After confirmation of the TET-1 insertion into the *fixN* gene (pHS17058H11Tc), a cloramphenicol cassette from pTnMod-OCm plasposon was inserted into the plasmid, outside the *fixN* gene, to yield the plasmid pHS17058H11TcCm, which was then electro-transformed into *H. seropedicae* SmR1. A transconjugant resistant to tetracycline and sensitive to cloramphenicol was selected and named RAM21. The double recombinant was confirmed by DNA hybridization (Supplementary Fig. S3.1-12).

3.1.5.6 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 total RNA was isolated using the RNA RiboPure™-Bacteria kit (Ambion). Two rounds of enrichment were performed using MICROB*Express*™ kit (Ambion) for removing ribosomal RNA from total RNA samples. Approximately 200 to 500 ng of mRNA enriched RNA were used for cDNA synthesis and library construction using the SOLiD™ Whole Transcriptome Analysis Kit (Life Technologies). The libraries were amplified by emulsion PCR using SOLiD ePCR kit and sequenced in a SOLiD 4 System (Life Technologies).

3.1.5.7 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 CLC Genomics Workbench package. Read counts table was exported into the RobiNA software (LOHSE et al., 2012) and both normalization and statistical evaluation of differential gene expression was performed by DESeq (ANDERS; HUBER, 2010) with a *p*-value cut-off of 0.05 using the Benjamini-Hochberg method for multiple testing correction (BENJAMINI; HOCHBERG, 1995). Genes with fold change lower than three were excluded from the analysis. Genes with fold change lower than 3 or *p*-value slightly higher than 0.05 were included in the analysis when genome context indicated they are part of operon with genes differentially expressed according to the above parameters.

Potential Fnr-binding sites were located using the sequence motif search facilities of PEPPER (JONG, DE et al., 2012) (http://pepper.molgenrug.nl/index.php) and Virtual Footprinting (MÜNCH et al., 2005) (http://prodoric.tu-bs.de/vfp/vfp_promoter.php) using default search parameters.

3.1.5.8 Construction of transcriptional fusions

To study the expression of *fixNOP* and *hemN-Hsero_3206-fixIS* operons, the putative promoter regions were amplified by PCR, cloned into pTZ57R/T and then subcloned into PstI and BglII sites of pPW452 to generate the plasmids pPWPFN (*fixN::lacZ* fusion) and pPWPHN (*hemN::lacZ* fusion) (Supplementary Table S3.1-1). All constructs were verified by DNA sequencing.

3.1.5.9 β-Galactosidase activity

To analyse the activities of *fixN::lacZ* and *hemN::lacZ* fusions, strains were grown to an $O.D_{600}$ = 0.3, collected and then resuspended in NFbHP Malate liquid medium supplemented with 20mM NH_4Cl to an $O.D_{600}$ = 0.05. The cells were incubated for 3 hours either under air or at initial concentrations of 2, 4 or 6% oxygen. After incubation samples were taken for β-galactosidase activity determination as described (MILLER, 1972). The results are expressed in Miller units (MU).

3.1.5.10 Acetylene reduction assay

The acetylene-reduction assay was used to determine nitrogenase activity on free-living cultures (DILWORTH, 1966; SCHÖLLHORN; BURRIS, 1967). Ethylene formation was determined either by using a Varian Star 3400 CX gas chromatograph equipped with a Porapak N column.

Freshly grown cultures were used for inoculating NFbHP-Malate semi solid medium (0.17% agar) containing 0.5 mM sodium glutamate followed by eighteen hours incubation at 30°C. Acetylene (10%) was injected and after incubation for one hour at 30°C, 0.5 mL samples were collected for determination of produced ethylene by gas chromatography. The same procedure was used for assaying nitrogenase activity in liquid media, except that the cultures were collected by centrifugation (3220 g, for 4 min at room temperature), re-suspended in NFbHP-Malate supplemented with 0.5 mM of NH_4Cl to an O.D of 0.2. The cells were incubated at 5.0% initial oxygen concentration in the gas phase as described. Nitrogenase activity is reported as nmol of C_2H_4 produced per minute per mg protein. Whole cell protein concentration was determined by the Bradford method (BRADFORD, 1976) after overnight lysis with 0.2 mM NaOH.

3.1.5.11 Reduced minus Oxidized Spectra and Heme Stain

For preparation of protein extracts *H. seropedicae* strains were cultured in 250 mL erlenmeyer flasks containing 50 mL of NFbHP-Malate supplemented with 20 mM NH_4Cl. After overnight incubation, the cells were collected by centrifugation and re-suspended in 2 mL of sonication buffer (100 mM NaCl and 50 mM Tris.HCl pH 7.5). Cells were broken by sonication, and cell debris was separated from the protein extract by centrifugation (6000 g, 30 min). The supernatant was collected and used for further analysis.

UV–visible difference spectra of 0.3 $mg.mL^{-1}$ protein extracts were recorded in 1 cm path-length quartz cuvettes at room temperature on a Shimadzu UV-2501 PC spectrophotometer. Reduced-minus-oxidized difference spectra of the protein fractions were recorded by measuring the dithionite-reduced spectrum of the sample against the air-oxidized one.

For the covalently bound heme stain, 50 μg of the protein extract, prepared as described above, was loaded without boiling onto a 12% Tris-Tricine SDS-PAGE gel (SCHÄGGER; JAGOW, VON, 1987), and stained using *o*-dianisidine (FRANCIS; BECKER, 1984).

3.1.6 Acknowledgments

This work was supported by the Brazilian Program of National Institutes of Science and Technology-INCT/Brazilian Research Council-CNPq/MCT, Fundac□ão Araucária and CAPES. We would like to thank Leonardo Magalhães Cruz for help with the use of MEGA 5.05 software. We are also thankful to Roseli A. Prado, Julieta Pie, Marilza D. Lamour and Valter A. de Baura for technical assistance.

3.1.7 Author contributions

M.B.B 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.

3.1.8 Competing financial interests

The authors declare no competing financial interests.

3.1.9 Figures

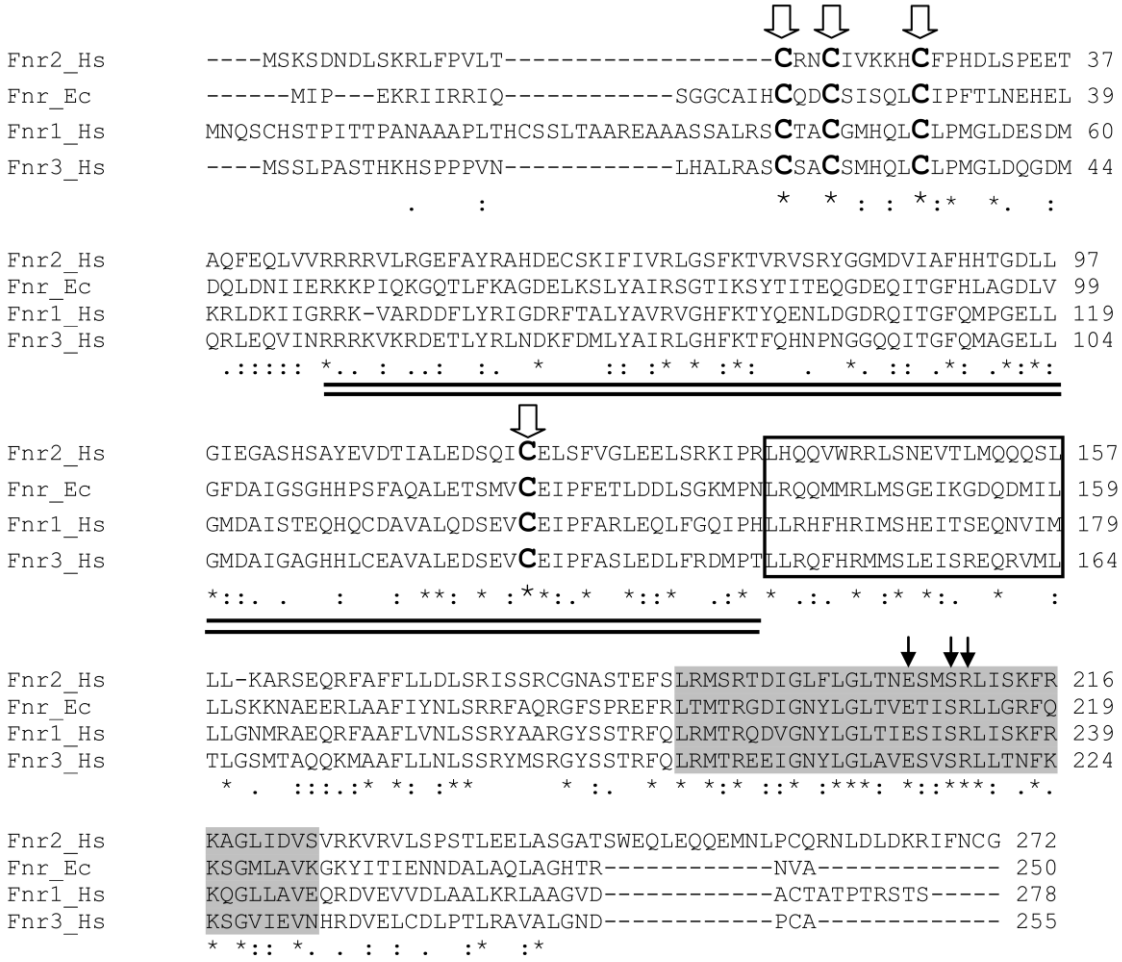


Figure 3.1-1. Alignment between *H. seropedicae* Fnr1, Fnr2 and Fnr3 proteins and *E. coli* Fnr. Identical amino acids are indicated by asterisks (*), high similarity amino acids are indicated by colons (:) and low similarity amino acids by dots (.). Conserved cysteines required for binding of the $[4Fe\text{-}4S]^{+}$ 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.

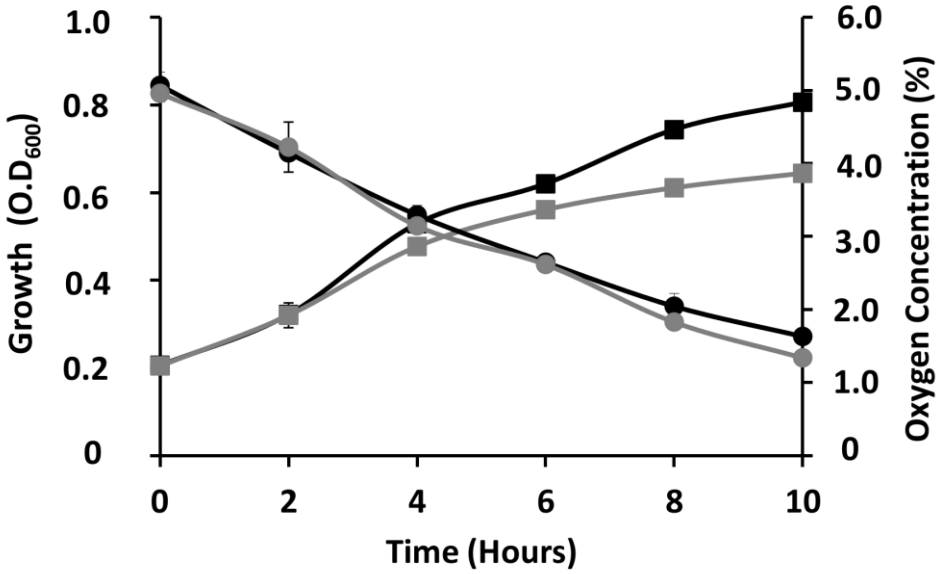


Figure 3.1-2. Influence of *fnr* genes on growth during oxygen limitation. The growth of *H. seropedicae* SmR1 (black squares) and MB231 (grey squares) strains were assayed in NFbHP-Malate minimal media supplemented with high ammonium concentration (20mM NH_4Cl) under 5 % initial oxygen concentration. 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.

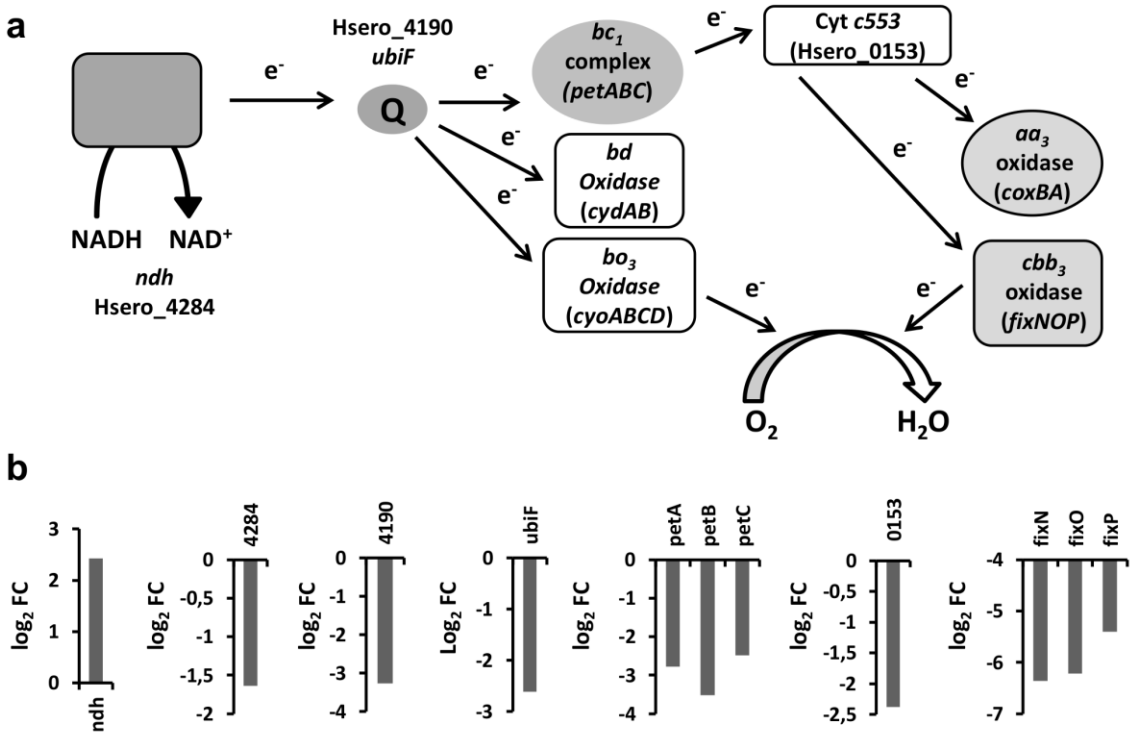


Figure 3.1-3. Fnr regulation of components of the electron transport chain (ETC) in *H. seropedicae* as determined by transcript profiling. (a) Schematic representation of the probable organization of ETC branches in *H. seropedicae* based on the genome annotation. (b) Influence of Fnr on differential expression of genes represented in (a). FC indicates fold change.

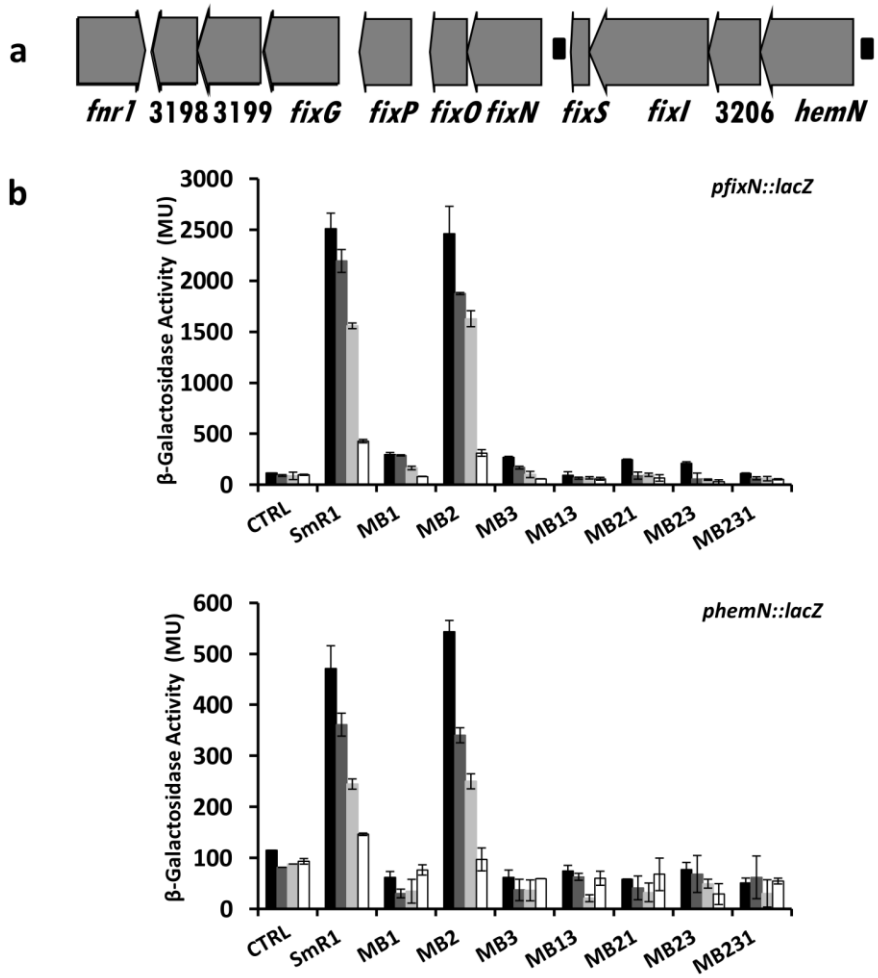


Figure 3.1-4. Effect of *fnr1, fnr2* and *fnr3* mutations on expression of the *fixNOP* and *hemN-Hsero_3206-fixIS* operons in *H. seropedicae.* (a) Schematic representation of genomic region encoding the *fixNOP* and *hemN-Hsero_3206-fixIS* operons. The genes and predicted functions in the locus are: Hsero_3198, transmembrane protein; Hsero_3199, FixH domain containing protein; *fixG*, iron-sulfur 4Fe-4S ferredoxin transmembrane protein; *fixP*, cbb_3-type cytochrome c oxidase-subunit III; *fixO*, cbb_3-type cytochrome oxidase-subunit II; *fixN*,cbb_3- type cytochrome c oxidase, subunit I; *fixS,* nitrogen fixation protein P-type ATPase protein; *fixI,* 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).

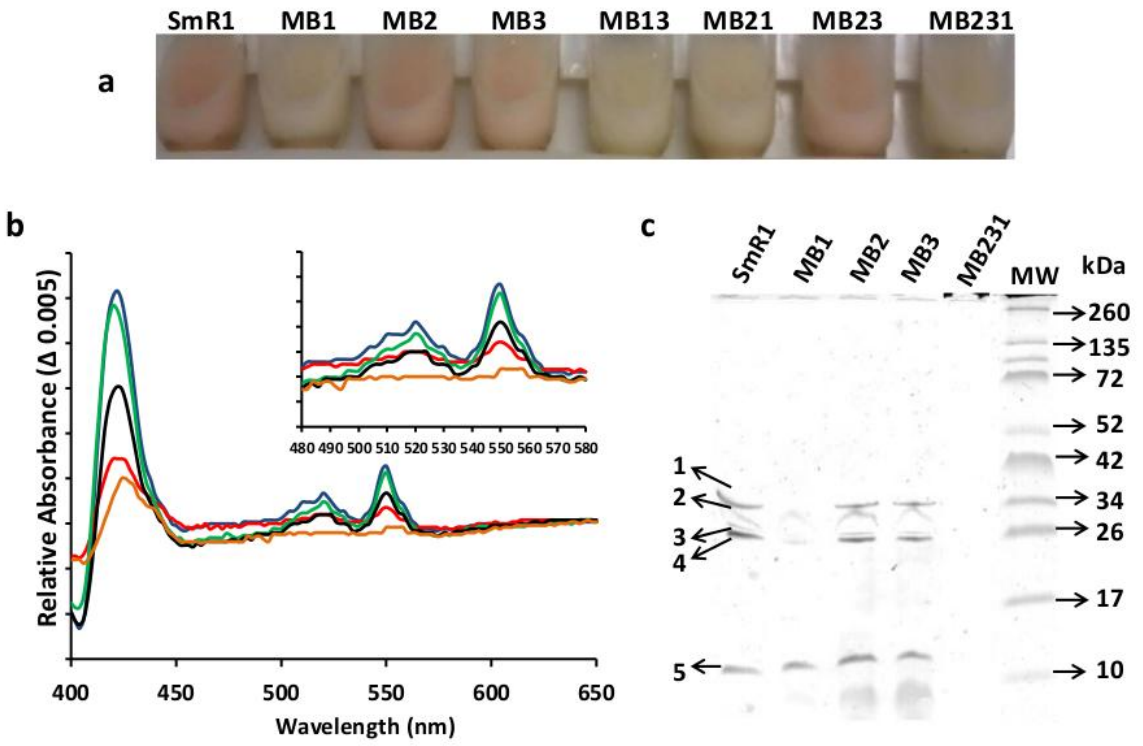


Figure 3.1-5. Fnr proteins influence the cytochrome content in *H. seropedicae*. (a) Bacterial suspensions of *H. seropedicae* SmR1 and *fnr* mutant strains. Cells, from 100 mL cultures, were collected by centrifugation and resuspended in 10 mL of buffer (100 mM NaCl and 50 mM Tris.HCl pH 7.5). (b) Reduced minus oxidized visible absorption spectra of protein extracts from *H. seropedicae* SmR1 and *fnr* mutant strains. For simplification, only the data for SmR1 (blue), MB1 (red), MB2 (green), MB3 (black) and MB231 (orange) are shown. (c) Heme stained gel of *H. seropedicae* protein extracts. Samples (50 μg protein per lane) from *H. seropedicae* SmR1 and *fnr* mutant strains were separated by 12% Tris-Tricine SDS-PAGE and stained for covalently bound heme with *o*-dianisidine. On the left the heme stained bands are labeled as 1, 2, 3, 4 and 5 from the top to the bottom of the gel. The apparent molecular masses of proteins (kDa) are indicated on the right. MW: Spectra™ Multicolor Broad Range Protein Ladder (Fermentas). The strains MB13, MB21 and MB23 gave similar spectra and heme stain profiles to MB231, MB1 and MB3, respectively (Supplementary Fig. S3.1-8).

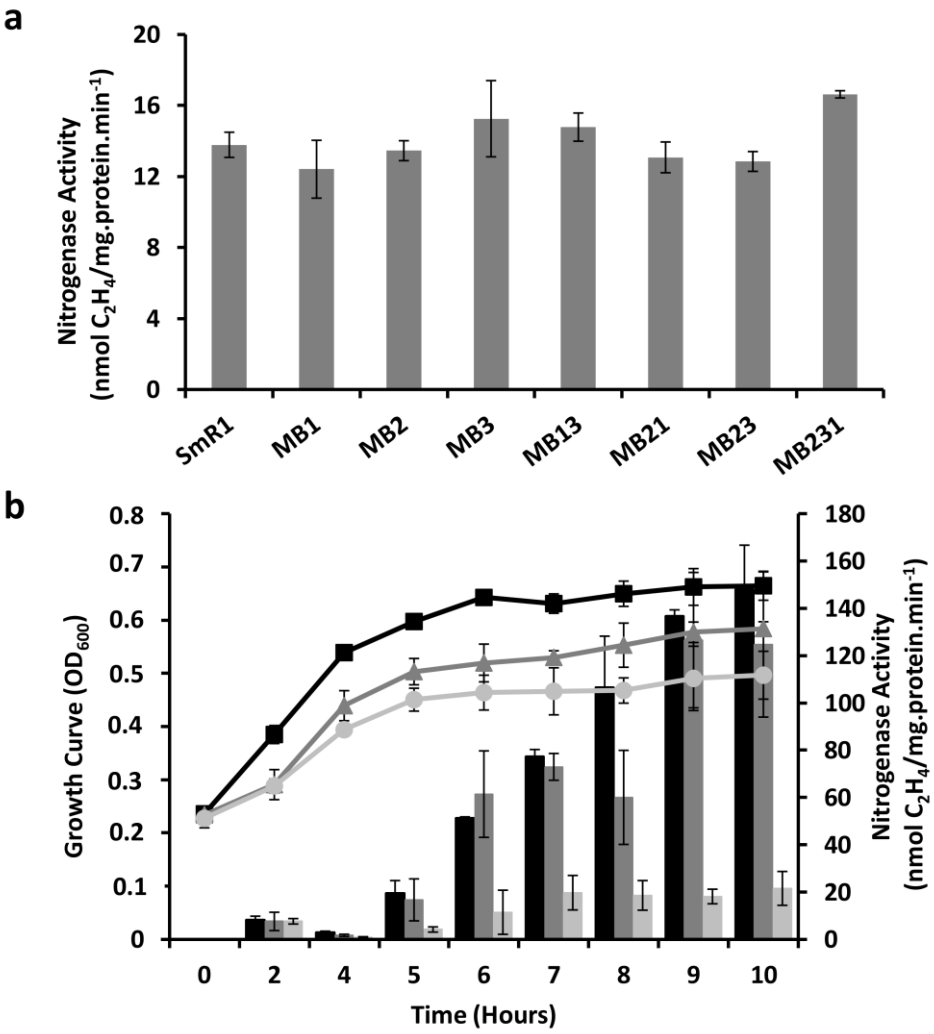


Figure 3.1-6. Influence of Fnr and FixN on nitrogenase activity. The acetylene reduction assay was performed as described in Methods using strains grown in (a) semi-solid medium supplemented with 0.5 mM of sodium glutamate or (b) liquid medium supplemented with 0.5 mM of ammonium chloride under 5 % initial oxygen in the gas phase. In (b) samples were taken from the culture to measure the growth curve (primary y axis) of SmR1 (black squares), RAM 21 (dark grey triangles) and MB231 (light grey circles). Black, dark grey and light grey bars (secondary y axis) indicate the nitrogenase activity of SmR1, RAM21 and MB231, respectively. Data represent the average of two independent experiments performed in duplicate. Error bars indicate standard deviations.

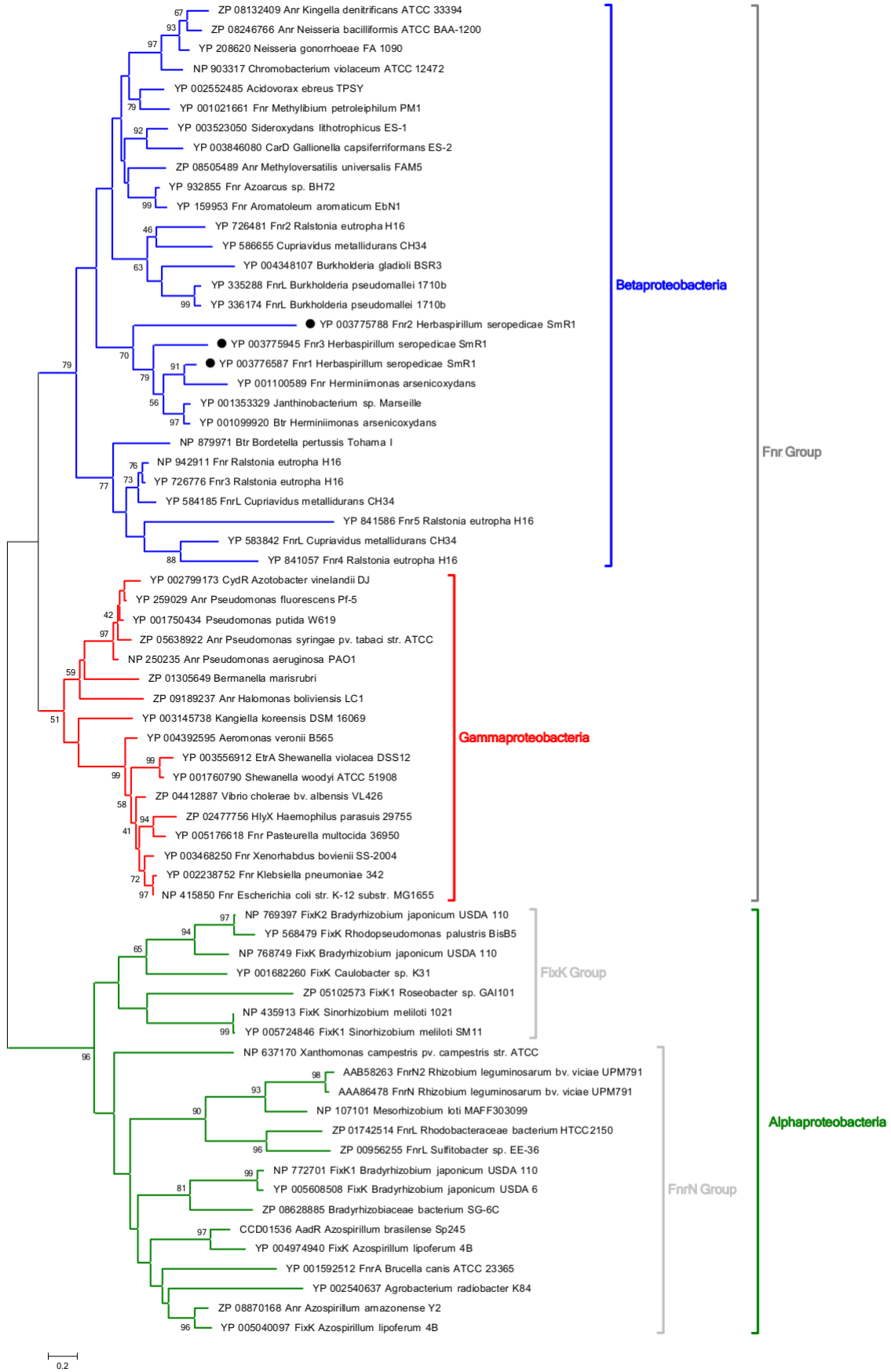
3.1.10 Tables

Table 3.1-1. Differential expression of a subset of genes required for, or implicated in, modulation of the composition of the electron transport chain and nitrate metabolism.

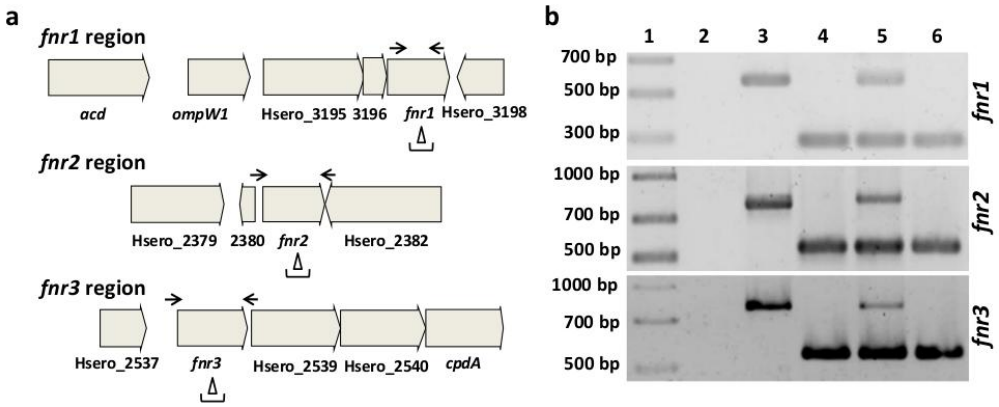
Feature ID[a]	Gene Description	COG[b]	FC[c]	Fnr Box[d]	Sequence[e]	Position	
						Start	End
ccmC	ABC-type transport system, permease component protein	O	-3.62	N	-	-	-
dsbC	thiol:disulfide interchange protein	O	-3.22	Y	TTGATTTGAATCAG$^{(P)}$	-171	-185
fnr1	Crp/Fnr family transcription regulator protein	T	-5.51	Y	TTGATCTGGGACAA$^{(VF)}$	-111	-97
fnr2	Crp/Fnr family transcription regulator protein	T	-4.48	Y	TTGACCTAGATCAG$^{(VF)}$	-185	-171
fnr3	Crp/Fnr family transcription regulator protein	T	0.39*	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	TTGATCTATGACAG$^{(P)(VF)}$	-98	-112
Hsero_4190	2-polyprenyl-6-methoxyphenol hydroxylase protein	C; H	-3.27	N	-	-	-
Hsero_4284	NADH-ubiquinone oxidoreductase protein	C	-1.64	Y	TTCCTTTCAATGAA $^{(VF)}$	-211	-197
ndh	NADH dehydrogenase, FAD-subunit cytochrome c reductase	C	2.43	Y	TTGAGGGACATCAA$^{(P)(VF)}$	-176	-190
resB	cytochrome C-type biogenesis transmembrane protein	O	-2.34	N	-	-	-
ubiF	2-polyprenyl-3-methyl-6-methoxy-1,4-benzoquinol hydroxylase	H	-2.61	N	-	-	-
cutA	periplasmic divalent cation tolerance protein	P	-2.12	N	-	-	-
dsbD	thiol:disulfide interchange transmembrane protein	O; C	-1.53	N	-	-	-
fixN	cbb3-type cytochrome c oxidase, subunit I	C	-6.36	Y	TTGATACACGTCAA$^{(P)(VF)}$	-143	-157
fixO	cbb3-type cytochrome c oxidase, subunit II	C	-6.22	N	In operon with *fixN*	-	-
fixP	cbb3-type cytochrome c oxidase, subunit III	C	-5.41	N	In operon with *fixN*	-	-
hemN	oxygen-independent coproporphyrinogen III oxidase protein	H	-4.17	Y	TTGATACACATCAA$^{(P)(VF)}$	-128	-142
Hsero_3206	conserved hypothetical protein	S	-4.44	N	In operon with *hemN*	-	-
fixI	cation transport P-type ATPase protein	P	-4.23	N	In operon with *hemN*	-	-
fixS	nitrogen fixation protein	P	-6.14	N	In operon with *hemN*	-	-
narG	respiratory nitrate reductase alpha chain oxidoreductase	C	-6.30	N	-	-	-
narH	respiratory nitrate reductase beta subunit protein	C	-5.16	N	-	-	-
narI	respiratory nitrate reductase transmembrane gamma subunit	C	-4.97	N	-	-	-
narJ	respiratory nitrate reductase protein	C	-5.41	N	-	-	-
moaA	molybdenum cofactor biosynthesis enzyme A protein	H	-4.00	N	-	-	-
narK1	nitrite/nitrate transporter protein	P	-6.23	Y	TTGGAAGTGCTCAC$^{(M)}$	-283	-269
narU	nitrite/nitrate transporter protein	P	-7.34	N	In operon with *narK1*	-	-
narX	nitrate/nitrite sensor histidine kinase	T	-7.11	Y	TTCATCTCACCCAT$^{(M)}$	-372	-358
narL	nitrate/nitrite response regulator transcription regulator protein	K; T	-6.08	N	In operon with *narX*	-	-
petA	ubiquinol-cytochrome C (Iron-sulfur) oxidoreductase	C	-2.78	Y	TTGATGGCGCGCAC$^{(VF)}$	-90	-103
petB	cytochrome b subunit transmembrane protein	C	-3.52	N	In operon with *petA*	-	-
petC	cytochrome c1 precursor transmembrane protein	C	-2.48	N	In operon with *petA*	-	-

[a] 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.[b] 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.[c] $\log_2$ fold change comparing RNA-Seq libraries of *H. seropedicae* MB231 (*fnr* ablated strain) and SmR1 (wild type) strain.
[d] 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$^{(P)}$ or Virtual Footprint$^{(VP)}$ as described in the Methods section. In some cases manual $^{(M)}$ predictions were also included.
[e] Start and End positions of Fnr boxes related to the predicted translational start site.
* Not statistically significant (p-value = 0.74).

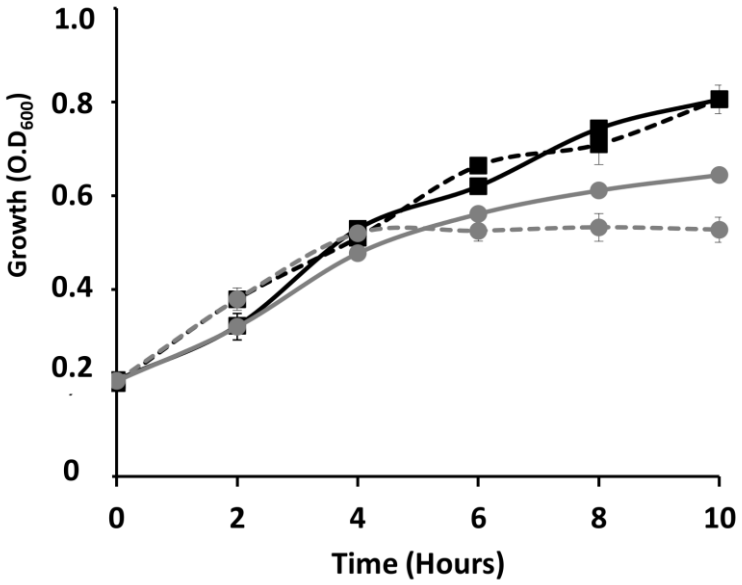
3.1.11 Supplementary Material



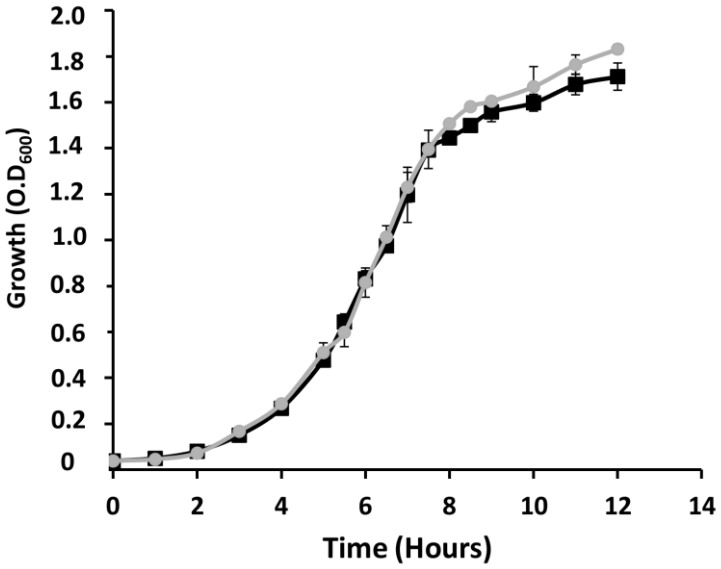
Supplementary Figure S3.1-1: Phylogenetic reconstruction of selected members of Fnr, FnrN and FixK groups of the CRP-FNR superfamily of transcriptional regulators. The unrooted, maximum-likelihood tree was constructed as described in the methods section. The tree with the highest log likelihood (-12984.9076) is shown. The members of the tree are identified by the NCBI Reference sequence identification number and by the name of the host bacterium. Drawings are to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 68 amino acid sequences. All positions containing gaps and missing data were eliminated. The branches of Alpha, Gamma and Betaproteobacteria are differentiated by the following colors: green, red and blue, respectively. *H. seropedicae* Fnr proteins are indicated with closed circles.



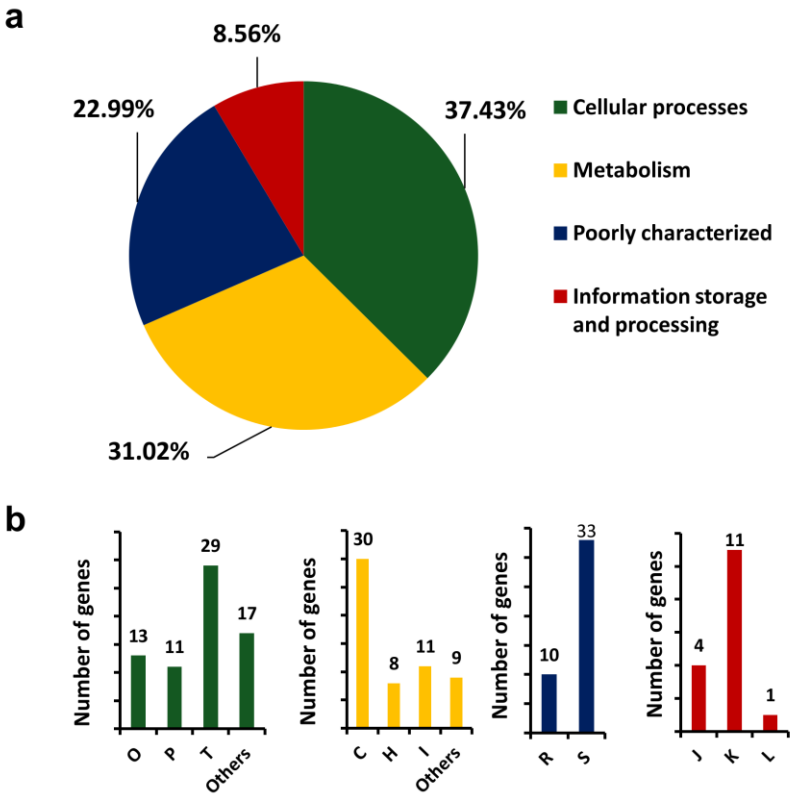
Supplementary Figure S3.1-2: Construction and validation of the *fnr1*, *fnr2* and *fnr3* mutant strains. (a) Chromosomal regions encoding *fnr1, fnr2* and *fnr3*. The genes and predicted functions in the *fnr1* locus are: *acd*, 1-aminocyclopropane-1-carboxylate deaminase; *ompW1*, outer membrane W protein; Hsero_3195, fatty acid desaturase protein; Hsero_3196 hypothetical protein; Hsero_3198, transmembrane protein. The genes and predicted functions in the *fnr2* locus are: Hsero_2379, AraC family transcription regulator protein; Hsero_2380, hypothetical protein; Hsero_2382, acyltransferase family protein. The genes and predicted functions in the *fnr3* locus are: Hsero_2537, nicotinamidase protein; Hsero_2539, type 2 phosphatidic acid phosphatase family protein; Hsero_2540, sphingosine kinase/eukaryotic diacylglicerol kinase protein; *cpdA*, 3'5' cyclic-nucleotide phosphodiesterase protein. The regions deleted in *fnr1*, *fnr2* and *fnr3* are indicated by Δ, as well the primers used for PCR genotyping are indicating by thin arrows external to the genes. (b) Genotypic validation of strains MB1 (*fnr1* deletion), MB2 (*fnr2* deletion) and MB3 (*fnr3* deletion). PCR was performed by using primers external to the targeted gene (as indicated in *A*). Lanes: 1, 1 Kb ladder Axygen; 2, water control; 3, SmR1; 4, suicide vector (pMBB1DS, pMBB2DS or pMBB3DS); 5, intermediate strain; 6, final deletion strain. Double and triple mutants were genotyped in the same fashion. On the left are indicated the length in base pairs (bp) of the DNA ladder.



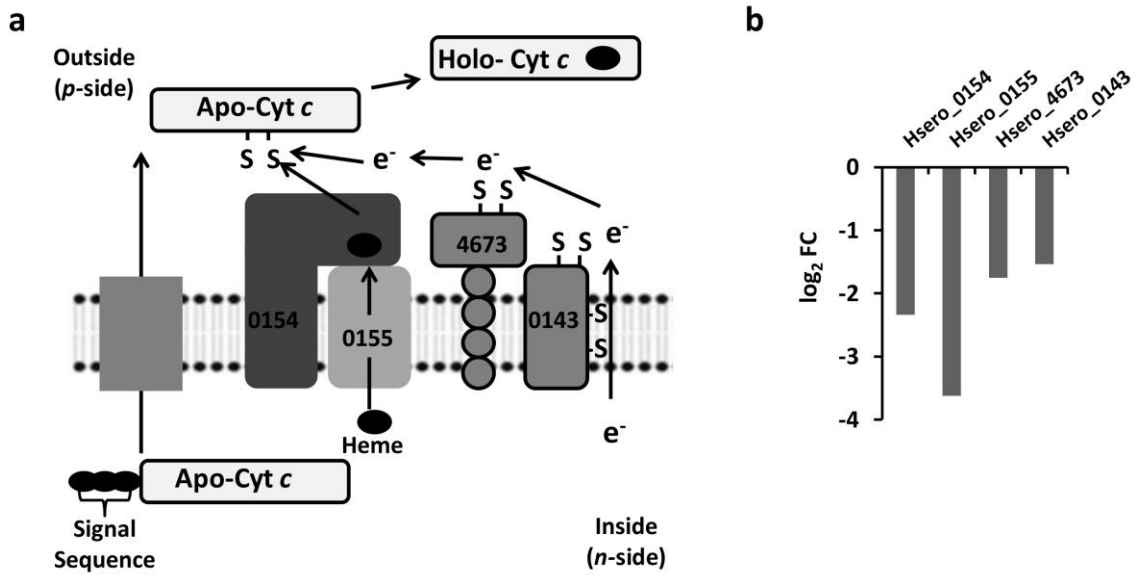
Supplementary Figure S3.1-3: Influence of *fnr* genes on growth under combined nitrogen and oxygen limitation. The growth of SmR1 (black squares) and MB231 (grey circles) were assayed in NFbHP-Malate minimal media supplemented with high (filled line) ammonium concentration (20mM NH4Cl) or low (dotted lines) ammonium concentration (2mM NH4Cl). 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.



