The Role of Di-iron Proteins in Pathogen Resistance

Joana Morais Baptista

Dissertation presented to obtain the Ph.D. degree in Biochemistry at the Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa.

Supervisor: Dr. Lígia M. Saraiva

From left to right: João Vicente (4th opponent), Ricardo Louro (3rd opponent), Simon Andrews (1st opponent), Joana Baptista, Paolo De Marco (2nd opponent), Carlos Romão (President) and Lígia Saraiva (Supervisor).

25th July 2012

Second edition, September 2012

Molecular Genetics of Microbial Resistance Laboratory

Instituto de Tecnologia Química e Biológica

Universidade Nova de Lisboa

2780-157

Portugal

À memória dos meus avós Norberto, Alice e António.

Aos meus pais.

Ao Pedro.

Learn from yesterday, live for today, hope for tomorrow.
The important thing is not to stop questioning.

Albert Einstein

Acknowledgments

This dissertation would not be possible without the aid, presence, support and friendship of the people to whom I will dedicate the following pages:

First of all, and most importantly, I would like to thank my supervisor **Dr. Lígia M. Saraiva**, who made all this possible. Without her constant support, resilience, encouragement, I would not be here now writing this dissertation. For the trust and believe in me, for always providing new ideas, for always pushing me to do better and for the 24 hours availability whenever a question or a problem emerged during experiments, I have to thank Dr. Lígia for always being there. I hope these 6 years were as good for You as it were for me... Thank You for the opportunity to work here!

I have also to thank **Prof. Miguel Teixeira** for his collaboration in this work, particularly for the expertise in the analysis of the EPR experiments, and especially for the very interesting and helpful discussions.

To **Dr. Ana Melo** for the cooperation in this work, for her expertise and interesting discussions.

With no doubt my lab colleagues are also responsible for my successful work. A big Thank You goes to **Marta**, who guide me in my first steps at the lab. For all the support during my PhD, for all the advices, for all the ideas (crazy or not…) and especially for the friendship. To **André** and also to **Sara** for all the help provided in the last months. To **Lígia Nobre** for all the work in YtfE, for always helping me when I needed and for being a friend.

To **Margarida**, **Fábio**, **Catarina** and **Luís** for all the lunch talks, for all the laughs and for contributing to a good environment. To **Susana** for always listening to my crazy talks and wild advices and always pretending I was saying something "smart". Thank you for being such a good friend and for all the support whenever I was down. To **Mafalda**, as a good friend is the one that without using words she knows how you feel. For always being there, for the shoulder when I needed and for understanding me. To **Filipa**, for being my lab "husband", as she was there in the good and in the bad times, inside and outside the lab, in sunny or rainy days. For providing me with her good mood and always making me laugh when I thought it would not be possible. To all my colleagues thank you for their friendship.

To our former group members **Vera** and **Cláudia** for their help during my first months at the lab and for being good friends.

To all the people in the third floor, present and past, especially to **Metalloproteins and Bioenergetics Lab** where I always felt welcome every time I had to purify a protein. To **Ana Paula**, **Patrícia**, **Sandra**, **Liliana**, **Miguel** e **Bruno** for always providing me such nice times when I visit their lab and for all their friendship. To **Filipa Sousa** for the good advices, for being a friend and for all the moments outside ITQB. To **Sofia Venceslau** and **Bárbara Henriques** for all the chats and for receiving me in their lab when I needed their assistance.

To **Filipa Pinto** for all the moments throughout these 10 years (and I am not tired… yet!), from the University, holidays and to ITQB. It has been fantastic and I hope it continues…

To my InTeraQB partners, **Neuza**, **Filipa N.**, **Cláudia**, **Bárbara**, **Sofia V.**, **Fábio**, **João D.** for providing me good moments and making ITQB not only a work but also a social place…. I hope our project continues… A special Thank You to **João Damas** for sharing good musics and concerts and for being a friend.

To my **PhD class colleagues** for making those early months easier and for sharing their experience with me. A big Thank you for **Sofia Leite** for not being only a colleague but also a friend, a *good* friend...

I have also to thank **Prof. Simon Andrews**, for the warm welcome at his lab, for his dedication and for the helpful conversations. To the people in Reading who helped me to integrate in UK: **Joanna** and **Helen**.