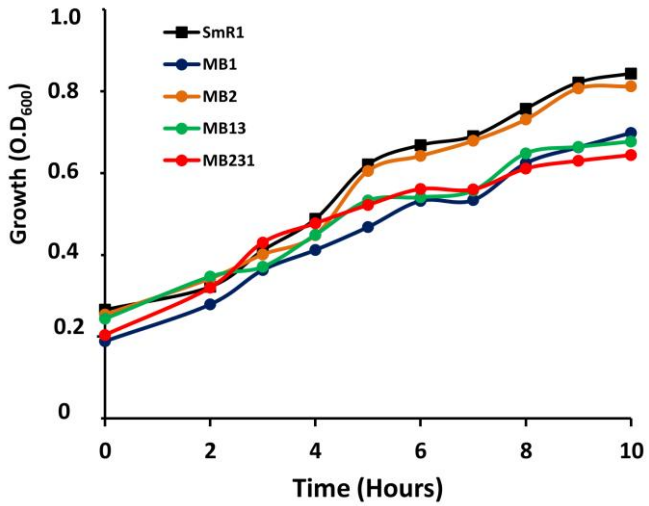
Supplementary Figure S3.1-4: Deletion of the three *fnr* genes does not affect growth under 20.8% oxygen. The growth of SmR1 (black squares) and MB231 (grey circles) were assayed in NFbHP-Malate minimal media supplemented with 20 mM ammonium chloride under air (20,8% oxygen). The data represents the mean of two independent assays performed in duplicate. Error bars indicate standard deviations. In some cases these are not visible as they are smaller than the graph points.



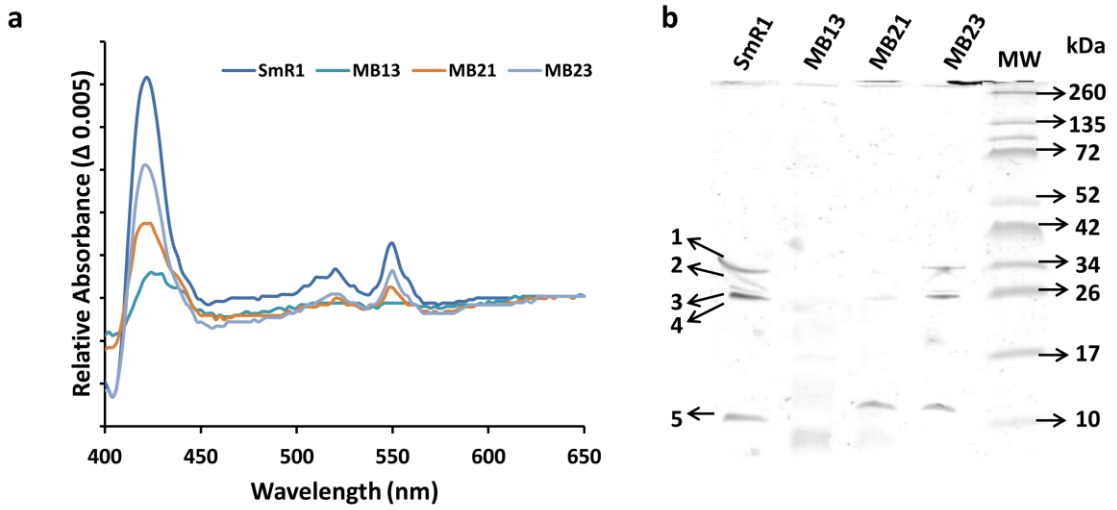
Supplementary Figure S3.1-5: Functional COG classification of all differentially expressed genes revealed by comparison of the RNA-Seq libraries of *H. seropedicae* MB231 (*fnr* ablated strain) and SmR1 (wild type) strains. (a) Distribution of regulated genes into four general categories. The categories were classified according to its abundance in the clockwise direction. (b) Detailed distribution of the most representative functional categories in the data. The letters referring to each specific category are: O, post-translational modification; protein turnover, chaperone functions; P, Inorganic ion transport and metabolism; T, signal transduction; C, energy production and conversion; H, coenzyme metabolism; I, lipid metabolism; R, general functional prediction only; S, function unknown; J, translation; K, transcription; L, replication and repair.



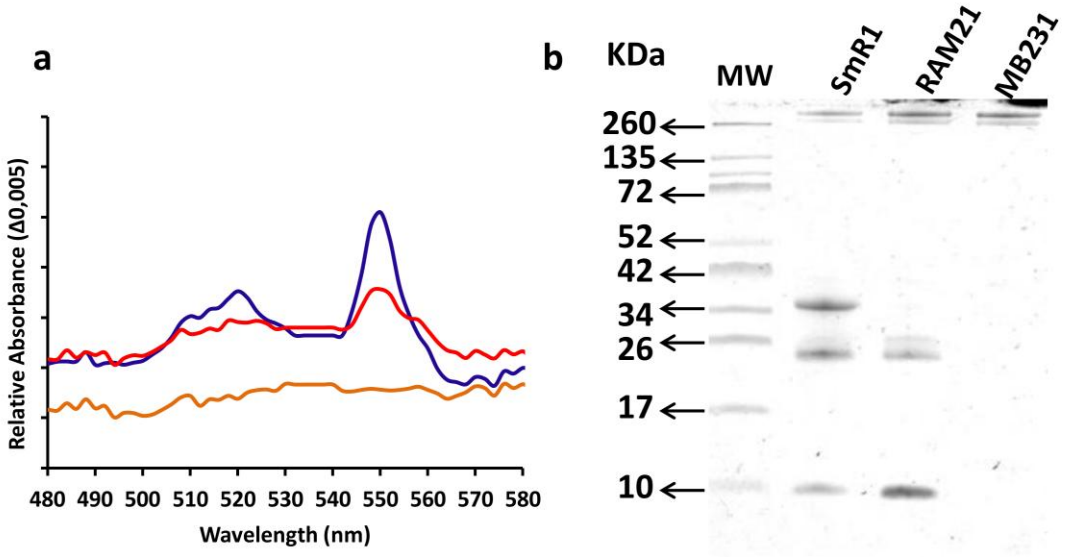
Supplementary Figure S3.1-6: The System II (CCS - Cytochrome C Synthesis) for cytochrome *c* maturation pathway in *H. seropedicae.* (a) Schematic representation of cytochrome *c* maturation based on genes present in the genome. The genes and predicted functions are: Hsero_0154: *resB* cytochrome *C*-type biogenesis transmembrane protein; Hsero_0155: *ccmC* (*resC*) ABC-type transport system, permease component protein; Hsero_4673: Hsero_4673 (*resA*) thioredoxin protein; Hsero_0143: *dsbD* thiol:disulfide interchange transmembrane protein. (b) Level of differential expression of genes coding for components of CCS. The genes were down regulated in the MB231 strain, indicating that they are Fnr activated. FC means fold change.



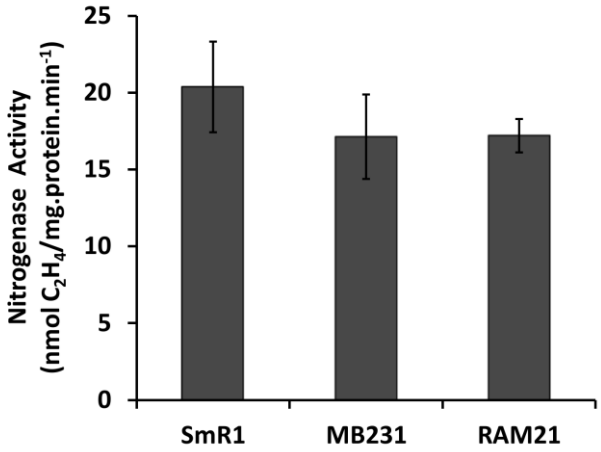
Supplementary Figure S3.1-7: The Fnr2 protein is not required for growth under oxygen-limiting conditions. The growth of SmR1 (black squares), MB1 (blue circles), MB2 (orange circles), MB13 (green circles) and MB231 (red circles) were assayed in NFbHP-Malate minimal media supplemented with 20 mM ammonium chloride under an oxygen concentration of 5.0 % in the gas phase of the culture flask.



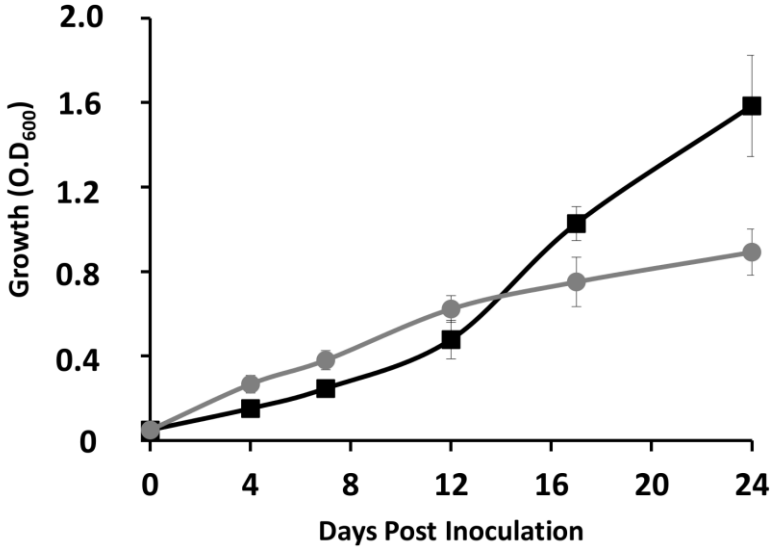
Supplementary Figure S3.1-8: The levels of *c*-type cytochromes in *H.seropedicae* strains MB13, MB21 and MB23 are similar to those of the strains, MB231, MB1 and MB3, respectively. (a) UV–visible reduced minus oxidized absorption spectra and (b) heme stain profile of protein extracts from *H. seropedicae* SmR1 (wild type), MB13, MB21 and MB23 mutant strains.



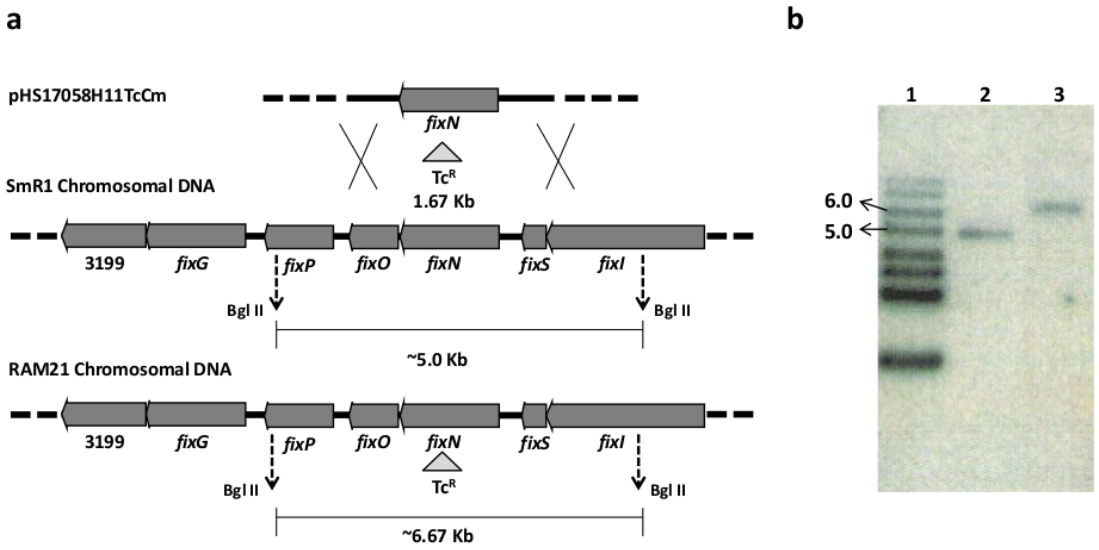
Supplementary Figure S3.1-9. The RAM21 strain is deficient in the levels of *c*-type cytochromes. (a) Reduced minus oxidized visible absorption spectra of protein extracts from *H. seropedicae* SmR1 (blue), RAM21 (red) and MB231 (orange) strains. The UV–visible difference spectra of 0.3 mg.mL^{-1} protein extracts was recorded as described in Methods. (b) Heme stain profile of protein extracts from *H. seropedicae* SmR1 (wild type), RAM21 (*fixN* mutant) and MB231(triple *fnr* mutant) strains. Samples (50 μg protein per lane) from all the strains were separated by 10% Tris-Tricine SDS-PAGE and stained for covalently bound heme with *o*-dianisidine as described in Methods.



Supplementary Figure S3.1-10. Influence of Fnr and FixN on nitrogenase activity in semi solid media. The acetylene reduction assay was performed as described in Methods using strains grown in semi-solid medium supplemented with 0.5 mM of sodium glutamate. Data represent the average of two independent experiments performed in duplicate. Error bars indicate standard deviations.



Supplementary Figure S3.1-11: Influence of *fnr* genes deletion on diazotrophic growth. The SmR1 (black squares) and MB231 (grey circles) strains were collected by centrifugation, washed with NFbHP-Malate minimal media without addition of nitrogen source and ressuspended back in the same media to an initial optical density of 0.05. Strains were incubated statically at 30°C. In some cases error bars are not visible as they are smaller than the graph points.



Supplementary Figure S3.1-12: Construction and validation of the *fixN* mutant strain. (a) Schematic representation of the mutagenic plasmid (pHS17058H11TcCm) and its integration into *H. seropedicae* SmR1 *fixN* chromosomal region. The genes and predicted functions in the locus are: Hsero_3199, FixH domain containing protein; *fixG*, iron-sulfur 4Fe-4S ferredoxin transmembrane protein; *fixP*, cbb_3-type cytochrome c oxidase-subunit III; *fixO*, cbb_3-type cytochrome oxidase-subunit II; *fixN*,cbb_3- type cytochrome c oxidase, subunit I; *fixS*, nitrogen fixation protein P-type ATPase protein; *fixI*, cation transport P-type ATPase protein. Genes are not drawn to scale. (b) Genotypic validation of RAM21 strain (insertion of Tc^R transposon into *fixN* gene) by DNA hybridization using a specific *fixN* probe. Lanes: 1, 1 Kb ladder (Fermentas); 2, *H. seropedicae* SmR1 chromosomal DNA digested with Bgl II; 3, *H. seropedicae* RAM21 chromosomal DNA digested with Bgl II. On the left are indicated the length in kilobase pairs of the DNA ladder.

Supplementary Table S3.1-1 - Strains and plasmids used in this study

Strains and Plasmids	**Relevant Characteristic**	**Source or Reference**
E. coli		
TOP 10	*hsd*R, *mcr*A, lacZΔM15, *rec*A	INVITROGEN
S17.1	Sm^R, Tra^+	(SIMON et al., 1983)
H. seropedicae		
SmR1	Z78 but Sm^R 100µg/mL, Nif+	(SOUZA et al., 2000)
MB1	Same as SmR1, but with a deletion at *fnr1* gene	This work
MB2	Same as SmR1, but with a deletion at *fnr2* gene	This work
MB3	Same as SmR1, but with a deletion at *fnr3* gene	This work
MB13	Same as SmR1, but with deletions at *fnr1* and *fnr3* genes	This work
MB21	Same as SmR1, but with deletions at *fnr2* and *fnr1* genes	This work
MB23	Same as SmR1, but with deletions at *fnr2* and *fnr3* genes	This work
MB231	Same as SmR1, but with deletions at *fnr1*, *fnr2* and *fnr3* genes	This work
RAM21	Same as SmR1, but with insertion of a TET-1 transposon at *fixN* gene	This work
Plasmids		
pTZ57R/T	Amp^R , TA cloning vector	FERMENTAS
pSUP202	Cb^R, Cm^R, Tc^R, Mob	(SIMON et al., 1983)
pMH1701	Km^R, contains the *nptI-sacR-sacB* cassete	(HYNES et al., 1989)
pPW452	Same as pMP220, but with inverted cloning site	(WOODLEY et al., 1996)
pTnMod-OCm	pMB1, Cam^R mob+ Tn5 tnp	(DENNIS; ZYLSTRA, 1998)
pHS17058H11	pUC18 derivative plasmid from *H. seropedicae* SmR1 genomic sequencing library containing the *fixN* gene	(PEDROSA et al., 2011)
pHS17058H11Tc	Same as pHS17058H11 with the TET-1 transposon inserted at the *fixN* gene	This work
pHS17058H11TcCm	Same as pHS17058H11Tc with the Cam^R cassete (from pTnMod-Com) inserted outside the *fixN* gene	This work
pTZFNR1A	Contains 198 bp upstream to *fnr1* plus 294 bp of the 5' coding region in pTZ57R/T vector.	This work
pTZFNR1B	Contains 245 bp downstream to *fnr1* plus 255 bp of the 3' coding region in pTZ57R/T vector	This work
pTZFNR2A	Contains 333 bp upstream to *fnr2* plus 279 bp of the 5' coding region in pTZ57R/T vector	This work
pTZFNR2B	Contains 357 bp downstream to *fnr2* plus 273 bp of the 3'	This work

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	coding region in pTZ57R/T vector	
pTZFNR3A	Contains 315 bp upstream to *fnr3* plus 255 bp of the 5' of the coding region in pTZ57R/T vector	This work
pTZFNR3B	Contains 315 bb downstream to *fnr3* plus 264 pb of the 3' coding region in pTZ57R/T vector	This work
pTZFNR1DEL	Contains the XhoI and HindIII fragment of pTZFNR1A vector in pTZFNR1B to yield a plasmid with a 279 bp deletion in *fnr1*	This work
pTZFNR2DEL	Contains the XhoI and HindIII fragment of pTZFNR2A vector in pTZFNR2B to yield a plasmid with a 276 bp deletion in *fnr2*	This work
pTZFNR3DEL	Contains the XhoI and HindIII fragment of pTZFNR3A vector in pTZFNR3B to yield a plasmid with a 267 bp deletion in *fnr3*	This work
pMBB1D	Contains the HindIII and BamHI fragment of pTZFNR1DEL vector in pSUP202 sucide vector	This work
pMBB2D	Contains the HindIII and BamHI fragment of pTZFNR2DEL vector in pSUP202 sucide vector	This work
pMBB3D	Contains the HindIII and BamHI fragment of pTZFNR3DEL vector in pSUP202 sucide vector	This work
pMBB1DS	Same as pMBB1D, but with the *nptI-sacR-sacB* cassete inserted at BamHI site	This work
pMBB2DS	Same as pMBB2D, but with the *nptI-sacR-sacB* cassete inserted at BamHI site	This work
pMBB3DS	Same as pMBB3D, but with the *nptI-sacR-sacB* cassete inserted at BamHI site	This work
pTZPFN	Contains the putative promoter region of *fixNOP* operon in pTZ57R/T vector	This work
pTZPHN	Contains the putative promoter region of *hemN-Hsero_3206-fixIS* operon in pTZ57R/T vector	This work
pPWPFN	TcR, Mob, *fixN::lacZ* fusion, PstI and BglII fragment of pTZPFN in pPW452 vector	This work
pPWPHN	TcR, Mob, *hemN::lacZ* fusion, PstI and BglII fragment of pTZPHN in pPW452 vector	This work

Supplementary Table S3.1-2 - Sequences Retrieved for Phylogenetic Analyses

Class	Clade	Order	Ref seq[a]	Locus Tag[b]	Definition	Gene	Organism
Betaproteobacteria	**Fnr Group**	**1**	ZP_08132409	HMPREF9098_0136	transcriptional regulator Anr	-	*Kingella denitrificans* ATCC 33394
		2	ZP_08246766	HMPREF9123_0193	transcriptional regulator Anr	-	*Neisseria bacilliformis* ATCC BAA-1200
		3	YP_208620	NGO1579	anaerobic transcriptional regulator	-	*Neisseria gonorrhoeae* FA 1090
		4	NP_903317	CV_3647	Crp/Fnr family transcriptional regulator	-	*Chromobacterium violaceum* ATCC 12472
		5	YP_002552485	Dtpsy_1007	Crp/Fnr family transcriptional regulator	-	*Acidovorax ebreus* TPSY
		6	YP_001021661	Mpe_A2471	transcription factor Fnr	*fnr*	*Methylibium petroleiphilum* PM1
		7	YP_003523050	Slit_0422	Crp/Fnr family transcriptional regulator	-	*Sideroxydans lithotrophicus* ES-1
		8	YP_003846080	Galf_0271	CarD family transcriptional regulator	*carD*	*Gallionella capsiferriformans* ES-2
		9	ZP_08505489	METUNv1_02552	Transcriptional activator protein anr	-	*Methyloversatilis universalis* FAM5
		10	YP_932855	azo1351	fnr gene product	-	*Azoarcus sp.* BH72
		11	YP_159953	ebA5149	transcription factor Fnr	-	*Aromatoleum aromaticum* EbN1
		12	YP_726481	H16_A2013	transcriptional regulator, FNR-like	*fnr2*	*Ralstonia eutropha* H16
		13	YP_586655	Rmet_4521	Crp/Fnr family transcriptional regulator	-	*Cupriavidus metallidurans* CH34
		14	YP_004348107	bgla_2g01140	Transcriptional regulator, Crp/Fnr family protein	-	*Burkholderia gladioli* BSR3
		15	YP_335288	BURPS1710b_A0129	fnrL gene product	*fnrL*	*Burkholderia pseudomallei* 1710b
		16	YP_336174	BURPS1710b_A1016	fnrL gene product	*fnrL*	*Burkholderia pseudomallei* 1710b
		17	YP_003775788	Hsero_2381	Crp/Fnr family transcription regulator	*fnr2*	*Herbaspirillum seropedicae* SmR1
		18	YP_003775945	Hsero_2538	Crp/Fnr family transcription regulator	*fnr3*	*Herbaspirillum seropedicae* SmR1
		19	YP_003776587	Hsero_3197	Crp/Fnr family transcription regulator	*fnr1*	*Herbaspirillum seropedicae* SmR1
		20	YP_001100589	HEAR2336	fnr gene product	*fnr*	*Herminiimonas arsenicoxydans*
		21	YP_001353329	mma_1639	Crp/FNR family transcriptional regulator	-	*Janthinobacterium. sp. Marseille*
		22	YP_001099920	HEAR1637	btr gene product	*btr*	*Herminiimonas arsenicoxydans*
		23	NP_879971	BP1197	btr gene product	*btr*	*Bordetella pertussis* Tohama I

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		24	NP_942911	PHG276	transcriptional regulator	*fnr*	*Ralstonia eutropha* H16
		25	YP_726776	H16_A2312	transcriptional regulator, FNR-like	*fnr3*	*Ralstonia eutropha* H16
		26	YP_584185	Rmet_2037	fnrL gene product	*fnrL*	*Cupriavidus metallidurans* CH34
		27	YP_841586	H16_B2074	transcriptional regulator, FNR-like	*fnr5*	*Ralstonia eutropha* H16
		28	YP_583842	Rmet_1692	fnr gene product	*fnr*	*Cupriavidus metallidurans* CH34
		29	YP_841057	H16_B1540	transcriptional regulator, FNR-like	*fnr4*	*Ralstonia eutropha* H16
Gammaproteobacteria		**30**	YP_002799173	Avin_19910	cydR gene product	*cydR*	*Azotobacter vinelandii* DJ
		31	YP_259029	PFL_1910	anr gene product	*anr*	*Pseudomonas fluorescens* Pf-5
		32	YP_001750434	PputW619_3583	Crp/FNR family transcriptional regulator	-	*Pseudomonas putida* W619
		33	ZP_05638922	PsyrptA_020100016591	transcriptional regulator Anr	-	*Pseudomonas syringae* pv. tabaci str. ATCC
		34	NP_250235	PA1544	anr gene product	*anr*	*Pseudomonas aeruginosa* PAO1
		35	ZP_01305649	RED65_09994	transcriptional regulator Anr	-	*Bermanella marisrubri*
		36	ZP_09189237	KUC_2859	Transcriptional activator protein anr	-	*Halomonas boliviensis* LC1
		37	YP_003145738	Kkor_0550	unnamed protein product	-	*Kangiella koreensis* DSM 16069
		38	YP_004392595	B565_1943	fumarate/nitrate reduction transcriptional regulator	-	*Aeromonas veronii* B565
		39	YP_003556912	SVI_2163	etrA gene product	*etrA*	*Shewanella violacea* DSS12
		40	YP_001760790	Swoo_2417	fumarate/nitrate reduction transcriptional regulator	-	*Shewanella woodyi* ATCC 51908
		41	ZP_04412887	VCA_001046	fumarate and nitrate reduction regulatory protein	-	*Vibrio cholerae* bv. albensis VL426
		42	ZP_02477756	HPS_05113	regulatory protein HlyX	-	*Haemophilus parasuis* 29755
		43	YP_005176618	Pmu_07350	fnr gene product	*fnr*	*Pasteurella multocida* 36950
		44	YP_003468250	XBJ1_2353	fnr gene product	*fnr*	*Xenorhabdus bovienii* SS-2004
		45	YP_002238752	KPK_2925	fnr gene product	*fnr*	*Klebsiella pneumoniae* 342
		46	NP_415850	b1334	DNA-binding transcriptional dual regulator	*fnr*	*Escherichia coli* str. K-12 substr. MG1655
Alfaproteobacteria	**Fixk Group**	**47**	NP_769397	bll2757	fixK gene product	*fixk*	*Bradyrhizobium japonicum* USDA 110
		48	YP_568479	RPD_1340	transcriptional regulator FixK	*fixk*	*Rhodopseudomonas palustris* BisB5
		49	NP_768749	bll2109	fixK gene product	*fixk*	*Bradyrhizobium japonicum* USDA 110

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		50	YP_001682260	Caul_0629	transcriptional regulator FixK	*fixk*	*Caulobacter sp*. K31
		51	ZP_05102573	RGAI101_3810	FixK1 Transcriptional activator	*fixk*	*Roseobacter sp*. GAI101
		52	NP_435913	SMa1225	transcriptional regulator FixK	*fixk*	*Sinorhizobium meliloti* 1021
		53	YP_005724846	SM11_pC0964	fixK1 gene product	*fixk1*	*Sinorhizobium meliloti* SM11
	FnrN Group	**54**	NP_637170	XCC1805	fumarate and nitrate reduction regulatory protein	*fnr*	*Xanthomonas campestris* pv. campestris str. ATCC
		55	AAB58263	1	Fnr-type transcriptional regulator FnrN2	-	*Rhizobium leguminosarum* bv. Viciae UPM791
		56	AAA86478	2	FnrN	-	*Rhizobium leguminosarum* bv. Viciae UPM791
		57	NP_107101	mll6632	Fnr-type transcriptional regulator	-	*Mesorhizobium loti* MAFF303099
		58	ZP_01742514	RB2150_17129	Crp-Fnr regulatory protein (FnrL)	-	*Rhodobacteraceae bacterium* HTCC2150
		59	ZP_00956255	EE36_11304	Crp-Fnr regulatory protein (FnrL)	-	*Sulfitobacter sp.* EE-36
		60	NP_772701	bll6061	fixK gene product	*fixk*	*Bradyrhizobium japonicum* USDA 110
		61	YP_005608508	BJ6T_36460	fixK gene product	*fixk*	*Bradyrhizobium japonicum* USDA 6
		62	ZP_08628885	CSIRO_1970	transcriptional regulator, Crp/Fnr family	-	*Bradyrhizobiaceae bacterium* SG-6C
		63	CCD01536	AZOBR_p210200	transcriptional activatory protein aadR	*aadR*	*Azospirillum brasilense* Sp245
		64	YP_004974940	AZOLI_p20689	Crp family transcriptional regulator	-	*Azospirillum lipoferum* 4B
		65	YP_001592512	BCAN_A0667	transcriptional activator protein fnrA	*fnrA*	*Brucella canis* ATCC 23365
		66	YP_002540637	Arad_7576	nitrogen fixation transcriptional regulator protein	*fixkf*	*Agrobacterium radiobacter* K84
		67	ZP_08870168	AZA_90412	Transcriptional activator protein Anr	-	*Azospirillum amazonense* Y2
		68	YP_005040097	AZOLI_2692	fixK gene product	*fixk*	*Azospirillum lipoferum* 4B

a NCBI reference sequence number

b Locus Tags are given for all organisms that have complete genome deposited in NCBI database until June 2012. The sequences identified with NCBI reference sequence numbers AAB58263 and AAA86478 were subject of independent deposition in NCBI database in 24-FEB-1997 (GUTIÉRREZ et al., 1997) and 15-DEC-1994 (HERNANDO et al., 1995), respectively.

Supplementary Table S3.1-3: Primers used in this study

Primer	Restriction Site	Sequence (5'-->3')[a]	Genome Position[b]	Aplication
1DA+	HindIII	TGCATC**AAGCTT**GTGTGGTA	From 3659172 to 3659191	Cloning 198 bp upstream to *fnr1* plus 294 bp of the 5' coding region
1DA-	XhoI	GCCGTC**CTCGAG**TTCCTGATA	From 3659664 to 3659684	
1DB+	XhoI	GTGAAC**CTCGAG**TCACGCTAC	From 3659952 to 3659972	Cloning 245 bp downstream to *fnr1* plus 255 bp of the 3' coding region
1DB-	BamHI	TCTTTT**GGATCC**TCAACCCG	From 3660432 to 3660451	
2DA+	HindIII	GGAACA**AAGCTT**TCAGCAGC	From 2710337 to 2710356	Cloning 333 bp upstream to *fnr2* plus 279 bp of the 5' coding region
2DA-	XhoI	GGTATG**CTCGAG**GGCAATCAC	From 2710928 to 2710948	
2DB+	XhoI	AATGCC**CTCGAG**GAATTCTCG	From 2711216 to 2711236	Cloning 357 bp downstream to *fnr2* plus 273 bp of the 3' coding region
2DB-	BamHI	AGGTTG**GGATCC**TGGTGGAAG	From 2711825 to 2711845	
3DA+	HindIII	ACTGGA**AAGCTT**GGCCTATG	From 2891852 to 2891871	Cloning 315 bp upstream to *fnr3* plus 255 bp of the 5' of the coding region
3DA-	XhoI	CTGGAAGGT**CTCGAG**ATGGCC	From 2892401 to 2892421	
3DB+	XhoI	ATGACGGCG**CTCGAG**AAGATG	From 2892671 to 2892691	Cloning 315 bb downstream to *fnr3* plus 264 pb of the 3' coding region
3DB-	BamHI	GTATAGCCC**GGATCC**AGTTCG	From 2893229 to 2893249	
PFNP	PstI	TGGAAG**CTGCAG**ATCTCCTCAT	From 3666293 to 3666314	Amplify 360 bp of the promoter region of *fixNXOP* operon
PFNB	BglII	ATTGGCGC**AGATCT**GTGTAGTT	From 3665955 to 3665976	
PHNP	PstI	TTAACATTG**CTGCAG**TTGGGCT	From 3671461 to 3671440	Amplify 367 bp of the promoter region of *hemNHsero_3206fixIS*operon
PHNB	BglII	TCTTGTTCA**AGATCT**CGAGACT	From 3671095 to 3671116	
1F	None	ACCAGTTGTGCCTGCCCA	From 3659509 to 3659526	Confirmation of cloning and mutations
1R	None	CTGCTTGCGGAACTTGGA	From 3660075 to 3660089	
2F	None	CAATGACCTGAGTAAGCGT	From 2710684 to 2710702	Confirmation of cloning and mutations
2R	None	GAACTTGCTGATGAGGCG	From 2711297 to 2711314	
3F	None	TTCCACCCACAAGCATTC	From 2892184 to 2892201	Confirmation of cloning and mutations
3R	None	TGAAGTTGGTCAGCAGGC	From 2892819 to 2892836	

[a]Highlighted in bold are the sequences of restriction sites inserted in the primers

[b]Positions of primers annealing are given for *H.seropedicae* SmR1 genome

Supplementary Dataset 3.1-1 - Differentially expressed genes revealed by comparison of the RNA-Seq libraries of *H. seropedicae* MB231 (*fnr* ablated strain) and SmR1 (wild type) strain.

(a) - Genes down-regulated in the MB231 strain or activated by Fnr.

Feature ID	**Locus Tag**	**FoldChange MB231/SmR1**	**$\log_2$ FoldChange**	***p*-value**	**Gene Description**
cutA	Hsero_0142	0.230230444	-2.11884948	0.04723319	*cutA* periplasmic divalent cation tolerance protein
dsbD	Hsero_0143	0.345116109	-1.534846277	0.098321666	*dsbD* thiol:disulfide interchange transmembrane protein
rpoE	Hsero_0150	0.173693724	-2.525382467	0.006082267	*rpoE* RNA polymerase sigma-24 factor protein
hemB	Hsero_0151	0.168568515	-2.568592999	0.002415662	*hemB* delta-aminolevulinic acid dehydratase protein
Hsero_0153	Hsero_0153	0.192822173	-2.374657136	0.010222833	Hsero_0153 cytochrome c553 protein
resB	Hsero_0154	0.197823243	-2.337716148	0.049515936	*resB* cytochrome C-type biogenesis transmembrane protein
ccmC	Hsero_0155	0.081201678	-3.622346648	2.67333E-05	*ccmC* ABC-type transport system, permease component protein
Hsero_0172	Hsero_0172	0.086992857	-3.522959241	0.020118366	Hsero_0172 AraC family transcription regulator protein
Hsero_0260	Hsero_0260	0.065262279	-3.937606819	0.000902533	Hsero_0260 GGDEF domain containing protein
Hsero_0261	Hsero_0261	0.013719553	-6.187622744	2.37622E-06	Hsero_0261 hypothetical protein
pta	Hsero_0263	0.023280705	-5.424721438	1.02783E-11	*pta* phosphate acetyltransferase protein
ackA	Hsero_0264	0.016882917	-5.888292006	3.00361E-12	*ackA* acetate kinase protein
phaC	Hsero_0265	0.033371626	-4.90523419	7.05189E-10	*phaC* poly(3-hydroxyalkanoate)synthetase protein
hpcH	Hsero_0323	0.263371075	-1.924831188	0.02379143	*hpcH* 2,4-dihydroxyhept-2-ene-1,7-dioic acid aldolase protein
dps	Hsero_0431	0.281377371	-1.829421786	0.016239293	*dps* DNA-binding ferritin-like protein
Hsero_0474	Hsero_0474	0.114720677	-3.123802651	0.000109159	Hsero_0474 conserved hypothetical protein
kdtA	Hsero_0475	0.11199294	-3.158520305	0.020080055	*kdtA* 3-deoxy-d-manno-octulosonic-acid (KDO) transferase protein
aerr	Hsero_0514	0.151176918	-2.725690212	0.04019552	*aer* aerotaxis sensor receptor (chemotaxis transducer) transmembrane protein
Hsero_0515	Hsero_0515	0.059624462	-4.067951853	3.1495E-06	Hsero_0515 hypothetical protein
Hsero_0623	Hsero_0623	0.304469318	-1.715631243	0.027873034	Hsero_0623 methyl-accepting chemotaxis transducer transmembrane protein

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cheW	Hsero_0624	0.099094141	-3.335056436	2.44495E-05	*cheW* positive regulator of CheA protein activity protein
cheR	Hsero_0625	0.085793624	-3.542985754	0.000124407	*cheR* chemotaxis protein methyltransferase protein
cheB	Hsero_0626	0.125545197	-2.993721263	0.003917035	*cheB* chemotaxis-specific methylesterase protein
Hsero_0627	Hsero_0627	0.186240075	-2.424764553	0.00301248	Hsero_0627 response regulator (EAL domain) protein
pilA	Hsero_0660	0.181502504	-2.461938644	0.032440429	*pilA* Type IV pilin A protein
cfa	Hsero_0681	0.239935687	-2.059280342	0.010711097	*cfa* cyclopropane-fatty-acyl-phospholipid synthase protein
pdxH	Hsero_0682	0.115790498	-3.110411224	9.61874E-05	*pdxH* pyridoxamine 5'-phosphate oxidase protein
Hsero_0962	Hsero_0962	0.022750017	-5.457988595	1.1583E-11	Hsero_0962 C4-dicarboxylate transporter
Hsero_0963	Hsero_0963	0.065908561	-3.923390307	0.000274149	Hsero_0963 helix-turn-helix motif containing protein
adhA	Hsero_0964	0.030811302	-5.020396554	0.013982814	*adhA* alcohol dehydrogenase protein
Hsero_1104	Hsero_1104	0.238110376	-2.070297607	0.005529194	Hsero_1104 cytochrome c551/c552 transmembrane protein
Hsero_1180	Hsero_1180	0.063996043	-3.965873479	0.000288134	Hsero_1180 small-conductance mechanosensitive channel protein
Hsero_1246	Hsero_1246	0.151861744	-2.719169614	0.000986812	Hsero_1246 beta-hydroxybutyrate dehydrogenase protein
Hsero_1247	Hsero_1247	0.080460736	-3.635571255	0.041588248	Hsero_1247 hemolysin protein
Hsero_1250	Hsero_1250	0.030561538	-5.032139042	0.000225343	Hsero_1250 methyl-accepting chemotaxis I protein
Hsero_1552	Hsero_1552	0.154380396	-2.695438535	0.023817366	Hsero_1552 conserved hypothetical protein
Hsero_1553	Hsero_1553	0.139391074	-2.842789914	0.020750516	Hsero_1553 2-polyprenyl-6-methoxyphenol hydroxylase protein
copA	Hsero_1601	0.113579791	-3.138221931	0.000792768	*copA* multicopper oxidase protein
copB	Hsero_1602	0.061633683	-4.020137192	0.000323044	*copB* copper tolerance protein
Hsero_1603	Hsero_1603	0.209785396	-2.253013842	0.014816882	Hsero_1603 hypothetical protein
copZ	Hsero_1609	0.154988759	-2.689764508	0.002602622	*copZ* heavy metal transport/detoxification protein
trxB	Hsero_1624	0.412559999	-1.277324147	0.094613546	*trxB* thioredoxin reductase oxidoreductase protein
Hsero_1625	Hsero_1625	0.115607383	-3.11269456	0.031599983	Hsero_1625 hypothetical protein
Hsero_1639	Hsero_1639	0.154942452	-2.690195623	0.000364531	Hsero_1639 phasin family protein protein
Hsero_1829	Hsero_1829	0.02720607	-5.199927619	5.75137E-08	Hsero_1829 outer membrane drug efflux lipoprotein
Hsero_1830	Hsero_1830	0.026275254	-5.250151467	6.22597E-11	Hsero_1830 type I antifreeze protein:HlyD family secretion protein
yhiH	Hsero_1831	0.024444437	-5.35435004	9.67502E-12	*yhiH* ABC-type multidrug transport system, ATPase component protein

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yhhJ	Hsero_1832	0.01885845	-5.728645081	3.52603E-07	*yhhJ* ABC-type multidrug transport system, permease component protein
clpB	Hsero_2110	0.143547922	-2.800395649	0.001764101	*clpB* ATP-dependent Clp protease subunit (heat-shock) protein
aceA	Hsero_2114	0.139565293	-2.840987873	0.003660524	*aceA* isocitrate lyase protein
Hsero_2263	Hsero_2263	0.192266899	-2.378817687	0.010276194	Hsero_2263 transmembrane protein
uspA	Hsero_2290	0.014516259	-6.106186482	1.02687E-15	*uspA* universal stress protein
Hsero_2379	Hsero_2379	0.039684318	-4.655287185	1.21084E-06	Hsero_2379 AraC family transcription regulator protein
fnr2	Hsero_2381	0.04466931	-4.484572216	0.055568016	*fnr2* Crp/Fnr family transcription regulator protein
Hsero_2383	Hsero_2383	0.1424221	-2.81175506	0.001493717	Hsero_2383 DNA polymerase IV protein
Hsero_2384	Hsero_2384	0.077577924	-3.688210023	0.004780962	Hsero_2384 cation transport P-type ATPase protein
Hsero_2387	Hsero_2387	0.016239428	-5.944355344	0.000342188	Hsero_2387 ABC-type export system, membrane fusion protein
Hsero_2388	Hsero_2388	0.020164537	-5.632035875	0.000132163	Hsero_2388 ABC-type export system, outer membrane channel protein
Hsero_2390	Hsero_2390	0.016582463	-5.914197893	4.64309E-05	Hsero_2390 flavodoxin protein
Hsero_2391	Hsero_2391	0.029054964	-5.105071523	0.000578851	Hsero_2391 conserved hypothetical protein
Hsero_2392	Hsero_2392	0.030523271	-5.033946608	1.87835E-11	Hsero_2392 molecular chaperone, small heat shock protein
Hsero_2393	Hsero_2393	0.019285602	-5.696332008	0.000489113	Hsero_2393 periplasmic or secreted lipoprotein
Hsero_2394	Hsero_2394	0.033845783	-4.884880102	1.81099E-07	Hsero_2394 hypothetical protein
Hsero_2395	Hsero_2395	0.007213297	-7.115125504	0.001005548	Hsero_2395 conserved hypothetical protein
Hsero_2396	Hsero_2396	0.009331621	-6.743656562	0.006058904	Hsero_2396 hypothetical protein
Hsero_2397	Hsero_2397	0.065141466	-3.940280011	0.00047144	Hsero_2397 hypothetical protein
Hsero_2398	Hsero_2398	0.014699644	-6.088075005	0.006770439	Hsero_2398 conserved hypothetical protein
Hsero_2399	Hsero_2399	0.02616795	-5.256055302	1.00845E-13	Hsero_2399 periplasmic or secreted lipoprotein
Hsero_2400	Hsero_2400	0.009837806	-6.667447626	2.94676E-12	Hsero_2400 universal stress protein
Hsero_2402	Hsero_2402	0.034090952	-4.874467304	1.44471E-08	Hsero_2402 hypothetical protein
Hsero_2403	Hsero_2403	0.02559612	-5.287931034	2.76262E-09	Hsero_2403 hemerythrin protein
dksA	Hsero_2404	0.010029916	-6.63954667	2.23539E-16	*dksA* DnaK suppressor protein
phbC	Hsero_2405	0.014191047	-6.138875195	1.55996E-16	*phbC* poly-beta-hydroxyalkanoate synthase protein

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fabI	Hsero_2406	0.007653677	-7.029631268	6.0166E-18	*fabI* NADH-dependent enoyl-[acyl-carrier-protein] reductase protein
Hsero_2407	Hsero_2407	0.008588539	-6.863371583	2.41751E-16	Hsero_2407 phosphate acetyl/butaryl transferase protein
cybB	Hsero_2437	0.0636715	-3.973208445	6.7648E-05	*cybB* cytochrome b561 protein
uspA	Hsero_2488	0.015687597	-5.99423178	2.81469E-15	*uspA* universal stress protein
Hsero_2510	Hsero_2510	0.009272663	-6.752800554	3.97972E-12	Hsero_2510 conserved hypothetical protein
ldhA	Hsero_2511	0.025871489	-5.272493107	5.98449E-10	*ldhA* D-lactate dehydrogenase protein
Hsero_2530	Hsero_2530	0.18838207	-2.408266438	0.003804295	Hsero_2530 hypothetical protein
lldD	Hsero_2594	0.129629855	-2.947530071	0.000289434	*lldD* L-lactate dehydrogenase protein
phnA	Hsero_2718	0.189798043	-2.39746298	0.01704267	*phnA* alkylphosphonate uptake protein
Hsero_2914	Hsero_2914	0.033485545	-4.900317721	1.43948E-08	Hsero_2914 methyl-accepting chemotaxis transducer transmembrane I protein
Hsero_2915	Hsero_2915	0.028066126	-5.155026239	6.22414E-08	Hsero_2915 methyl-accepting chemotaxis transducer transmembrane I protein
phbC	Hsero_2999	0.38920097	-1.36141279	0.049750856	*phbC* poly(3-hydroxyalkanoate) synthetase protein
aer	Hsero_3072	0.049991362	-4.322177356	0.043008347	*aer* aerotaxis sensor recptor (chemotaxis transducer)
ompW1	Hsero_3194	0.007320211	-7.093899128	0.0001025	*ompW1* outer membrane W protein
Hsero_3195	Hsero_3195	0.00825993	-6.91965469	0.000199126	Hsero_3195 fatty acid desaturase protein
Hsero_3196	Hsero_3196	0.063083022	-3.98660442	0.002535303	Hsero_3196 hypothetical protein
fnr1	Hsero_3197	0.021969098	-5.508380529	5.57747E-07	*fnr1* Crp/Fnr family transcription regulator protein
Hsero_3198	Hsero_3198	0.099721743	-3.325948095	0.214078089	Hsero_3198 transmembrane protein
Hsero_3199	Hsero_3199	0.01393582	-6.165058314	5.64919E-13	Hsero_3199 conserved hypothetical protein
fixG	Hsero_3200	0.012456601	-6.326945724	2.37237E-15	*fixG* iron-sulfur 4Fe-4S ferredoxin transmembrane protein
fixP	Hsero_3201	0.023582506	-5.406139142	1.67836E-14	*fixP* cbb3-type cytochrome c oxidase, subunit III transmembrane protein
fixO	Hsero_3202	0.013399429	-6.221684695	2.08034E-17	*fixO* cbb3-type cytochrome c oxidase, subunit II transmembrane protein
fixN	Hsero_3203	0.012198901	-6.357105009	5.35955E-18	*fixN* cbb3-type cytochrome c oxidase, subunit I transmembrane protein
fixS	Hsero_3204	0.014146259	-6.143435625	0.001235458	*fixS* nitrogen fixation protein
fixI	Hsero_3205	0.053338329	-4.228683564	0.005987544	*fixI* cation transport P-type ATPase protein
Hsero_3206	Hsero_3206	0.046064874	-4.440189113	0.083549068	Hsero_3206 conserved hypothetical protein
hemN	Hsero_3207	0.055667835	-4.167012211	1.7214E-07	*hemN* oxygen-independent coproporphyrinogen III oxidase protein

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Hsero_3223	Hsero_3223	0.222242676	-2.169792217	0.005792512	Hsero_3223 arginyl-tRNA:protein arginylyltransferase protein
Hsero_3256	Hsero_3256	0.054743197	-4.191176488	0.010058032	Hsero_3256 PKHD-type hydroxylase protein
Hsero_3273	Hsero_3273	0.126856156	-2.978734561	0.018725157	Hsero_3273 conserved hypothetical protein
ardA	Hsero_3370	0.149229347	-2.744396815	0.015147595	*ardA* anti-restriction ArdA protein
spoVK	Hsero_3473	0.217651866	-2.199905709	0.006721183	*spoVK* AAA+ class ATPase protein
Hsero_3572	Hsero_3572	0.219313709	-2.188932101	0.006543228	Hsero_3572 sulfurtransferase (Rhodanese-like) protein
Hsero_3575	Hsero_3575	0.264195753	-1.92032082	0.075485484	Hsero_3575 transcription regulator protein
gltX	Hsero_3576	0.244632687	-2.031310911	0.036140158	*gltX* glutamyl/glutaminyl-tRNA synthetase protein
Hsero_3626	Hsero_3626	0.140470077	-2.831665254	0.01157042	Hsero_3626 extracellular polysaccharide synthase protein
narK1	Hsero_3638	0.013355267	-6.226447323	1.79365E-14	*narK1* nitrite/nitrate transporter protein
narU	Hsero_3639	0.006156032	-7.34378352	9.71216E-15	*narU* nitrite/nitrate transporter protein
narG	Hsero_3640	0.012702708	-6.298720093	4.92609E-15	*narG* respiratory nitrate reductase alpha chain oxidoreductase protein
narH	Hsero_3641	0.027968726	-5.160041627	7.09537E-09	*narH* respiratory nitrate reductase beta subunit protein
narJ	Hsero_3642	0.023470906	-5.412982655	0.034600002	*narJ* respiratory nitrate reductase protein
narI	Hsero_3643	0.031906235	-4.970017822	0.004498163	*narI* respiratory nitrate reductase transmembrane gamma subunit protein
moaA	Hsero_3644	0.062377322	-4.002834572	0.002976713	*moaA* molybdenum cofactor biosynthesis enzyme A protein
narX	Hsero_3645	0.00726157	-7.105502724	4.30185E-08	*narX* transmembrane nitrate/nitrite sensor histidine kinase G143
narL	Hsero_3646	0.014746647	-6.083469182	3.05333E-12	*narL* nitrate/nitrite response regulator transcription regulator protein
Hsero_3790	Hsero_3790	0.134757952	-2.891557685	0.000800667	Hsero_3790 conserved hypothetical
oxyR	Hsero_3791	0.283620435	-1.817966611	0.017787296	*oxyR* LysR family transcription regulator protein
glcF	Hsero_3825	0.154383859	-2.695406171	0.00371256	*glcF* glycolate oxidase, iron-sulfur subunit oxidoreductase protein
glcE	Hsero_3826	0.284224224	-1.814898574	0.041127669	*glcE* glycolate oxidase iron-sulfur subunit oxidoreductase protein
glcD	Hsero_3827	0.123133903	-3.021700051	0.000595189	*glcD* glycolate oxidase (FAD-linked subunit) oxidoreductase protein
ubiF	Hsero_3852	0.163837885	-2.609659101	0.028493927	*ubiF* ubiquinone biosynthesis protein
pyrC	Hsero_3853	0.200733602	-2.316645956	0.005775378	*pyrC* dihydroorotase protein
Hsero_3877	Hsero_3877	0.052392244	-4.254502929	8.51801E-05	Hsero_3877 fumarylacetoacetate hydrolase family protein

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fabG	Hsero_3878	0.055905938	-4.160854659	0.08732927	*fabG* short-chain alcohol dehydrogenase protein
uspA	Hsero_3887	0.049780753	-4.328268145	3.16856E-07	*uspA* universal stress protein
petC	Hsero_4047	0.179314409	-2.47943667	0.001337011	*petC* cytochrome c1 precursor transmembrane protein
petB	Hsero_4048	0.087378875	-3.516571653	0.040609588	*petB* cytochrome b subunit transmembrane protein
petA	Hsero_4049	0.145418654	-2.781715743	0.00928671	*petA* transmembrane ubiquinol-cytochrome C reductase
dsbC	Hsero_4189	0.106976397	-3.224635576	0.001225182	*dsbC* thiol:disulfide interchange protein
Hsero_4190	Hsero_4190	0.10394935	-3.266047354	0.0002482	Hsero_4190 2-polyprenyl-6-methoxyphenol hydroxylase protein
Hsero_4240	Hsero_4240	0.171411173	-2.544466944	0.017496062	Hsero_4240 AraC family transcription regulator protein
Hsero_4241	Hsero_4241	0.271830591	-1.879220271	0.019026335	Hsero_4241 conserved hypothetical protein
cca	Hsero_4282	0.339146771	-1.560018339	0.038501814	*cca* tRNA nucleotidyltransferase protein
gst	Hsero_4283	0.321136957	-1.638739394	0.033122972	*gst* glutathione S-transferase protein
Hsero_4284	Hsero_4284	0.321145849	-1.638699446	0.040312402	Hsero_4284 NADH-ubiquinone oxidoreductase protein
Hsero_4673	Hsero_4673	0.296310689	-1.754817426	0.021033989	Hsero_4673 thioredoxin protein
crp	Hsero_4703	0.176641178	-2.501106395	0.006431329	*crp* cyclic nucleotide-binding protein
Hsero_4704	Hsero_4704	0.112889243	-3.147020075	0.000729664	Hsero_4704 acyl-CoA synthetase (AMP-forming)/AMP-acid ligase II protein
rhtB	Hsero_4787	0.175287928	-2.512201454	0.044863801	*rhtB* threonine efflux protein
blc	Hsero_4788	0.19152638	-2.38438498	0.007106997	*blc* outer membrane lipoprotein (lipocalin)

(b) - Genes up-regulated in the MB231 strain or repressed by Fnr.

Feature ID	**Locus Tag**	**FoldChange MB231/SmR1**	**log_2 FoldChange**	***p*-value**	**Gene Description**
Hsero_0266	Hsero_0266	4.603448113	2.202714885	0.014089851	Hsero_0266 transcription regulator protein
cheD1	Hsero_0394	4.208864259	2.073430982	0.030645068	*cheD1* methyl-accepting chemotaxis sensory transducer protein
nifR3	Hsero_0413	4.841816481	2.275548398	0.021259264	*nifR3* tRNA-dihydrouridine synthase protein or NifR3-like protein
Hsero_0638	Hsero_0638	15.74018646	3.976380726	0.042787665	Hsero_0638 glutathione S-transferase protein
Hsero_0947	Hsero_0947	7.254361004	2.858848541	0.023025569	Hsero_0947 dienelactone hydrolase (Esterase_Lipase superfamily) protein
Hsero_1022	Hsero_1022	5.815042934	2.539789843	0.008827137	Hsero_1022 NAD-dependent aldehyde dehydrogenase protein
hppA	Hsero_1172	3.133369133	1.647714739	0.010204968	*hppA* V-type H(+)-translocating pyrophosphatase protein
Hsero_1271	Hsero_1271	16.68043031	4.060084602	0.033949892	Hsero_1271 conserved hypothetical protein
Hsero_1278	Hsero_1278	9.389720271	3.231082179	0.012408768	Hsero_1278 AraC family transcription regulator protein
Hsero_1337	Hsero_1337	7.668858748	2.939011897	0.001452696	Hsero_1337 two component response regulator protein
Hsero_1591	Hsero_1591	2.929967993	1.550884905	0.037191387	Hsero_1591 integral membrane protein
Hsero_1698	Hsero_1698	31.03164619	4.955668327	1.53906E-08	Hsero_1698 methyl-accepting chemotaxis protein
cheW	Hsero_1699	8.070951789	3.012738817	0.001921494	*cheW* chemotaxis signal transduction protein
Hsero_1708	Hsero_1708	10.34917795	3.371444271	7.20193E-05	Hsero_1708 methyl-accepting chemotaxis transducer transmembrane protein
Hsero_1709	Hsero_1709	4.338222285	2.117103977	0.007962687	Hsero_1709 conserved hypothetical protein
ndh	Hsero_1756	5.375891211	2.426503943	0.000371061	*ndh* NADH dehydrogenase
ilvA	Hsero_1809	5.136743719	2.360854097	0.029818893	*ilvA* threonine dehydratase protein
tar	Hsero_2019	6.217563132	2.636349251	0.002521838	*tar* methyl-accepting chemotaxis protein
rsuA	Hsero_2175	4.902670676	2.293567856	0.026798593	*rsuA* RNA pseudouridylate synthase protein
Hsero_2228	Hsero_2228	8.635715951	3.110315791	3.38427E-06	Hsero_2228 permease of the major facilitator superfamily protein
Hsero_2304	Hsero_2304	2.731491574	1.449688973	0.028793258	Hsero_2304 nodulin 21 protein
Hsero_2458	Hsero_2458	8.244586272	3.043447099	0.003415437	Hsero_2458 two component response regulator protein
caiD	Hsero_2549	5.180260168	2.373024556	0.000223033	*caiD* enoyl-CoA hydratase protein

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caiA	Hsero_2550	4.135970113	2.048225761	0.003896082	*caiA* acyl-CoA dehydrogenase protein
Hsero_2554	Hsero_2554	12.81043279	3.679247312	0.008134987	Hsero_2554 short-chain alcohol dehydrogenase protein
Hsero_2566	Hsero_2566	4.026682908	2.009591868	0.056887232	Hsero_2566 ABC-type amino acid transporter, ATPase component protein
fadD	Hsero_2567	4.695142009	2.231168794	0.001438356	*fadD* long-chain acyl-CoA synthetase protein
Hsero_2622	Hsero_2622	4.50806561	2.172508512	0.046832761	Hsero_2622 conserved hypothetical protein
emrA	Hsero_2704	8.229956905	3.040884876	0.004911818	*emrA* multidrug resistance A (translocase) transmembrane secretion protein
Hsero_3064	Hsero_3064	3.932312227	1.975377877	0.042982571	Hsero_3064 GntR family transcription regulator protein
Hsero_3168	Hsero_3168	10.3519783	3.371834593	0.001575435	Hsero_3168 ABC-type antimicrobial peptide transporter ATPase component
Hsero_3169	Hsero_3169	8.23119248	3.041101454	0.090943308	Hsero_3169 ABC-type antimicrobial peptide transporter, permease component
Hsero_3170	Hsero_3170	4.41418801	2.142148079	0.043842171	Hsero_3170 conserved hypothetical protein
atzB	Hsero_3260	4.716113462	2.237598428	0.02658532	*atzB* hydroxy-atrazine ethyl amino hydrolase protein
dctD	Hsero_3357	7.682278449	2.941534256	0.043570748	*dctD* C4-dicarboxylate transport transcription regulator protein
wrbA	Hsero_3573	3.081730146	1.623740537	0.035558929	*wrbA* tryptophan repressor binding protein
Hsero_4082	Hsero_4082	3.760172865	1.910798988	0.025865377	Hsero_4082 conserved hypothetical protein
Hsero_4083	Hsero_4083	22.3364012	4.481324855	0.004617405	Hsero_4083 conserved hypothetical protein
Hsero_4098	Hsero_4098	5.658640747	2.500455547	0.000196286	Hsero_4098 hypothetical protein
hipA	Hsero_4099	5.695774017	2.509891908	0.00037306	*hipA* HipA domain-containing protein
Hsero_4100	Hsero_4100	13.17558809	3.719795452	0.003481117	Hsero_4100 conserved hypothetical protein
Hsero_4163	Hsero_4163	4.652082329	2.217876629	0.034119509	Hsero_4163 SAM-dependent methyltransferase protein
mqo	Hsero_4225	6.722401052	2.748976615	0.003204626	*mqo* malate:quinone oxidoreductase protein
groEL	Hsero_4441	3.161901911	1.660792613	0.04787784	*groEL* chaperonin GroEL protein

The full Supplementary dataset including the COG classification is available on line at: http://www.nature.com/srep/2013/130902/srep02544/full/srep02544.html

3.2 - Enhanced oxygen consumption in *Herbaspirillum seropedicae fnr* mutants leads to increased NifA mediated transcriptional activation

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3.2.1 Abstract

3.2.1.1 Background

Orthologous proteins of the Crp/Fnr family have been previously implicated in controlling expression and/or activity of the NifA transcriptional activator in some diazotrophs. This study aimed to address the role of three Fnr-like proteins from *H. seropedicae* SmR1 in controlling NifA activity and consequent NifA-mediated transcription activation.

3.2.1.2 Results

The activity of NifA-dependent transcriptional fusions (*nifA::lacZ* and *nifB::lacZ*) was analysed in a series of *H. seropedicae fnr* deletion mutant backgrounds. We found that combined deletions in both the *fnr1* and *fnr3* genes lead to higher expression of both the *nifA* and *nifB* genes and also an increased level of *nifH* transcripts. Expression profiles of *nifB* under different oxygen concentrations, together with oxygen consumption measurements suggest that the triple *fnr* mutant has higher respiratory activity when compared to the wild type, which we believe to be responsible for greater stability of the oxygen sensitive NifA protein. This conclusion was further substantiated by measuring the levels of NifA protein and its activity in *fnr* deletion strains in comparison with the wild-type.

3.2.1.3 Conclusions

Fnr proteins are indirectly involved in controlling the activity of NifA in *H. seropedicae,* probably as a consequence of their influence on respiratory activity in relation to oxygen availability. Additionally we can suggest that there is some redundancy in the physiological function of the three Fnr paralogs in this organism, since altered respiration and effects on NifA activity are only observed in deletion strains lacking both *fnr1* and *fnr3*.

3.2.1.4 Key words:

Herbaspirilum seropedicae, NifA, Fnr.

3.2.2 Background

The endophytic diazotroph *Herbaspirillum seropedicae* SmR1 is a Beta-proteobacterium found in association with economically important crops such as rice, maize, sugar cane and sorghum (MONTEIRO et al., 2012; CHUBATSU et al., 2012). *H. seropedicae* can fix nitrogen under micro-oxic and nitrogen limiting conditions and expression of *H. seropedicae nif* genes inside plant tissues has been demonstrated (RONCATO-MACCARI et al., 2003). The σ^{54}-dependent transcriptional activator, NifA, a member of the bacterial enhancer binding family (BUSH; DIXON, 2012) is a master regulator of *nif* gene expression in *H. seropedicae* SmR1 (CHUBATSU et al., 2012). NifA responds to both nitrogen and oxygen levels, being activated in response to limitation of these resources (SOUZA et al., 1999). Once active, NifA activates transcription of the σ^{54}-dependent *nif* promoters (DIXON; KAHN, 2004).

The Fnr protein, is a widespread transcriptional regulator that binds a $[4Fe–4S]^{2+}$ cluster to monitor the oxygen status in the cell (LAZAZZERA, B. A et al., 1996), and regulates the transcription of genes required for the metabolic switch in response to decreasing oxygen levels (SPIRO, STEPHEN; GUEST, 1990; SPIRO, 1994; KORNER et al., 2003). Orthologous proteins of the Crp/Fnr family (KORNER et al., 2003; MATSUI et al., 2013) have been previously implicated in controlling expression and/or activity of the NifA transcriptional activator in some diazotrophs (GRABBE et al., 2001; MESA et al., 2008; MATSUI et al., 2013). In *Klebsiella pneumoniae* Fnr influences NifA activity through modulation of the mechanism by which the NifL repressor protein is sequestered to the membrane (GRABBE et al., 2001). In *Bradyrhizobium japonicum* the Fnr-like protein, $FixK_{1,}$ negatively controls genes that are subject to NifA activation (MESA et al., 2008) suggesting that $FixK_{1,}$ can repress transcription at NifA-dependent promoters. Another precedent for Fnr involvement in NifA activity was observed by Monteiro and co-workers (MONTEIRO et al., 2003), who showed that the activity of an amino-terminally truncated form of *H. seropedicae* NifA was influenced by Fnr when expressed in an *Escherichia coli fnr*- background.

The *H. seropedicae* genome (PEDROSA et al., 2011) has three genes encoding for Fnr-like proteins (CHUBATSU et al., 2012) and a role for these Fnr orthologs in controlling the expression of the complete cytochrome *c* branch of the electron transport chain has been demonstrated (BATISTA et al., 2013). In this study we aimed to investigate the potential involvement of *H. seropedicae* Fnr proteins in the expression

and activity of NifA and the consequences for transcriptional activation of other *nif* genes.

We found that combined deletions in both the *fnr1* and *fnr3* genes lead to higher expression of *nifB::lacZ* and *nifA::lacZ* transcriptional fusions and increased *nifH* transcription. We also show that the oxygen consumption rate in multiple *fnr* deletion strains is higher than in the wild-type, which we believe to result in either higher stability or activity of the oxygen sensitive NifA protein and consequently increased transcriptional activation of *nif* genes.

3.2.3 Results and Discussion

To analyze if the three *fnr* genes in *H. seropedicae* influence either the expression level or the activity of NifA, we monitored expression of a *nifB::lacZ* fusion, as a reporter of NifA activity. We compared *nifB* expression in the wild-type strain (SmR1) with a double deletion strain, which lacks both *fnr1* and *fnr3* (MB13) and a strain carrying deletions in all three *fnr* genes (MB231). Multiple *fnr* deletion strains were not analysed in these experiments since single gene deletions did not influence NifA activity (see Figure 3.2-2). As *nifB* gene expression is tightly regulated by nitrogen and oxygen levels in the cell (REGO et al., 2006), the activity of the *nifB::lacZ* reporter fusion is only observed when the cultures exhaust the supply of fixed nitrogen and oxygen becomes limited, as the culture reaches a high cell density. Although the *fnr* deletions impaired growth under these conditions as observed previously (BATISTA et al., 2013), the activity of the *nifB::lacZ* fusion was significantly higher after 12-16 hours incubation in the strain lacking both *fnr1* and *fnr3* (MB13) and also in the triple *fnr* deletion strain (MB231) when compared with the wild-type (Figure 3.2-1A). This suggests that either NifA expression or its activity is more highly induced in cultures of these multiple *fnr* deletion strains.

We considered the possibility that the multiple *fnr* deletion strains exhaust dissolved oxygen in the media faster than the wild type strain, thus leading to higher activity or stability of NifA in cultures of the *fnr* deletion mutants. To examine this further, we assayed *nifB::lacZ* activity in cultures grown in the absence of fixed nitrogen under defined initial oxygen concentrations of oxygen in the gas phase (Figure3.2-1B). As anticipated, *nifB* expression was not detected under either 8% or 20.8% oxygen in both wild-type and the *fnr* triple deletion mutant, presumably because

H. seropedicae NifA is inactivated at high oxygen concentrations (SOUZA et al., 1999; OLIVEIRA et al., 2009). However, the activity of the *nifB::lacZ* promoter fusion was markedly higher in the triple *fnr* deletion strain (MB231) compared with the parental strain, when cultures were incubated under an initial oxygen concentration of 4% or 6% in the gas phase (Figure 3.2-1B). To ensure that the increase of *nifB* expression observed in the mutant strains was NifA-dependent, we prepared single *nifA*$^-$ and multiple deletion strains carrying a *nifA* deletion in addition to the *fnr* mutations (Supplementary Fig. S3.2-1) and confirmed that the influence of Fnr proteins on *nifB* promoter activation requires NifA protein (Supplementary Fig. S3.2-2).

Since expression of the *nifA* gene itself is subject to autoactivation in *H. seropedicae* (WASSEM et al., 2002), we tested the influence of *fnr* deletions on *nifA* expression using various *nifA::lacZ* promoter constructs (Figure 3.2-2). Transcriptional regulation of *nifA* is complex, since this σ^{54}-dependent promoter is subject to nitrogen regulation by the enhancer binding protein NtrC in addition to autogenous activation by NifA under oxygen-limiting conditions (see Figure 3.2-2A). Notably, single deletions in each of the three *fnr* genes had no apparent influence on *nifA* expression. However, as in the case of *nifB,* an increase in promoter activation was apparent in the double *fnr1, fnr3* deletion mutant (MB13) and the triple *fnr* deletion strain (MB231) (Figure 3.2-2B). In all cases, promoter activation significantly decreased when cultures were grown in the presence of a high concentration of fixed nitrogen (Figure 3.2-2C), or when the -24 to -12 region of the promoter was disrupted (plasmid pRW22, Figure 3.2-2B), indicating that activation is *rpoN*-dependent and subject to nitrogen regulation by NtrC as expected [20]. In all cases, irrespective of the presence of *fnr* mutations, *nifA* expression decreased when promoter constructs (plasmids pRWC and pRW3) carried mutations in the upstream activation sequence (UAS2) of the promoter (Figure 3.2-2B), presumably as a consequence of decreased autoactivation by NifA [20]. Overall, these results demonstrate that in the absence of both *fnr1* and *fnr3,* activation of the *nifA* promoter is increased. Since higher expression of the *nifA::lacZ* fusion is not observed when the NifA binding site (UAS2) is deleted, it is likely that the increased expression results from autoactivation of the *nifA* promoter due to increased activity or stability of NifA protein.

Given that the *nifA* promoter is subjected to complex regulation, we designed experiments to confirm that NifA activity is enhanced in *fnr* mutant strains. Firstly, using the combined *fnr*$^-$ and *nifA* deletion strains described above (Supplementary Figs.

S3.2-1 and S3.2-2) we complemented the *nifA* mutation with NifA expressed ectopically from the *lac* promoter (plasmid pRAMM1), which is constitutive in *H. seropedicae*. In this complementation assay we observed that the levels of *nifH* mRNA were higher in the in the *fnr* deletion strains complemented with constitutively expressed NifA in comparison with the complemented strain containing wild-type *fnr* alleles (Figure 3.2-3). This implies that an increase in NifA activity, rather than its expression, is responsible for increased activation of *nif* promoters in the *fnr* deletion mutants. Secondly, we constructed strains expressing NifA fused to a 3XFlag peptide to allow detection of the protein in both wild type and *fnr* mutant backgrounds (Supplementary Fig. S3.2-3). Western blots of strains carrying the *nifA*-3Xflag allele revealed higher levels of NifA expression in the double *fnr1, fnr3* deletion (MBN5) and also in the triple *fnr* deletion (MBN6) backgrounds compared with the strain wild-type *fnr* alleles (MBN4) (Figure 3.2-4). This confirms the additional level of autoactivation of the *nifA* promoter conferred by the multiple *fnr* deletions (Figure 3.2-2A), again indicating that NifA activity is higher in the *fnr* mutant strains.

As the *H. seropedicae* NifA protein is sensitive to oxygen, being inactivated and degraded upon exposure to O_2 (SOUZA et al., 1999; OLIVEIRA et al., 2009), we hypothesized that NifA might be protected in its active form in *fnr* deletion strains if these strains exhibit a higher oxygen consumption rate. To further test this hypothesis we measured oxygen depletion during the growth of bacterial cultures in the same growth conditions as described for the assay of the *nifA::lacZ* fusions. We first analyzed the decrease in oxygen concentration in the gas phase of Suba-seal stoppered flasks (Figure 3.2-5A) and additionally compared the profiles of dissolved oxygen consumption using a Clark type electrode (Figure 3.2-5B). These assays revealed that the consumption of oxygen was higher in multiple *fnr* deletion strains, implying that these strains have a higher respiratory rate when compared to the wild type (Figure 3.2-5). Notably, the oxygen consumption data in Figure 3.2-5A directly correlates with the increased activity of NifA observed in strains lacking both Fnr1 and Fnr3 (Figure 3.2-2B) implying that the absence of both these transcription factors results in higher respiration rates.

In a previous study, we showed that the triple *fnr* mutant is deficient in the expression of the cytochrome *c*-type branch of the electron transport chain (BATISTA et al., 2013). An alternative route of electron transport from the quinol pool to oxygen via the terminal quinol oxidases is likely to occur in the triple *fnr* mutant. As the quinol

branch of the respiratory chain results in a lower number of proton-translocation events it is conceivable that the activity of this branch is enhanced in the *fnr* mutant strains to compensate for the lower level of energy production. This may result in increased electron flux through the respiratory chain and hence enhanced oxygen consumption as observed in our experiments.

We demonstrated previously that nitrogenase activity is severely impaired when the triple *fnr* deletion strain is cultured in ammonium-limiting liquid medium, potentially as a consequence of energy depletion (BATISTA et al., 2013). We also showed that diazotrophic growth is impaired in the *fnr* ablated strain, after subjecting cultures to severe nitrogen starvation (BATISTA et al., 2013). Under these conditions, cultures divide at extremely low growth rates, requiring 24 days post-inoculation to achieve an OD_{600} of ~ 1.6 (Supplementrary Fig. S3.2-4). However, it is notable that the triple *fnr* mutant grew faster than the wild-type for the first 12 days of incubation under these conditions. Potentially, the enhanced rate of O_2 consumption by the triple *fnr* deletion allows higher levels of NifA activity and consequently higher nitrogenase activity during the 'early' stages of growth. However, it is possible that as the bacterial population increases and the oxygen levels in the culture drops further, the triple *fnr* mutant strain can no longer maintain the necessary electron flux to support nitrogenase activity and as a consequence, diazotrophic growth is impaired.

In summary, these studies have not identified a direct role for the *H. seropedicae* Fnr proteins in regulating NifA activity and nitrogen fixation, but rather suggest that they may influence both, by means of altering the composition of the electron transport chain and the oxygen consumption rate. Since we only observe such effects in strains deleted for both *fnr1* and *fnr3*, there is apparently some redundancy in the physiological functions of the three *fnr* paralogs in *H. seropedicae.* It is feasible that *H. seropedicae* can take advantage of the three *fnr* genes to differentially modulate respiratory chain composition. This is likely to influence nitrogen fixation during different phases of growth and enable efficient adaptation during plant-bacterial colonization.

3.2.4 Conclusions

In this study we have used a combination of transcriptional and physiological approaches to address the role of the *H. seropedicae* Fnr proteins in influencing the expression and activity of NifA. In summary we found that Fnr1 and Fnr3 participate

indirectly in modulating NifA stability as a consequence of alterations in the rate of O_2 consumption. This mechanism can potentially allow the bacteria to fine tune nitrogen fixation in response to environmental cues.

3.2.5 Methods

3.2.5.1 Plasmids, bacterial strains and Growth conditions

Plasmids and bacterial strains used are listed in Table 1. *H. seropedicae* strains were grown at 30 °C in NFbHP-Malate medium (KLASSEN et al., 1997) supplemented with NH_4Cl as indicated. The antibiotics used were tetracycline (10 µg mL^{-1}), streptomycin (80 µg mL^{-1}), kanamycin (500 µg mL^{-1} for *H. seropedicae* and 50 µg mL^{-1} for *E. coli*), gentamicin (500 µg mL^{-1} for *H. seropedicae*) and nalidixic acid (5 µg mL^{-1}).

3.2.5.2 Construction of *nifA* deletion and 3xFlag tagged strains

To construct the C-terminal 3xFlag tagged NifA strains, we generated a *nifA*-3XFlag gene by cloning the complete *nifA* gene (1629 bp) in frame with the 3xFlag sequence from a vector synthesized by the GenScript® Corporation (Table 1). To assist homologous recombination, a fragment of 647 bp downstream of *nifA* was cloned adjacent to the 3xFlag tag sequence to generate an approximately 2.4 Kb fragment containing the *nifA*-3xFlag allele plus the downstream region. This fragment was then digested with BamHI, and subcloned into pK18mobsacBKm vector (SCHÄFER et al., 1994) to generate the suicide vector pK18nifAflag. A similar approach was used to generate a vector for C-terminal 3xFlag tagging of the *fnr1* gene, but a fragment of 1002 bp downstream of *fnr1* gene was cloned adjacent to the 3xFlag tag sequence to generate a fragment of approximately 1.95 kb containing the *fnr1*-3xFlag allele plus the downstream region. This fragment was then subcloned into pJQ200SK suicide vector (QUANDT, JÜRGEN; HYNES, 1993) to generate pJQfnr1Flag. To generate the *nifA* deletion vector, plasmid pRAM1T7 was digested with EcoRI and re-ligated to yield the vector pRAM1T7del containing a deleted copy of *nifA* lacking 576 bp. Then an XbaI/BamHI fragment from pRAM1T7del was cloned into pK18mobsacB vector to generate the pK18nifAdel suicide vector.

The suicide plasmids generated for both tagging of *nifA* and *fnr1* and also for deletion of *nifA* gene were transferred to wild type *H. seropedicae* SmR1, and the *fnr* deletion strains MB13 and MB231 strains by conjugation as described (SOUZA et al., 2000; BATISTA et al., 2013). Single crossover strains were selected by antibiotic resistance. Double crossover strains were selected on plates containing 5% sucrose and then tested for antibiotic marker sensitivity. The mutant strains sensitive to kanamycin or gentamicin and resistant to sucrose were analysed by PCR using specific primers as described (Supplementrary Figs. S3.2-1, S3.2-3 and S3.2-5). All primers used are listed in the Table S3.2-1.

3.2.5.3 β-Galactosidase activity and transcriptional fusions

β-Galactosidase activities of various *nif promoter:lacZ* transcriptional fusions were assayed in *H. seropedicae* strains as previously described (WASSEM et al., 2002; REGO et al., 2006), except that the strains were grown in NFbHP-Malate liquid medium supplemented with 5 mM NH_4Cl (6 ml in 25 ml cylindrical bottles under air). Under these conditions, the cultures exhaust the supply of fixed nitrogen and become oxygen limited resulting in formation of active NifA and *nif* gene expression.

Alternatively, *H. seropedicae* strains carrying the *nifB::lacZ* fusion were assayed for β-galactosidase activity after incubation under defined initial oxygen concentrations. In summary, cultures with an $O.D_{600}$ adjusted to 0.2, were incubated for six hours in NFbHP-Malate without addition of fixed nitrogen and under the initial oxygen concentrations of 4%, 6% or air (20.8%).

3.2.5.4 RNA extraction and RT-PCR

Strains were grown under 4% of oxygen for six hours. Cells from 30 mL of culture were collected by centrifugation (7000 rpm, 4°C, 5 minutes) and re-suspended in 200 μL of 10 mM Trizma® (Sigma# T-2694). The cells were then mixed with 700 μL of RLT Buffer (Qiagen Rneasy Mini Kit #74104) containing 1% of β-mercaptoethanol and added to lysing tubes containing zirconia and silica/glass beads in the proportion of 2:1 (Thistle Scientific Ltd). Lysis was carried out with 3 pulses (speed 6.5 with 30 seconds on/1.5 minutes off) using the Thermo Savant FastPrep 120 Cell Disrupter System. Beads and cellular debris were collected by centrifugation (17000 x

g, 4°C, 5 min). The supernatant (900 µL) was transferred to a new RNase free tube and 450 µL of ethanol (Sigma #459844) was then added. The samples were applied to the RNeasy columns (Qiagen RNeasy Mini Kit #74104) and total RNA was recovered after *on column* DNAse treatment with the Qiagen RNase-Free DNase set (#79254) following the manufacturer's instructions. The quality of purified RNA was accessed by electrophoresis in a 1% agarose gel. RNA was treated with Turbo DNase (Ambion#AM1907) following manufacturer's instructions and further purified with Qiagen RNeasy columns to avoid carryover of divalent cations.

Approximately 0.25 ng/µL of total RNA was used for direct RT-PCR using the One-Step RT-PCR kit (Qiagen #210210) according to the manufacturer's instructions. Expression of *nifH* gene was evaluated using 16S rRNA as endogenous control. The primers are listed in the Table S1.

3.2.5.5 Preparation of protein extracts and western blotting

H. seropedicae cultures adjusted to an $O.D_{600}$ of 0.2 were grown under 4% of oxygen for six hours. After incubation, approximately 3 mL of cells (volumes were adjusted as necessary) were collected by centrifugation (17000 X g, 2 min), re-suspended in 100 µL of protein sample buffer (120 mM of Tris-HCl pH 6.8, 2% SDS, 20% Glycerol, 9% β-mercaptoethanol and 0.03% bromophenol blue) and boiled for 5 minutes. Subsequently, 10 µL of the resulting extract was loaded onto 12% SDS-PAGE for resolution of the proteins, which were immediately transferred to a PVDF membrane and then hybridized with ANTI-FLAG® (Sigma #7425) primary antibody (1/2500 dilution), followed by secondary anti-rabbit-HRP conjugated antibody (1/10000 dilution). The HRP activity was detected using the ECL Plus Western Blotting detection kit (GE Healthcare #RPN2132) as indicated by the manufacturer and the UVP® gel imaging system.

3.2.5.6 Oxygen consumption measurements

For determination of the oxygen consumption we designed two assays. First we evaluated the depletion of the oxygen levels in the gas phase of culture flasks sealed with Suba-seal septa. Every hour we took a 0.5 ml gas sample from the growing culture and analyzed the oxygen concentration by gas chromatography (Varian GC-450)

coupled to a molecular sieve column and a TCD detector. Oxygen depletion was linear until 10 hours growth. The rate of consumption was calculated as the amount of oxygen consumed in the gas phase normalized by the protein concentration of the culture. A measurement of the dissolved oxygen consumption was also carried out with a Clark-type electrode. After addition of 100 µl of bacterial culture into the chamber, containing 1.6 ml of NFbHP-Malate at 30 ºC, the consumption of dissolved oxygen in the medium was recorded until the polarizing voltage reached 0 (i.e. 0% oxygen saturation).

3.2.6 Competing interests

The authors declare that they have no competing interests.

3.2.7 Author contributions

MBB designed the study, performed experiments, analysed the data and wrote the manuscript. RW and FOP participated in study design and data analysis. EMS, RD and RAM designed the study, analysed the data and wrote the manuscript.

3.2.8 Acknowledgments

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3.2.9 Figures

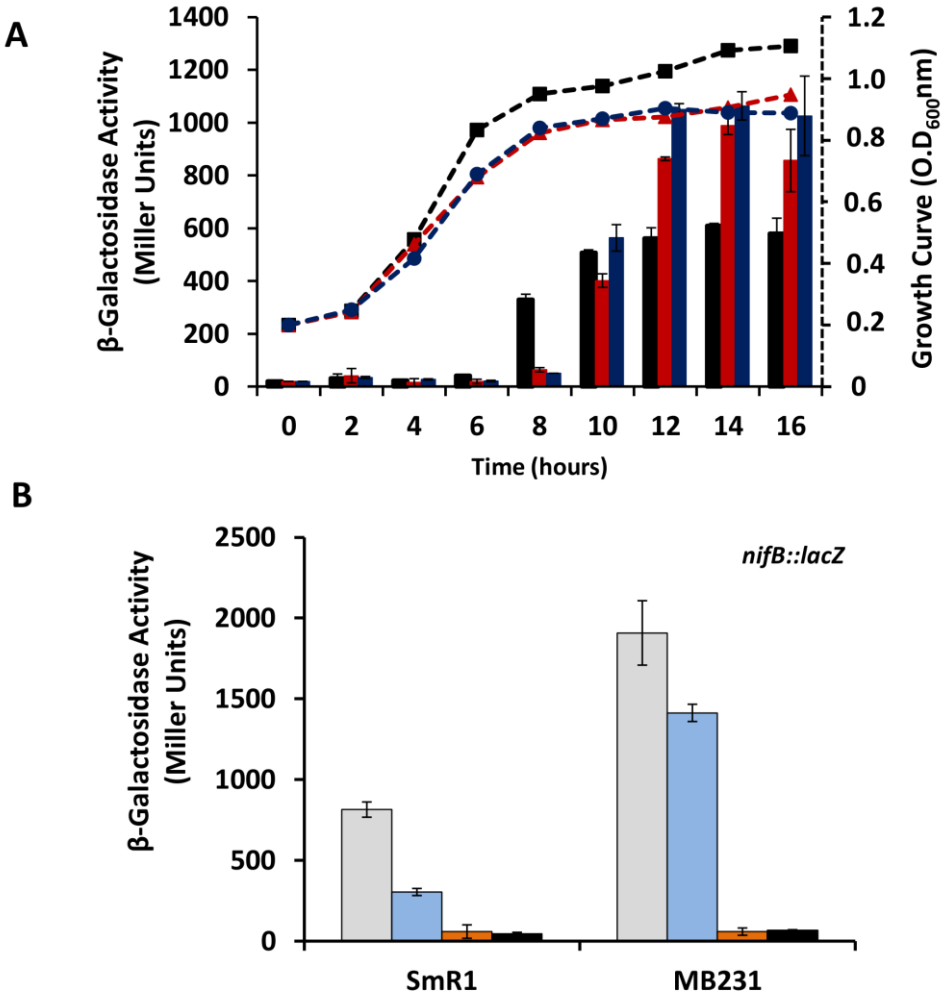


Figure 3.2-1. The *nifB* gene expression is enhanced in the *fnr* mutant strains from *H. seropedicae.* (A) The activity of the *nifB::lacZ* fusion (primary y axis) was assayed using cells cultured in NFbHP-Malate liquid medium supplemented with 5mM NH_4Cl (6 ml in 25 ml bottles under air). Every two hours samples were taken for determination of β-galactosidase activity of the wild type strain (SmR1) (black bars), the double *fnr1* and *fnr3* deletion strain (MB13) (red bars) and the triple *fnr* deletion strain (MB231) (blue bars). Samples were also taken for measuring the growth ($O.D_{600\text{-nm}}$ – secondary y axis) of the strains SmR1 (dotted black lines – squares), MB13 (dotted red lines – triangles) and MB231 (dotted blue lines – circles). (B) Activity of the *nifB::lacZ* fusion measured under the initial oxygen concentrations of 4.0% (light grey bars), 6.0% (blue bars), 8.0% (orange bars) and 20.8% (black bars) in liquid medium without addition of fixed nitrogen as describe in Methods.

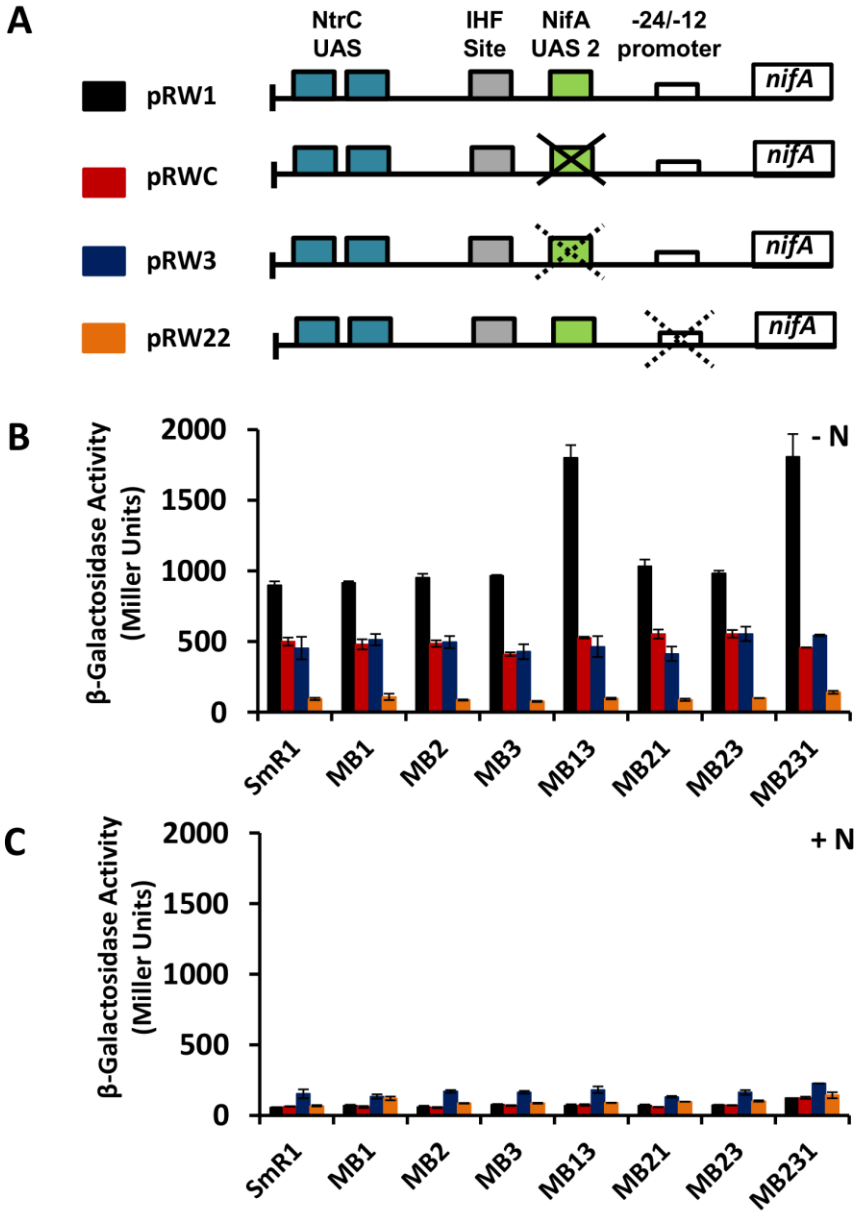


Figure 3.2-2. The *nifA* gene expression is enhanced in the *fnr* mutant strains from *H. seropedicae*. (A) Schematic representation of native and mutant *nifA::lacZ* fusions assayed for β-Galactosidase activity in *fnr* mutant strains is showed. Full crosses indicate deletions, while dotted crosses indicate point mutations. The B-Galactosidase activity of different *nifA::lacZ* transcriptional fusions in *H. seropedicae* SmR1 and *fnr* mutant strains were assayed under 5 mM (B) or 20 mM of NH_4Cl (C). The colour code used for each transcriptional fusion in the bar graphs in B and C is represented in A. Results showed are representative of two independent experiments.