Por último não posso esquecer as pessoas que sempre me apoiaram durante o meu percurso pessoal e profissional. Às minhas amigas **Filipa P.**, **Xala**, **Magui**, **Mimosa**, **Figuita**, **Russa**, **Raquel** e **Cata** por serem as minhas amigas de sempre e *para sempre*, por poder contar sempre com elas em todos os momentos, por se preocuparem comigo e por serem como são. **À Royal Family**, por serem a minha segunda família durante estes anos todos, porque nunca nos fartamos uns dos outros e porque sei que estarão lá sempre para mim. À **Cristina G.**, por ser aquela amiga que aparece à distância de um telefonema…
Sem nunca esquecer as pessoas que sempre estiveram presentes nos meus 28 anos de vida, agradeço aos meus **Tios**, **Tias**, **Primos**, **Primas**, **Priminhos** e **Priminhas**, por fazerem do meu mundo, um Mundo Melhor…

À minha **Avó Domitília** que sempre me apoiou incondicionalmente e sempre zelou pelos meus interesses. Por todos os telefonemas ao longo de todos estes anos, preocupada com o meu bem-estar mesmo quando não tinha tempo para falar. Obrigada Avó por sempre cuidares de mim…

Aos meus **avós** que já cá não estão por contribuírem para o que sou hoje…

Finalmente, às três pessoas mais importantes da minha vida: **Mãe**, **Pai** e **Pedro**. Ao meu **Pai**, pelo seu apoio incondicional, por todos os momentos marcantes da minha vida, por querer fazer de mim uma pessoa sempre melhor e por todo o seu amor. À minha **Mãe**, por me aturar todos os meus maus humores, por tornar os meus sonhos realidade, por acreditar em mim e por estar sempre mas mesmo Sempre lá.
Ao **Pedro**, por saber sempre o que fazer para me pôr feliz, por toda a sua Grande paciência, por ser o meu melhor amigo, por todo o seu amor incondicional, por toda a sua alegria de viver contagiante. Por tudo! ... E por mais ainda…

Thank U ALL!!!

Thesis **P**ublications

This dissertation is based on original publications, listed by chronological order:

1. Overton, T. W., Justino, M. C., Li, Y., **Baptista, J. M.**, Melo, A. M., Cole, J. A., Saraiva, L. M. (2008) "Widespread distribution in pathogenic bacteria of di-iron proteins that repair oxidative and nitrosative damage to iron-sulphur centres" Journal of Bacteriology, 190 (6): 2004-2013
2. Justino, M. C., **Baptista, J. M.**, Saraiva, L. M. (2009) "Di-iron proteins of the Ric family are involved in iron-sulfur cluster repair" Biometals, 22 (1): 99-108
3. **Baptista, J. M.**, Justino, M. C., Melo, A. M., Teixeira, M., Saraiva, L. M. (2012) "Oxidative stress modulates the nitric oxide defense promoted by *E. coli* flavorubredoxin " Journal of Bacteriology, 194 (14): 3611-3617

Abstract

Reactive oxygen and nitrogen species (ROS and RNS) are produced by phagocytic cells of the human immune system to attack invading pathogens due to their ability to damage DNA and the metal centres of proteins. In order to survive inside the host, bacteria activate genes that encode detoxifier enzymes, like the *Escherichia coli* nitric oxide-reductase flavodiiron protein, also known as flavorubredoxin (FlRd), and repairing proteins, such as the *E. coli* YtfE di-iron protein involved in the recovery of damaged Fe-S centres. Using *E. coli* and *Staphylococcus aureus*, the work presented in this thesis aimed at unravelling: i) the role of *E. coli* FlRd in bacteria exposed to a combination of oxidative and nitrosative stresses, ii) the identification and characterisation of *S. aureus* YtfE homologue, iii) the study of *E. coli* YtfE mechanisms that allow the repair of damaged Fe-S clusters, and iv) the identification of proteins that interact with *E. coli* YtfE.