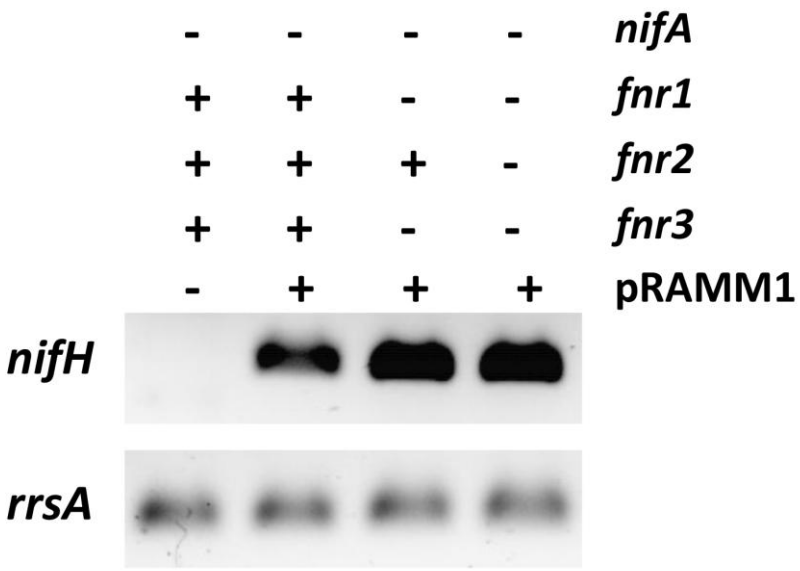


Figure 3.2-3. The NifA activity is higher in *H. seropedicae* strains lacking both Fnr1 and Fnr3. The RNA from the strains MBN1 (*nifA* deletion), MBN2 (*nifA* deletion in the double *fnr1*, *fnr3* deletion background) and MBN3 (*nifA* deletion in the triple *fnr* deletion background) complemented with the plasmid pRAMM1 (expressing *H. seropedicae* NifA from *lac* promoter) was purified and submitted to direct RT-PCR amplification of *nifH* gene as described in Methods. The 16S rRNA (*rrsA*) was used as an endogenous expression control. A representative gel from two independent RNA extractions is showed.

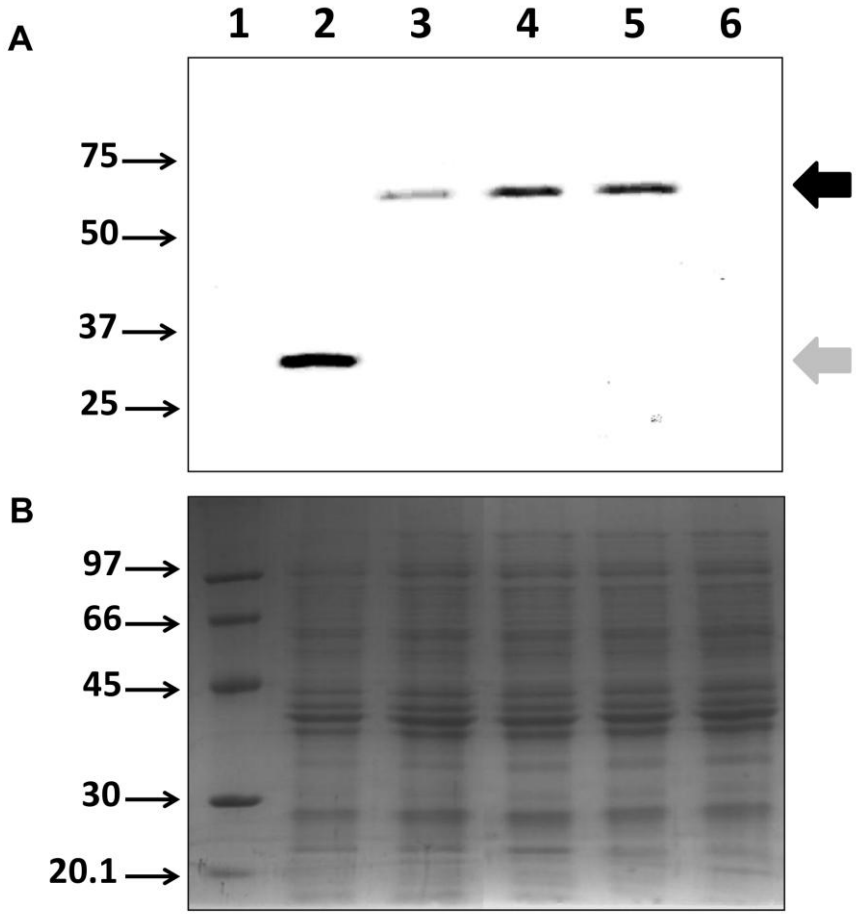


Figure 3.2-4. The NifA protein levels are higher in *H. seropedicae* strains lacking both Fnr1 and Fnr3. Protein extracts from different *H. seropedicae* strains were prepared and then hybridized with ANTI-FLAG antibodies as described in Methods. (A) Western hybridization of protein samples after resolution on 12% SDS-PAGE and transfer to PVDF membrane. (B) Protein loading control SDS-PAGE of samples used for hybridization in A. Lanes are as follows: 1, Protein molecular weight standard (MW); 2, *H. seropedicae* MBF1 (*fnr1-3xFlag*)*;* 3, *H. seropedicae* MBN4 (*nifA*-3xFlag)*;* 4, *H. seropedicae* MBN5 (*nifA-3xFlag* in the double *fnr1*, *fnr3* deletion background)*;* 5, *H. seropedicae* MBN6 (*nifA-3XFlag* in the triple *fnr* mutant background)*;* 6, *H. seropedicae* SmR1 (no Flag protein control). In A, the MW used was Precision Plus Protein WesternC™ Pack (BioRad #161-0385), while in B, the MW used was the LMW-SDS Marker Kit (GE healthcare# 17-0446-01). Thin arrows indicate the molecular weight in KDa of protein standards. The black thick arrow indicates the NifA-3xFlag protein (~64 KDa), while the light grey thick arrow indicates the Fnr1-3xFlag protein (~34KDa). A representative result is show from three independent protein extract preparations.

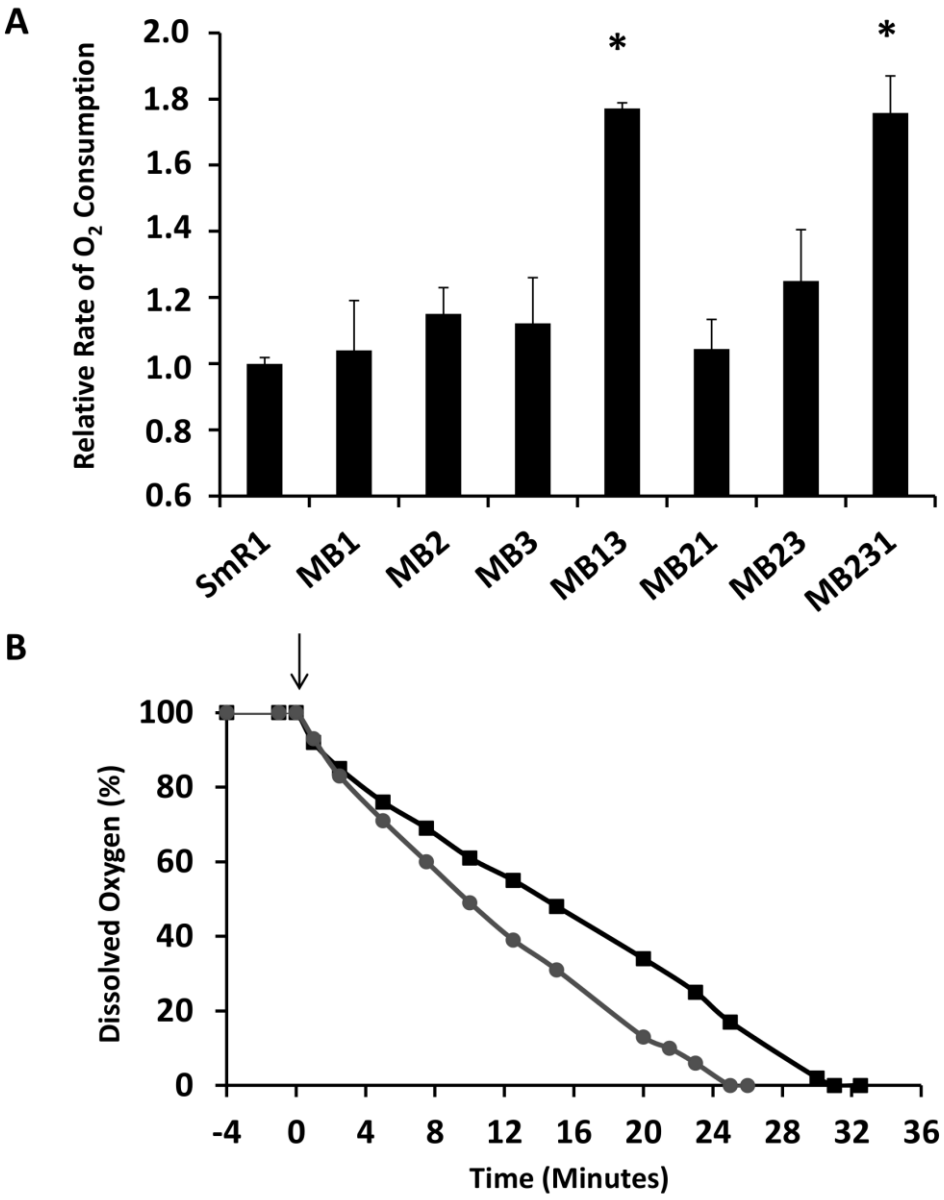


Figure 3.2-5. The oxygen consumption rate is higher in the *H. seropedicae fnr* mutant strains. (A) Gas phase oxygen consumption in *H. seropedicae* SmR1 and *fnr* mutant strains. The asterisks indicates statistical significance according to the Student's T-test ($p<0,05$). (B) Consumption of dissolved oxygen in liquid media of *H. seropedicae* strains SmR1 (black squares) and MB231 (grey circles) using a clark-type electrode. The arrow indicates addition of 100 μL of cells into the electrode chamber containing 1.6 ml of NfbHP-Malate supplemented with 5.0 mM of ammonium chloride.

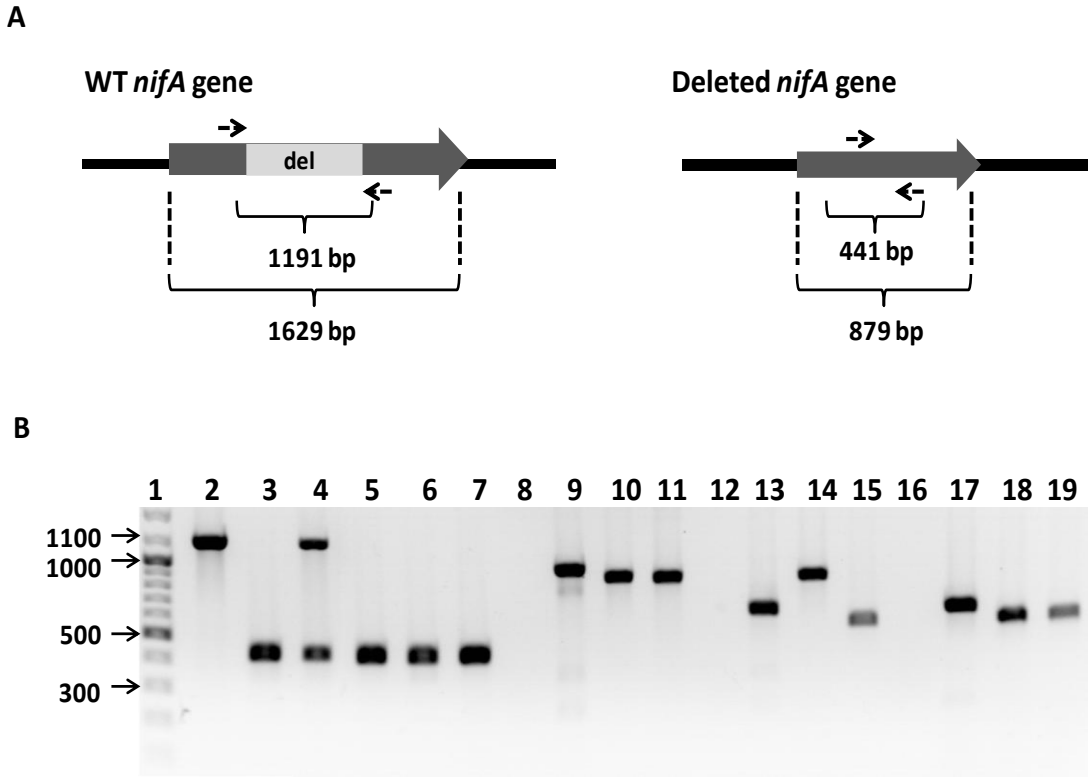
3.2.10 Tables

Table 3.2-1. Plasmids and strains used in this study

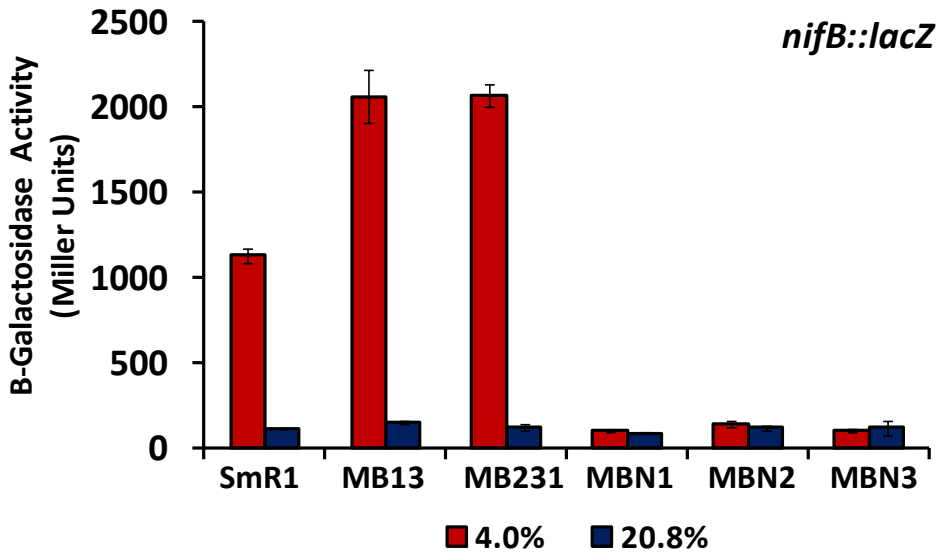
Plasmids	**Relevant Characteristic**	**Source/Reference**
pLAFR3.18	pLAFR vector containing the policloning site of pTZ18R (Tc^R, Cm^R)	(SOUZA et al., 1999)
pRAMM1	*H.seropedicae* NifA expressed from *lac* promoter	(NOINDORF et al., 2011)
pK18mobsacBKm	Allelic exchange suicide vector; mobilized by *E. coli* S17-1 λpir, *sacB*,Km^R	(SCHÄFER et al., 1994)
pJQ200SK	Allelic exchange suicide vector; mobilized by *E. coli* S17-1, *sacB*,Gm^R	(QUANDT, JÜRGEN; HYNES, 1993)
pRAM1T7	*H.seropedicae* NifA in pT7-7 vector	This study
pRAM1T7del	*H.seropedicae* NifA with a deletion of 576 bp	This study
pK18nifAdel	XbaI/BamHI fragment from pram1t7del	This study
pUC57Simple-3xFlag	5'-BamHI, KpnI, XhoI – 3xFlag-Stop – HindIII, SalI, XmaI-3', Amp^R	Genscript® Corporation
pUC57nifAFlag	*H. serpopedicae nifA-3xFlag* gene plus 647 bp of the downstream region, Amp^R	This study
pK18nifAFlag	*H. serpopedicae nifA-3xFlag* gene plus 647 bp of the downstream region, Mob, SacB, Km^R	This study
pUC57fnr1Flag	*H. serpopedicae fnr1-3xFlag* gene plus 1000 bp of the downstream region, Amp^R	This study
pJQfnr1Flag	*H. serpopedicae fnr1-3xFlag* gene plus 1000 bp of the downstream region, Gm^R	This study
pPW452	*lacZ* fusion vector, Tc^R, Mob	(WOODLEY et al., 1996)
pEMS140	Tc^R, Mob, *nifB::lacZ* fusion, *nifB* promoter cloned in pPW452	(REGO et al., 2006)
pRW1	Tc^R, Mob, *nifA::lacZ* fusion, *nifA* promoter cloned in pMP220	(WASSEM et al., 2002)
pRWC	Same as pRW1, but with a 49 bp deletion including the UAS 2 site for NifA	(WASSEM et al., 2002)
pRW3	Same as pRW1, but with a double mutation at the UAS 2 site for NifA (TGT -> TCT and ACA -> AGA)	(WASSEM et al., 2002)
pRW22A	Same as pRW1, but with a single mutation at the -24/-12 promoter (G -> T at -25 residues)	(WASSEM et al., 2002)
Strains	**Relevant Characteristic**	**Source/Reference**
SmR1	*Herbaspirillum seropedicae* Z78 but Sm^R 100µg/mL, Nif+	(SOUZA et al., 2000)

MB1	Derived from SmR1 *fnr1 deletion*	(BATISTA et al., 2013)
MB2	Derived from SmR1, *fnr2* deletion	(BATISTA et al., 2013)
MB3	Derived from SmR1, *fnr3* deletion	(BATISTA et al., 2013)
MB13	Derived from MB1, *fnr1* and *fnr3* double deletion	(BATISTA et al., 2013)
MB21	Derived from MB2, *fnr1* and *fnr1* double deletion	(BATISTA et al., 2013)
MB23	Derived from MB2, *fnr2* and *fnr3* double deletion	(BATISTA et al., 2013)
MB231	Derived from MB23, *fnr1*, *fnr2* and *fnr3* triple deletion	(BATISTA et al., 2013)
MBN1	Derived from SmR1, but with 576 bp deletion in the *nifA* gene	This study
MBN2	Double *fnr1*, *fnr3* deletion, plus a 576 bp deletion in the *nifA* gene	This study
MBN3	Triple *fnr* deletion, plus a 576 bp deletion in the *nifA* gene	This study
MBN4	Derived from SmR1, but with a C-terminal 3xFlag *nifA* gene	This study
MBN5	Double *fnr1*, *fnr3* deletion, plus a C-terminal 3xFlag *nifA* gene	This study
MBN6	Triple *fnr* deletion, plus a C-terminal 3xFlag *nifA* gene	This study
MBF1	Derived from SmR1, but with a C-terminal 3xFlag *fnr1* gene	This study

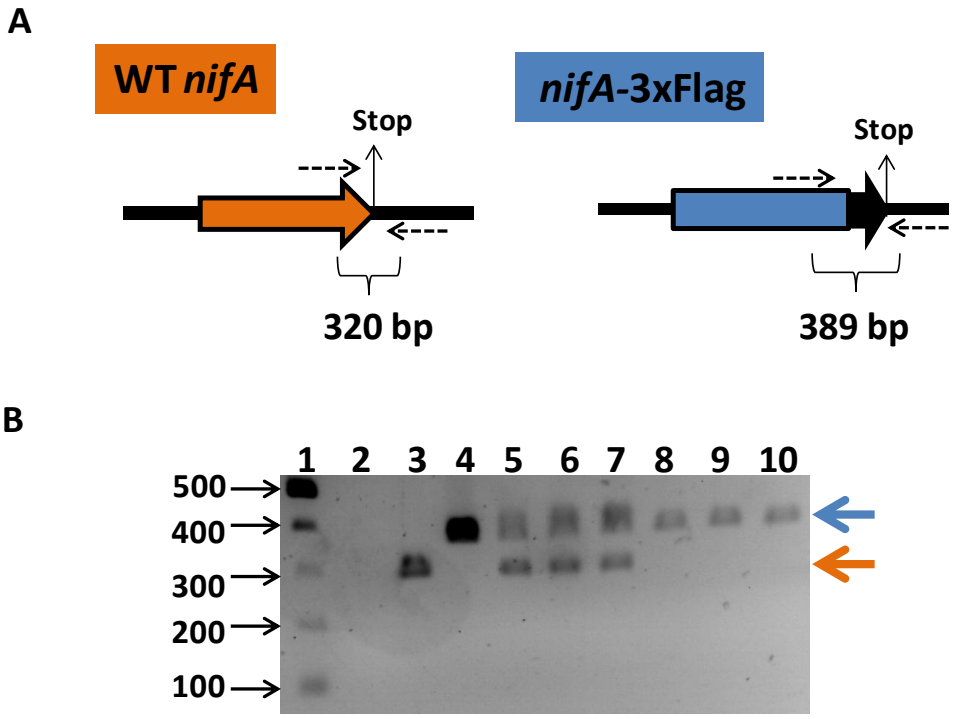
3.2.11 Supplementary Material



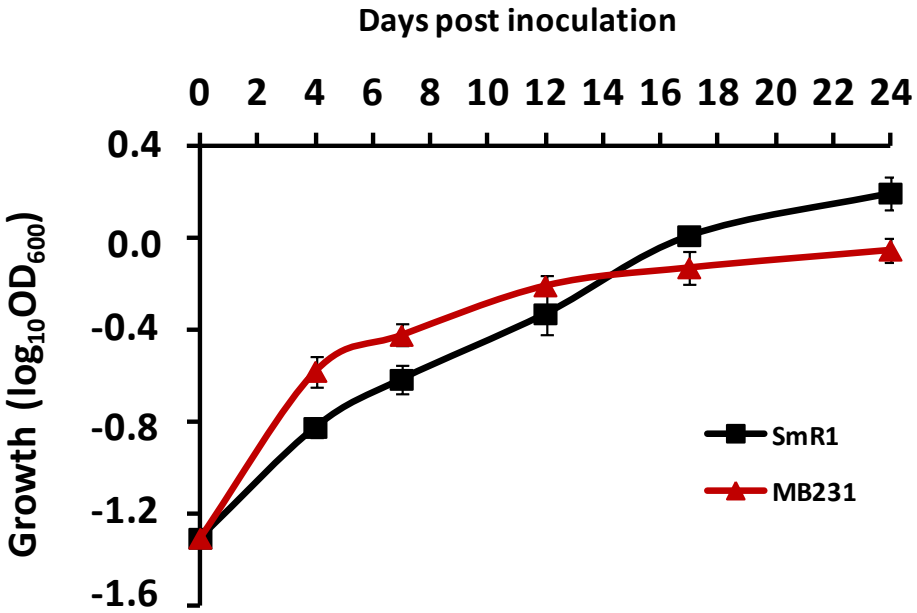
Supplementary Figure S3.2-1. Construction and validation of *nifA* deletion strains in different *H. seropedicae* backgrounds. (A) Schematic representation of the *nifA* deletion construct and primers (dotted arrows) designed to validate the mutants. Drawings are not to scale. (B) Genotypic validation of strains MBN1 (*nifA* deletion), MBN2 (*nifA* deletion in the double *fnr1* and *fnr3* deletion background) and MBN3 (*nifA* deletion in the triple *fnr* deletion background). PCR was performed by using primers flanking the region of deletion (as indicated in A). Lanes: 1, 1 Kb ladder Fermentas; 2, SmR1 (Wild type *nifA*); 3 suicide vector (pK18nifAdel);4, intermediate strain for SmR1 background; 5, 6, and 7, final *nifA* deleted strains for SmR1, MB13 and MB231 backgrounds, respectively; 8, no template control using primers to *fnr1* gene; 9,10 and 11, SmR1 genotyping of *fnr1*, *fnr2* and *fnr3* genes; 12, no template control using primers to *fnr2* gene; 13, 14 and 15, MB13 genotyping of *fnr1*, *fnr2* and *fnr3* genes; 16, no template control using primers to *fnr3* gene; 17, 18 and 19, MB231 genotyping of *fnr1*, *fnr2* and *fnr3* genes. On the left are indicated the length in base pairs (bp) of the DNA ladder.



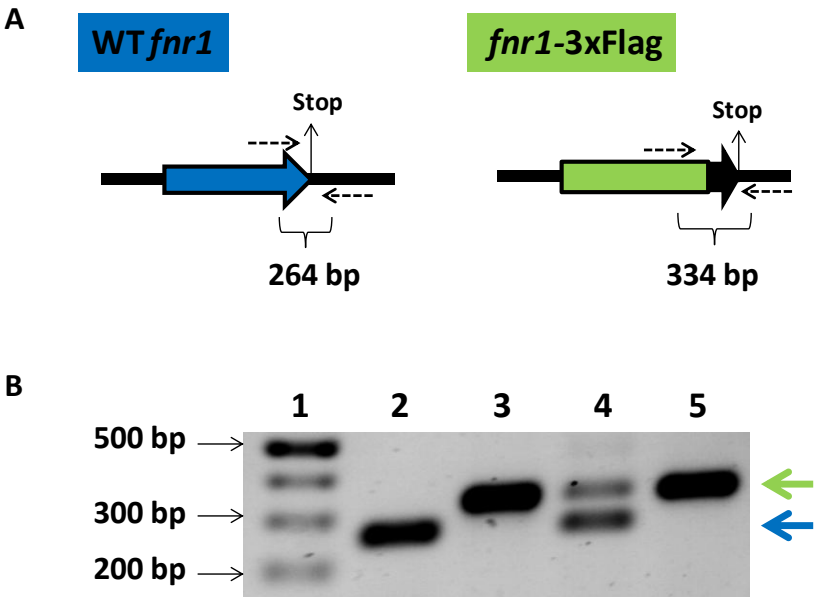
Supplementary Figure S3.2-2. The enhanced *nifB::lacZ* promoter activity in strains lacking both Fnr1 and Fnr3 is dependent upon NifA protein. The *H.seropedicae* strains, SmR1 (wild type), MB13 (*fnr1* and *fnr3* deletion), MB231 (triple *fnr* deletion), MBN1 (*nifA* deletion), MBN2 (*nifA* deletion in the *fnr1*, *fnr3* deletion background) and MBN3 (*nifA* deletion in the triple *fnr* deletion background) harbouring the plasmid pEMS140 (*nifB::lacZ*) were assayed for β-Galactosidase activity under nitrogen deficient media and 4.0% (red bars) or 20.8% (blue bars) of oxygen as described in Methods.



Supplementary Figure S3.2-3. Construction and validation of *nifA*-3xFlag strains in different *H. seropedicae* backgrounds. (A) Schematic representation of C-terminally 3xFlag tagged construct and primers (dotted arrows) designed to validate the mutants. Drawings are not to scale. (B) Genotypic validation of strains MBN4 (*nifA*-3xFlag), MBN5 (*nifA*-3xFlag in the double *fnr1* and *fnr3* deletion background) and MBN6 (*nifA*-3xFlag in the triple *fnr* mutant background). PCR was performed by using primers flanking the C-terminal region around the insertion of the 3xFlag (as indicated in *A*). Lanes: 1, 1 Kb ladder Fermentas; 2, no template control; 3, SmR1; 4, suicide vector (pK18nifAFlag); 5, 6 and 7, intermediate strains for SmR1, MB13 and MB231 backgrounds; 8,9 and 10, final 3xFlag tagged strains for SmR1, MB13 and MB231 backgrounds. The *fnr* genotypes on different *nifA*-3XFlag backgrounds were verified as showed on Supplementrary Fig. S3.2-1. On the left are indicated the length in base pairs (bp) of the DNA ladder.



Supplementary Figure S3.2-4. Deletion of *fnr* genes influences the diazotrophic growth profile. Both *H. seropedicae* wild type strain (SmR1) (black squares) and the triple *fnr* mutant strain (MB231) (red triangles) were incubated statically at 30°C in NFbHP-Malate minimal media without addition of nitrogen source.



Supplementary Figure S3.2-5. Construction and validation of *fnr1*-3xFlag strain from *H. seropedicae*. (A) Schematic representation of C-terminally 3xFlag tagged construct and primers (dotted arrows) designed to validate the mutant. Drawings are not to scale. (B) Genotypic validation of the strain MBF1 (*fnr1*-3xFlag). PCR was performed by using primers flanking the C-terminal region around the insertion of the 3xFlag (as indicated in A). Lanes: 1, 1 Kb ladder Fermentas; 2, SmR1; 3, suicide vector (pJQfnr1Flag); 4, intermediate strain and 5 final *fnr1*-3xFlag tagged strain (MBF1). On the left are indicated the length in base pairs (bp) of the DNA ladder.

Table S3.2-1. Primers used in this study.

Primer	RS[a]	Sequence (5'-->3')[b]	Aplication
CCFlagF	BamHI	*ATATTA***GGATCC**ATGGCCACTATTCTCGAC	Amplification of 1629 bp corresponding to the coding region of the *nifA* gene
CCFlagR	KpnI	*ATATTA***GGTACC**GAACTTCTTGACCTCGAT	
DCFlagF	HindIII	*ATATTA***AAGCTT**AACTCATCTGCGAGCGTG	Amplification of 647 bp corresponding to the downstream region of the *nifA* gene
DCFlagR	XmaI	*ATATTA***CCCGGG**GTTGCACTGGATGTT	
DBamCFlagR	BamHI	*ATATTA***GGATCC**GTTGCACTGGATGTT	Introduce BamHI site at 3' of 3xFlag construct
NifACFlagF	None	ATGGCCAAGGACAAGCCA	Genotypic validation of *nifA*-3XFlag strains
NifACFlagR	None	AATGCGAGGACGTGCCAG	
Fnr1CodF#	BamHI	*TATATA***GGATCC**TTGCCACATGTCCGCCGG	Amplification of 885 bp covering to the coding region of the *fnr1* gene
Fnr1CodR#	Xho I	*ATATAT***CTCGAG**GCTGGTGCTGCGGGTCG	
Fnr1DownF#	HindIII	*TATATA***AAGCTT**GGGCTGGCCTCATCG	Amplification of 1002 bp corresponding to the downstream region of the *fnr1* gene
Fnr1DownR#	XmaI	*ATATAT***CCCGGG**CGCTGATCGGACCCG	
1FlagF	None	ATTGAAAGCATCAGCCGGCTGATC	Genotypic validation of *fnr1*-3XFlag strains
2FlagR	None	CAGACCTTGCCCAGGGAACGT	
NifAdelF	None	ATGGCCACTATTCTCGACGAC	Genotypic validation of *nifA* deletion strains
NifAdelR	None	CTGGTTCTCCACACGGAAT	
nifH-RT-F	None	AAGTCCACCACCTCGCAAAA	RT-PCR
nifH-RT-R	None	TTCCAGCTCCAGATCCTCCA	
16S-RT-F[c]	None	TGTCAGGGAAGAAACGGTTTTG	RT-PCR
16S-RT-R[c]	None	AGTTAGCCGGTGCTTATTCTTCA	

All primers were designed for this study, except when stated.

[a] Restriction site

[b] When present, the restriction sites and non-homologous regions are in bold and italicized, respectively.

[C] Michelle Zibetti Tadra Sfeir, personal communication.

3.3 - ChIP-Seq and RNA-Seq analysis unravel the role of three Fnr proteins in the adaptation of *Herbaspirillum seropedicae* SmR1 to low oxygen levels

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3.3.1 Abstract

H. seropedicae SmR1 is an endophytic aerobic bacterium capable of fixing atmospheric nitrogen under conditions of nitrogen and oxygen limitation. To efficiently adapt to low O_2 levels, *H. seropedicae,* as in the case of other bacteria, takes advantage of a branched respiratory chain comprising different types of terminal oxidases, including oxidases predicted to have high affinity for oxygen. Remarkably this organism has genes coding for three Fnr proteins, designated as Fnr1, Fnr2 and Fnr3. Using genome-wide transcriptional profiling in combination with physiological characterisation, we previously observed that efficient reconfiguration of the respiratory chain in *H. seropedicae* under low oxygen availability relies on transcriptional regulation of gene expression by these Fnr proteins. However, we were not able to define the specific regulons and functional roles of each of the three Fnr transcription factors. In this study we have used a combination of RNA-Seq transcriptional profiling of single *fnr* mutants together with a ChIP-Seq approach to address the functions of three Fnr orthologs encoded by *H. seropedicae* genome. Although Fnr1 and Fnr3 regulate discrete classes of genes, we have identified another group of genes that are jointly regulated by both transcription factors. Promoters in this class are bound by both Fnr1 and Fnr3, potentially indicative of regulation by Fnr1-Fnr3 heterodimers. In contrast Fnr2 is apparently an oxygen insensitive protein responsible for the transcriptional activation of the bo_3-type respiratory oxidase. Our current data suggest that Fnr2 is responsive to nitrosative stress.

3.3.2 Key words:

H.seropedicae, Fnr, RNA-Seq, ChIP-Seq.

3.3.3 Introduction

H. seropedicae SmR1 is a nitrogen-fixing bacterium able to interact with many important agricultural crops (MONTEIRO et al., 2012; CHUBATSU et al., 2012). This diazotroph encodes three transcriptional regulators sharing homology with *E. coli* Fnr, previously designated as Fnr1, Fnr2 and Fnr3 (BATISTA et al., 2013). The Fnr protein is a widespread transcriptional regulator in Bacteria, where it evolved to allow efficient adaptation to low O_2 levels (SPIRO, S; GUEST, 1990; KORNER et al., 2003; MATSUI et al., 2013). Under conditions of oxygen limitation, the *E. coli* Fnr protein binds one $[4Fe\text{-}4S]^{2+}$ cluster per subunit which promotes conformational changes to allow protein dimerization and consequently the binding at gene promoters (LAZAZZERA et al., 1993; LAZAZZERA, B. A. et al., 1996; CRACK et al., 2008; GREEN et al., 2009). Various transcriptional profiling studies showed that *E.coli* Fnr can either activate or repress the transcription of a large number of target genes (SALMON et al., 2003; KANG et al., 2005; CONSTANTINIDOU et al., 2006).

RNA-Seq analysis of a *H. seropedicae* triple *fnr* deletion mutant (MB231) in comparison with the wild-type strain (SmR1) revealed that the Fnr proteins are important for the synthesis and activity of the cytochrome *c*-type branch of the respiratory chain. As a consequence, the triple *fnr* mutant strain was deficient in growth and had impaired nitrogenase activity under oxygen-limiting conditions (BATISTA et al., 2013). Although this previous study gave us an indication of the global role of Fnr proteins in *H. seropedicae*, we were unable to precisely determine specific sets of genes regulated by each transcription factor.

The rational for the existence of three discrete Fnr paralogs in *H. seropedicae* is not understood. Potentially each transcriptional regulator may target different sub sets of genes and may have differential sensitivity towards oxygen so that a hierarchy of oxygen-dependent transcriptional regulation is achieved. To systematically address the regulatory role of the three paralogs, we have performed RNA-Seq transcriptional profiling of single *fnr1, fnr2* and *fnr3* deletion mutants in comparison with the wild type. In parallel, we also have also performed chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-Seq) to identify binding sites of each Fnr protein in the genome, taking advantage of C-terminally 3xFlag alleles expressed from the chromosome. In combination, these analyses have enabled us to unambiguously

determine the specific regulons of Fnr1 and Fnr3, which are regulated in response to switches in oxygen concentration in *H. seropedicae.* We have also identified a subset of genes that are apparently co-regulated by Fnr1 and Fnr3. Under oxygen-limiting conditions, the promoters of such genes are bound simultaneously by both transcription factors, perhaps indicative of heterodimer formation. In contrast, we observe that Fnr2 is not apparently an oxygen responsive transcriptional regulator and it may regulate expression of the *cyt* bo_3-type respiratory oxidase in response to nitrosative stress.

3.3.4 Results

3.3.4.1 Overview of the effects of oxygen and the Fnr proteins on the transcriptome of *H. seropedicae*

Previously we analyzed the transcriptome of a triple *fnr* mutant strain (MB231) in comparison with the wild type strain (SmR1) of *H. seropedicae* after switching the cells from 20.8% to 5.0% of O_2 in the gas phase, while maintaining a constant shaking rate (120 rpm) (BATISTA et al., 2013). Under these conditions we identified 187 differentially expressed genes, with 143 down-regulated and 44 up-regulated genes being identified in the triple *fnr* mutant strain. Whilst this study provided an overview of Fnr function in controlling genes essential for adaptation to low oxygen in *H. seropedicae*, it did not examine the level of transcription under different oxygen regimes and furthermore, the discrete role of each Fnr paralog was not determined. In the current study, we have compared the transcription profiles of single *fnr1*, *fnr2* and *fnr3* deletion mutants with the wild type strain under a new oxygen switch protocol. In these experiments cells were grown to mid log phase at very high aeration rates (350 rpm, to ensure aerobic conditions) and duplicate cultures were then switched to oxygen-limiting conditions (120 rpm) for 30 minutes prior to RNA extraction. Under these conditions, the switch in oxygen availability has minimal impact on growth rates of either the wild-type or the *fnr* mutant strains, enabling comparison of transcript profiles between “high O2” (350 rpm) and “low O2” (120 rpm) conditions (see Materials and Methods).

Comparison of the RNA-seq datasets of the wild type strain under high O_2 and low O_2 conditions revealed that 252 genes were differentially regulated in response to the change in oxygen concentration. Analysis of the transcript profiles of the *fnr1*, *fnr2*

and *fnr3* single deletion strains revealed that 128 of these genes are either directly of indirectly regulated by the Fnr proteins. Most of the 109 genes up-regulated under low O_2 conditions (97 of which were identified in our previous analysis of the triple *fnr* mutant strain (BATISTA et al., 2013) are dependent on either Fnr1 or Fnr3 (Table S3.3-1). Following the switch from "high to low" O_2 46 genes were down regulated in the wild-type strain; 25 of these were up-regulated in the *fnr1* or in the *fnr3* deletion mutants (6 of which were identified previously (BATISTA et al., 2013)), indicative of repression by these Fnr proteins (Table S3.3-2). The remaining 21 genes repressed under low oxygen were not dependent on Fnr proteins (Table S3.3-3), suggesting that an additional mechanism of gene repression in response to oxygen limitation is present in *H. seropedicae*.

In comparison with our previous RNA-seq analysis of the ablated *fnr* strain (triple *fnr* deletion mutant) (BATISTA et al., 2013) we have found new targets for the *H. seropedicae* Fnr proteins, probably as a consequence of the different physiological conditions used in the current analysis. Looking at the transcription profiles at the whole genome level, the most highly induced transcripts after the switch to low oxygen conditions were those of the structural genes coding for the cbb_3-type (*fixNOP*) terminal respiratory oxidase and the *ompW1* gene that encodes an outer membrane protein; both genes are located in the neighbourhood of the *fnr1* gene (Figure 3.3-1). Other notably highly-induced genes under oxygen limitation were the genes encoding the cytochrome bc_1-complex (*petABC*) and also Hsero_2290 (*uspA*) coding for a universal stress protein. Both the bc_1 and cbb_3 complexes are likely to be essential targets for re-configuration of the cytochrome *c*-type electron transport chain, when oxygen is limiting in *H. seropedicae*. As a consequence the triple *fnr* mutant strain was found to be deficient in growth and also had impaired nitrogenase activity under oxygen limiting conditions (BATISTA et al., 2013).

3.3.4.2 RNA-Seq combined with ChIP-Seq unambiguously reveals the Fnr targets in *H. seropedicae*

To systematically address the role of the Fnr proteins, we have correlated the transcriptional changes with ChIP-Seq analysis, taking advantage of C-terminally 3xFlag *fnr* alleles engineered in the *H. seropedicae* genome. The Flag tags did not influence the activity of Fnr proteins as judged by the transcription activation of the

fixN::lacZ fusion (previously identified as dependent upon Fnr1 and Fnr3(BATISTA et al., 2013)) in 3xFlag strains in comparison with the wild type (Figure S3.3-1). Chip-seq data was obtained from cultures grown under limited oxygen availability using the same conditions used for the RNA-seq analysis. Using this approach, DNA-binding targets for the *H. seropedicae* Fnr proteins were unambiguously revealed and correlated with transcript profiles. The Fnr1-3xFlag protein bound to 57 promoters with associated changes in the transcriptome data, while the Fnr3-3xFlag protein bound to 36 promoters (Figure 3.3-2). 39 promoters were only bound by Fnr1 (Table 3.3-1) while 23 promoters were specifically bound by Fnr3 (Table 3.3-2), leaving a remaining set of 18 promoters that were bound by both Fnr1 and Fnr3 proteins (Table 3.3-3). Both proteins seem to act mainly as activators, since only a total of 7 promoters were repressed by either Fnr1 or Fnr3 (Figure 3.3-2). Additionally all the promoters containing sites for binding for both Fnr1 and Fnr3 were down-regulated in the single *fnr1* and *fnr3* deletion strains indicating that these promoters are activated by Fnr1 and/or Fnr3.

A subset of selected direct targets for each promoter binding pattern category is presented in Figure 3.3-3. The genomic location of *fnr1* is reflected by its direct activation of both *ompW1* and the *fixNOQP* operon (Figure 3.3-2). Fnr1 also directly activates promoters of genes coding for proteins that are related to the taxis response, such as *cheW* (Hsero_0624) and *cheY* (Hsero_1976). Other activated genes related to taxis include homologs of MCPs (Methyl Accepting Proteins), *aer* (Hsero_3072) and *tsr* (Hsero_3488). Another *cheY* –like gene (Hsero_2983) is directly activated by Fnr3. Genes related to the metabolism of polyhydroxyalkanoates (PHAs) in *H. seropedicae* are also directly activated by Fnr1. These include two genes encoding PhbC synthases, Hsero_2999 (*phbC1*) and Hsero_2405 (*phbC*) (the later organized as the operon *dksAphbCfabI-Hsero_2407*) and also an acetyl-CoA acetyltransferase encoded by *phbA2* (Hsero_0239). PHA metabolism may also be influenced by Fnr3, since a phasin protein encoded by Hsero_1639 is directly activated by this paralog. This phasin was previously shown to be the most abundant protein associated with polyhydroxybutyrate (PHB) granules in *H. seropedicae* (TIRAPELLE et al., 2013) and might be responsible for preventing granule coalescence (JURASEK; MARCHESSAULT, 2002; TIRAPELLE et al., 2013). The PhbC1 synthase, encoded by Hsero_2999, is essential for PHB production since an *phbC*- mutant strain was not able to accumulate PHB (TIRAPELLE et al., 2013).

Fnr3 directly activates the *adhA* (Hsero_0964) and *ldhA* (Hsero_2511) genes coding for alcohol dehydrogenase and a D-lactate dehydrogenase and also the *cydAB* genes, coding for *bd*-type quinol oxidase, which are likely to organized as an operon *Hsero_3055-3056cydAB*. Notably, most of the direct targets for Fnr3, encode transcription factors, including regulators of the TetR/AcrR, LysR, DeoR, GntR and MerR families. The functions of this transcription regulators remains to be elucidated, but potentially may allow *H.seropedicae* to spread the low O_2 signal through another regulons. Additionally the Fnr3 protein is important for the regulation of the Fnr1 protein itself and also the NarXL two component system.

Another set of promoters appear to be bound by both Fnr1 and Fnr3, including the genes coding for cytochrome *c*553 (Hsero_0153), cytochrome *c*551/*c*552 (Hsero_1104), the bc_1-complex (*petA*) and also for two universal stress proteins, annotated as *uspA* (Hsero_2290 and Hsero_2488). A third copy of *uspA* (Hsero_3887) is a target for activation by Fnr1.

The correlation of the ChIP-Seq and RNA-Seq data allowed us to identify the direct target for Fnr regulation and then also to predict the Fnr binding motifs for Fnr1 and Fnr3 specific promoters. Motifs were equally predicted for the promoters that were jointly bound by both Fnr1 and Fnr3 (Figure 3.3-4). The motif prediction for Fnr1, Fnr3 and Fnr1-Fnr3 regulatory categories used 39, 23 and 18 promoters respectively (Figure 3.3-2). All promoters within a regulation category had strong ChIP-Seq peaks (Figure 3.3-4B) with an associated change in the RNA-Seq transcripts mapping (Figure 3.3-4C). The promoters selected were submitted for MEME search as described in Materials and Methods. This approach allowed the finding of discrete binding motifs for each regulation category which is likely to reflect the binding specificity of each of the Fnr proteins. The Fnr1 binding motif reveals that the Fnr1 protein seems to recognize binding motifs more similar to the canonical binding site described for the Fnr proteins of other organisms such as *E. coli* (SCOTT et al., 2003) . In the other hand, the motif found for Fnr3, reveals that this protein might recognize less conserved motif sequences. Comparison of Fnr1 and Fnr3 motifs with the Fnr1-Fnr3 motif clearly reveals that the later motif seems to have mixed characteristics between Fnr1 and Fnr3 motifs. This finding may allow us to speculate that the promoters within the Fnr1-Fnr3 category are regulated by the binding of heterodimers between Fnr1 and Fnr3 to these promoters.