To analyse the role of *E. coli* FlRd in cells submitted to both hydrogen peroxide and nitric oxide, the transcription and expression of *norV* was explored by means of β-galactosidase and immunoblotting assays, respectively. Under these conditions, it was observed that the *norV* transcription and expression were hindered. To identify if the lack of *norV* expression was related to its regulator, the NorR transcription factor, the gene was cloned and expressed, and the protein was purified and the binding of nitric oxide to NorR in the presence of hydrogen peroxide was studied. EPR experiments revealed that upon incubation of NorR with nitric oxide and hydrogen peroxide the oxidation promoted by H_2O_2 of the mono-iron centre of NorR impairs the ligation of nitric oxide. We also exploited the NorR ATPase activity, a requisite for FlRd activation, in the presence of

oxidative and nitrosative stress. The results revealed that, under these conditions, the ATPase activity was not triggered. The role of *E. coli* FlRd was also examined *in vivo* upon infection of macrophages with the *E. coli* wild type and the *norV* mutant strain. We showed that the contribution of flavorubredoxin to survival of *E. coli* depends on the stage of macrophage infection, and that the absence of protection observed at the early phase is related to the inhibition of the NorR activity by the oxidative burst.

In this dissertation, a homologue of *E. coli* YtfE was found in *Staphylococcus aureus* encoded by the *scdA* gene. To address its role in *S. aureus*, the *scdA* gene was disrupted and the viability of the mutant assessed, which resulted in a strain more sensitive to oxidative stress. Furthermore, upon exposure to nitric oxide and hydrogen peroxide, the *scdA* staphylococcal mutant strain exhibited more pronounced inhibition of the aconitase activity, an enzyme dependent on the integrity of [4Fe-4S]$^{2+/1+}$ clusters. *S. aureus scdA* was able to complement the *E. coli ytfE* mutant strain as it rescued the ability of the mutant to repair damaged Fe-S clusters. In contrast, the Isc (*I*ron-*s*ulphur *c*luster) or Suf (*S*ul*f*ur formation) systems, which contain proteins involved in the assembly of Fe-S clusters, were unable to complement the same mutant strain. Hence, it was concluded that *S. aureus* ScdA is involved in the repair of Fe-S clusters. A comprehensive search of the amino acid sequence database revealed that homologues of *E. coli* YtfE and *S. aureus* ScdA are found in the proteomes of a wide range of bacteria, fungi, protozoa, and in several pathogens including *Neisseria gonorrhoeae*, *Haemophilus influenzae* and *Bacillus anthracis*. Thus, this family of proteins was named Ric for *R*epair of *i*ron *c*entres.

The role of *E. coli* YtfE in the repair of oxidatively and nitrosatively damaged Fe-S clusters was also addressed by analysing the assembly of Fe-

S centres in the scaffold protein IscU and in the $[2Fe\text{-}2S]^{2+/1+}$ cluster-containing ferredoxin in the presence of YtfE. For this purpose, the cysteine desulphurase IscS, the scaffold IscU and the *E. coli* holo-YtfE proteins were recombinantly produced and purified. UV-visible and resonance Raman studies demonstrated that holo-YtfE promotes the assembly of Fe-S clusters in IscU and in the apoform of ferredoxin.

In order to identify the proteins that interact *in vivo* with *E. coli* YtfE, the bacterial adenylate cyclase two-hybrid system (BACTH) was used. This study was performed in two ways: in the first, the interaction of YtfE with specific proteins was analysed; in the second, the *E. coli* proteome was screened for YtfE interactants. Since the assembly of Fe-S clusters requires cysteine desulphurases as sulphur donors and scaffold proteins to assemble the centre, it was analysed whether YtfE interacts *in vivo* with these proteins. Hence, the cysteine desulphurases IscS and SufS, and the scaffold protein IscU were cloned in the BACTH system plasmids. The results showed that *E. coli* YtfE is able to interact with both IscS and SufS. In the second part of this study, using two libraries that covered approximately 30% of *E. coli* genome, we detected protein interactants that were confirmed by further BACTH assays to interact with *E. coli* YtfE.

Overall, this thesis has contributed to enlarge our understanding of the role of two di-iron proteins in the resistance of microbial pathogens to oxidative and nitrosative stresses which are inflicted by the host during the infection process.