3.3.4.3 Some promoters bound by Fnr1 or Fnr3 were silent for regulation

During the analyses of Fnr binding targets we observed that some promoters with an associated ChIP-Seq peak exhibited no change in expression in the RNA-Seq data from the cognate *fnr* deletion strain; 15 promoters bound by Fnr1 belonged to this class (Table S3.3-4). However, the promoters of *fnr2*, *oxyR* and the Hsero_3790 were previously identified as being down-regulated in the triple *fnr* mutant strain (BATISTA et al., 2013) (Table S3.3-4). Amongst the 12 other promoters bound by Fnr1 with no apparent regulation according to the transcript profiles, the promoter upstream of the *Hsero_4385-4384-4383* operon had a strong ChIP-Seq peak with an extremely good p-value (4.50E-58) comparable to the *p*-values observed for canonical Fnr1 regulated promoters such as the *fixN* and *ompW1* genes (ChIP-Seq p-values of 1.36E-59 and 1.31E-36, respectively). This operon encodes an ABC-transporter, with homology to the Fe^{3+}-hydroxamate siderophore transport system that might be important for iron uptake in *H. seropedicae.* In *E. coli* the genes coding for this transporter are under the control of the Fur repressor (BAGG, ANNE; NEILANDS, 1987; BAGG, A; NEILANDS, 1987). Accordingly, *in silico* analysis of this promoter revealed a motif for binding of the Fur protein in addition to an Fnr motif. It is therefore conceivable, that repression by the *H. seropedicae* Fur homolog is masking regulation of the *Hsero_4385-4384-4383* operon by Fnr1. Similarly, as observed for Fnr1, we found 13 promoters that were bound by Fnr3 but had no differential expression when we compared the transcripts from the wild type and the *fnr3* mutant (Table S3.3-5). Notably, promoters of the operon *Hsero_1502-etfA* and the divergent promoter driving expression of Hsero_1783 (*etf*) and Hsero_1784 exhibited low ChIP-Seq p-values for Fnr3 (9.34E-15 and 5,16E-11, respectively). Hsero_1502 and the associated *etfA* gene encode the β and α subunits of an electron transfer flavoprotein-ubiquinone oxidoreductase respectively and might be important for electron transfer from the ubiquinone to the *bo3*-type and *bd*-type quinol oxidases, during aerobic and micro-aerobic respiration respectively. At least two hypotheses may explain why Fnr regulation is not observed at target promoters with clearly enriched ChIP-Seq peaks: (i) some promoters may require co-regulation with another transcription factor, which was probably not expressed or not active under our experimental conditions and (ii) the binding of a transcriptional regulator that acts as a repressor might prevent Fnr-dependent activation.

3.3.4.5 Estimation of whole genome Fnr binding occupancy

Apart from defining the specific regulons of Fnr1 and Fnr3, the ChIP-Seq data also enabled the prediction of consensus binding sites for these proteins. Fnr-binding motifs were determined by submitting the sequences of the regulated promoters associated with a ChIP-Seq peak to a motif search using MEME (BAILEY et al., 2009) as described above (Figure 3.3-4). Considering that some Fnr binding sites identified by the presence of an enriched ChIP-Seq peak were apparently silent in terms of transcriptional regulation, we sought to determine in which extent the *in silico* predicted Fnr binding motifs in the genome were reached by the ChIP-Seq analyses. Using the MEME motifs identified in Figure 3.3-4 we performed a search for Fnr binding sites throughout all intergenic regions in the *H. seropedicae* genome and then compared these *in silico* predicted Fnr binding motifs with those determined experimentally. The *in silico* motif search led to the identification of 328 putative Fnr binding sites in the intergenic regions of the *H. seropedicae* genome. Of these, 57 promoters were enriched by our ChIP-Seq analyses, although 45 ChIP-Seq enriched sequences were not predicted by the *in silico* Fnr motif search. This can probably be explained by the observation that some of the Fnr enriched binding sites are located within the coding regions of predicted open reading frames, which were not included in the determination of our DNA sequence motifs. Moreover, in some cases ChIP-Seq enriched sequences were not associated with a very well conserved Fnr-box, which also would not be predicted by our *in silico* motif search. A clear example of this is the promoter of the *Hsero_0964* gene, which although associated with a ChIP-Seq enriched peak, has a poor match (ATGAC-N4-ATGAT) to the Fnr binding site consensus, while the best possible match is TTGA-N6-TCAA according to the motif used for Fnr binding site prediction.

Conversely we also verified if promoters with an *in silico* predicted Fnr binding motif, were apparently regulated by Fnr under conditions of oxygen limitation, even though they were not identified in the ChIP-Seq assay. This analysis identified a further 18 putative target promoters where transcript changes were detected in the RNA-Seq datasets of the *fnr* mutants (Table S3.3-6). Notably the promoter of the *ndh* gene, which encodes for the non-coupling NADH dehydrogenase II enzyme, was not associated with a strong ChIP-Seq peak, since it was outside the cut-off parameters for promoter enrichment used in this study. Nevertheless, the *ndh* gene was previously identified as

an Fnr repression target (BATISTA et al., 2013) and a very good consensus binding site for Fnr is present in its promoter (Table S3.3-6).

3.3.4.6 Insights into the function of the Fnr2 protein

In the results presented so far, we have only discussed data relating to Fnr1 and Fnr3, as these proteins seems to be responsible for the majority of the transcriptional changes observed in response to changes in oxygen availability. However, in a few cases the Fnr2 protein appears to participate in the regulation of a subset of genes that were identified as targets of Fnr1 or Fnr3 (Table S3.3-1 and S3.3-2).

Under the conditions employed for both the RNA-Seq and the ChIP-Seq, the Fnr2 protein seems to be expressed at lower levels when compared to both Fnr1 and Fnr3 (Figure S3.3-2). Perhaps as a consequence of the low levels of expression, the recovery of Fnr2-3xFlag-DNA from immunoprecipitation assays was not very efficient. However, the ChIP-Seq of the Fnr2-3xFlag strain allowed us to predict one binding site for Fnr2 at the promoter of the putative *cyoABCDshb1hydH* operon (Figure 3.3-5A). This ChIP-Seq peak was associated with an 4-fold decrease in the expression of this operon in the *fnr2* deletion mutant suggesting that this promoter is directly activated by Fnr2 (Figure 3.3-5 and Table 3.3-4). Interestingly the promoter upstream of *cyoA* was not predicted to have a canonical Fnr binding site, by our *in silico* approach. Accordingly, manual inspection of the promoter failed to predict an Fnr binding site at this promoter. Remarkably, the *cyoABCDshb1hydH* operon was down regulated in the *fnr2* mutant irrespective of oxygen availability (Figure 3.3-5B and 3.3-5C), thus indicating that Fnr2 may mediate activation of this promoter in an oxygen independent manner. Hence it its likely either that Fnr2 is not responsive to oxygen or that it is far more tolerant to oxygen compared with Fnr1 and Fnr3. In addition, we observed that some genes were up-regulated (Table 3.3-4) in the *fnr2* mutant strain (for example the operon *lldD-Hsero_0243-0242-0241-0240*), indicating that these genes are repressed either directly or indirectly by this transcription factor. Interestingly these genes were also regulated in an oxygen independent manner, reinforcing our hypothesis that Fnr2 may not be responsive to oxygen under physiological conditions.

As we observed that the Fnr2 protein is responsible for the activation of the bo_3-type oxidase in an oxygen independent manner, and as the activation of this oxidase is induced under conditions of nitrosative stress in other bacteria (ARAI et al., 2014) we

were interested to investigate whether or not the expression of the *fnr2* gene is induced in the presence of nitrate and nitrite, which may promote nitrosative stress through activity of nitrate and nitrite reductases (POOLE, 2005; GILBERTHORPE; POOLE, 2008) . To do that, we evaluated the activity of an *fnr2::lacZ* fusion as a measure of the *fnr2* expression under the presence of 10 or 20 mM of nitrate as the sole nitrogen source or under 20 mM of ammonium chloride plus 0.25 mM of nitrite (Figure S3.3-3). We found that the expression of the *fnr2* gene was induced at least 2-fold in the presence of nitrite, and approximately 2.5 and 3-fold under 10 and 20 mM of nitrate, respectively. Under the presence of nitrate and limited oxygen, the *H. seropedicae* was shown to produce nitric oxide NO (BONATO *et al., in preparation*). As the Fnr2 protein expression seems to be stimulated under such conditions we can speculate that this protein might function as a sensor of NO rather than O_2.

3.3.5 Discussion

In this study we have expanded our knowledge about the role of the three Fnr proteins in the adaptation of *H. seropedicae* to oxygen availability by using a combination of RNA-Seq and ChIP-Seq analysis. The Fnr3 seems to be a primary sensor of the oxygen status in the cell, since it appears to become active in early stages during the growth to activate the Fnr1 expression, which then expands the regulatory cascade when the oxygen reaches very low levels. Here we found that Fnr1 and Fnr3 regulate discrete classes of genes required for different cellular processes, including genes coding for important components of the electron transport chain (Figure 3.3-6). Apart from this we have identified a group of genes that are jointly regulated by both transcription factors. Promoters in this class are bound by both Fnr1 and Fnr3, potentially indicative of regulation by Fnr1-Fnr3 heterodimers.

The correlation of the ChIP-Seq with transcriptional changes in the transcription profile of *fnr* mutants allowed us to determine the motifs for binding of Fnr1 and Fnr3. This motif analyses also revealed that the 18 promoters jointly bound by Fnr1 and Fnr3 have mixed motif characteristics between the Fnr1 and Fnr3 motifs (Figure 3.3-4). In addition to the correlation of the experimentally identified binding sites with the transcriptional changes prior to distinguish the direct from indirect targets, we have also used the motifs found to perform a whole genome search for Fnr binding sites *in silico*. Surprisingly we found that only a subset of the *in silico* predicted Fnr binding sites were

occupied experimentally. The opposite situation was also observed. There were cases in which a very well conserved *in silico* Fnr binding motif was not associated with a strong ChIP-Seq peak. These observations can lead us to speculate that as observed by (MYERS et al., 2013) other DNA binding proteins such as nucleoid associated proteins (NAPs) (BROWNING et al., 2010; RIMSKY; TRAVERS, 2011) may interfere in the Fnr binding occupancy at some promoters. Another potential explanation for this relatively low Fnr occupancy can be related to the short time of incubation after the oxygen switch we have used for recovering the cross linked complexes. It is possible that the concentration of Fnr dimers were not high enough to allow occupation of all potential Fnr binding sites. We also cannot exclude the possibility that cross linking of Fnr to some target promoters failed for other unknown reasons. Finally, the correlation of the RNA-Seq with the ChIP-Seq revealed that under physiological conditions the Fnr2 is apparently an oxygen insensitive protein responsible for the transcriptional activation of an operon encoding the bo_3-type respiratory oxidase. Apart from this, the promoters of *ompW1* and the *fixNOP* operon, which were assigned as specific targets of Fnr1 and the promoter upstream of *narXL*, which was identified as an Fnr3 target, appear to be under the influence of the Fnr2, since 7, 2.1 and 2.3-fold change in expression was observed, respectively, when we compared the RNA seq data from the *fnr2* mutant strain compared with the wild type (Table S3.3-1). The mechanism by which Fnr2 protein influences the expression of Fnr1 and Fnr3 targets remains to be elucidated, since we were not able to determine whether or not Fnr2 binds at these promoters. Furthermore, no evidence for the participation of the Fnr2 in controlling the expression of either Fnr1 or Fnr3 was found. In addition the effect of the *fnr2* deletion on the expression of *fixNOP* seems to be overcome by Fnr1 and/or Fnr3 proteins. Notably, the *fnr2* deletion strain (strain MB2) had the same heme stainable *c*-type cytochromes content as the wild type strain (SmR1), including the FixP protein (which is encoded by the *fixNOP* operon), as judged by *o*-dianisidine heme staining and also by reduced minus oxidized spectra analysis (BATISTA et al., 2013).

3.3.5.1 Fnr3 is a primary oxygen sensor for reconfiguration of the electron transport chain

Our data establish that Fnr3 binds to the *fnr1* promoter and that transcription of *fnr1* is dependent on Fnr3. As a consequence all genes in the *fnr1* regulon are hierarchically dependent on Fnr3. However, the Fnr1 protein was also found to bind to its own promoter. Fnr3 appears be essential for Fnr1 expression after the switch from "high to low" oxygen levels. Potentially it may be activated at higher levels of oxygen than Fnr1. In this scenario as the cell density increases and the O_2 levels decrease further, Fnr1 is activated, which may result, in displacement of Fnr3, to enable binding of Fnr1 to the promoter either as a homodimer or as a heterodimer with Fnr3. In either case it appears that activation of Fnr1 favours a more productive interaction with RNA polymerase since we observe that expression of a *pfnr1::lacZ* fusion is significantly impaired after the switch to low oxygen in strains carrying a deletion in *fnr1* (Figure S3.3-4). In contrast, expression from the *fnr1* promoter in strains that lack *fnr3* (Δ*fnr3* and Δ*fnr2*/Δ*fnr3*) increases to 50% of wild type activity, 4 hours after the switch to low oxygen. It is, therefore, conceivable that in the 'early' stages of growth, Fnr3 acts as a primary sensor of the adaptation to low oxygen levels, since it switches on the initial expression of Fnr1, which will then expand the regulatory cascade. Apart from its role in Fnr1 activation, Fnr3 also directly activates the *bd*-type quinol oxidase, encoded by the *cydAB* genes, which we anticipate to be the oxidase responsible for respiration under intermediate levels of oxygen.

According to our model, when the oxygen concentration decreases further for example, as cultures reach high cell densities, expression of the cytochrome *c* branch of the electron transport chain is activated (Figure 3.3-6). Remarkably, we observed that both the Fnr1 and Fnr3 proteins are able to bind to the promoters of the *petABC* operon and also the promoter of the Hsero_0153 gene, which probably codes for the cytochrome responsible for electron transport from the bc_1 complex to the cbb_3 oxidase. This may indicate that these promoters are regulated in a similar fashion to that discussed for the *fnr1* promoter. As a consequence, the activation of Fnr1 protein could result in a burst in the expression of the *fixNOP* operon, encoding the high affinity cbb_3-type oxidase.

The observation that both Fnr1 and Fnr3 are required for the expression of key complexes of the electron transport chain may suggest a fine tuning mechanism for gene

expression in which one regulator, Fnr3, which we anticipate to be less oxygen sensitive than Fnr1, first enables initial induction of the genes required for the adaptation to low O_2. As the oxygen concentration further decreases, Fnr1 is activated. This protein may have higher affinity for the promoter than Fnr3 or has the capacity to form Fnr1-Fnr3 heterodimers with increased affinity for the promoter, leading to increased expression levels. This mechanism is likely to optimize the electron flux towards a more efficient terminal oxidase in fluctuating oxygen concentrations.

3.3.5.2 Fnr2 is potentially an NO rather than an O_2 sensor.

The correlation of the RNA-Seq with the ChIP-Seq revealed that under physiological conditions the Fnr2 is apparently an oxygen insensitive protein responsible for the transcriptional activation of the *bo*$_3$-type respiratory oxidase. An alignment of the Fnr2 protein from *H. seropedicae* with *E.coli* Fnr (Figure S3.3-5) reveals that Fnr2 has an interesting substitution at an amino acid residue adjacent to one of the cysteine ligands of the $[4Fe\text{-}4S]^{2+}$ cluster, which may influence oxygen sensitivity. Notably, histidine 26 in the Fnr2 protein may stabilize the cluster. In *E. coli* Fnr, the substitution of the leucine at the position 28 by a histidine (position equivalent to 26 in the Fnr2) decreases inactivation of the iron-sulfur cluster upon exposure to oxygen (BATES et al., 2000). Another interesting substitution occurs within the dimerization helix of Fnr2. At position 152, the *H. seropedicae* Fnr2 protein, has a methionine residue, which is normally occupied by acidic amino acid residues such as aspartate in *E.coli* Fnr (position 154) or glutamate in *H.seropedicae* Fnr1 (position 174) and Fnr3 (position 159). In *E.coli* Fnr, the substitution of an aspartate to an alanine residue at position 154, influences the dimerization properties of Fnr, enabling dimer formation under aerobic conditions (LAZAZZERA et al., 1993; BATES et al., 1995). These observations, can therefore suggest that some amino acid substitutions might have occurred across evolution of the *H. seropedicae* Fnr proteins to allow the three different proteins to tune their response across towards different environmental niches. In the case of the Fnr2 protein amino acid substitutions may have led to the appearance of a protein that is far less oxygen sensitive or with increased propensity to dimerize under aerobic conditions.

In *P. aeruginosa* the *bo*$_3$-type oxidase encoded by the *cyo* genes are up-regulated in the presence of the nitric oxide donor *S*-nitrosoglutathione (GSNO), indicating that

the Cyo oxidase can be utilized under NO-stress conditions (ARAI et al., 2014). Unpublished results from our laboratory indicates that under nitrate and limited oxygen *H. seropedicae* SmR1 is able to produce high amounts of NO and also induction of *cyoABCD* operon expression was observed under these conditions (BONATO *et al*., *in preparation*). These observations may indicate that the Cyo oxidase can be utilized under NO-stress conditions in *H. seropedicae* as described for *P. aeruginosa*. Further we observed that the expression of the *fnr2* gene is induced in the presence of nitrate and nitrite (Figure S3.3-3). As the respiratory oxidase encoded by the *cyoABCD* operon seems to be activated by Fnr2 in an oxygen independent manner and considering that both *cyoABCD* and *fnr2* expression are activated under conditions of NO production we can speculate that the Fnr2 protein might function as an NO sensor.

3.3.6 Materials and Methods

3.3.6.1 Growth conditions

E. coli strains were grown at 37°C in LB medium (Sambrook). *H.seropedicae* strains were grown at 30°C in NFbHP-Malate medium (39) supplemented with 20 mM of NH_4Cl. The antibiotics used were ampicillin (250 µg $mL^{-1}$for *E. coli*), streptomycin (80 µg mL^{-1}), nalidixic acid (5 µg mL^{-1}), tetracycline (10 µg mL^{-1}), kanamycin (50 µg mL^{-1} for *E. coli* and 500 µg mL^{-1}L for *H. seropedicae*) and gentamycin (50 µg mL^{-1} for *E. coli* and 500 µg mL^{-1}L for *H. seropedicae*). To evaluate the adaptation of *H. seropedicae* SmR1 to low oxygen levels we developed an oxygen switch protocol, in which the *H. seropedicae* strains were grown under 350 rpm (high oxygen) until an $O.D_{600}$ of 0.36±0.02 was reached and then cultures were switched to 120 rpm (low oxygen) for 30 minutes or until cells reach an $O.D_{600}$ of 0.43±0.02. The activation of the Fnr1 protein after the high to low oxygen switch was validated by checking the activity of the *fixN* promoter before and after switch (Figure S3.3-6).

3.3.6.2 Construction of *H.seropedicae fnr* deletions and *fnr* 3xFlag tagged strains.

To construct complete deletions of *fnr* genes we first amplified by PCR upstream and downstream fragments flanking all three *fnr* genes. Both upstream and downstream fragments were ligated together by overlapping PCR (URBAN et al., 1997)

and then digested with *Bam*HI and *Hind*III enzymes and finally cloned into pK18mobSacB vector (QUANDT, J; HYNES, 1993), to generate the suicide vectors pMBBR1D (*fnr1* deletion), pMBBR2D (*fnr2* deletion) and pMBBR3D (*fnr3* deletion). For tagging the *fnr* genes with the 3xFlag at the C-terminal of the Fnr proteins, we cloned the whole coding region of the *fnr* genes upstream of 3xFlag sequence vector synthesized by the GenScript Corporation to give us restriction enzyme compatibility for cloning. Then a fragment of approximately 1.0 Kb downstream of the cognate *fnr* gene was also combined with the 3xFlag tag sequence to generate an approximately 2.0 Kb fragment containing the 3xFlag tag sequence flanked by the coding and downstream regions of each *fnr* gene. The ~2.0 Kb fragments for *fnr1* and *fnr2* genes were then digested with *Bam*HI and *Xma*I, whereas the *fnr3* fragment was digested with *Not*I/*Xba*I and cloned into pJQ200SK vector (QUANDT, J; HYNES, 1993) to generate the suicide vectors pMBUK1T (*fnr1* tagging), pMBUK2T (*fnr2* tagging) and pMBUK3T (*fnr3* tagging). The suicide plasmids generated for both deletion and tagging of *fnr* genes were transferred for *H. seropedicae* SmR1 by conjugation as described (BATISTA et al., 2013). Single crossover strains for the deletion and tagging strategies were selected by kanamycin and gentamycin antibiotic resistance, respectively. Double crossover strains were selected on plates containing 5% sucrose and then tested for specific antibiotic sensitivity. The final mutant candidate strains, sensitive to either kanamycin or gentamycin and resistant to sucrose, were analysed by PCR using primers external to *fnr1*, *fnr2* and *fnr3*, whilst the candidate 3xFlag tagged strains were analysed using primers flanking the 3xFlag tag insertion region.

3.3.6.3 RNA purification and high throughput sequencing (RNA- Seq)

H. seropedicae SmR1 (wt), MB1Del (Δ*fnr1*), MB2Del (Δ*fnr2*) and MB3Del (Δ*fnr3*) strains were grown in 50 mL of Nfb-MalateHPN in either “high” oxygen or switched from “high” to “low” conditions as described above. After incubation, 30 mL of RNA Later solution was added to 50 mL cultures and then split into falcon tubes containing 40 mL each. Cells were collected by centrifugation (7000 rpm, 4°C, 5 minutes) and ressuspended to a final volume of 200 μL with 10 mM Trizma® (Sigma# T-2694) prepared in RNase free water. All subsequent steps were performed as described (Item 3.2.5.4). rRNA depletion was performed with Ribo-Zero Gram-negative Bacteria kit (Epicentre#MRZGN126) using 4 μg of total RNA as recommended by

manufacturer. The mRNA enriched samples were sent for library construction and sequencing by The Genome Analysis Centre (TGAC), Norwich Research Park, Norwich, United Kingdom.

3.3.6.4 Chromatin immunoprecipitation followed by high throughput sequencing (ChIP-Seq)

H. seropedicae SmR1 (wt) and MB1Flag (*fnr1::[Leu-Glu]-3XFlag*), MB2Flag (*fnr2::[Leu-Glu]-3XFlag*) and MB3Flag (*fnr3:: [Leu-Glu]- 3XFlag*) were grown in two 50 mL volumes of Nfb-MalateHPN under 350 rpm until an O.D600 of 0.33 to 0.35 and switched for 120 rpm for 30 minutes. The cells were cross linked by the addition of 1% (vol/vol) formaldehyde (Sigma#F8775) to the cultures and further incubation for 25 min. Glycine (final concentration 125mM) was added to quench the cross linking, and samples were then incubated for 5 min on ice. Cells were collected by centrifugation and washed twice in 20 mL of PBS buffer pH 7.4 (Sigma#P4417). The washed pellets were resuspended in 1.0 mL of IP lysis buffer (10mMTris-Cl, pH 8.0, 50 mM NaCl), containing 1x protease cocktail inhibitor (Roche#11836170001). Lysis and combined DNA shearing were performed by sonication (Soniprep 150 – Sanyo) in an ice/water bath at 10 microns (50% of amplitude) as follows: 5 pulses of 15 seconds on and 15 seconds off followed by 4 minutes incubation on ice and 5 more pulses of 15 seconds on and 15 seconds off. After clarification of lysates by centrifugation at 4°C, 17000 x g for 5 min, 25 µL of supernant combined with 75 µL of TE buffer (10 mM Tris-Cl, pH 7.4, 1 mM EDTA), was treated with 1 µL of RNase (Sigma#R4642) and extracted once with phenol:chlorofrom:isoamyl alcohol (25:24:1) followed by chloroform extraction. Verification of DNA shearing was confirmed by loading 10 µL of purified DNA onto a 1% agarose gel. Fragments ranged from 100 to 500 bp, centered on 300 bp.

Subsequently, 525 µL of IP Buffer (50 mM Tris-Cl, pH 8.0, 250 mM NaCl, 0.8% [vol/vol] Triton X-100), containing 1x protease cocktail inhibitor, was added, to the remainder of the lysate (975 µL) and samples were chilled on ice. 50 µL of each lysate was set aside for total-DNA extraction. The remaining lysates were added to 45 µL of EZview Red ANTI-FLAG M2 affinity gel (Sigma#F2426) prepared and washed with TBS buffer (Sigma# T5030) as described in the manufacturer's instructions. The cross-linked lysates were immuno precipitated by incubation for 20 hours on a rotating wheel at 4°C. The samples were then centrifuged for 1 min at 4°C at 4,500 X *g*, and the pellets

were washed once with 1.5 mL of IP Buffer and then three times more with 1 mL of IP buffer. After washing, the affinity gel pellets and 50 μL of total cell extracts (set aside earlier) were eluted overnight at 65°C in 100 μL of IP elution buffer (50mMTris-Cl, pH 7.6, 10mMEDTA, 1% SDS). After elution, samples were centrifuged at 16,000X*g* for 5 min to remove the affinity gel. The eluted DNA was transferred to a new tube and the affinity gel was re-extracted with 50 μL of TE buffer followed by a further 5 min incubation at 65°C. Both total DNA and IP DNA samples were adjusted to 200 μL TE buffer and incubated with 3 μL of 10 mg/mL of proteinase K (Roche# 03115879001) for 2 h at 55°C. The samples were extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform and further purified using Macherey-Nagel NucleoSpin® columns (Catalog#740609.50). DNA was eluted in 50 μL of nuclease-free H_2O and quantified by nanodrop (Thermo Scientific).

The purified DNA was sent for library preparation and sequencing by The Genome Analysis Centre (TGAC), Norwich Research Park, Norwich, United Kingdom. The TruSeq ChIP sample preparation kit from Illumina Inc. was used as indicated by the manufacturer (BUSH et al., 2013).

3.3.6.5 ChIP-Seq and RNA-Seq Data analysis

The reads in the fastq files received from the sequencing contractor were aligned to the *H. seropedicae* genome (GenBank accession number: NC_014323.1) using the bowtie2 software (LANGMEAD; SALZBERG, 2012), which resulted in one SAM (.sam) file for each fastq file. All further operations for the ChIP-Seq analysis were carried out using a combination of Perl scripts dependent on the BioPerl toolkit (STAJICH et al., 2002) and R scripts essentially as described previously (BUSH et al., 2013). The differential expression analyses from the RNA-Seq assay was performed using edgeR (ROBINSON et al., 2010).

3.3.6.6 Determination of Fnr DNA-binding motifs and whole genome prediction of Fnr binding motifs occurrence

For identification of the Fnr binding motifs, the promoters with an associated ChIP-Seq peak and combined differential expression in the RNA-Seq analyses were

selected and submitted to MEME search (BAILEY et al., 2009). Briefly, a sequence of 300 bp surrounding the centre of the ChIP-Seq peak (150 bp upstream and 150 bp downstream from the center of the peak) was selected in a strand specific manner. A fasta file containing all sequences for a given regulatory category was generated and then submitted for MEME search using the on line submission plataform (BAILEY et al., 2009). 39 sequences were submitted for the Fnr1 motif search, while 23 and 18 sequences were submitted for the Fnr3 and Fnr1-Fnr3 categories, respectively. The occurrence of the Fnr binding motifs in the whole *H. seropedicae* genome was performed by submitting the Fnr binding motifs found by MEME to FIMO search using the intergenic regions of *H.seropedicae* SmR1 genome as a query sequence database. The output returned a number of 1123 Fnr binding motifs occurring in both strands. Only strand specific binding motifs within 400 base pairs from the start codon were considered as potential functional Fnr binding motifs, thus reducing the number of potentially Fnr binding motifs to 328.

3.3.7 Figures

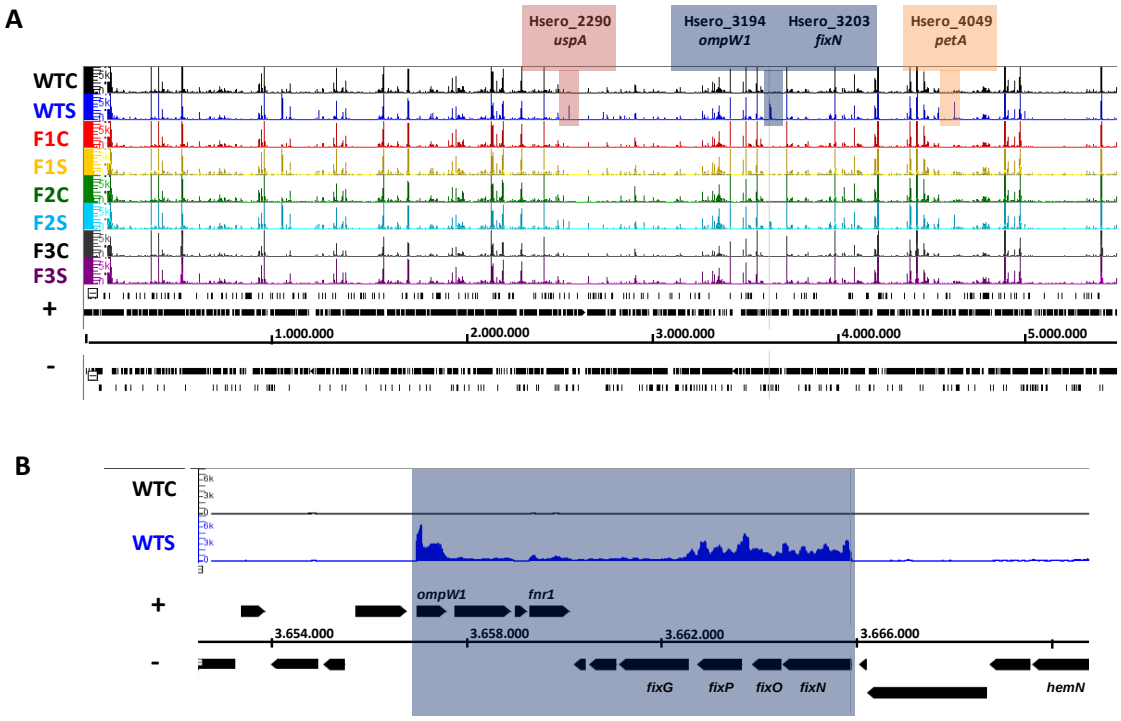

Figure 3.3-1. Transcriptional profiling reveals genes highly induced after the switch from aerobic to micro-aerobic conditions. In panel **A**, transcripts mapping to the *H. seropedicae* genome under different conditions are showed using the IGB viewer (ref). The lanes are colour coded as follows: WTC, wild type under 350 rpm (black); WTS, wild type after switch to 120 rpm (blue); F1C, *fnr1* deletion under 350 rpm (red); F1S, *fnr1* deletion after switch to 120 rpm (yellow); F2C, *fnr2* deletion under 350 rpm (green); F2S, *fnr2* deletion after switch to 120 rpm (light blue); F3C, *fnr3* deletion under 350 rpm (dark blue); F3S, *fnr3* deletion after switch to 120 rpm . Examples of genes that are highly expressed after the switch to micro-aerobic conditions are shown above the lanes. In panel **B**, highly induced genes in the genomic region of the *fnr1* gene are zoomed in.

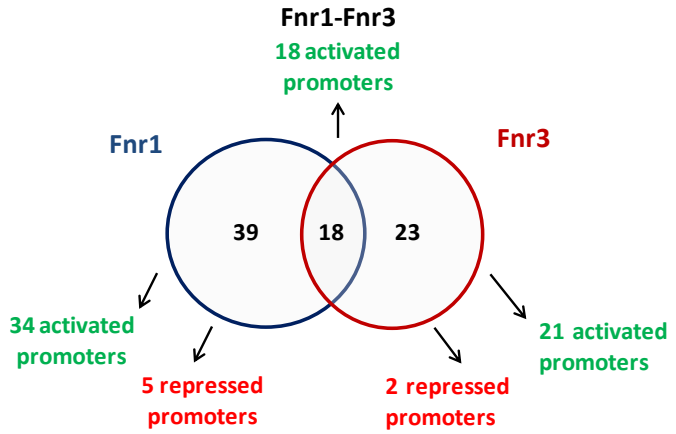

Figure 3.3-2. Overview of the direct targets identified for the Fnr1 and Fnr3 proteins. For identification of the specific targets for each Fnr, ChIP-Seq peaks were correlated with the differential expression profile revealed by the transcriptome data.

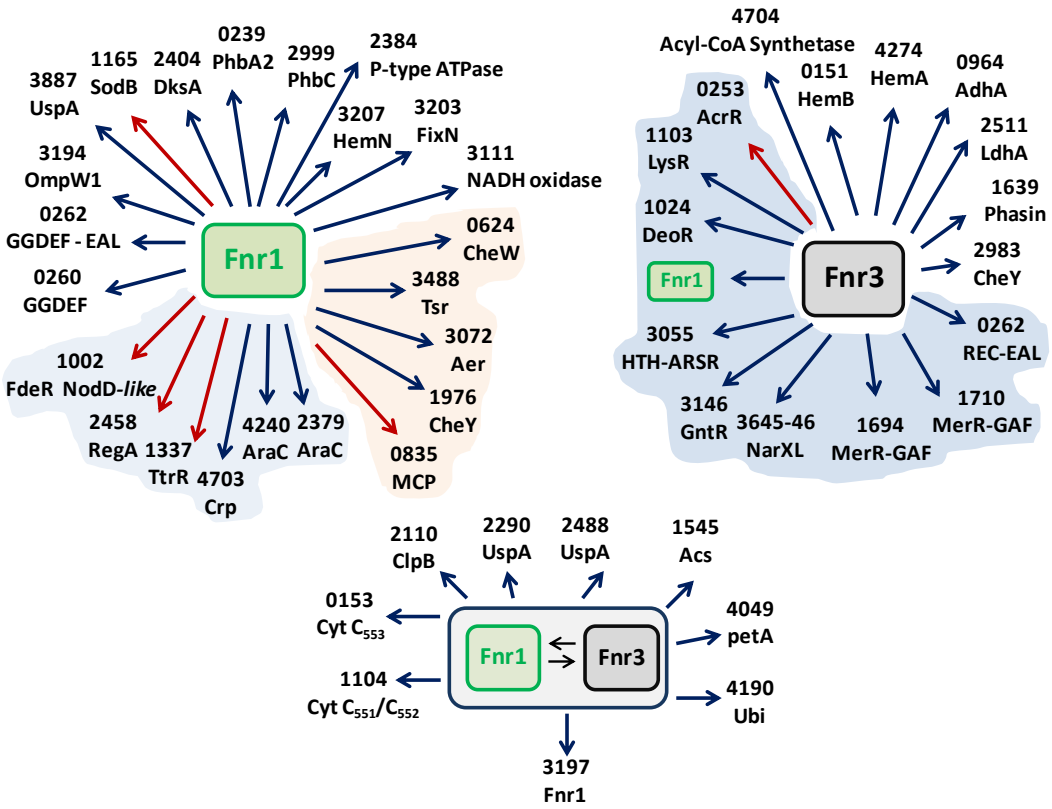

Figure 3.3-3. Selected targets for Fnr1 and Fnr3 regulation. The upper right panel shows direct targets for Fnr1, the upper left panel shows direct targets for Fnr3 and the lower panel shows targets for both Fnr1 and Fnr3. Blue arrows indicates activation while red arrows indicate repression. The numbers represent the locus tag IDs. Gene products are indicated below each number.

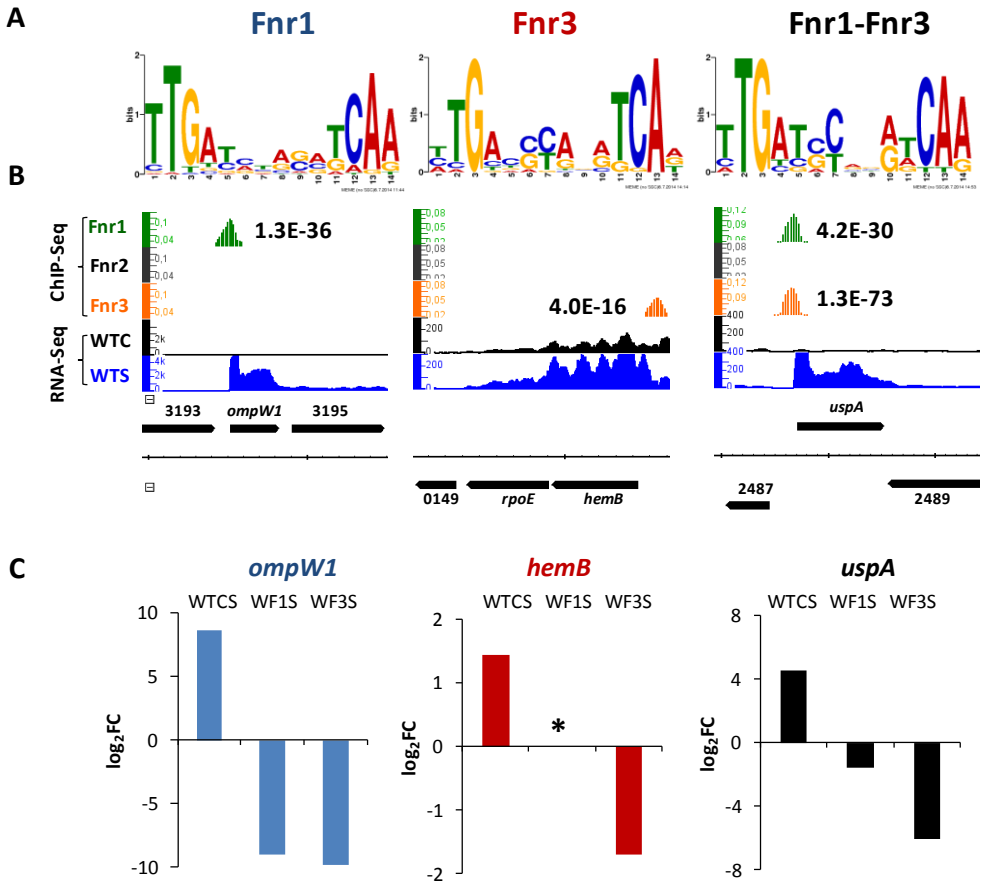

Figure 3.3-4. ChIP-Seq leads to the identification of Fnr1 and Fnr3 binding motifs. Panel **A,** motifs for Fnr1, Fnr3, and Fnr1-Fnr3 identified by MEME searches are shown. Panel **B**, ChIP-Seq peaks and transcripts mapping to representative genes regulated by each regulatory class are shown in addition to the respective p-value for each ChIP-Seq peak. The ChIP-Seq data for Fnr1, Fnr2 and Fnr3 3xFlag strains is presented in green, black and orange, respectively. Abbreviations and colour coding for the RNA-Seq data are as follows: WTC, wild type under 350 rpm (blue); WTS, wild type after switch to 120 rpm (black). Panel C, the log_2FC of the genes in each regulatory group are shown. Abbreviations indicates the following library comparisons for differential expression: WTCS, wild type control (350 rpm) x wild type after switch to 120 rpm (blue); WF1S, wild type after switch x *fnr1* mutant after switch (red); WF3S, wild type after switch x *fnr3* mutant after switch (black). The asterisk indicates a statistically insignificant fold change.

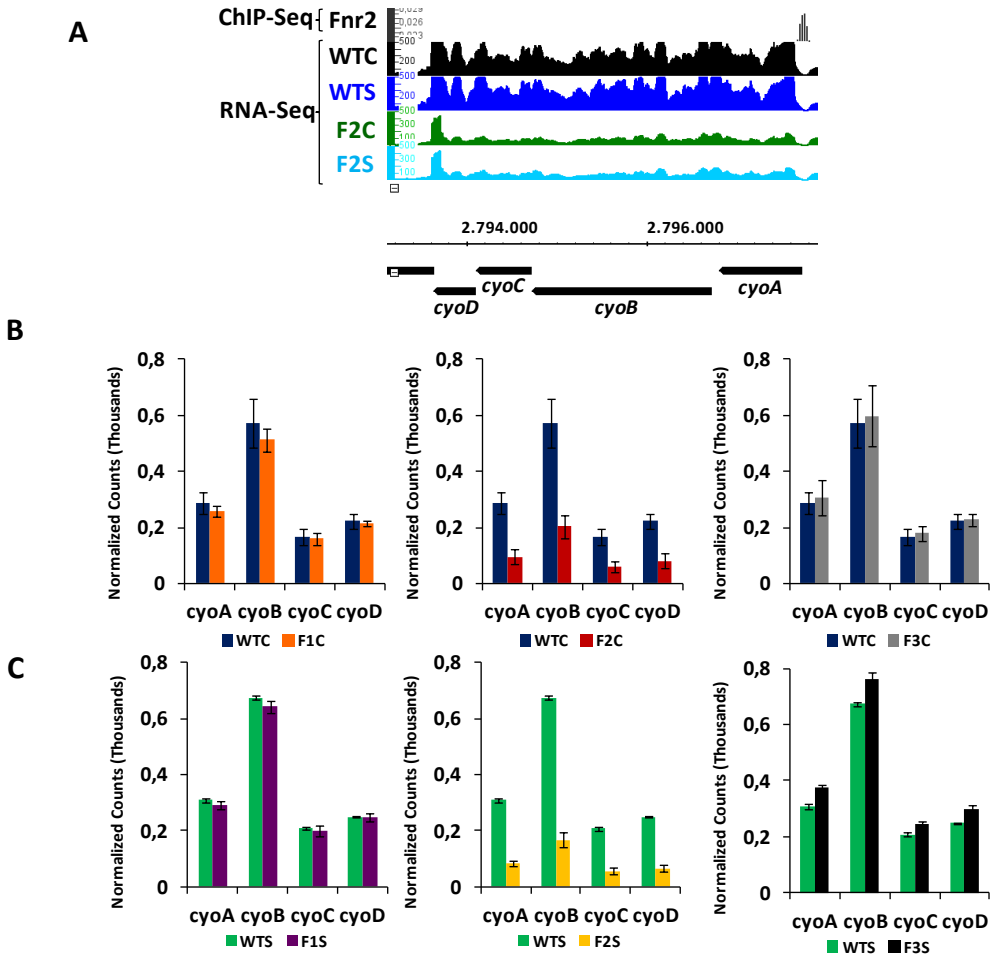

Figure 3.3-5. Fnr2 regulates the *cyo* genes in an oxygen independent manner. Panel **A**, the ChIP-Seq peak for the Fnr2-3xFlag protein and the transcripts mapping to *H. seropecidae cyo* genes region are shown. The abbrevations are as follows: WTC, wild type under 350 rpm ; WTS, wild type after switch to 120 rpm; F1C, *fnr1* deletion under 350 rpm; F1S, *fnr1* deletion after switch to 120 rpm; F2C, *fnr2* deletion under 350 rpm; F2S, *fnr2* deletion after switch to 120 rpm. Panel **B**, normalized counts under high oxygen (350 rpm) of each *cyo* gene for the wild type (blue bars) in comparison with the *fnr1* deletion (orange bars), *fnr2* deletion (red bars) and *fnr3* deletion (grey bars) are shown. Panel C, normalized counts under low oxygen (after the switch from 350 to 120 rpm) of each *cyo* gene for the wild type (green bars) in comparison with the *fnr1* deletion (violet bars), *fnr2* deletion (yellow bars) and *fnr3* (black bars) deletion is shown.