Resumo

As espécies reativas de oxigénio e de azoto (ERO e ERA) são produzidas por células fagocíticas do sistema imunitário para atacar os agentes patogénicos devido à sua capacidade de danificar o ADN e os centros metálicos das proteínas. Para conseguirem sobreviver dentro do hospedeiro, as bactérias ativam genes que codificam enzimas destoxificantes, tais como a proteína flavo-di-férrica que reduz o óxido nítrico em *Escherichia co*li, também conhecida como flavorubredoxina (FlRd), e proteínas reparadoras, como a proteína di-férrica YtfE de *E. coli* que está envolvida na recuperação de centros ferro-enxofre danificados. Utilizando *E. coli* e *Staphylococcus aureus*, o trabalho apresentado nesta tese pretendeu determinar: i) o papel de FlRd de *E. coli* em bactérias expostas a uma combinação de stress oxidativo e nitrosativo; ii) a identificação e caracterização do homólogo da YtfE em *S. aureus*; iii) o estudo de mecanismos usados por YtfE de *E. coli* para reparar os centros danificados de Fe-S; e iv) a identificação de proteínas que interagem com YtfE de *E. coli*.

Para avaliar-se o papel de FlRd de *E. coli* em células tratadas com peróxido de hidrogénio e óxido nítrico, a transcrição e a expressão do gene *norV* foi analisado usando-se ensaios de β-galactosidase e immunoblotting, respectivamente. Nestas condições, observou-se que a transcrição e expressão de *norV* foram eliminadas. Para identificar se a falta de expressão de FlRd estava relacionada com o seu regulador, o fator de transcrição NorR, a proteína foi clonada, expressa, purificada e a ligação de óxido nítrico à NorR na presença de peróxido de hidrogénio foi estudada. Experiências de EPR revelaram que, após a incubação de NorR com óxido

nítrico e peróxido de hidrogénio, a oxidação do centro mono-férrico de NorR promovida por H_2O_2, prejudica a ligação do óxido nítrico. Também explorámos a atividade ATPase de NorR, um requisito para a ativação de FlRd, na presença do stress oxidativo e nitrosativo. Os resultados mostraram que, sob estas condições, a atividade ATPase não foi despoletada. O papel de FlRd de *E. coli* foi também examinado *in vivo* após a infeção dos macrófagos com a estirpe selvagem de *E. coli* e com a estirpe mutada no gene *norV*. Mostrámos assim que a contribuição de flavorubredoxina para a sobrevivência de *E. coli* depende da fase de infeção dos macrófagos, e que a ausência de proteção observada na fase inicial está relacionada com a inibição da atividade de NorR durante o stress oxidativo.

Nesta dissertação, foi encontrado um homólogo de YtfE de *E. coli* em *S. aureus* codificado pelo gene *scdA*. Para estudar a sua função em *S. aureus*, o gene *scdA* foi interrompido e a viabilidade do mutante avaliada, o que resultou numa estirpe mais sensível ao stress oxidativo. Para além disso, após exposição ao óxido nítrico e ao peróxido de hidrogénio, esta estirpe estafilocócica mutada no gene *scdA* exibiu uma maior inibição da atividade de aconitase, uma enzima dependente da integridade dos seus centros $[4Fe\text{-}4S]^{2+/1+}$. A proteína ScdA de *S. aureus* conseguiu complementar a estirpe de *E. coli* mutada no gene *ytfE* resgatando a capacidade do mutante para reparar os centros Fe-S danificados. Em contrapartida, os sistemas Isc (*I*ron-*s*ulphur *c*luster) e Suf (*Sulf*ur formation), que contém proteínas envolvidas na montagem dos centros Fe-S, foram incapazes de complementar a mesma estirpe mutante. Assim, conclui-se que a proteína ScdA de *S. aureus* participa na reparação dos centros Fe-S. Uma análise global da base de dados de amino ácidos revelou que homólogos de YtfE de *E. coli* e de ScdA de *S. aureus* existem nos proteomas de várias bactérias, fungos, protozoários, e em vários

organismos patogénicos incluindo *Neisseria gonorrhoeae*, *Haemophilus influenzae* e *Bacillus anthracis*. Deste modo, esta família de proteínas foi chamada Ric (*R*epair of *i*ron *c*entres).