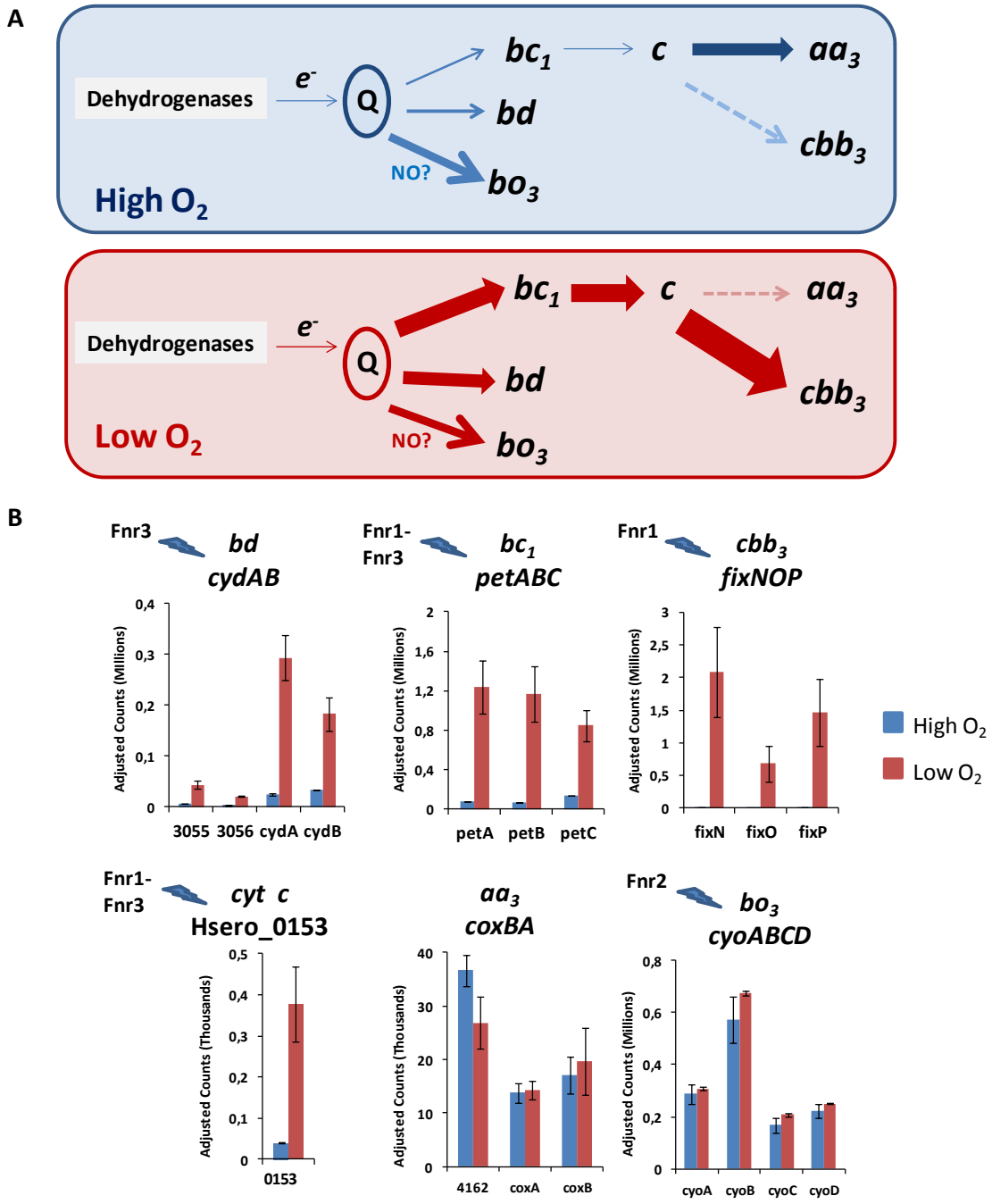

Figure 3.3-6. Main transcriptional changes observed in the electron transport chain upon switch from high to low oxygen levels. Panel A, a model for the predicted electron flow under high oxygen (blue) or low oxygen (red) is shown, with the arrow thickness representing the favoured route for the electron flux based on the transcriptional changes observed. Panel B, the adjusted counts of the genes coding for the main respiratory complexes represented in A are shown. The high O_2 condition is represented in blue, while the low O_2 is represented in red. The binding of an Fnr protein to the specified operon or gene promoter is indicated.

3.3.8 Tables

Table 3.3-1 – Genes with an upstream Fnr1 ChIP-Seq peak and altered expression in the *fnr1* deletion mutant. (39 promoters).

		RNA-Seq						ChIP-Seq	
		WF1S		WF2S	WF3S	WTCS	WT/MB231[a]	Fnr1	
Locus Tag	**Gene ID**	**log2FC**	**p-value**	**log2FC**	**log2FC**	**log2FC**	**logFC**	**Peak**	**p-value**
Hsero_0239	phbA2	-1.22	1.59E-14	-	-1.03	1.05	-	+	4.73E-13
Hsero_0260	Hsero_0260	-1.66	3.61E-27	-	-1.70	1.50	-3.94	+	3.30E-10
Hsero_0262	Hsero_0262	-0.85	1.21E-12	-	-1.07	0.84	-	+	3.30E-10
Hsero_0371	Hsero_0371	-0.75	4.36E-08	-	-0.74	0.72	-	+	5.72E-21
Hsero_0624	cheW	-0.72	2.14E-05	-	-0.70	0.64	-3.34	+	6.04E-05
Hsero_0660	pilA	-1.38	8.58E-19	-	-1.42	1.38	-2.46	+	7.57E-11
Hsero_0835	***Hsero_0835***	1.08	1.49E-07	-	1.34	-1.24	-	+	4.24E-11
Hsero_1002	***nodD***	0.59	1.40E-04	-	-	-	-	+	1.10E-15
Hsero_1165	***sodB***	1.16	2.44E-20	-	1.31	-1.34	-	+	4.25E-05
Hsero_1250	Hsero_1250	-1.49	8.93E-25	-	-6.32	4.44	-5.03	+	7.77E-05
Hsero_1337	***Hsero_1337***	1.73	5.64E-22	1.12	1.58	-1.91	2.94	+	7.52E-05
Hsero_1446	Hsero_1446	-1.43	1.66E-26	-	-1.68	1.60	-	+	6.20E-06
Hsero_1552	Hsero_1552	-3.75	1.47E-07	-	-4.16	3.15	-2.70	+	7.51E-05
Hsero_1880	Hsero_1880	-0.97	1.76E-16	-	-1.09	0.86	-	+	1.30E-12
Hsero_1957	Hsero_1957	-1.06	1.14E-11	-	-1.55	1.64	-	+	1.40E-15
Hsero_1976	cheY	-0.44	2.84E-06	-	-0.52	0.70	-	+	2.53E-11
Hsero_2144	Hsero_2144	-0.76	9.28E-11	-	-0.65	0.38	-	+	5.63E-07
Hsero_2379	Hsero_2379	-2.67	2.27E-23	-1.04	-3.42	3.40	-4.66	+	1.29E-06
Hsero_2384	Hsero_2384	-2.09	6.43E-10	-	-3.19	3.23	-3.69	+	1.54E-11
Hsero_2403	Hsero_2403	-1.60	2.25E-02	-	-2.57	2.58	-5.29	+	2.95E-08
Hsero_2404	dksA	-4.60	5.43E-15	-	-6.43	8.35	-6.64	+	2.95E-08
Hsero_2458	***Hsero_2458***	1.02	5.08E-13	-	1.16	-1.40	3.04	+	5.80E-14
Hsero_2909	Hsero_2909	-1.25	4.68E-20	-	-1.46	1.45	-	+	1.92E-30
Hsero_2999	phbC	-0.63	4.77E-07	-	-0.70	0.45	-1.36	+	1.19E-39
Hsero_3072	era	-4.15	2.16E-15	-1.40	-4.04	4.22	-4.32	+	5.93E-17
Hsero_3111	Hsero_3111	-1.68	1.95E-21	-	-1.94	1.15	-	+	3.17E-07
Hsero_3194	ompW1	-8.96	4.76E-77	-2.88	-9.79	8.60	-7.09	+	1.31E-36
Hsero_3203	fixN	-6.57	1.44E-118	-1.13	-9.55	8.64	-6.36	+	1.36E-59
Hsero_3207	hemN	-4.78	6.29E-52	-	-7.42	5.94	-4.17	+	5.93E-39
Hsero_3246	Hsero_3246	-5.57	9.71E-07	-	-2.71	4.11	-	+	2.27E-05
Hsero_3273	Hsero_3273	-1.01	1.62E-17	-	-1.31	1.17	-2.98	+	1.04E-10
Hsero_3488	tsr	-1.22	8.86E-10	-	-1.34	1.38	-	+	5.67E-23
Hsero_3532	Hsero_3532	-1.76	2.80E-12	-	-3.80	3.57	-	+	0.00E+00
Hsero_3853	pyrC	-0.56	2.37E-10	-	-0.72	0.62	-2.32	+	2.38E-23

Continues in the next page

Hsero_3887	uspA	-1.53	8.45E-12	-	-4.42	3.57	-4.33	+	4.14E-13
Hsero_3965	Hsero_3965	-0.87	1.14E-10	-	-1.11	0.84	-	+	5.80E-10
Hsero_3974	yhbH	-1.29	4.59E-13	-	-1.07	1.04	-	+	4.29E-19
Hsero_4240	Hsero_4240	-1.01	3.00E-15	-	-1.25	0.98	-2.54	+	2.35E-31
Hsero_4703	crp	-1.18	3.42E-11	-	-1.66	1.22	-2.50	+	7.30E-06

RNA-Seq library comparisons are as follows: WF1S: wild type after switch to low O_2 x *fnr1* mutant after switch to low O_2; WF2S: wild type after switch to low O_2 x *fnr2* mutant after switch to low O_2; WF3S: wild type after switch to low O_2 x *fnr3* mutant after switch to low O_2; WTCS: wild type before switch or high O_2 x wild type after switch to low O_2; [a]WT/MB231: wild type under low O_2 x triple *fnr* mutant after under low O_2 (BATISTA et al., 2013).

Table 3.3-2 – Genes with an upstream Fnr3 ChIP-Seq peak and altered expression in the *fnr3* deletion mutant (23 promoters).

		RNA-Seq						ChIP-Seq	
		WF3S		WF1S	WF2S	WTCS	WT/MB231[a]		Fnr3
Locus Tag	**Gene ID**	**log2FC**	**p-value**	**log2FC**	**log2FC**	**log2FC**	**log2FC**	**Peak**	**p-value**
Hsero_0151	hemB	-1.70	3.74E-43	-	-	-1.44	-2.57	+	4.00E-16
Hsero_0253	***Hsero_0253***	0.42	2.16E-03	-	-	-	-	+	3.32E-05
Hsero_0627	Hsero_0627	-2.16	1.61E-72	-	-	-1.85	-2.42	+	3.45E-42
Hsero_0962	Hsero_0962	-6.91	1.70E-150	-	-	-4.14	-5.46	+	1.22E-75
Hsero_0964	adhA	-8.87	0.00E+00	-	-	-3.05	-5.02	+	1.17E-111
Hsero_1024	deoR	-1.40	4.26E-23	-	-	-1.17	-	+	2.32E-06
Hsero_1103	Hsero_1103	-0.61	6.41E-04	-	-	-0.67	-	+	2.50E-18
Hsero_1639	Hsero_1639	-1.09	3.51E-32	-	-	-	-2.69	+	4.77E-14
Hsero_1694	Hsero_1694	-2.85	2.58E-167	-	-	-2.05	-	+	1.69E-73
Hsero_1710	Hsero_1710	-0.67	3.72E-11	-	-	-0.51	-	+	5.87E-91
Hsero_2263	Hsero_2263	-1.96	1.74E-21	-	-	-1.78	-2.38	+	2.92E-29
Hsero_2511	ldhA	-5.36	1.10E-253	-	-	-3.66	-5.27	+	6.94E-15
Hsero_2530	Hsero_2530	-3.46	6.65E-86	-	-	-2.91	-2.41	+	5.06E-24
Hsero_2983	cheY	-0.46	1.22E-05	-	-	-0.42	-	+	7.24E-27
Hsero_3055	Hsero_3055	-3.32	2.83E-75	-	-	-2.84	-	+	8.70E-143
Hsero_3146	Hsero_3146	-0.80	1.39E-15	-	-	-0.56	-	+	6.84E-34
Hsero_3223	Hsero_3223	-1.68	1.44E-21	-	-	-1.48	-2.17	+	1.06E-77
Hsero_3327	***argM***	0.69	8.05E-20	-	-	-	-	+	6.96E-06
Hsero_3370	ardA	-2.25	7.31E-35	-	-	-1.57	-2.74	+	8.52E-47
Hsero_3472	Hsero_3472	-1.83	7.73E-45	-	-	-1.83	-	+	4.24E-78
Hsero_3645	narX	-6.28	3.95E-198	-	-1.24	-3.83	-7.11	+	1.19E-13
Hsero_4274	hemA	-1.54	8.61E-65	-	-	-	-	+	5.48E-67
Hsero_4704	Hsero_4704	-1.49	3.69E-29	-	-	-	-3.15	+	5.25E-09

RNA-Seq library comparisons are as follows: WF3S: wild type after switch to low O_2 x *fnr3* mutant after switch to low O_2; WF1S: wild type after switch to low O_2 x *fnr1* mutant after switch to low O_2; WF2S: wild type after switch to low O_2 x *fnr2* mutant after switch to low O_2; WTCS: wild type before switch or high O_2 x wild type after switch to low O_2; [a]WT/MB231: wild type under low O_2 x triple *fnr* mutant after under low O_2 (BATISTA et al., 2013).

Table 3.3-3 - Genes with an upstream ChIP-Seq peak for Fnr1 and Fnr3 and altered expression in both *fnr1* and *fnr3* deletion mutants. (18 promoters).

		RNA-Seq							Chip-Seq			
		WF1S		WF3S		WF2S	WTCS	WT/MB231[a]	Fnr1	Fnr1	Fnr3	Fnr3
Locus Tag	**Gene ID**	**log2FC**	**p-value**	**log2FC**	**p-value**	**log2FC**	**log2FC**	**log2FC**	**Peak**	**p-value**	**Peak**	**p-value**
Hsero_0005	Hsero_0005	-0.86	1.66E-19	-1.10	1.51E-30	-	-0.91	-	+	2.30E-08	+	2.59E-06
Hsero_0153	Hsero_0153	-2.37	2.86E-44	-3.41	1.84E-82	-	-3.24	-2.37	+	2.33E-49	+	4.97E-16
Hsero_0998	mdpB	-0.61	1.15E-05	-1.80	2.36E-36	-	-1.36	-	+	5.38E-30	+	5.18E-38
Hsero_1104	Hsero_1104	-1.47	2.85E-24	-4.08	1.90E-141	-1.00	-3.73	-2.07	+	4.87E-32	+	2.50E-18
Hsero_1175	Hsero_1175	-0.48	1.97E-06	-1.38	8.97E-41	-	-1.03	-	+	9.61E-29	+	2.47E-82
Hsero_1383	hflB	-0.24	5.56E-03	-0.49	3.17E-08	-	-0.36	-	+	5.24E-18	+	1.36E-26
Hsero_1545	acs	-0.60	6.41E-05	-2.04	3.19E-39	-	-1.69	-	+	7.01E-15	+	8.33E-44
Hsero_1829	Hsero_1829	-1.39	2.37E-09	-6.74	2.48E-116	-1.05	-4.90	-5.20	+	1.55E-18	+	1.96E-22
Hsero_2110	clpB	-1.37	1.83E-15	-2.28	6.88E-38	-	-2.00	-2.80	+	6.24E-30	+	4.03E-40
Hsero_2290	uspA	-6.39	7.02E-42	-9.64	3.39E-71	-	-8.78	-6.11	+	4.10E-73	+	9.38E-24
Hsero_2488	uspA	-1.62	2.17E-13	-6.05	2.27E-113	-1.23	-4.54	-5.99	+	4.24E-30	+	1.28E-73
Hsero_2510	Hsero_2510	-0.92	7.41E-08	-6.07	6.38E-182	-	-3.93	-6.75	+	3.86E-41	+	2.43E-36
Hsero_2530	Hsero_2530	-0.84	1.99E-07	-3.46	6.65E-86	-	-2.91	-2.41	+	7.72E-07	+	5.06E-24
Hsero_2915	Hsero_2915	-0.63	5.11E-04	-6.53	5.07E-179	-1.36	-4.65	-5.16	+	1.10E-23	+	3.88E-66
Hsero_3148	Hsero_3148	-0.68	2.90E-10	-1.26	3.86E-31	-	-1.24	-	+	5.41E-36	+	3.05E-38
Hsero_3197	fnr1	-	-	-6.49	2.05E-169	-	-3.12	-5.51	+	1.43E-65	+	1.40E-94
Hsero_4049	petA	-3.22	2.27E-109	-4.33	2.78E-177	-	-3.99	-2.78	+	3.38E-63	+	5.98E-19
Hsero_4190	Hsero_4190	-1.31	4.50E-27	-1.95	1.03E-55	-	-1.69	-3.27	+	2.68E-10	+	2.94E-06

RNA-Seq library comparisons are as follows: WF1S: wild type after switch to low O_2 x *fnr1* mutant after switch to low O_2; WF3S: wild type after switch to low O_2 x *fnr3* mutant after switch to low O_2; WF2S: wild type after switch to low O_2 x *fnr2* mutant after switch to low O_2; WTCS: wild type before switch or high O_2 x wild type after switch to low O_2; [a]WT/MB231: wild type under low O_2 x triple *fnr* mutant after under low O_2 (BATISTA et al., 2013).

Table 3.3-4 – Genes exclusively regulated by the Fnr2 protein in an oxygen independent manner.

		RNA-Seq			
		WF2S		WF2C	
Locus Tag	**Gene ID**	**log2FC**	**p-value**	**log2FC**	**p-value**
Hsero_0181	putA	1.21	8.3E-07	1.11	5.9E-06
Hsero_0240	Hsero_0240	4.53	4.4E-174	4.91	2.2E-197
Hsero_0241	Hsero_0241	4.98	4.1E-92	5.51	9.1E-107
Hsero_0242	Hsero_0242	5.40	6.5E-76	5.63	1.8E-80
Hsero_0243	Hsero_0243	5.51	1.0E-92	5.65	4.0E-96
Hsero_0244	lldD	6.29	3.4e-311	6.52	0.0E+00
Hsero_1874	Hsero_1874	1.11	5.4E-11	1.02	2.1E-09
Hsero_2459	hydH	-1.75	1.6E-26	-0.97	2.0E-09
Hsero_2460	shb1	-1.91	7.6E-36	-1.40	1.2E-20
Hsero_2461	cyoD	-1.93	3.1E-32	-1.44	2.5E-19
Hsero_2462	cyoC	-1.90	5.4E-25	-1.44	1.8E-15
Hsero_2463	cyoB	-2.01	2.6E-38	-1.49	1.4E-22
Hsero_2464	cyoA	-1.87	6.3E-31	-1.58	5.5E-23
Hsero_3000	Hsero_3000	1.48	1.4E-23	1.12	2.3E-14
Hsero_3119	prpC	1.07	1.4E-10	1.23	2.2E-13
Hsero_3389	tauA	1.44	5.1E-12	1.04	4.6E-07
Hsero_3390	tauA	1.40	6.7E-15	1.19	2.3E-11
Hsero_3452	chrA	1.49	2.6E-11	1.46	6.5E-11
Hsero_3453	chrA	1.45	1.0E-14	1.48	3.3E-15
Hsero_3541	Hsero_3541	1.25	1.1E-06	1.37	8.9E-08
Hsero_3542	Hsero_3542	1.77	1.3E-06	1.39	1.2E-04
Hsero_4460	Hsero_4460	1.17	2.5E-05	1.95	7.0E-12
Hsero_4461	menG	1.07	3.3E-05	2.07	6.5E-15

RNA-Seq library comparisons are as follows: WF2S: wild type after switch to low O_2 x *fnr2* mutant after switch to low O_2; WF2C: wild type under high O_2 x *fnr2* mutant under high O_2.

3.3.9 Supplemental Material

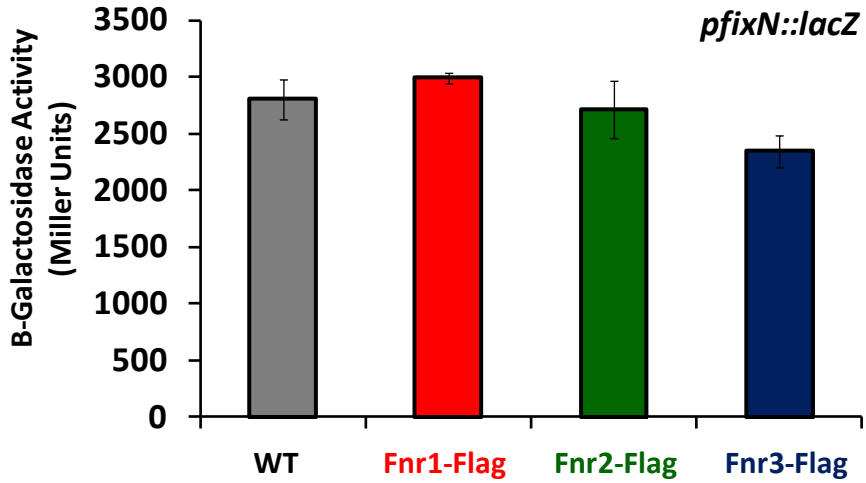

Figure S3.3-1. The activity of the Fnr1-3xFlag and Fnr3-3xFlag proteins is equivalent to the activity of the native proteins. The *pfixN::lacZ* fusion was assayed for β-galactosidase activity in NFbHP-Malate media supplemented with 20 mM of ammonium chloride 4 hours after switch to low oxygen in the *H. seropedicae* wild type (grey bar), Fnr1-3xFlag (red bar), Fnr2-3xFlag (green bar) and Fnr3-3xFlag (blue bar) strains.

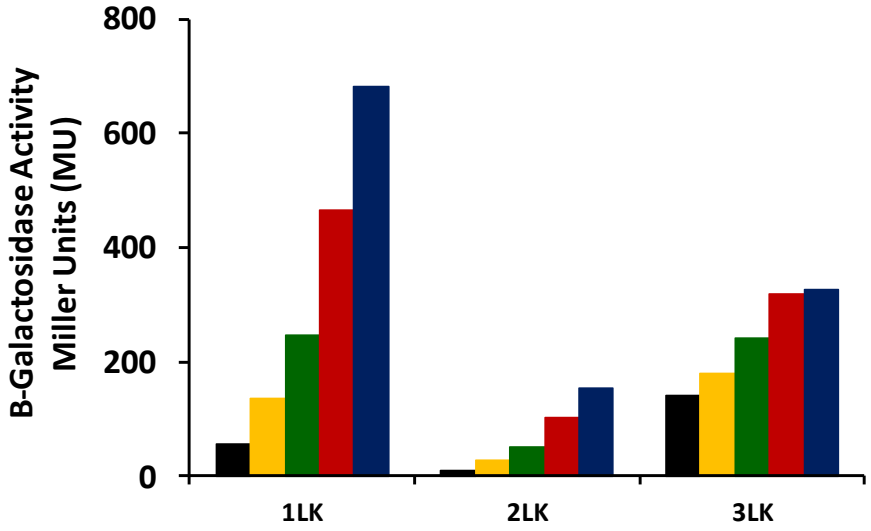

Figure S3.3-2. Expression profile of *fnr1 fnr2* and *fnr3* genes. *H. seropedicae* strains 1LK (chromosomal *fnr1::lacZKm* fusion), 2LK (*fnr2::lacZKm*) and 3LK (*fnr3::lacZKm*) were assayed for β-galactosidase activity in NFbHP-Malate media supplemented with 20 mM of ammonium chloride under high oxygen or 350 rpm (black bars) or after 1 hour of switch to 120 rpm (yellow bars), 2 hours after switch (green bars), 4 hours after switch (red bars) and 6 hours after switch (blue bars). The activity of the promoter less vector (pPW452) was measured in the same conditions and was equivalent to aproximately 150 MU in all the measurement points.

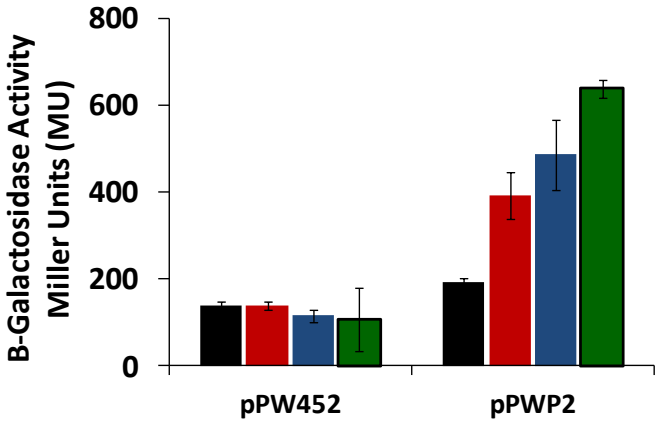

Figure S3.3-3. Expression of the *fnr2* gene is induced in the presence of nitrite or nitrate. The *pfnr2::lacZ* fusion (plasmid pPWP2) was assayed for β-galactosidase activity in NFbHP-Malate media supplemented with 20 mM of ammonium chloride (black bars), 20 mM of ammonium chloride plus 0.25 mM of nitrite (red bars), 10 mM of nitrate (blue bars) or 20 mM of nitrate (green bars). Cells were grown under 120 rpm until an $O.D_{600}$ of 1.2. As a control, the activity of the promoter less vector (pPW452) was measured in the same conditions.

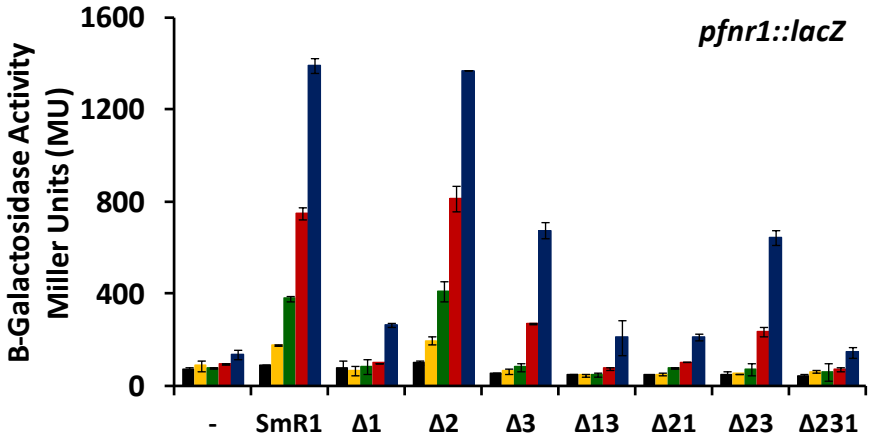

Figure S3.3-4. Expression of the *fnr1* gene is dependent upon Fnr1 and Fnr3. The *pfnr1::lacZ* fusion was assayed for β-galactosidase activity in NFbHP-Malate media supplemented with 20 mM of ammonium chloride under high oxygen (350 rpm, black bars) or after 1 hour of switch to 120 rpm (yellow bars), 2 hours after switch (green bars), 3 hours after switch (red bars) and 4 hours after switch (blue bars) in the wild type and in a series of *fnr* deletion mutants as indicated. As a control (-), the activity of the promoter less vector (pPW452) was measured under the same conditions.

Figure S3.3-5. Amino acid substitutions in Fnr2 that may indicate it is less sensitive to oxygen than *E. coli* Fnr. The leucine to histidine substitution in a position adjacent to the third cysteine predicted to bind the [4Fe-4S]2+ is indicated by the red arrow. The aspartate to methionine substitution within the dimerization helix (shaded in grey) is indicated by green arrow.

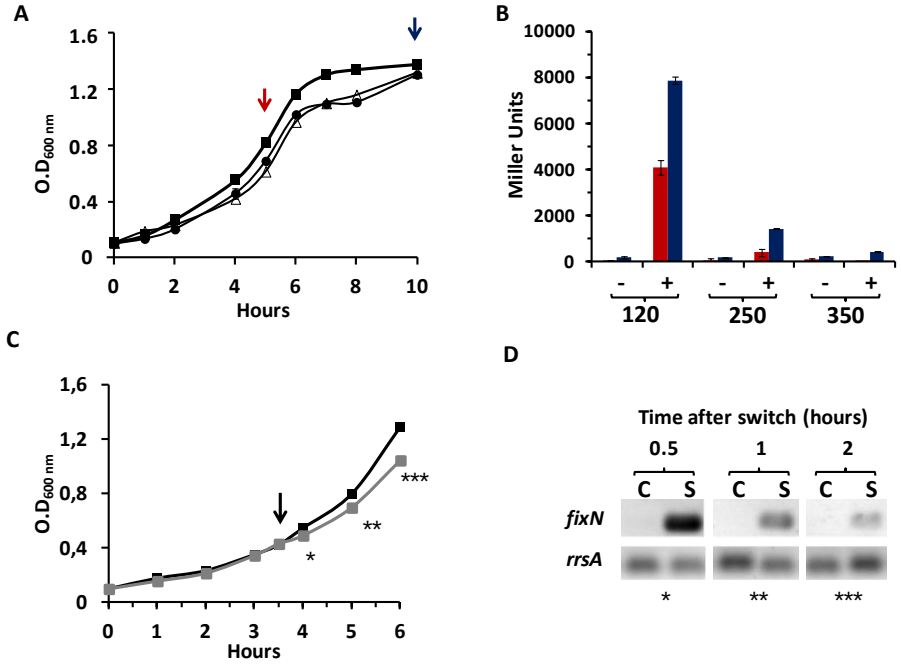

Figure S3.3-6. Fnr1 is activated 30 minutes after the switch from 350 to 120 rpm. Panel **A,** comparison of the growth profile of the *H. seropedicae* SmR1 strain under different aeration regimes. The growth curve was performed under 350 rpm (squares), 250 rpm (open triangles) and 120 rpm (circles). Arrows indicate the time points when β-Galactosidase activity was measured. . Panel **B**, β-Galactosidase activity of the *pfixN::lacZ* fusion (+) under different aeration regimes as indicated. The red bars indicate activity in the exponential phase of growth (red arrow in **A**) while the blue bars indicate the activity in the late stationary phase (blue arrow in **B**). The promoter less vector (-) was as used as a control. Panel **C**, growth profile of the *H. seropedicae* SmR1 strain under 350 rpm (black line) and after the switch from 350 to 120 rpm (grey line). The arrow indicates the time of the switch and the asterisks the points of sample collection for RNA extraction prior to performing RT-PCR. Panel **D**, RT-PCR of an Fnr1 target gene (*fixN*) after different times after the switch as indicated by the asterisks in **C**. The *rrsA* gene, coding for 16S rRNA was used as an endogenous control.

Table S3.3-1 – Genes with Fnr related activation under low oxygen.

		RNA-Seq libraries						ChIP-Seq		
		WTCS		WF1S	WF2S	WF3S	WT/MB231[a]	Fnr1	Fnr2	Fnr3
Locus Tag	**Gene ID**	**log2FC**	**p-value**	**log2FC**	**log2FC**	**log2FC**	**log2FC**	**p-value**	**p-value**	**p-value**
Hsero_0005	Hsero_0005	0.91	1.1E-21	-0.86	-	-1.10	-	2.3E-08	-	2.6E-06
Hsero_0006	Hsero_0006	1.13	4.7E-16	-1.20	-	-1.37	-	-	-	-
Hsero_0007	Hsero_0007	1.31	6.7E-19	-1.30	-	-1.19	-	-	-	-
Hsero_0044	Hsero_0044	2.31	6.5E-05	-2.67	-	-2.83	-	-	-	-
Hsero_0150	rpoE	1.18	1.7E-12	-	-	-1.98	-2.53	-	-	-
Hsero_0151	hemB	1.44	3.7E-32	-	-	-1.70	-2.57	-	-	4.0E-16
Hsero_0153	Hsero_0153	3.24	7.8E-76	-2.37	-	-3.41	-2.37	2.3E-49	-	5.0E-16
Hsero_0154	resB	1.58	9.1E-40	-1.01	-	-1.98	-2.34	-	-	-
Hsero_0155	ccmC	1.25	1.2E-22	-0.96	-	-1.59	-3.62	-	-	-
Hsero_0156	Hsero_0156	0.90	4.4E-10	-0.92	-	-1.55	-	-	-	-
Hsero_0239	phbA2	1.05	3.6E-11	-1.22	-	-1.03	-	4.7E-13	-	-
Hsero_0256	phbA	1.05	1.0E-03	-1.12	-	-	-	-	-	-
Hsero_0257	Hsero_0257	1.28	1.3E-03	-1.40	-	-	-	-	-	-
Hsero_0260	Hsero_0260	1.50	1.0E-22	-1.66	-	-1.70	-3.94	3.3E-10	-	-
Hsero_0261	Hsero_0261	7.27	1.6E-24	-7.18	-	-8.53	-6.19	-	-	-
Hsero_0262	Hsero_0262	0.84	1.5E-12	-0.85	-	-1.07	-	3.3E-10	-	-
Hsero_0263	pta	3.88	2.4E-50	-1.40	-1.14	-4.86	-5.42	-	-	-
Hsero_0264	ackA	4.39	1.5E-69	-1.42	-1.19	-4.48	-5.89	-	-	-
Hsero_0265	phaC	3.62	3.2E-57	-1.56	-1.15	-3.91	-4.91	-	-	-
Hsero_0371	Hsero_0371	0.72	1.7E-07	-0.75	-	-0.74	-	5.7E-21	-	-
Hsero_0440	Hsero_0440	1.00	4.2E-06	-	-	-1.35	-	-	-	-
Hsero_0441	Hsero_0441	1.56	1.3E-12	-	-	-1.74	-	-	-	-
Hsero_0442	Hsero_0442	1.94	1.2E-03	-	-	-2.42	-	-	-	-
Hsero_0443	Hsero_0443	2.35	9.8E-07	-	-	-2.47	-	-	-	-
Hsero_0444	Hsero_0444	1.74	2.7E-12	-	-	-2.51	-	-	-	-
Hsero_0445	dalD	2.17	2.9E-24	-	-	-2.35	-	-	-	-
Hsero_0447	Hsero_0447	1.55	8.2E-34	-	-	-2.09	-	-	-	-
Hsero_0448	era	2.19	2.1E-99	-	-	-2.81	-	-	-	-
Hsero_0474	Hsero_0474	2.55	1.0E-88	-2.68	-	-2.69	-3.12	-	-	-
Hsero_0475	kdtA	2.60	4.0E-88	-2.64	-	-2.66	-3.16	-	-	-
Hsero_0476	galE	1.12	8.1E-20	-1.33	-	-1.25	-	-	6.1E-08	-
Hsero_0514	aerr	0.88	5.2E-08	-0.79	-	-0.89	-2.73	-	-	-
Hsero_0515	Hsero_0515	2.59	3.5E-27	-1.76	-	-2.77	-4.07	-	-	-
Hsero_0624	cheW	0.64	1.5E-04	-0.72	-	-0.70	-3.34	6.0E-05	-	-
Hsero_0626	cheB	2.13	9.2E-46	-	-	-2.40	-2.99	-	-	-
Hsero_0627	Hsero_0627	1.85	8.9E-55	-	-	-2.16	-2.42	-	-	3.5E-42
Hsero_0660	pilA	1.38	6.6E-19	-1.38	-	-1.42	-2.46	7.6E-11	-	-
Hsero_0681	cfa	1.76	1.1E-28	-1.13	-	-2.50	-2.06	-	-	-

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Hsero_0682	pdxH	2.27	3.1E-45	-1.29	-	-2.97	-3.11	-	-	-
Hsero_0855	Hsero_0855	1.34	9.9E-30	-	-	-1.34	-	-	-	-
Hsero_0936	qbdB	1.06	1.3E-04	-1.18	-	-1.28	-	-	-	-
Hsero_0962	Hsero_0962	4.14	4.3E-71	-	-	-6.91	-5.46	-	-	1.2E-75
Hsero_0963	Hsero_0963	2.84	8.9E-34	-	-	-5.36	-3.92	-	-	-
Hsero_0964	adhA	3.05	3.2E-74	-	-	-8.87	-5.02	-	-	1.2E-111
Hsero_0969	Hsero_0969	0.74	1.2E-03	-	-	-1.06	-	-	-	-
Hsero_0998	mdpB	1.36	8.7E-22	-	-	-1.80	-	5.4E-30	-	5.2E-38
Hsero_0999	Hsero_0999	1.71	2.5E-24	-	-	-2.12	-	-	-	-
Hsero_1000	fhaB	1.74	3.1E-19	-	-	-1.96	-	-	-	-
Hsero_1001	fhaC	1.52	5.8E-16	-	-	-1.87	-	-	-	-
Hsero_1013	Hsero_1013	2.67	2.9E-26	-	-1.45	-3.38	-	-	-	-
Hsero_1014	Hsero_1014	2.29	1.1E-33	-	-1.22	-2.63	-	-	-	-
Hsero_1024	deoR	1.17	7.8E-17	-	-	-1.40	-	-	-	2.3E-06
Hsero_1103	Hsero_1103	0.67	1.8E-04	-	-	-0.61	-	-	-	2.5E-18
Hsero_1104	Hsero_1104	3.73	1.5E-122	-1.47	-1.00	-4.08	-2.07	4.9E-32	-	2.5E-18
Hsero_1105	tktC	2.80	3.4E-35	-1.08	-1.30	-3.18	-	-	-	-
Hsero_1106	tktB	1.96	1.6E-06	-	-1.06	-2.41	-	-	-	-
Hsero_1175	Hsero_1175	1.03	9.7E-24	-	-	-1.38	-	9.6E-29	-	2.5E-82
Hsero_1176	Hsero_1176	1.05	1.5E-19	-	-	-1.07	-	-	-	-
Hsero_1180	Hsero_1180	0.80	1.0E-10	-	-	-1.22	-3.97	-	-	-
Hsero_1246	Hsero_1246	0.62	7.6E-08	-0.63	-	-0.56	-2.72	-	-	-
Hsero_1250	Hsero_1250	4.44	1.7E-161	-1.49	-	-6.32	-5.03	7.8E-05	-	-
Hsero_1251	cheD	2.03	7.3E-38	-	-1.27	-2.53	-	-	-	-
Hsero_1383	hflB	0.36	4.7E-05	-0.24	-	-0.49	-	5.2E-18	-	1.4E-26
Hsero_1446	Hsero_1446	1.60	1.7E-32	-1.43	-	-1.68	-	6.2E-06	-	-
Hsero_1528	cirA	1.14	2.3E-19	-	-	-1.40	-	-	-	-
Hsero_1529	Hsero_1529	1.15	3.0E-13	-	-	-1.48	-	-	-	-
Hsero_1530	Hsero_1530	0.92	9.9E-04	-	-	-1.17	-	-	-	-
Hsero_1531	Hsero_1531	0.87	1.1E-03	-	-	-1.06	-	-	-	-
Hsero_1532	Hsero_1532	1.01	1.5E-12	-	-	-1.28	-	-	-	-
Hsero_1533	Hsero_1533	0.93	6.1E-03	-	-	-1.18	-	-	-	-
Hsero_1545	acs	1.69	5.6E-28	-	-	-2.04	-	7.0E-15	-	8.3E-44
Hsero_1552	Hsero_1552	3.15	5.7E-06	-3.75	-	-4.16	-2.70	7.5E-05	-	-
Hsero_1553	Hsero_1553	1.57	1.8E-15	-1.52	-	-1.96	-2.84	-	-	-
Hsero_1581	Hsero_1581	1.58	3.2E-03	-	-	-1.72	-	-	-	-
Hsero_1639	Hsero_1639	0.91	3.3E-23	-	-	-1.09	-2.69	-	-	4.8E-14
Hsero_1693	Hsero_1693	1.82	4.4E-42	-	-	-2.68	-	-	-	-
Hsero_1694	Hsero_1694	2.05	6.8E-93	-	-	-2.85	-	-	-	1.7E-73
Hsero_1710	Hsero_1710	0.51	4.7E-07	-	-	-0.67	-	-	-	5.9E-91
Hsero_1744	Hsero_1744	0.98	8.7E-18	-	-	-1.24	-	-	-	-
Hsero_1829	Hsero_1829	4.90	7.1E-74	-1.39	-1.05	-6.74	-5.20	1.6E-18	-	2.0E-22
Hsero_1830	Hsero_1830	5.01	7.6E-25	-1.51	-1.25	-6.36	-5.25	-	-	-

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Hsero_1831	yhiH	5.27	1.2E-37	-1.51	-1.11	-7.20	-5.35	-	-	-
Hsero_1832	yhhJ	4.49	1.8E-47	-1.65	-	-5.44	-5.73	-	-	-
Hsero_1880	Hsero_1880	0.86	2.3E-13	-0.97	-	-1.09	-	1.3E-12	-	-
Hsero_1880	Hsero_1880	0.86	2.3E-13	-0.97	-	-1.09	-	-	-	-
Hsero_1881	Hsero_1881	0.96	1.2E-14	-0.85	-	-1.00	-	-	-	-
Hsero_1896	exaC	1.83	1.5E-08	-	-	-	-	-	-	-
Hsero_1957	Hsero_1957	1.64	3.3E-25	-1.06	-	-1.55	-	1.4E-15	-	-
Hsero_1976	cheY	0.70	1.0E-13	-0.44	-	-0.52	-	2.5E-11	-	-
Hsero_2110	clpB	2.00	2.7E-30	-1.37	-	-2.28	-2.80	6.2E-30	-	4.0E-40
Hsero_2144	Hsero_2144	0.38	1.3E-03	-0.76	-	-0.65	-	5.6E-07	-	-
Hsero_2263	Hsero_2263	1.78	2.7E-18	-	-	-1.96	-2.38	-	-	2.9E-29
Hsero_2290	uspA	8.78	2.3E-63	-6.39	-	-9.64	-6.11	4.1E-73	-	9.4E-24
Hsero_2375	Hsero_2375	2.40	9.4E-05	-1.55	-	-	-	-	-	-
Hsero_2379	Hsero_2379	3.40	7.8E-35	-2.67	-1.04	-3.42	-4.66	1.3E-06	-	-
Hsero_2383	Hsero_2383	2.05	2.3E-08	-1.30	-	-2.96	-2.81	-	-	-
Hsero_2384	Hsero_2384	3.23	9.7E-20	-2.09	-	-3.19	-3.69	1.5E-11	-	-
Hsero_2398	Hsero_2398	1.85	7.9E-03	-2.74	-	-2.56	-6.09	-	-	-
Hsero_2399	Hsero_2399	3.40	5.1E-06	-3.36	-	-3.37	-5.26	-	-	-
Hsero_2400	Hsero_2400	4.05	1.6E-06	-4.47	-	-2.71	-6.67	-	-	-
Hsero_2401	Hsero_2401	1.87	3.0E-06	-2.03	-	-1.92	-	-	-	-
Hsero_2402	Hsero_2402	2.56	2.0E-10	-2.46	-	-2.83	-4.87	-	-	-
Hsero_2403	Hsero_2403	2.58	3.6E-04	-1.60	-	-2.57	-5.29	2.9E-08	-	-
Hsero_2404	dksA	8.35	9.1E-33	-4.60	-	-6.43	-6.64	2.9E-08	-	-
Hsero_2405	phbC	4.95	2.9E-34	-3.87	-1.45	-5.65	-6.14	-	-	-
Hsero_2406	fabI	5.78	5.9E-16	-4.84	-1.69	-5.88	-7.03	-	-	-
Hsero_2407	Hsero_2407	4.33	5.7E-23	-3.80	-1.39	-4.48	-6.86	-	-	-
Hsero_2437	cybB	1.63	8.5E-23	-1.63	-	-1.93	-3.97	-	-	-
Hsero_2438	Hsero_2438	2.01	3.4E-29	-1.54	-	-1.85	-	-	-	-
Hsero_2488	uspA	4.54	2.6E-74	-1.62	-1.23	-6.05	-5.99	4.2E-30	-	1.3E-73
Hsero_2489	Hsero_2489	1.41	5.6E-15	-1.09	-1.17	-2.20	-	-	-	-
Hsero_2508	Hsero_2508	1.99	2.7E-07	-	-	-2.50	-	-	-	-
Hsero_2509	Hsero_2509	1.40	1.2E-03	-	-	-2.10	-	-	-	-
Hsero_2510	Hsero_2510	3.93	3.1E-94	-	-	-6.07	-6.75	3.9E-41	-	2.4E-36
Hsero_2511	ldhA	3.66	6.5E-140	-	-	-5.36	-5.27	-	-	6.9E-15
Hsero_2530	Hsero_2530	2.91	2.7E-64	-	-	-3.46	-2.41	7.7E-07	-	5.1E-24
Hsero_2531	Hsero_2531	0.88	4.2E-03	-	-	-1.41	-	-	-	-
Hsero_2544	Hsero_2544	1.67	1.4E-08	-	-	-2.23	-	-	-	-
Hsero_2717	Hsero_2717	1.66	1.2E-55	-	-	-1.74	-	-	-	-
Hsero_2718	phnA	2.10	3.2E-46	-	-	-1.80	-2.40	-	-	-
Hsero_2909	Hsero_2909	1.45	4.7E-26	-1.25	-	-1.46	-	1.9E-30	-	-
Hsero_2913	Hsero_2913	1.22	8.1E-25	-	-	-1.19	-	-	-	-
Hsero_2914	Hsero_2914	4.51	3.7E-117	-	-1.43	-5.57	-4.90	-	-	-
Hsero_2915	Hsero_2915	4.65	5.3E-109	-	-1.36	-6.53	-5.16	1.1E-23	-	3.9E-66

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Hsero_2983	cheY	0.42	6.0E-05	-	-	-0.46	-	-	-	7.2E-27
Hsero_2999	phbC	0.45	2.9E-04	-0.63	-	-0.70	-1.36	1.2E-39	-	-
Hsero_3044	Hsero_3044	1.42	1.4E-03	-1.18	-	-1.51	-	-	-	-
Hsero_3055	Hsero_3055	2.84	1.0E-57	-	-	-3.32	-	-	-	8.7E-143
Hsero_3056	Hsero_3056	2.79	1.6E-22	-	-	-3.85	-	-	-	-
Hsero_3057	cydA	3.62	4.7E-130	-	-1.01	-4.13	-	-	-	-
Hsero_3058	cydB	2.45	3.5E-65	-	-	-3.67	-	-	-	-
Hsero_3067	mmsA	1.12	5.7E-04	-	-	-1.24	-	-	-	-
Hsero_3072	era	4.22	9.4E-16	-4.15	-1.40	-4.04	-4.32	5.9E-17	-	-
Hsero_3111	Hsero_3111	1.15	4.8E-11	-1.68	-	-1.94	-	3.2E-07	-	-
Hsero_3117	prpE	1.61	1.1E-19	-	-	-1.56	-	-	-	-
Hsero_3146	Hsero_3146	0.56	1.8E-08	-	-	-0.80	-	-	-	6.8E-34
Hsero_3148	Hsero_3148	1.24	3.8E-30	-	-	-1.26	-	5.4E-36	-	3.0E-38
Hsero_3164	Hsero_3164	1.27	2.2E-25	-	-	-1.30	-	-	-	-
Hsero_3165	Hsero_3165	1.11	2.6E-15	-	-	-1.32	-	-	-	-
Hsero_3166	Hsero_3166	1.32	1.4E-24	-	-	-1.49	-	-	-	-
Hsero_3167	Hsero_3167	1.17	6.9E-12	-	-	-1.14	-	-	-	-
Hsero_3168	Hsero_3168	0.84	4.7E-05	-	-	-1.40	3.37	-	-	-
Hsero_3169	Hsero_3169	1.11	1.4E-13	-	-	-1.17	3.04	-	-	-
Hsero_3190	Hsero_3190	1.24	1.6E-12	-1.27	-	-1.09	-	-	-	-
Hsero_3194	ompW1	8.60	3.8E-73	-8.96	-2.88	-9.79	-7.09	1.3E-36	-	-
Hsero_3195	Hsero_3195	4.58	2.3E-97	-3.95	-2.45	-4.33	-6.92	-	-	-
Hsero_3197	fnr1	3.12	6.7E-55	-	-	-6.49	-5.51	1.4E-65	-	1.4E-94
Hsero_3198	Hsero_3198	2.45	7.7E-50	-	-	-2.99	-3.33	-	-	-
Hsero_3199	Hsero_3199	6.94	7.9E-62	-3.82	-	-7.75	-6.17	-	-	-
Hsero_3200	fixG	7.24	9.2E-103	-5.31	-	-8.14	-6.33	-	-	-
Hsero_3201	fixP	7.99	1.4E-88	-6.57	-1.16	-8.74	-5.41	-	-	-
Hsero_3202	fixO	8.51	2.0E-79	-6.78	-1.20	-10.00	-6.22	-	-	-
Hsero_3203	fixN	8.64	1.9E-170	-6.57	-1.13	-9.55	-6.36	1.4E-59	-	-
Hsero_3204	fixS	1.45	8.6E-06	-1.56	-	-2.10	-6.14	-	-	-
Hsero_3205	fixI	5.15	7.5E-73	-4.76	-	-5.59	-4.23	-	-	-
Hsero_3206	Hsero_3206	6.95	4.6E-38	-5.01	-	-7.37	-4.44	-	-	-
Hsero_3207	hemN	5.94	3.8E-71	-4.78	-	-7.42	-4.17	5.9E-39	-	-
Hsero_3223	Hsero_3223	1.48	2.9E-17	-	-	-1.68	-2.17	-	-	1.1E-77
Hsero_3246	Hsero_3246	4.11	1.0E-04	-5.57	-	-2.71	-	2.3E-05	-	-
Hsero_3256	Hsero_3256	1.97	9.5E-11	-2.06	-	-2.03	-4.19	-	-	-
Hsero_3273	Hsero_3273	1.17	8.2E-23	-1.01	-	-1.31	-2.98	1.0E-10	-	-
Hsero_3370	ardA	1.57	1.2E-18	-	-	-2.25	-2.74	-	-	8.5E-47
Hsero_3434	Hsero_3434	1.26	1.9E-14	-	-	-1.42	-	-	-	-
Hsero_3471	Hsero_3471	1.75	8.9E-35	-	-	-1.55	-	-	-	-
Hsero_3472	Hsero_3472	1.83	7.3E-45	-	-	-1.83	-	-	-	4.2E-78
Hsero_3473	spoVK	1.48	1.9E-65	-	-	-1.24	-2.20	-	-	-
Hsero_3488	tsr	1.38	6.4E-12	-1.22	-	-1.34	-	5.7E-23	-	-

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Hsero_3532	Hsero_3532	3.57	7.0E-40	-1.76	-	-3.80	-	0.0E+00	-	-
Hsero_3572	Hsero_3572	1.91	1.0E-98	-	-	-2.34	-2.19	-	-	-
Hsero_3575	Hsero_3575	0.69	1.7E-05	-0.43	-	-0.53	-1.92	-	-	-
Hsero_3645	narX	3.83	4.4E-94	-	-1.24	-6.28	-7.11	-	-	1.2E-13
Hsero_3646	narL	4.41	4.1E-20	-	-1.31	-6.30	-6.08	-	-	-
Hsero_3732	Hsero_3732	1.54	3.0E-08	-1.77	-	-2.02	-	-	-	-
Hsero_3852	ubiF	0.99	2.5E-15	-1.07	-	-1.12	-2.61	-	-	-
Hsero_3853	pyrC	0.62	2.5E-12	-0.56	-	-0.72	-2.32	2.4E-23	-	-
Hsero_3887	uspA	3.57	4.6E-49	-1.53	-	-4.42	-4.33	4.1E-13	-	-
Hsero_3965	Hsero_3965	0.84	4.7E-10	-0.87	-	-1.11	-	5.8E-10	-	-
Hsero_3973	rpoN	0.87	2.1E-13	-1.04	-	-0.88	-	-	-	-
Hsero_3974	yhbH	1.04	4.4E-09	-1.29	-	-1.07	-	4.3E-19	-	-
Hsero_4003	pcaR	1.27	2.1E-25	-	-	-1.64	-	-	-	-
Hsero_4004	ompC	0.84	5.8E-04	-	-	-1.25	-	-	-	-
Hsero_4047	petC	2.64	1.6E-87	-2.56	-	-3.03	-2.48	-	-	-
Hsero_4048	petB	4.10	2.4E-121	-3.29	-	-4.61	-3.52	-	-	-
Hsero_4049	petA	3.99	1.1E-155	-3.22	-	-4.33	-2.78	3.4E-63	-	6.0E-19
Hsero_4189	dsbC	0.90	2.4E-16	-0.80	-	-0.98	-3.22	-	-	-
Hsero_4190	Hsero_4190	1.69	6.4E-43	-1.31	-	-1.95	-3.27	2.7E-10	-	2.9E-06
Hsero_4240	Hsero_4240	0.98	1.1E-14	-1.01	-	-1.25	-2.54	2.3E-31	-	-
Hsero_4241	Hsero_4241	0.99	1.4E-14	-0.80	-	-1.08	-1.88	-	-	-
Hsero_4274	hemA	0.96	5.7E-27	-	-	-1.54	-	-	-	5.5E-67
Hsero_4275	prfA	0.95	1.6E-21	-	-	-1.15	-	-	-	-
Hsero_4279	Hsero_4279	0.73	1.1E-03	-0.76	-	-1.15	-	-	-	-
Hsero_4282	cca	0.57	4.7E-05	-0.63	-	-0.41	-1.56	-	-	-
Hsero_4283	gst	0.53	6.5E-05	-0.65	-	-0.53	-1.64	-	-	-
Hsero_4284	Hsero_4284	0.74	6.0E-10	-0.89	-	-0.84	-1.64	-	-	-
Hsero_4356	priA	1.23	6.5E-23	-	-	-1.50	-	-	-	-
Hsero_4357	hemE	1.37	4.4E-34	-	-	-1.51	-	-	-	-
Hsero_4358	pheC	1.16	1.2E-13	-	-	-1.24	-	-	-	-
Hsero_4359	Hsero_4359	1.55	7.5E-24	-	-	-1.99	-	-	-	-
Hsero_4360	Hsero_4360	1.35	9.7E-12	-	-	-1.93	-	-	-	-
Hsero_4387	Hsero_4387	2.81	7.1E-73	-3.05	-	-3.21	-	-	-	-
Hsero_4440	groES	0.58	6.7E-03	-0.98	-	-1.23	-	-	-	-
Hsero_4673	Hsero_4673	0.79	7.8E-11	-0.51	-	-0.89	-1.75	-	-	-
Hsero_4703	crp	1.22	6.5E-12	-1.18	-	-1.66	-2.50	7.3E-06	-	-
Hsero_4704	Hsero_4704	0.98	7.6E-14	-	-	-1.49	-3.15	-	-	5.2E-09
Hsero_4710	Hsero_4710	0.68	1.7E-05	-	-	-1.04	-	-	-	-
Hsero_4762	Hsero_4762	0.99	2.4E-16	-1.04	-	-1.13	-	-	-	-

RNA-Seq library comparisons are as follows: WTCS: wild type before switch or high O_2 x wild type after switch to low O_2; WF1S: wild type after switch to low O_2 x *fnr1* mutant after switch to low O_2; WF2S: wild type after switch to low O_2 x *fnr2* mutant after switch to low O_2; WF3S: wild type after switch to low O_2 x *fnr3* mutant after switch to low O_2;[a]WT/MB231: wild

type under low O_2 x triple *fnr* mutant after under low O_2 (BATISTA et al., 2013). When present, the p-value for a ChIP-seq peak identified is given for a defined Fnr protein. The hyphen (-) indicates no diferential expression in the RNA-Seq or absence of a ChIP-Seq peak.

Table S3.3-2 – Genes with Fnr related repression under low O2

		RNA-Seq libraries						Chip-Seq		
		WTCS		WF1S	WF2S	WF3S	WT/MB231[a]	Fnr1	Fnr2	Fnr3
Locus Tag	**Gene ID**	**log2FC**	**p-value**	**log2FC**	**log2FC**	**log2FC**	**log2FC**	**p-value**	**p-value**	**p-value**
Hsero_0835	Hsero_0835	-1.24	1.3E-09	1.08	-	1.34	-	4.2E-11	-	-
Hsero_1165	sodB	-1.34	1.7E-26	1.16	-	1.31	-	4.3E-05	-	-
Hsero_1337	Hsero_1337	-1.91	4.6E-26	1.73	1.12	1.58	2.94	7.5E-05	-	-
Hsero_1343	Hsero_1343	-1.37	6.6E-19	1.42	-	1.62	-	-	-	-
Hsero_1474	Hsero_1474	-1.26	2.9E-24	1.37	-	1.45	-	-	-	-
Hsero_1604	zntA	-1.28	5.0E-18	-	-	1.39	-	-	-	-
Hsero_1670	Hsero_1670	-1.32	3.9E-18	1.02	-	1.21	-	-	-	-
Hsero_1671	Hsero_1671	-1.26	1.1E-19	-	-	1.27	-	-	-	-
Hsero_1672	Hsero_1672	-1.44	1.2E-12	1.14	-	1.47	-	-	-	-
Hsero_1698	Hsero_1698	-0.94	1.3E-06	-	-	3.04	4.96	-	-	-
Hsero_1708	Hsero_1708	-1.22	9.9E-05	-	-	3.04	3.37	-	-	-
Hsero_1756	ndh	-1.33	3.6E-17	1.31	-	1.48	2.43	-	-	-
Hsero_2458	Hsero_2458	-1.40	6.4E-23	1.02	-	1.16	3.04	5.8E-14	-	-
Hsero_2672	Hsero_2672	-1.97	5.5E-05	-	-	2.45	-	-	-	-
Hsero_2723	Hsero_2723	-1.40	5.2E-32	-	1.02	1.16	-	-	-	-
Hsero_2724	Hsero_2724	-1.55	1.4E-30	-	1.03	1.30	-	-	-	-
Hsero_3188	Hsero_3188	-0.92	5.1E-06	1.00	-	1.02	-	-	-	-
Hsero_3228	Hsero_3228	-1.53	1.2E-15	-	1.14	1.52	-	-	-	-
Hsero_3327	argM	-0.26	5.3E-04	0.67	-	0.69	-	-	-	7.0E-06
Hsero_3372	mgtA	-0.84	4.1E-11	2.81	-	3.29	-	-	-	-
Hsero_3845	Hsero_3845	-1.34	2.3E-44	-	-	1.05	-	-	-	-
Hsero_3892	Hsero_3892	-0.62	9.2E-09	1.27	-	1.32	-	-	-	-
Hsero_4225	mqo	-1.41	1.1E-16	1.28	1.04	1.41	2.75	-	-	-
Hsero_4759	Hsero_4759	-0.90	4.2E-09	-	-	1.14	-	-	-	-
Hsero_4803	Hsero_4803	-1.44	1.8E-28	-	1.17	1.36	-	-	-	-

RNA-Seq library comparisons are as follows: WTCS: wild type before switch or high O_2 x wild type after switch to low O_2; WF1S: wild type after switch to low O_2 x *fnr1* mutant after switch to low O_2; WF2S: wild type after switch to low O_2 x *fnr2* mutant after switch to low O_2; WF3S: wild type after switch to low O_2 x *fnr3* mutant after switch to low O_2;[a]WT/MB231: wild type under low O_2 x triple *fnr* mutant after under low O_2 (BATISTA et al., 2013). When present, the p-value for a ChIP-seq peak identified is given for a defined Fnr protein. The hyphen (-) indicates no diferential expression in the RNA-Seq or absence of a ChIP-Seq peak.

Table S3.3-3 – Genes with Fnr independent repression under low O_2.

		RNA-Seq libraries						Chip-Seq		
		WTCS		WF1S	WF2S	WF3S	WT/MB231[a]	Fnr1	Fnr2	Fnr3
Locus Tag	**Gene ID**	**log2FC**	**p-value**	**log2FC**	**log2FC**	**log2FC**	**log2FC**	**p-value**	**p-value**	**p-value**
Hsero_1202	Hsero_1202	-1.66	6.41E-06	-	-	-	-	-	-	-
Hsero_1344	ompC	-1.05	2.32E-05	-	-	-	-	-	-	-
Hsero_2497	pgi	-1.21	7.25E-07	-	-	-	-	-	-	-
Hsero_2506	Hsero_2506	-1.08	2.77E-11	-	-	-	-	-	-	-
Hsero_3060	osmC	-1.32	4.84E-22	-	-	-	-	-	-	-
Hsero_3061	marR	-1.42	1.90E-26	-	-	-	-	-	-	-
Hsero_3136	Hsero_3136	-1.24	4.41E-26	-	-	-	-	-	-	-
Hsero_3137	Hsero_3137	-1.00	7.58E-14	-	-	-	-	-	-	-
Hsero_3138	fdx	-1.03	5.86E-10	-	-	-	-	-	-	-
Hsero_3139	Hsero_3139	-1.11	7.81E-25	-	-	-	-	-	-	-
Hsero_3140	rhtB	-1.30	2.48E-19	-	-	-	-	-	-	-
Hsero_3141	Hsero_3141	-1.02	1.40E-17	-	-	-	-	-	-	-
Hsero_3142	Hsero_3142	-1.07	4.86E-20	-	-	-	-	-	-	-
Hsero_3143	iscU	-1.09	8.21E-20	-	-	-	-	-	-	-
Hsero_3144	iscS1	-1.17	8.76E-33	-	-	-	-	-	-	-
Hsero_3145	iscR	-1.07	1.74E-24	-	-	-	-	-	-	-
Hsero_3341	Hsero_3341	-1.03	9.45E-23	-	-	-	-	-	-	-
Hsero_3342	betA	-1.13	2.26E-20	-	-	-	-	-	-	-
Hsero_3343	Hsero_3343	-1.02	9.64E-14	-	-	-	-	-	-	-
Hsero_3344	Hsero_3344	-1.05	3.31E-20	-	-	-	-	-	-	-
Hsero_3768	pcaR	-1.04	3.46E-13	-	-	-	-	-	-	-

RNA-Seq library comparisons are as follows: WTCS: wild type before switch or high O_2 x wild type after switch to low O_2; WF1S: wild type after switch to low O_2 x *fnr1* mutant after switch to low O_2; WF2S: wild type after switch to low O_2 x *fnr2* mutant after switch to low O_2; WF3S: wild type after switch to low O_2 x *fnr3* mutant after switch to low O_2;[a]WT/MB231: wild type under low O_2 x triple *fnr* mutant after under low O_2 (BATISTA et al., 2013). The hyphen (-) indicates no diferential expression in the RNA-Seq or absence of a ChIP-Seq peak.

Table S3.3-4 – Genes with an upstrean Fnr1 ChIP-Seq peak but with no apprarent regulation in the current RNA-Seq analysis.

		RNA-Seq						ChIP-Seq	
		WF1S		WTCS		WT/MB231[a]			Fnr1
Locus Tag	**Gene ID**	**log2FC**	**p-value**	**log2FC**	**p-value**	**log2FC**	**p-value**	**Peak**	**p-value**
Hsero_0457	Hsero_0457	-	-	-	-	-	-	+	8.60E-16
Hsero_1171	adk	-	-	-	-	-	-	+	4.63E-20
Hsero_1221	Hsero_1221	-	-	-	-	-	-	+	1.23E-12
Hsero_2279	Hsero_2279	-	-	-	-	-	-	+	8.39E-12
Hsero_2280	Hsero_2280	-	-	-	-	-	-	+	8.39E-12
Hsero_2381	fnr2	-	-	-	-	-4.48	0.055568	+	8.65E-07
Hsero_2519	Hsero_2519	-	-	-	-	-	-	+	9.97E-06
Hsero_2520	eutR	-	-	-	-	-	-	+	9.97E-06
Hsero_2667	Hsero_2667	-	-	-	-	-	-	+	2.08E-12
Hsero_3571	dnaE	-	-	-	-	-	-	+	4.46E-13
Hsero_3790	Hsero_3790	-	-	-	-	-2.89	0.0008007	+	3.91E-09
Hsero_3791	oxyR	-	-	-	-	-1.82	0.0177873	+	3.91E-09
Hsero_3953	Hsero_3953	-	-	-	-	-	-	+	1.16E-34
Hsero_3954	Hsero_3954	-	-	-	-	-	-	+	1.16E-34
Hsero_4385	Hsero_4385	-	-	-	-	-	-	+	4.47E-58

RNA-Seq library comparisons are as follows: WF1S: wild type after switch to low O_2 x *fnr1* mutant after switch to low O_2; WTCS: wild type before switch or high O_2 x wild type after switch to low O_2; [a]WT/MB231: wild type under low O_2 x triple *fnr* mutant after under low O_2 (BATISTA et al., 2013). The symbol (+) indicates the presence of an Fnr1 ChIP-seq peak followed by its respective p-value. The hyphen (-) indicates no diferential expression in the RNA-Seq library.

Table S3.3-5 - Genes with an upstrean Fnr3 ChIP-Seq peak but with no regulation in any of the RNA-Seq analysis.

		RNA-Seq						ChIP-Seq	
		WF3S		WTCS		WT/MB231[a]		Fnr3	Fnr3
Locus Tag	**Gene ID**	**log2FC**	**p-value**	**log2FC**	**p-value**	**log2FC**	**p-value**	**Peak**	**p-value**
Hsero_0981	Hsero_0981	-	-	-	-	-	-	+	6.24E-06
Hsero_0982	cysM	-	-	-	-	-	-	+	6.24E-06
Hsero_1502	Hsero_1502	-	-	-	-	-	-	+	9.34E-15
Hsero_1783	etf	-	-	-	-	-	-	+	5.16E-11
Hsero_1784	Hsero_1784	-	-	-	-	-	-	+	5.16E-11
Hsero_2560	Hsero_2560	-	-	-	-	-	-	+	1.03E-05
Hsero_2956	ndk	-	-	-	-	-	-	+	7.27E-07
Hsero_2957	Hsero_2957	-	-	-	-	-	-	+	7.27E-07
Hsero_3160	Hsero_3160	-	-	-	-	-	-	+	7.45E-10
Hsero_3162	Hsero_3162	-	-	-	-	-	-	+	7.45E-10
Hsero_3742	Hsero_3742	-	-	-	-	-	-	+	8.27E-06
Hsero_3743	Hsero_3743	-	-	-	-	-	-	+	8.27E-06
Hsero_4162	Hsero_4162	-	-	-	-	-	-	+	9.50E-11

RNA-Seq library comparisons are as follows: WF3S: wild type after switch to low O_2 x *fnr3* mutant after switch to low O_2; WTCS: wild type before switch or high O_2 x wild type after switch to low O_2; [a]WT/MB231: wild type under low O_2 x triple *fnr* mutant after under low O_2 (BATISTA et al., 2013). The symbol (+) indicates the presence of an Fnr3 ChIP-seq peak followed by its respective p-value. The hyphen (-) indicates no diferential expression in the RNA-Seq library.

Table S3.3-6 – Genes that were not associated with an upstream ChIP-Seq peak, but were predict to have a motif for Fnr binding by *in silico* motif search, and also were differentially expressed in response to low O_2 and in an *fnr* mutant.

	in silico motif prediction					RNA-Seq				
Locus Tag	**Start**	**Stop**	**Motif Sequence**	**Score**	**p-value**	**WTCS**	**WF1S**	**WF2S**	**WF3S**	**WT/MB231[a]**
Hsero_0474	29	42	TTGAAGCAGATCAA	15.85	1.83E-06	2.55	-2.68	-	-2.69	-3.12
Hsero_0514	42	55	TTGATGCCGGGAAA	10.43	7.95E-05	0.88	-0.79	-	-0.89	-2.73
Hsero_0515	72	85	CTGTTCCAGATCAA	13.95	8.26E-06	2.59	-1.76	-	-2.77	-4.07
Hsero_1344	176	189	TTGCGTCAGATCAA	10.95	5.88E-05	-1.05	-	-	-	-
Hsero_1670	115	128	CTGCTGCAGATCAA	11.81	3.51E-05	-1.32	1.02	-	1.21	-
Hsero_1756	176	189	TTGAGGGACATCAA	15.35	2.82E-06	-1.33	1.31	-	1.48	2.43
Hsero_2375	146	159	TTGATATAAATCAG	17.38	4.55E-07	2.40	-1.55	-	-	-
Hsero_2399	45	58	TTGATGCTCCTCAG	11.38	4.58E-05	3.40	-3.36	-	-3.37	-5.26
Hsero_2400	148	161	CTGATGTGGATCAA	15.56	2.43E-06	4.05	-4.47	-	-2.71	-6.67
Hsero_2406	113	126	GTGACCTGGCTCAA	11.73	3.70E-05	5.78	-4.84	-1.69	-5.88	-7.03
Hsero_3117	110	123	TTGACTTGCATCAA	15.99	1.62E-06	1.61	-	-	-1.56	-
Hsero_3256	11	24	TTGATCTGCCTCAG	15.83	1.88E-06	1.97	-2.06	-	-2.03	-4.19
Hsero_3473	153	166	TTTCTAAACAACAA	10.16	9.27E-05	1.48	-	-	-1.24	-2.20
Hsero_3575	186	199	ATGATCGACCGCAA	11.17	5.17E-05	0.69	-0.43	-	-0.53	-1.92
Hsero_3845	40	53	TTGAGGCGGATCAA	14.68	4.77E-06	-1.34	-	-	1.05	-
Hsero_3852	128	141	TTGATCACGCGCAG	11.27	4.88E-05	0.99	-1.07	-	-1.12	-2.61
Hsero_4189	171	184	CTGATTCAAATCAA	12.72	1.96E-05	0.90	-0.80	-	-0.98	-3.22
Hsero_4673	21	34	CTGATGCAAGTCAA	12.47	2.32E-05	0.79	-0.51	-	-0.89	-1.75

The motifs were predicted as described in materials and methods. The localization of the motifs in relation to the inital codon is showed as well as the matched sequence. The score and the p-value for each motif given by MEME are also showed. RNA-Seq library comparisons are as follows: WF3S: wild type after switch to low O_2 x *fnr3* mutant after switch to low O_2; WTCS: wild type before switch or high O_2 x wild type after switch to low O_2; [a]WT/MB231: wild type under low O_2 x triple *fnr* mutant after under low O_2 (BATISTA et al., 2013). The symbol (+) indicates the presence of an Fnr3 ChIP-seq peak followed by its respective p-value. The hyphen (-) indicates no diferential expression in the RNA-Seq library.

4 CONSIDERAÇÕES FINAIS

A presença de oxidases respiratórias alternativas com diferentes afinidades pelo oxigênio é amplamente distribuída em bactérias. Estas oxidases dão às bactérias uma grande flexibilidade respiratória, permitindo que possam rapidamente adaptar-se às condições prevalentes de oxigênio em seu ambiente para que a produção de ATP pela cadeia transportadora de elétrons aeróbia não seja prejudicada. Para assegurar uma adaptação eficiente, as bactérias precisaram desenvolver estratégias para monitorar os níveis de O_2 e transmitir esse sinal para o aparato transcricional afim de que as proteínas necessárias sejam sintetizadas em resposta aos diferentes níveis de oxigênio. Duas principais estratégias para o sensoriamento dos níveis de oxigênio são baseadas na habilidade de algumas proteínas reguladoras transcricionais em ligar um grupo Fe-S ou um cofator do tipo heme (revisado em GREEN et al., 2009). Os sensores que contém um grupo Fe-S exploram a capacidade do O_2 em oxidá-lo resultando na desmontagem do grupo e inativação da proteína. Já os sensores baseados no cofator heme, exploram a capacidade do átomo de Fe presente no grupamento heme de ligar a molécula de O_2 e comutar a proteína reguladora entre seu estado ativo ou inativo.

H. seropedicae possui três genes homólogos ao gene *fnr* de *E.coli.* A proteína Fnr é um regulador transcricional que contém um grupamento $[4Fe\text{-}4S]^{2+}$, sensível ao O_2. Em *E. coli,* Fnr é responsável por controlar a adaptação de ao baixo O_2. A ocorrência de 3 homólogos de Fnr em *H. seropedicae* coloca a intrigante questão sobre o papel das proteínas Fnr1, Fnr2 e Fnr3 de *H. seropedicae* na regulação da expressão gênica global e adaptação de *H. seropedicae* aos baixos níveis de oxigênio. O emprego de técnicas como RNA-Seq e ChIP-Seq associada ao uso de outras caracterizações fisiológicas e bioquímicas nos permitiu ter um panorama sobre a função das três proteínas.

A análise do transcriptoma da estirpe mutante para os três genes *fnr* revelou que as proteínas Fnr1 e Fnr3 são essenciais para o rearranjo da cadeia respiratória e do fluxo de elétrons durante a limitação dos níveis de oxigênio. Além de regular a expressão das oxidades respiratórias terminais, estas proteínas também são responsáveis pela expressão de proteínas essenciais para a síntese e maturação de proteínas que contém o

citocromo do tipo *c*, que estão presentes em diferentes componentes da cadeia de transporte de elétrons.

Além disso, o emprego da técnica de ChIP-Seq, aliada a uma nova análise transcriptômica de mutantes simples para cada gene *fnr* de *H.seropedicae* nos permitu expandir o nosso conhecimento sobre o conjunto específico de genes regulado pelas três proteínas Fnr. Ainda, baseado nos promotores alvo das proteínas Fnr, identificados através da análise do ChIP-Seq, pudemos sugerir que as proteínas Fnr1 e Fnr3 apresentam motivos de ligação específicos e ainda há a probabilidade de existência de um grupo de promotores que é regulado através de um mecanismo de formação de heterodímeros entre as proteínas Fnr1 e Fnr3. Este mecanismo ainda carece de melhores evidências experimentais para ser validado, porém pode potencialmente fornecer uma estratégia para o ajuste fino da regulação da expressão gênica em resposta aos níveis de oxigênio.

Devido ao seu papel na modulção da composição da cadeia respiratória, as proteínas Fnr1 e Fnr3 influenciam indiretamente fixação de nitrogênio e a atividade da proteína NifA em *H. seropedicae.* Sob determinadas condições, Fnr1 e Fnr3 parecem modular a taxa respiratória de afim de prover um mecanismo para proteção da proteína NifA da inativação por oxigênio, tornando-a mais ativa ou mais estável. A fixação de nitrogênio é severamente afetada no mutante triplo *fnr* sob condições de limitação de oxigênio, provavelmente como uma consequência da depleção dos níveis de proodução energia no mutante *fnr*. Porém quando as estirpes selvagem e triplo mutante *fnr* foram cultivadas diazotróficamente em um meio sem adição de nitrogênio fixado, ficou evidente que a taxa de crescimento do mutante *fnr* foi maior nos primeiros dias de crescimento. É possível que a elevada taxa de consumo de O_2 no triplo mutante *fnr* permita uma maior ativadade da proteína NifA e consequentemente maior atividade da nitrogenase durante os estágios iniciais de crescimento. No entanto, quando os nívies de oxigênio caem mais, o tripo mutante *fnr* não pode mais manter um fluxo de elétrons adequado para suportar a atividade da nitrogenase e como consequência o crescimento diazotrófico é prejudicado.

Vale destacar que este trabalho revelou uma atividade inédita para a proteína Fnr2. Durante análise do ChIP-Seq somente um promotor foi identificado como sendo um potencial alvo de regulação para a proteína Fnr2, o promotor *cyoABCDshb1hydH*,

que codifica para a oxidase respiratória do tipo *bo*$_3$. Este promotor foi reprimido no mutante *fnr2* de *H. seropedicae* independetemente das concentrações de oxigênio empregadas neste estudo, sugerindo que Fnr2 não é sensível ao O_2. Considerando que a expressão de *fnr2* em *H.seropedicae* e da oxidase do tipo *bo*$_3$ são induzidas sob condições de estresse nitrosativo, pode-se sugerir que talvez Fnr2 seja um sensor de óxido nítrico (NO) ao invés de oxigênio.

Por fim, este estudo permitiu concluir que as proteínas Fnr1 e Fnr3 são as principais reguladoras transcricionais durante a mudança dos níveis de oxigênio, sendo que Fnr3 seria o sensor primário que ativa a expressão de alguns genes necessários à adaptação a níveis intermediários de O_2, incluindo Fnr1. Quando os níveis de O_2 caem mais, Fnr1 se torna ativa e então novos genes são ativados. Aparentemente Fnr1 é capaz de deslocar Fnr3 de determinados promotores alvo, para ligar-se como um homodímero ou como um heterodímero com Fnr3, afim de provocar uma aumento abrupto nos níveis de expressão de promotores Fnr dependentes, garantido deste modo uma eficiente expressão de proteínas essenciais à adaptação aos baixos níveis de oxigênio.

5 CONCLUSÕES

- As proteínas Fnr1 e Fnr3 são proteínas reguladoras transcricionais requeridas para eficiente adaptação de *H. seropedicae* aos baixos níveis de oxigênio, atuando principalmente como ativadotes transcricionais;
- A adaptação aos baixos níveis de oxigênio requer a reconfiguração da cadeia respiratória. A expressão de diferentes oxidades respiratórias terminais e de proteínas essenciais para a síntese e maturação de proteínas que contém o citocromo do tipo *c*, presentes em diferentes componentes da cadeia de transporte de elétrons, é dependente de Fnr1 e Fnr3;
- A proteína Fnr3 parece ser menos sensível ao oxigênio em relação à proteína Fnr1, pois Fnr3 é requerida para a ativação da expressão de Fnr1 durante as fases iniciais do crescimento sob níveis intermediários de oxigênio;
- O regulon Fnr1 é composto por 112 genes. Destes, 54 genes (39 promotores) são regulados diretamente e 58 genes são regualdos indiretamente;
- O regulon Fnr3 é composto por 206 genes. Destes, 35 genes (21 promotores) são regulados diretamente e 171 genes são regualdos indiretamente;
- A proteína Fnr3 regula a expressão de uma série de outros fatores de regulação de transcrição, incluindo reguladores das famílias DeoR, LysR, MarR, GntR, o sistema de dois componentes NarXL e também Fnr1. Isto pode indicar que Fnr3 atue na expansão do sinal de baixo oxigênio através da atividade destes outros reguladores transcricionais. Assim, pode-se justificar o grande número de genes indiretamente regulados por Fnr3;
- 21 genes foram regulados em resposta à diminuição nos níveis de oxigênio de maneira Fnr independente. Isto sugere a presença de um mecanismo adicional de regulação da expressão gênica em resposta aos baixos níveis de oxigênio, dependente de um regulador transcricional ainda não identificado;
- Todos os genes que compõem o regulon Fnr1 são indiretamente dependentes de Fnr3 como consequência do requerimento de Fnr3 para a expressão de Fnr1;

- O regulon Fnr2 é compostor por 23 genes. Destes, 6 genes são ativados e 17 são reprimidos. O resultados de ChIP-Seq mostraram que Fnr2 liga-se ao promotor do operon *cyoABCDshb1hydH.* Este promotor, que é compostos por 6 genes, é diretamente ativado por Fnr2 de maneira independente dos níveis de oxigênio*;*
- A proteína Fnr2 é uma proteína reguladora transcricional não sensível ao oxigênio sob as condições fisiológicas empregadas nesse estudo;
- A proteína Fnr2 parece atuar principalmente como um repressor transcricional;
- A expressão da proteína Fnr2 é ativada na presença de nitrato e nitrito, sugerindo que esta proteína possa ser um sensor das condições de estresse nitrosativo;
- As proteínas Fnr1 e Fnr3 provavelmente formam heterodímeros para regulação de 18 promotores alvo (24 genes);
- O motivo de ligação para Fnr1 é: TTGnnnnnnnCAA;
- O motivo de ligação para Fnr3 é: nTGnnnC-TnnnTCAn;
- Os promotores provavelmente regulados por heterodímeros entre Fnr1 e Fnr3 contém um motivo de ligação híbrido, que é: nTGnnnC-TnnnnCAA;
- O motivo de ligação para Fnr2 não pôde ser definido devido à baixa eficiência da imunoprecipitação sob as condições empregadas neste estudo;
- As proteínas Fnr1 e Fnr3 influenciam indiretamente a atividade da proteína NifA, pois regulam a expressão de genes codificando para oxidases terminais de alta afinidade e de citocromos necessários para modular a taxa de consumo de oxigênio de modo a proteger a proteína NifA da inativação;

REFERÊNCIAS

ALTSCHUL, S. F.; MADDEN, T. L.; SCHÄFFER, A. A.; et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. **Nucleic Acids Res.** 25 (17), 3389–3402, 1997.

ALWINE, J. C.; KEMP, D. J.; STARK, G. R. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. **Proc. Natl. Acad. Sci. USA**, 74 (12), 5350–5354, 1977.

ANDERS, S.; HUBER, W. Differential expression analysis for sequence count data. **Genome Biol.** 11(10), R106–R106, 2010.

ARAI, H.; KAWAKAMI, T.; OSAMURA, T.; et al. Enzymatic Characterization and In Vivo Function of Five Terminal Oxidases in *Pseudomonas aeruginosa*. **J. Bacteriol.** 196 (24), 4206–15, 2014.

ARCONDÉGUY, T.; JACK, R.; MERRICK, M. PII Signal Transduction Proteins, Pivotal Players in Microbial Nitrogen Control. **Microbiol. Mol. Biol. Rev.** 65 (1), 80–105, 2001.

AXELROD, J. D.; MAJORS, J. An improved method for photofootprinting yeast genes in vivo using Taq polymerase. **Nucleic Acids Res.** 17 (1), 171–183, 1989.

BAGG, A.; NEILANDS, J. B. Ferric uptake regulation protein acts as a repressor, employing iron(II) as a cofactor to bind the operator of an iron transport operon in *Escherichia coli*. **Biochemistry**, 26 (17), 5471–5477, 1987.

BAGG, A.; NEILANDS, J. B. Molecular mechanism of regulation of siderophore-mediated iron assimilation. **Microbiol. Rev.** 51 (4), 509–518, 1987.

BAILEY, T. L.; BODEN, M.; BUSKE, F. A.; et al. MEME Suite: tools for motif discovery and searching. **Nucleic Acids Res.** 37, p. W202–W208, 2009.

BALDANI, J. I.; BALDANI, V. L. D.; SELDIN, L.; DOBEREINER, A. N. D. J. Characterization of *Herbaspirillum seropedicae* gen. nov., sp. nov., a Root- Associated Nitrogen-Fixing Bacterium. **Int. J. Syst. Bacteriol.** v. 36, n. 1, p. 86–93, 1986.

BALDANI, J.; POT, B.; KIRCHHOF, G.; et al. Emended Description of Herbaspirillum; Inclusion of [Pseudomonas] rubrisubalbicasn, a Mild Plant Pathogen, as Herbaspirillum rubrisubalbicans comb. nov.; and Classification of a Group of Clinical Isolates (EF Group 1) as Herbaspirillum Species 3. **Int. J. Syst. Evol. Microbiol.** 46 (3), 802–810, 1996.

BATES, D. M.; LAZAZZERA, B. A; KILEY, P. J. Characterization of FNR* mutant proteins indicates two distinct mechanisms for altering oxygen regulation of the *Escherichia coli* transcription factor FNR. **J. Bacteriol.** 177(14), 3972–8, 1995.

BATES, D. M.; POPESCU, C. V; KHOROSHILOVA, N.; et al. Substitution of leucine 28 with histidine in the *Escherichia coli* transcription factor FNR results in increased stability of the [4Fe-4S](2+) cluster to oxygen. **J. Biol Chem.** 275 (9), 6234–40, 2000.

BATISTA, M. B.; SFEIR, M. Z. T.; FAORO, H.; et al. The *Herbaspirillum seropedicae* SmR1 Fnr orthologs controls the cytochrome composition of the electron transport chain. **Sci. Rep.** 3, p. 2544, 2013.

BATUT, J.; BOISTARD, P. Oxygen control in Rhizobium. **Antonie van Leeuwenhoek**, 66 (1-3), 129–50, 1994.

BATUT, J.; DAVERAN-MINGOT, M. L.; DAVID, M.; et al. fixK, a gene homologous with fnr and crp from *Escherichia coli*, regulates nitrogen fixation genes both positively and negatively in *Rhizobium meliloti*. **EMBO J.** 8 (4), 1279–86, 1989.

BECKER-ANDRÉ, M.; HAHLBROCK, K. Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATTY). **Nucleic Acids Res.** 17(22), 9437–9446, 1989.

BENJAMINI, Y.; HOCHBERG, Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. **J. R. Stat. Soc. Ser. B .** 57 (1), 289–300, 1995.

BES, M. T.; PARISINI, E.; INDA, L. A; et al. Crystal structure determination at 1.4 A resolution of ferredoxin from the green alga *Chlorella fusca*. **Structure**, v. 7, n. 10, p. 1201–11, 1999.

BOBIK, C.; MEILHOC, E.; BATUT, J. FixJ: a Major Regulator of the Oxygen Limitation Response and Late Symbiotic Functions of *Sinorhizobium meliloti*. **J. Bacteriol. 1**88 (13), 4890–4902, 2006.

BOGUSKI, M. S. The turning point in genome research. **Trends Biochem. Sci.** 20 (8), 295–296, 1995.

BONNET, M.; KURZ, M.; MESA, S.; et al. The Structure of *Bradyrhizobium japonicum* Transcription Factor FixK2 Unveils Sites of DNA Binding and Oxidation. **J. Biol. Chem.** 288 (20), 14238–14246, 2013.

BORNEMAN, A. R.; GIANOULIS, T. A.; ZHANG, Z. D.; et al. Divergence of Transcription Factor Binding Sites Across Related Yeast Species. **Science**, v. 317, n. 5839, p. 815–819, 2007.

BRADFORD, M. M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. **Anal. Biochem.** 72 (1-2), 248–254, 1976.

BRDLIK, C. M.; NIU, W.; SNYDER, M. Chromatin immunoprecipitation and multiplex sequencing (ChIP-Seq) to identify global transcription factor binding sites in the nematode Caenorhabditis elegans**. Methods Enzymol.** 539, 89-111, 2014.

BROWNING, D. F.; GRAINGER, D. C.; BUSBY, S. J. W. Effects of nucleoid-associated proteins on bacterial chromosome structure and gene expression. **Curr. Opin. Microbiol.** 13 (6), 773–780, 2010.

BUENO, E.; MESA, S.; BEDMAR, E. J.; RICHARDSON, D. J.; DELGADO, M. J. Bacterial adaptation of respiration from oxic to microoxic and anoxic conditions: redox control. **Antioxid. Redox Signal.** 16 (8), 819–52, 2012.

BURRIS, R. H. Nitrogenases. **J Biol Chem**, v. 266, n. 15, p. 9339–9342, 1991.

BUSH, M.; DIXON, R. The role of bacterial enhancer binding proteins as specialized activators of σ54-dependent transcription. **Microbiol. Mol. Biol. Rev.** 76 (3), 497–529, 2012.

BUSH, M. J.; BIBB, M. J.; CHANDRA, G. Genes Required for Aerial Growth , Cell Division , and Chromosome Segregation Are Targets of WhiA before Sporulation in *Streptomyces venezuelae*. **mBio**, 4(5) e00684-13, 2013.

CARRO, L.; RIVAS, R.; LEÓN-BARRIOS, M.; et al. *Herbaspirillum canariense* sp. nov., *Herbaspirillum aurantiacum* sp. nov. and *Herbaspirillum soli* sp. nov., isolated from volcanic mountain soil, and emended. **Int J. Syst. Evol. Microbiol.** 62 (6), 1300–6, 2012.

CHANG, H.-Y.; AHN, Y.; PACE, L. A.; et al. The diheme cytochrome c(4) from Vibrio cholerae is a natural electron donor to the respiratory cbb(3) oxygen reductase. **Biochemistry**, v. 49, n. 35, p. 7494–7503, 2010.

CHEN, J.; SU, Z.; LIU, Y.; et al. Herbaspirillum Species: A Potential Pathogenic Bacteria Isolated from Acute Lymphoblastic Leukemia Patient. **Curr. Microbiol.** 62 (1), 331–333, 2011.

CHUBATSU, L. S.; MONTEIRO, R. A.; SOUZA, E. M.; et al. Nitrogen fixation control in *Herbaspirillum seropedicae*. **Plant Soil**, v. 356, n. 1-2, p. 197–207, 2012.