O papel de YtfE de *E. coli* na reparação dos centros Fe-S danificados pelo stress oxidativo e nitrosativo foi também abordado ao analisar-se a formação de centros Fe-S na proteína molde IscU e na ferredoxina que contém centros [2Fe-2S] $^{2+/1+}$ na presença de YtfE. Com este propósito, a cisteína desulfurase IscS, a proteína molde IscU e a holo-proteína YtfE foram produzidas recombinantemente e purificadas. Estudos espectroscópicos de UV-visível e ressonância Raman demonstraram que a holo-YtfE promove a montagem de centros Fe-S na IscU e na apo-forma de ferredoxina.

Para se poder identificar as proteínas que interagem *in vivo* com YtfE de *E. coli* usou-se o sistema BACTH (*B*acterial *A*denylate *C*yclase *T*wo-*H*ybrid). Este estudo foi efetuado de duas maneiras: na primeira foi analisada a interação de YtfE com proteínas específicas; de seguida, o proteoma de *E. coli* foi sondado para se encontrar as proteínas que interagem com YtfE. Uma vez que a montagem de centros Fe-S requer cisteínas desulfurases como dadores de enxofre e proteínas molde para montar o centro, analisou-se se YtfE interage *in vivo* com estas proteínas. Assim, as proteínas cisteínas desulfurases IscS e SufS e a proteína molde IscU foram clonadas nos plasmídeos do sistema BACTH. Os resultados mostraram que YtfE de *E. coli* é capaz de interagir com IscS e SufS. Na segunda parte deste estudo, usando duas bibliotecas que cobrem aproximadamente 30% do genoma de *E. coli*, detetámos proteínas interagentes cuja interação com YtfE de *E. coli* foi confirmada por ensaios adicionais de BACTH.

No geral, esta tese contribuiu para aumentar o nosso conhecimento acerca do papel de duas proteínas que contém centros di-férricos na resistência de micróbios patogénicos aos stresses oxidativo e nitrosativo que são provocados pelo hospedeiro durante a infeção.

Abbreviations

Abs	Absorbance
AhpCF	Alkyl hydroperoxide reductase
Amp	Ampicillin
Asp	Aspartic acid
ATP	Adenosine tryphosphate
ATPase	ATP hydrolase
B.	Bacillus
BACTH	Bacterial adenylate cyclase two-hybrid system
Bfr	Bacterioferritin
CLR	C-type lectin receptor
CO	Carbon monoxide
Cya	Adenylate cyclase
Cys	Cysteine
Da	Dalton
DNA	Deoxyribonucleic acid
DNIC	Dinitrosyl-iron complex
Dps	DNA protein from starved cells
DTT	Dithiothreitol
E.	Escherichia
e.g.	*exempli gratia*, for example
eNOS	endothelial NOS
EPR	Electron paramagnetic resonance
Erm	Erythromycin
et al.	*et alia*, and other people
EXAFS	Extended x-ray absorption fine structure
FDP	Flavodiiron proteins

Fe(II)	Ferrous iron
Fe(III)	Ferric iron
Fe-S	Iron-sulphur
FlRd	Flavorubredoxin
FMN	Flavin mononucleotide
FNR	Fumarate and Nitrate reduction Regulator
Ftn	Ferritin
Fur	Ferric uptake regulator
Glu	Glutamic acid
GMP	Guanosine monophosphate
GTP	Guanosine tryphosphate
H_2O	Water molecule
H_2O_2	Hydrogen peroxide
His	Histidine
Hmp	Flavohaemoglobin
HO^-	Hydroxyl anion
$HO^\bullet$	Hydroxyl radical
HOCl	Hypochlorous acid
i.e.	*id est*, that is to say
IFNγ	Interferon γ
IHF	histone-like integration host factor
IL	Interleukin
iNOS	inducible NOS
IPTG	Isopropyl-β-D-thiogalactopyranoside
IRP	Iron-regulatory protein
Isc	Iron-sulphur cluster system
Kan	Kanamycin
kDa	kilo Dalton
LPS	Lipopolysaccharide
MerR	Mercury resistance