COENYE, T.; GORIS, J.; SPILKER, T.; VANDAMME, P.; LIPUMA, J. J. Characterization of Unusual Bacteria Isolated from Respiratory Secretions of Cystic Fibrosis Patients and Description of Inquilinus limosus gen. nov., sp. nov. **J. Clin. Microbiol.** 40 (6), 2062–2069, 2002.

CONSTANTINIDOU, C.; HOBMAN, J. L.; GRIFFITHS, L.; et al. A Reassessment of the FNR Regulon and Transcriptomic Analysis of the Effects of Nitrate, Nitrite, NarXL, and NarQP as *Escherichia coli* K12 Adapts from Aerobic to Anaerobic Growth. **J. Biol. Chem.** 281 (8), 4802–4815, 2006.

COSSEAU, C.; BATUT, J. Genomics of the *ccoNOQP*-encoded *cbb3* oxidase complex in bacteria. **Arch. Microbiol.** 181 (2), 89–96, 2004.

COTTER, P. A.; CHEPURI, V.; GENNIS, R. B.; GUNSALUS, R. P. Cytochrome o (*cyoABCDE*) and d (*cydAB*) oxidase gene expression in *Escherichia coli* is regulated by oxygen, pH, and the *fnr* gene product. **J. Bacteriol.** 172 (11), 6333–6338, 1990.

CRACK, J. C.; GASKELL, A. A.; GREEN, J.; et al. Influence of the Environment on the $[4Fe{-}4S]^{2+}$ to $[2Fe{-}2S]^{2+}$ Cluster Switch in the Transcriptional Regulator FNR. **J. Am. Chem Soc.** 130 (5), 1749–1758, 2008.

CROOKE, H.; COLE, J. The biogenesis of c-type cytochromes in *Escherichia coli* requires a membrane-bound protein, DipZ, with a protein disulphide isomerase-like domain. **Mol. Microbiol.** 15 (6), p. 1139–1150, 1995.

DENNIS, J. J.; ZYLSTRA, G. J. Plasposons: Modular Self-Cloning Minitransposon Derivatives for Rapid Genetic Analysis of Gram-Negative Bacterial Genomes. **Appl. Envir. Microbiol.** 64 (7), 2710–2715, 1998.

DILWORTH, M. J. Acetylene reduction by nitrogen-fixing preparations from *Clostridium pasteurianum*. **Biochim. Biophys. Acta - Gen. Subj.** 127 (2), 285–294, 1966.

DING, L.; YOKOTA, A. Proposals of Curvibacter gracilis gen. nov., sp. nov. and Herbaspirillum putei sp. nov. for bacterial strains isolated from well water and reclassification of [Pseudomonas] huttiensis, [Pseudomonas] lanceolata, [Aquaspirillum] delicatum and [Aquaspirillum. **Int. J. Syst. Evol. Microbiol.** 54, 2223–2230, 2004.

DIXON, R.; KAHN, D. Genetic regulation of biological nitrogen fixation. **Nat. Rev. Microbiol.** 2 (8), 621–31, 2004.

DÖBEREINER, J.; REIS, V. M.; PAULA, M. A.; OLIVARES, F. Endophytic Diazotrophs in Sugar Cane, Cereals and Tuber Plants. In: R. Palacios; J. Mora; W. Newton (Eds.); **New Horizons in Nitrogen Fixation SE - 55**, Current Plant Science and Biotechnology in Agriculture. v. 17, p.671–676, 1993.

DOBRITSA, A. P.; REDDY, M. C. S.; SAMADPOUR, M. Reclassification of *Herbaspirillum putei* as a later heterotypic synonym of *Herbaspirillum huttiense*, with the description of *H. huttiense subsp. huttiense subsp. nov.* and *H. huttiense subsp. putei subsp. nov., comb. nov.*, and description of *Herbaspirillum*. **Int. J. Syst. Evol. Microbiol.** 60 (6), 1418–26, 2010.

DOPAZO, J. Estimating errors and confidence intervals for branch lengths in phylogenetic trees by a bootstrap approach. **J. Mol. Evol.** 38 (3), 300–304, 1994.

DUFOUR, Y. S.; KILEY, P. J.; DONOHUE, T. J. Reconstruction of the Core and Extended Regulons of Global Transcription Factors. **PLoS Genetics**, v. 6, n. 7, p. e1001027, 2010.

EDGAR, R. C. MUSCLE: multiple sequence alignment with high accuracy and high throughput. **Nucleic Acids Res.** 32 (5), 1792–1797, 2004.

ELMERICH, C. Historical Perspective: From Bacterization to Endophytes. In: C. Elmerich; W. Newton (Eds.); **Associative and Endophytic Nitrogen-fixing Bacteria and Cyanobacterial Associations SE - 1**, Nitrogen Fixation: Origins, Applications, and Research Progress. v. 5, p.1–20, 2007.

EMMER, M.; DECROMBRUGGHE, B.; PASTAN, I.; PERLMAN, R. Cyclic AMP Receptor Protein of E. coli: Its Role in the Synthesis of Inducible Enzymes. **Proc. Natl. Acad. Sci. USA** 66 (2), 480–487, 1970.

FINN, R. D.; MISTRY, J.; TATE, J.; et al. The Pfam protein families database. **Nucleic Acids Res.** 38 (Database issue), D211–D222, 2010.

FISCHER, H. M. Genetic regulation of nitrogen fixation in rhizobia. **Microbiol. Rev.** 58 (3), 352–86, 1994.

FLEISCHHACKER, A. S.; KILEY, P. J. Iron-containing transcription factors and their roles as sensors. **Curr. Opin. Chem. Biol.** 15 (2), 335–341, 2011.

FRANCIS, R. J.; BECKER, R. Specific indication of hemoproteins in polyacrylamide gels using a double-staining process. **Anal. Biochem.** 14, 509–514, 1984.

GILBERTHORPE, N. J.; POOLE, R. K. Nitric oxide homeostasis in Salmonella typhimurium: roles of respiratory nitrate reductase and flavohemoglobin. **J. Biol. Chem.** 283 (17), 11146–54, 2008.

GODDARD, A. D.; STEVENS, J. M.; RONDELET, A.; et al. Comparing the substrate specificities of cytochrome c biogenesis Systems I and II. **FEBS J.** 277 (3), 726–737, 2010.

GRABBE, R.; KLOPPROGGE, K. A. I.; SCHMITZ, R. A. Fnr Is Required for NifL-Dependent Oxygen Control of nif Gene Expression in Klebsiella pneumoniae. **J.Bacteriol.** 183 (4), 1385–1393, 2001.

GRAINGER, D. C.; AIBA, H.; HURD, D.; BROWNING, D. F.; BUSBY, S. J. W. Transcription factor distribution in Escherichia coli: studies with FNR protein. **Nucleic Acids Res.** 35 (1), 269–278, 2007.

GREEN, J.; CRACK, J. C.; THOMSON, A. J.; LEBRUN, N. E. Bacterial sensors of oxygen. **Curr. Opin. Microbiol.** 12 (2), 145–151, 2009.

GREEN, J.; SCOTT, C.; GUEST, J. R. Functional versatility in the CRP-FNR superfamily of transcription factors: FNR and FLP. **Adv. Microb. Physiol**. 44, 1-34, 2001.

GUTIÉRREZ, D.; HERNANDO, Y.; PALACIOS, J. M.; IMPERIAL, J.; RUIZ-ARGÜESO, T. FnrN controls symbiotic nitrogen fixation and hydrogenase activities in Rhizobium leguminosarum biovar viciae UPM791. **J. Bacteriol.** 179 (17), 5264–5270, 1997.

HALLMANN, J.; QUADT-HALLMANN, A.; MAHAFFEE, W. F.; KLOEPPER, J. W. Bacterial endophytes in agricultural crops. **Can. J. Microbiol.** 43 (10), 895–914, 1997.

HERNANDO, Y.; PALACIOS, J. M.; IMPERIAL, J.; RUIZ-ARGÜESO, T. The hypBFCDE operon from Rhizobium leguminosarum biovar viciae is expressed from an Fnr-type promoter that escapes mutagenesis of the fnrN gene. **J. Bacteriol.** 177 (19), p. 5661–9, 1995.

HILL, S.; VIOLLET, S.; SMITH, A T.; ANTHONY, C. Roles for enteric d-type cytochrome oxidase in N2 fixation and microaerobiosis. **J. Bacteriol.** 172 (4), 2071–8, 1990.

HORAK, C. E.; SNYDER, M. ChIP-chip: A genomic approach for identifying transcription factor binding sites. In: C. G. and G. R. F. B. T.-M. in Enzymology (Ed.); **Guide to Yeast Genetics and Molecular and Cell Biology - Part B**. v. Volume 350, p.469–483, 2002.

HYNES, M. F.; QUANDT, J.; O'CONNELL, M. P.; PÜHLER, A. Direct selection for curing and deletion of Rhizobium plasmids using transposons carrying the Bacillus subtilis sacB gene. **Gene**, 78 (1), 111–120, 1989.

IM, W.; BAE, H.; YOKOTA, A.; LEE, S. Herbaspirillum chlorophenolicum sp. nov., a 4-chlorophenol-degrading bacterium. **Int. J. Syst. Evol. Microbiol.** 54 (3), 851–855, 2004.

JAMES, E. K.; GYANESHWAR, P.; MATHAN, N.; et al. Infection and Colonization of Rice Seedlings by the Plant Growth-Promoting Bacterium Herbaspirillum seropedicae Z67. **Mol. Plant-Microbe. Interact.** 15 (9), 894–906, 2002.

JAMES, E. K.; OLIVARES, F. L. Infection and Colonization of Sugar Cane and Other Graminaceous Plants by Endophytic Diazotrophs. **Crit. Rev. Plant. Sci.** 17 (1), 77–119, 1998.

JANSSEN, P. J.; HOUDT, R. VAN; MOORS, H.; et al. The complete genome sequence of Cupriavidus metallidurans strain CH34, a master survivalist in harsh and anthropogenic environments. **PloS one**, 5 (5), e10433, 2010.

JOHNSON, D. S.; MORTAZAVI, A.; MYERS, R. M.; WOLD, B. Genome-Wide Mapping of in Vivo Protein-DNA Interactions. **Science**, v. 316, n. 5830, p. 1497–1502, 2007.

JONES, D. T.; TAYLOR, W. R.; THORNTON, J. M. The rapid generation of mutation data matrices from protein sequences. **Comput. Appl. Biosci. CABIOS.** 8 (3), 275–282, 1992.

JONG, A. DE; PIETERSMA, H.; CORDES, M.; KUIPERS, O.; KOK, J. PePPER: a webserver for prediction of prokaryote promoter elements and regulons. **BMC Genomics**, 13 (1), 299, 2012.

JUNG, S.-Y.; LEE, M.-H.; OH, T.-K.; YOON, J.-H. Herbaspirillum rhizosphaerae sp. nov., isolated from rhizosphere soil of Allium victorialis var. platyphyllum. **Int. J. Syst. Evol. Microbiol.** 57(10), 2284–8, 2007.

JURASEK, L.; MARCHESSAULT, R. H. The Role of Phasins in the Morphogenesis of Poly(3-hydroxybutyrate) Granules. **Biomacromolecules**, v. 3, n. 2, p. 256–261, 2002.

KAHRAMANOGLOU, C.; SESHASAYEE, A. S. N.; PRIETO, A. I.; et al. Direct and indirect effects of H-NS and Fis on global gene expression control in Escherichia coli. **Nucleic Acids Res.** 39 (6), 2073–2091, 2011.

KAMINSKI, P. A.; KITTS, C. L.; ZIMMERMAN, Z.; LUDWIG, R. A. Azorhizobium caulinodans uses both cytochrome bd (quinol) and cytochrome cbb3 (cytochrome c) terminal oxidases for symbiotic N2 fixation. **J. Bacteriol.** 178 (20), 5989–5994, 1996.

KANG, Y.; WEBER, K. D.; QIU, Y.; KILEY, P. J.; BLATTNER, F. R. Genome-Wide Expression Analysis Indicates that FNR of Escherichia coli K-12 Regulates a Large Number of Genes of Unknown Function. **J. Bacteriol.**187 (3), 1135–1160, 2005.

KELLY, M. J.; POOLE, R. K.; YATES, M. G.; KENNEDY, C. Cloning and mutagenesis of genes encoding the cytochrome bd terminal oxidase complex in Azotobacter vinelandii: mutants deficient in the cytochrome d complex are unable to fix nitrogen in air. **J. Bacteriol.** 172 (10), 6010–9, 1990.

KILEY, P. J.; BEINERT, H. Oxygen sensing by the global regulator, FNR: the role of the iron-sulfur cluster. **FEMS Microbiol. Rev.** 22 (5), 341–352, 1998.

KIRCHHOF, G.; ECKERT, B.; STOFFELS, M.; et al. Herbaspirillum frisingense sp. nov., a new nitrogen-fixing bacterial species that occurs in C4-fibre plants. **Int. J Syst. Evol. Microbiol.** 51, p. 157–168, 2001.

KLASSEN, G.; PEDROSA, F. O.; SOUZA, E. M. DE; YATES, M. G.; RIGO, L. U. Nitrogenase activity of Herbaspirillum seropedicae grown under low iron levels requires the products of nifXorf1 genes. **FEMS Microbiol. Lett.** 224 (2), 255–259, 2003.

KLASSEN, G.; PEDROSA, F. O.; SOUZA, E. M.; FUNAYAMA, S.; RIGO, L. U. Effect of nitrogen compounds on nitrogenase activity in Herbaspirillum seropedicae SMR1. **Canadian J. Microbiol.** 43 (9), 887–891, 1997.

KLASSEN, G.; PEDROSA, F. O.; SOUZA, E. M.; YATES, M. G.; RIGO, L. U. Sequencing and functional analysis of the nifENXorf1orf2 gene cluster of Herbaspirillum seropedicae. **FEMS Microbiol. Lett.** 181 (1), 165–170, 1999.

KLOEPPER, J. W.; BEAUCHAMP, C. J. A review of issues related to measuring colonization of plant roots by bacteria. **Canadian J. Microbiol.** 38 (12), 1219–1232, 1992.

KOBOLDT, D. C.; STEINBERG, K. M.; LARSON, D. E.; WILSON, R. K.; MARDIS, E. R. The next-generation sequencing revolution and its impact on genomics. **Cell**, v. 155, n. 1, p. 27–38, 2013.

KOCH, H. G.; HWANG, O.; DALDAL, F. Isolation and characterization of Rhodobacter capsulatus mutants affected in cytochrome cbb3 oxidase activity. **J. Bacteriol.** 180 (4), p. 969–78, 1998.

KOCH, H. G.; WINTERSTEIN, C.; SARIBAS, A S.; ALBEN, J. O.; DALDAL, F. Roles of the ccoGHIS gene products in the biogenesis of the cbb(3)-type cytochrome c oxidase. **J. Mol. Biol.** 297 (1), p. 49–65, 2000.

KORNER, H.; SOFIA, H. J.; ZUMFT, W. G. Phylogeny of the bacterial superfamily of Crp-Fnr transcription regulators: exploiting the metabolic spectrum by controlling alternative gene programs. **FEMS Microbiol. Rev.** 27 (5) p. 559–592, 2003.

KUO, M.-H.; ALLIS, C. D. In Vivo Cross-Linking and Immunoprecipitation for Studying Dynamic Protein:DNA Associations in a Chromatin Environment. **Methods**, v. 19, n. 3, p. 425–433, 1999.

LAGIER, J.-C.; GIMENEZ, G.; ROBERT, C.; RAOULT, D.; FOURNIER, P.-E. Non-contiguous finished genome sequence and description of Herbaspirillum massiliense sp. nov. **Stand Gen. Sci.** 7 (2), p. 200–209, 2012.

LANGMEAD, B.; SALZBERG, S. L. Fast gapped-read alignment with Bowtie 2. **Nat. Methods.** 9 (4), p. 357–359, 2012.

LAZAZZERA, B. A; BATES, D. M.; KILEY, P. J. The activity of the Escherichia coli transcription factor FNR is regulated by a change in oligomeric state. **Genes Dev.** 7 (. 10), 1993–2005, 1993.

LAZAZZERA, B. A; BEINERT, H.; KHOROSHILOVA, N.; KENNEDY, M. C.; KILEY, P. J. DNA binding and dimerization of the Fe-S-containing FNR protein from Escherichia coli are regulated by oxygen. **J. Biol. Chem.** 271 (5), p. 2762–8, 1996.

LEFRANÇOIS, P.; ZHENG, W.; SNYDER, M. ChIP-Seq using high-throughput DNA sequencing for genome-wide identification of transcription factor binding sites. **Methods Enzymol.** 470 (10), p. 77–104, 2010.

LIEB, C.; SIDDIQUI, R. A.; HIPPLER, B. The Alcaligenes eutrophus hemN gene encoding the oxygen-independent coproporphyrinogen III oxidase, is required for heme biosynthesis during anaerobic growth. **Arch. Microbiol.** 169 (1), 52–60, 1998.

LOHSE, M.; BOLGER, A. M.; NAGEL, A.; et al. RobiNA: a user-friendly, integrated software solution for RNA-Seq-based transcriptomics. **Nucleic Acids Res.** 40(Web Server issue), p. W622–W627, 2012.

MACHADO, I. M.; YATES, M. G.; MACHADO, H. B.; SOUZA, E. M.; PEDROSA, F. O. Cloning and sequencing of the nitrogenase structural genes nifHDK of Herbaspirillum seropedicae. **Braz. J. Med. Biol. Res.** 29 (12), p. 1599–1602, 1996.

MANDON, K.; KAMINSKI, P. A; ELMERICH, C. Functional analysis of the fixNOQP region of Azorhizobium caulinodans. **J. Bacteriol.** 176 (9), p. 2560–8, 1994.

MARCHAL, K.; VANDERLEYDEN, J. The "oxygen paradox" of dinitrogen-fixing bacteria. **Biol. Fertil. Soils.** 30 (5-6), 363–373, 2000.

MARGULIES, M.; EGHOLM, M.; ALTMAN, W. E.; et al. Genome sequencing in microfabricated high-density picolitre reactors. **Nature**. 437, 376–380, 2005.

MARQUES DA SILVA, R.; CAUGANT, D. A.; ERIBE, E. R. K.; et al. Bacterial diversity in aortic aneurysms determined by 16S ribosomal RNA gene analysis. **J. Vasc. Surg.** v. 44, n. 5, p. 1055–1060, 2006.

MARTONE, R.; EUSKIRCHEN, G.; BERTONE, P.; et al. Distribution of NF-κB-binding sites across human chromosome 22. **Proc. Natl. Acad. Sci. USA.** 100 (21), p. 12247–12252, 2003.

MATSUI, M.; TOMITA, M.; KANAI, A. Comprehensive Computational Analysis of Bacterial CRP/FNR Superfamily and Its Target Motifs Reveals Stepwise Evolution of Transcriptional Networks. **Genome Biol. Evol.** 5 (2), p. 267–282, 2013.

MCKAY, D. B.; STEITZ, T. A. Structure of catabolite gene activator protein at 2.9 [angst] resolution suggests binding to left-handed B-DNA. **Nature**, v. 290, n. 5809, p. 744–749, 1981.

MENG, W.; GREEN, J.; GUEST, J. R. FNR-dependent repression of ndh gene expression requires two upstream FNR-binding sites. **Microbiology.** 143, 1521–32, 1997.

MERRICK, M. J.; EDWARDS, R. A. Nitrogen control in bacteria. **Microbiol. Rev.** 59 (4), 604–622, 1995.

MESA, S.; HAUSER, F.; FRIBERG, M.; et al. Comprehensive assessment of the regulons controlled by the FixLJ-FixK2-FixK1 cascade in Bradyrhizobium japonicum. **J. Bacteriol.** 190 (20), p. 6568–79, 2008.

MESA, S.; HENNECKE, H.; FISCHER, H. A multitude of CRP / FNR-like transcription proteins in Bradyrhizobium japonicum. **Bioch. Soc. Trans.** 34, 156–159, 2006.

MESA, S.; REUTIMANN, L.; FISCHER, H.-M.; HENNECKE, H. Posttranslational control of transcription factor FixK(2), a key regulator for the Bradyrhizobium japonicum–soybean symbiosis. **Proc. Natl. Acad. Sci. USA.** 106 (51), 21860–21865, 2009.

METTERT, E. L.; KILEY, P. J. ClpXP-dependent Proteolysis of FNR upon Loss of its O 2 -sensing $[4Fe–4S]^{2+}$ Cluster. **J. Mol. Biol.** 354(2), 220-232, 2005.

MILLER, J. **Experiments in Molecular Genetics**. New York: Cold Spring Harbor Laboratory Press, 1972.

MONTEIRO, R. A.; BALSANELLI, E.; WASSEM, R.; et al. Herbaspirillum-plant interactions: microscopical, histological and molecular aspects. **Plant Soil**. 356 (1-2), 175–196, 2012.

MONTEIRO, R. A.; SOUZA, E. M. DE; YATES, M. G.; PEDROSA, F. O.; CHUBATSU, L. S. Fnr Is Involved in Oxygen Control of Herbaspirillum seropedicae N-Truncated NifA Protein Activity in Escherichia coli. **Appl. Env. Microbiol.** 69 (3), 1527–1531, 2003.

MONTEIRO, R. A.; SOUZA, E. M.; FUNAYAMA, S.; et al. Expression and functional analysis of an N-truncated NifA protein of Herbaspirillum seropedicae. **FEBS Lett.** 447 (2–3), 283–286, 1999.

MONTEIRO, R. A.; SOUZA, E. M.; YATES, M. G.; PEDROSA, F. O.; CHUBATSU, L. S. In-trans regulation of the N-truncated-NIFA protein of Herbaspirillum seropedicae by the N-terminal domain. **FEMS Microbiol. Lett.** 180, n. 2, p. 157–161, 1999.

MOROZOVA, O.; HIRST, M.; MARRA, M. A. Applications of New Sequencing Technologies for Transcriptome Analysis. **Ann. Rev. Genomics. Hum. Genet.** 10 (1), 135–151, 2009.

MULLER, D.; MÉDIGUE, C.; KOECHLER, S.; et al. A tale of two oxidation states: bacterial colonization of arsenic-rich environments. **PLoS Genet.** 3 (4), e53, 2007.

MÜNCH, R.; HILLER, K.; GROTE, A.; et al. Virtual Footprint and PRODORIC: an integrative framework for regulon prediction in prokaryotes. **Bioinformatics** , v. 21 , n. 22 , p. 4187–4189, 2005.

MYERS, K. S.; YAN, H.; ONG, I. M.; et al. Genome-scale analysis of escherichia coli FNR reveals complex features of transcription factor binding. **PLoS Genet.** 9 (6), e1003565, 2013.

NAGALAKSHMI, U.; WAERN, K.; SNYDER, M. RNA-Seq□ : A Method for Comprehensive Transcriptome Analysis. **Curr. Protoc. Mol. Biol.,** p. 1–13, 2010.

NELLEN-ANTHAMATTEN, D.; ROSSI, P.; PREISIG, O.; et al. Bradyrhizobium japonicum FixK2, a crucial distributor in the FixLJ-dependent regulatory cascade for control of genes inducible by low oxygen levels. **J. Bacteriol.** 180 (19), p. 5251–5, 1998.

NINFA, A. J.; JIANG, P.; ATKINSON, M. R.; PELISKA, J. A. Integration of antagonistic signals in the regulation of nitrogen assimilation in *Escherichia coli*. **Curr Top Cell Regul.** 36, 31-75, 2001.

NOINDORF, L.; BONATTO, A. C.; MONTEIRO, R. A; et al. Role of PII proteins in nitrogen fixation control of Herbaspirillum seropedicae strain SmR1. **BMC Microbiol.** 11 (1), p. 8, 2011.

NOONAN, K. E.; BECK, C.; HOLZMAYER, T. A.; et al. Quantitative analysis of MDR1 (multidrug resistance) gene expression in human tumors by polymerase chain reaction. **Proc. Natl. Acad. Sci. USA.** 87 (18), 7160–7164, 1990.

OLDROYD, G. E. D.; DOWNIE, J. A. Coordinating nodule morphogenesis with rhizobial infection in legumes. **Annu. Rev. Plant. Biol.** 59, p. 519–46, 2008.

OLIVARES, F. L.; BALDANI, V. L. D.; REIS, V. M. Occurrence of the endophytic diazotrophs Herbaspirillum spp . in roots , stems , and leaves , predominantly of Gramineae. **Biol. Fertil. Soils.** 21, p. 197–200, 1996.

OLIVEIRA, M. A S.; BAURA, V. A; AQUINO, B.; et al. Role of conserved cysteine residues in Herbaspirillum seropedicae NifA activity. **Res. Microbiol.** 160 (6), 389–95, 2009.

OLIVEIRA, M. A. S.; AQUINO, B.; BONATTO, A. C.; et al. Interaction of GlnK with the GAF domain of Herbaspirillum seropedicae NifA mediates NH4+-regulation. **Biochimie**, v. 94, n. 4, p. 1041–1047, 2012.

OP DEN CAMP, R. H. M.; POLONE, E.; FEDOROVA, E.; et al. Nonlegume Parasponia andersonii Deploys a Broad Rhizobium Host Range Strategy Resulting in Largely Variable Symbiotic Effectiveness. **Mol. Plant-Microbe. Interact.** 25(7), p. 954–963, 2012.

ORLANDO, V.; STRUTT, H.; PARO, R. Analysis of Chromatin Structure byin VivoFormaldehyde Cross-Linking. **Methods**, v. 11, n. 2, p. 205–214, 1997.

PARK, P. J. ChIP-seq: advantages and challenges of a maturing technology. **Nat. Rev. Genet.** 10 (10), 669–680, 2009.

PEDROSA, F. O.; BENELLI, E. M.; YATES, M. G.; et al. Recent developments in the structural organization and regulation of nitrogen fixation genes in Herbaspirillum seropedicae. **J. Biotech.** 91 (2–3), 189–195, 2001.

PEDROSA, F. O.; MONTEIRO, R. A.; WASSEM, R.; et al. Genome of Herbaspirillum seropedicae strain SmR1, a specialized diazotrophic endophyte of tropical grasses. **PLoS Genet.** 7 (5), e1002064, 2011.

PEREZ, J. C.; GROISMAN, E. A. Evolution of transcriptional regulatory circuits in bacteria. **Cell**, v. 138, n. 2, p. 233–44, 2009.

POHLMANN, A.; FRICKE, W. F.; REINECKE, F.; et al. Genome sequence of the bioplastic-producing "Knallgas" bacterium Ralstonia eutropha H16. **Nat. Biotech.** 24 (10). 1257–62, 2006.

POOLE, R. K. Nitric oxide and nitrosative stress tolerance in bacteria. **Biochem. Soc. Trans.** 33 (1), 176–80, 2005.

POOLE, R. K.; HILL, S. Respiratory protection of nitrogenase activity in Azotobacter vinelandii--roles of the terminal oxidases. **Biosci. Rep.** 17 (3), 303–17, 1997.

PREISIG, O.; ANTHAMATTEN, D.; HENNECKE, H. Genes for a microaerobically induced oxidase complex in Bradyrhizobium japonicum are essential for a nitrogen-fixing endosymbiosis. **Proc. Natl. Acad. Sci. USA.** 90 (8), 3309–13, 1993.

PRICE, C. E.; DRIESSEN, A. J. M. Biogenesis of membrane bound respiratory complexes in Escherichia coli. **Biochim. Biophys. Acta - Mol. Cell Res.** 1803 (6), 748–766, 2010.

QUANDT, J.; HYNES, M. F. Versatile suicide vectors which allow direct selection for gene replacement in gram-negative bacteria. **Gene**, v. 127, n. 1, p. 15–21, 1993.

REGO, F. G. M.; PEDROSA, F. O.; CHUBATSU, L. S.; et al. The expression of nifB gene from Herbaspirillum seropedicae is dependent upon the NifA and RpoN proteins. **Can. J. Microbiol.** 52 (12), 1199–1207, 2006.

REINHOLD-HUREK, B.; HUREK, T. Life in grasses: diazotrophic endophytes. **Trends Microbiol.** 6 (4), 139–144, 1998.

REINHOLD-HUREK, B.; HUREK, T. Living inside plants: bacterial endophytes. **Curr. Opin. Plant Biol.** 14 (4), 435–443, 2011.

REIS, V. M.; BALDANI, J. I.; BALDANI, V. L. D.; DOBEREINER, J. Biological Dinitrogen Fixation in Gramineae and Palm Trees. **Critical Reviews in Plant Sciences**, v. 19, n. 3, p. 227–247, 2000.

RICHARDSON, A.; BAREA, J.-M.; MCNEILL, A.; PRIGENT-COMBARET, C. Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. **Plant Soil.** 321 (1-2), 305–339, 2009.

RICHARDSON, D. J. Bacterial respiration : a flexible process for a changing environment. **Microbiology.** 146 (3), 551–571, 2000.

RIMSKY, S.; TRAVERS, A. Pervasive regulation of nucleoid structure and function by nucleoid-associated proteins. **Curr. Opin. Microbiol.** 14 (2), 136–141, 2011.

ROBERTSON, A. G.; BILENKY, M.; TAM, A.; et al. Genome wide relationship between histone H3 lysine 4 mono- and tri-methylation and transcription factor binding. **Genome Res.** 2008.

ROBINSON, M. D.; MCCARTHY, D. J.; SMYTH, G. K. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. **Bioinformatics.** 26 (1), 139–40, 2010.

RODGERS, K. R.; LUKAT-RODGERS, G. S. Insights into heme-based O2 sensing from structure–function relationships in the FixL proteins. **J. Inorg Biochem.** 99 (4), 963–977, 2005.

RONCATO-MACCARI, L. D. B.; RAMOS, H. J. O.; PEDROSA, F. O.; et al. Endophytic Herbaspirillum seropedicae expresses nif genes in gramineous plants. **FEMS Microbiol. Ecol.** 45 (1), 39–47, 2003.

ROTHBALLER, M.; SCHMID, M.; KLEIN, I.; et al. Herbaspirillum hiltneri sp. nov., isolated from surface-sterilized wheat roots. **Int. J. Syst. Evol. Microbiol.** 56 (6), 1341–8, 2006.

SALMON, K.; HUNG, S.; MEKJIAN, K.; et al. Global Gene Expression Profiling in Escherichia coli K12: THE EFFECTS OF OXYGEN AVAILABILITY AND FNR. **J. Biol. Chem.** 278 (32), 29837–29855, 2003.

SAMBROOK, J.; FRITSCH, E. F.; MANIATIS, T. **Molecular cloning : a laboratory manual**. 2nd ed. New York: Cold Spring Harbor Laboratory Press, 1989.

SANGER, F.; NICKLEN, S.; COULSON, A. R. DNA sequencing with chain-terminating inhibitors. **Proc. Natl. Acad. Sci. USA.** 74 (12), 5463–5467, 1977.

SANTI, C.; BOGUSZ, D.; FRANCHE, C. Biological nitrogen fixation in non-legume plants. **Annals of botany**, v. 111, n. 5, p. 743–67, 2013.

SCHÄFER, A.; TAUCH, A.; JÄGER, W.; et al. Small mobilizable multi-purpose cloning vectors derived from the Escherichia coli plasmids pK18 and pK19: selection of defined deletions in the chromosome of Corynebacterium glutamicum. **Gene**, v. 145, n. 1, p. 69–73, 1994.

SCHÄGGER, H.; JAGOW, G. VON. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. **Analytical Biochemistry**, v. 166, n. 2, p. 368–379, 1987.

SCHENA, M.; SHALON, D.; DAVIS, R. W.; BROWN, P. O. Quantitative Monitoring of Gene Expression Patterns with a Complementary DNA Microarray. **Science**, v. 270, n. 5235, p. 467–470, 1995.

SCHMID, M.; HARTMANN, A. Molecular Phylogeny and Ecology of Root Associated Diazotrophic α- and β-Proteobacteria. In: C. Elmerich; W. Newton (Eds.); **Associative and Endophytic Nitrogen-fixing Bacteria and Cyanobacterial Associations SE - 2**, Nitrogen Fixation: Origins, Applications, and Research Progress. v. 5, p.21–40, 2007. Springer Netherlands.

SCHÖLLHORN, R.; BURRIS, R. H. Acetylene as a competitive inhibitor of N_2 fixation. **Proc. Natl. Acad. Sci. USA.** 58 (1), 213–216, 1967.

SCHULTZ, S. C.; SHIELDS, G. C.; STEITZ, T. A. Crystal structure of a CAP-DNA complex: the DNA is bent by 90 degrees. **Science**, v. 253, n. 5023, p. 1001–1007, 1991.

SCHUSTER, S. C. Next-generation sequencing transforms today's biology. **Nature methods**, v. 5, n. 1, p. 16–8, 2008.

SCIOTTI, M.-A.; CHANFON, A.; HENNECKE, H.; FISCHER, H.-M. Disparate Oxygen Responsiveness of Two Regulatory Cascades That Control Expression of Symbiotic Genes in Bradyrhizobium japonicum. **Journal of Bacteriology**, v. 185, n. 18, p. 5639–5642, 2003.

SCOTT, C.; PARTRIDGE, J. D.; STEPHENSON, J. R.; GREEN, J. DNA target sequence and FNR-dependent gene expression. **FEBS Letters**, v. 541, n. 1, p. 97–101, 2003.

SEKI, M.; NARUSAKA, M.; KAMIYA, A.; et al. Functional Annotation of a Full-Length Arabidopsis cDNA Collection. **Science**, v. 296, n. 5565, p. 141–145, 2002.

SHARROCKS, A. D.; GREEN, J.; GUEST, J. R. In vivo and in vitro mutants of FNR the anaerobic transcriptional regulator of E.coli. **FEBS Letters**, v. 270, n. 1–2, p. 119–122, 1990.

SHIMADA, T.; FUJITA, N.; YAMAMOTO, K.; ISHIHAMA, A. Novel Roles of cAMP Receptor Protein (CRP) in Regulation of Transport and Metabolism of Carbon Sources. **PLoS ONE**, v. 6, n. 6, p. e20081, 2011.

SIGRIST, C. J. A.; CERUTTI, L.; CASTRO, E. DE; et al. PROSITE, a protein domain database for functional characterization and annotation. **Nucleic Acids Research**, v. 38, n. Database issue, p. D161–D166, 2010.

SIMON, J.; HEDERSTEDT, L. Composition and function of cytochrome c biogenesis System II. **FEBS Journal**, v. 278, n. 22, p. 4179–4188, 2011.

SIMON, R.; PRIEFFER, U.; PUHLER, A. A Broad Host Range Mobilization System for In Vivo Genetic Engineering: Transposon Mutagenesis in Gram Negative Bacteria. **Nat. Biotech.** 1, 784–791, 1983.

SOLOMON, M. J.; LARSEN, P. L.; VARSHAVSKY, A. Mapping proteinDNA interactions in vivo with formaldehyde: Evidence that histone H4 is retained on a highly transcribed gene. **Cell**, 53 (6), 937–947, 1988.

SOLOMON, M. J.; VARSHAVSKY, A. Formaldehyde-mediated DNA-protein crosslinking: a probe for in vivo chromatin structures. **Proc. Natl. Acad. Sci. USA.** 82 (19), 6470–6474, 1985.

SOUZA, A. L. F.; INVITTI, A. L.; REGO, F. G. M.; et al. The involvement of the nif-associated ferredoxin-like genes fdxA and fdxN of Herbaspirillum seropedicae in nitrogen fixation. **J. Microbiol.** 48 (1), 77–83, 2010.

SOUZA, E. M.; FUNAYAMA, S.; RIGO, L. U.; PEDROSA, F. O. Cloning and characterization of the *nifA* gene from *Herbaspirillum seropedicae* strain Z78. **Can. J. Microbiol.** 37 (6), p. 425–429, 1991.

SOUZA, E. M.; PEDROSA, F. O.; DRUMMOND, M.; RIGO, L. U.; YATES, M. G. Control of *Herbaspirillum seropedicae* NifA Activity by Ammonium Ions and Oxygen. **J. Bacteriol.** 181 (2), p. 681–684, 1999.

SOUZA, E. M.; PEDROSA, F. O.; RIGO, L. U.; MACHADO, H. B.; YATES, M. G. Expression of the *nifA* gene of *Herbaspirillum seropedicae*: role of the NtrC and NifA binding sites and of the −24/−12 promoter element. **Microbiology**, v. 146, n. 6, p. 1407–1418, 2000.

SPILKER, T.; ULUER, A. Z.; MARTY, F. M.; et al. Recovery of Herbaspirillum Species from Persons with Cystic Fibrosis. **J. Clin. Microbiol.** 46 (8), p. 2774–2777, 2008.

SPIRO, S. The FNR family of transcriptional regulators. **Antonie van Leeuwenhoek**, v. 66, n. 1-3, p. 23–36, 1994.

SPIRO, S.; GASTON, K. L.; BELL, A I.; et al. Interconversion of the DNA-binding specificities of two related transcription regulators, CRP and FNR. **Mol. Microbiol.** 4 (11), p. 1831–8, 1990.

SPIRO, S.; GUEST, J. R. FNR and its role in oxygen-regulated gene expression in Escherichia coli. **FEMS Microbiol. Rev.** 6 (4), p. 399–428, 1990.

SPIRO, S.; ROBERTS, R. E.; GUEST, J. R. FNR-dependent repression of the ndh gene of Escherichia coli and metal ion requirement for FNR-regulated gene expression. **Mol. Microbiol.** 3 (5), p. 601–608, 1989.

STAJICH, J. E.; BLOCK, D.; BOULEZ, K.; et al. The Bioperl Toolkit: Perl Modules for the Life Sciences. **Genome Res.** 12 (10), p. 1611–1618, 2002.

STÜLKE, J.; HILLEN, W. Carbon catabolite repression in bacteria. **Curr. Opin. Microbiol.** 2(2), p. 195–201, 1999.

TAMURA, K.; PETERSON, D.; PETERSON, N.; et al. MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. **Mol. Biol. Evol.** 28 (10), 2731–2739, 2011.

TATUSOV, R. L.; FEDOROVA, N. D.; JACKSON, J. D.; et al. The COG database: an updated version includes eukaryotes. **BMC Bioinformatics**, v. 4, p. 41, 2003.

TERPE, K. Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. **Appl. Microbiol. Biotech.** 60 (5), 523–33, 2003.

TIRAPELLE, E. F.; MÜLLER-SANTOS, M.; TADRA-SFEIR, M. Z.; et al. Identification of proteins associated with polyhydroxybutyrate granules from Herbaspirillum seropedicae SmR1-old partners, new players. **PloS one**, v. 8, n. 9, p. e75066, 2013.

TOLLA, D. A.; SAVAGEAU, M. A. Regulation of Aerobic-to-Anaerobic Transitions by the FNR Cycle in Escherichia coli. **J. Mol. Biol.** 397 (4), 893–905, 2010.

TOLLA, D. A.; SAVAGEAU, M. A. Phenotypic Repertoire of the FNR Regulatory Network in Escherichia coli. **Mol. Microbiol.** 79 (1), 149–165, 2011.

TOMPA, M.; LI, N.; BAILEY, T. L.; et al. Assessing computational tools for the discovery of transcription factor binding sites. **Nat. Biotech.** 23 (1), 137–144, 2005.

TUANYOK, A.; LEADEM, B. R.; AUERBACH, R. K.; et al. Genomic islands from five strains of Burkholderia pseudomallei. **BMC Genomics**, v. 9, p. 566, 2008.

UGIDOS, A.; MORALES, G.; RIAL, E.; WILLIAMS, H. D.; ROJO, F. The coordinate regulation of multiple terminal oxidases by the Pseudomonas putida ANR global regulator. **Environ. Microbiol.** 10 (7), 1690–702, 2008.

UNDEN, G.; BONGAERTS, J. Alternative respiratory pathways of Escherichia coli: energetics and transcriptional regulation in response to electron acceptors. **Biochim. Biophys. Acta - Bioenerg.** 1320 (3), 217–234, 1997.

URBAN, A; NEUKIRCHEN, S.; JAEGER, K. E. A rapid and efficient method for site-directed mutagenesis using one-step overlap extension PCR. **Nucleic Acids Res.** 25 (11), 2227–8, 1997.

VALVERDE, A.; VELÁZQUEZ, E.; GUTIÉRREZ, C.; et al. Herbaspirillum lusitanum sp. nov., a novel nitrogen-fixing bacterium associated with root nodules of Phaseolus vulgaris. **Int. J. Syst. Evol. Microbiol.** 53 (6), 1979–1983, 2003.

VELCULESCU, V. E.; ZHANG, L.; VOGELSTEIN, B.; KINZLER, K. W. Serial Analysis of Gene Expression. **Science**, v. 270, n. 5235, p. 484–487, 1995.

VERGHESE, S.; MISRA, A. Frankia-actinorhizal symbiosis with special reference to host-microsymbiont relationship. **Curr. Sci.** 83 (4), 404-408, 2002.

VESSEY, J. K. Plant growth promoting rhizobacteria as biofertilizers. **Plant Soil**, 255 (2), 571–586, 2003.

WANG, Z.; GERSTEIN, M.; SNYDER, M. RNA-Seq: a revolutionary tool for transcriptomics. **Nat Rev Genet.** 10 (1) : 57-63, 2009.

WASSEM, R.; PEDROSA, F. O.; YATES, M. G.; et al. Control of autogenous activation of Herbaspirillum seropedicae nifA promoter by the IHF protein. **FEMS Microbiol. Lett.** 212 (2), 177–82, 2002.

WASSEM, R.; SOUZA, E. M. DE; YATES, M. G.; PEDROSA, F. DE O.; BUCK, M. Two roles for integration host factor at an enhancer-dependent nifA promoter. **Mol. Microbiol.** 35 (4), 756–764, 2000.

WIGNESHWERARAJ, S.; BOSE, D.; BURROWS, P. C.; et al. Modus operandi of the bacterial RNA polymerase containing the σ54 promoter-specificity factor. **Mol. Microbiol.** 68 (3), 538–546, 2008.

WOODLEY, P.; BUCK, M.; KENNEDY, C. Identification of sequences important for recognition of vnf genes by the VnfA transcriptional activator in Azotobacter vinelandii. **FEMS Microbiol. Lett.** 135 (2-3), 213–221, 1996.

WU, G.; CRUZ-RAMOS, H.; HILL, S.; et al. Regulation of Cytochrome bd Expression in the Obligate Aerobe Azotobacter vinelandii by CydR (Fnr). **J. Biol. Chem.** 275 (7), 4679-4686, 2000.

YANG, C. H.; AZAD, H. R.; COOKSEY, D. A. A chromosomal locus required for copper resistance, competitive fitness, and cytochrome c biogenesis in Pseudomonas fluorescens. **Proc. Natl. Acad. Sci. USA.** 93 (14), 7315–7320, 1996.

YOUNG, J. P. W. Phylogenetic classification of nitrogen-fixing organisms. In: G. Stacey; R. H. Burris; H. J. Evans (Eds.); **Biological Nitrogen Fixation**. p.43–86, 1992. London: Chapman & Hall, New York.

ZENG, W.; MORTAZAVI, A. Technical considerations for functional sequencing assays. **Nat. Immunol.** 13 (9), 802–7, 2012.

ZHENG, D.; CONSTANTINIDOU, C.; HOBMAN, J. L.; MINCHIN, S. D. Identification of the CRP regulon using in vitro and in vivo transcriptional profiling. **Nucleic Acids Res.** 32 (19), 5874–5893, 2004.

ZIGA, E. D.; DRULEY, T.; BURNHAM, C.A. D. Herbaspirillum Species Bacteremia in a Pediatric Oncology Patient. **J. Clin. Microbiol.** 48 (11), 4320–4321, 2010.

ZUBAY, G.; SCHWARTZ, D.; BECKWITH, J. Mechanism of Activation of Catabolite-Sensitive Genes: A Positive Control System. **Proc. Natl. Acad. Sci. USA**, 66 (1), 104–110, 1970.

ZUFFEREY, R.; PREISIG, O.; HENNECKE, H.; THÖNY-MEYER, L. Assembly and function of the cytochrome *cbb3* oxidase subunits in *Bradyrhizobium japonicum*. **J. Biol. Chem.** 271 (15), 9114–9, 1996.