MPO	Myeloperoxidase
MW	Molecular weight
N.	Neisseria
NADH	β-Nicotinamide adenine dinucleotide, reduced form
NADPH	β-Nicotinamide adenine dinucleotide phosphate, reduced form
Nfu	NifU-like
Nif	Nitrogen fixation system
NK	Natural killer
NLR	Nod-like receptor
nNOS	neuronal NOS
NO	Nitric oxide
NO_2^-	Nitrite
NOR	Nitric oxide reductase
NorR	Nitric oxide reduction regulator
NOS	Nitric oxide synthase
NrfA	cytochrome c nitrite reductase formate-dependent A
NsrR	Nitrite sensitive repressor regulator
O_2	Oxygen
O_2^-	Superoxide anion
OD	Optical density
$ONOO^•$	Peroxynitrite
ONPG	o-nitrophenyl-β-D-galactopyranoside
OxyR	Oxygen stress regulator
PAMP	Pathogen-associated molecular pattern
PerR	Peroxide regulator
pH	potential Hydrogen
Phox	NADPH oxidase
PLP	Pyridoxal-phosphate
PRR	Pattern recognition receptor
Ric	Repair of iron centres

RLH	RIG-like helicases
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROO	Rubredoxin:oxygen oxidoreductase
ROS	Reactive oxygen species
RR	Resonance Raman
S.	Staphylococcus
SOD	Superoxide dismutase
SOR	Superoxide reductase
SoxRS	Superoxide regulator and sensor
Suf	Sulfur assimilation
TCA	Tricarboxylic acid
TLR	Toll-like receptor
TNF	Tumor necrosis factor
UV	Ultra-violet
XAS	X-ray absorption spectroscopy

Table of Contents

Introduction

Chapter I

Nitric Oxide, an antibacterial molecule of the immune system

Chapter II

Iron, an essential metal for the living systems

Results

Chapter III

Oxidative stress modulates the nitric oxide defense promoted by E. coli

Chapter IV

Widespread distribution in pathogenic bacteria of di-iron proteins that repair oxidative and nitrosative damage to iron-sulphur centres

Chapter V

Escherichia coli YtfE is able to promote the formation of iron-sulphur clusters

Chapter VI

Searching for YtfE interactions in the Escherichia coli proteome

Discussion

Chapter VII

Discussion and general conclusions

Introduction

Chapter I

Nitric Oxide,
an antibacterial molecule of the immune system

I **Nitric Oxide**, an antibacterial molecule of the immune system

Chapter I

Nitric Oxide, an antibacterial molecule of the immune system

"Though NO's structure is simple, nitric oxide is now regarded as the most significant molecule in the body, absolutely crucial to your well-being."

Louis Ignarro, Nobel Prize (1998)

I.1 – The innate immune system

The immune system is a mechanism involving several biological structures and processes that protects an organism against disease through recognition and elimination of the infectious agent (*1*). The immune system is divided in three levels: anatomic and physiological barriers, innate immunity and adaptive immunity (*2*).

The anatomic and physiological barriers are the first line of defense against invading microorganisms and include intact skin, surface of mucous membranes, mucociliary clearance mechanisms and low stomach pH (*1, 2*).

In contrast, the adaptive immunity occurs after several days of infection and the peak of primary adaptive response, which is characterized by lymphocyte activation and proliferation, only takes place after 5-7 days post-infection (*3*). The adaptive immune response exhibits four immunological attributes: specificity, diversity, memory and self/nonself recognition. Moreover, the adaptive immune system is centralized in two

classes of specialized lymphocytes, the T and B cells, which display an extremely diverse repertoire of antigen-specific recognition receptors that enable specific identification and elimination of pathogens, as well as adaptive immune methods that ensure tailored immune responses and long lived immunological memory against reinfection (*1*). The adaptive immunity is divided into humoral immunity, which is involved in the eradication of microbes present in the blood or fluid by generating antibodies which are produced by B-cells, and cellular immunity, that involves the suppression of cancer cells and microbes hidden inside cells mediated by killer T-cells (*3*).

While the adaptive immune system is a later response found only in vertebrates, the innate immunity occurs in eukaryotes, from humans to earthworms, acting within seconds upon encounter with a pathogen, generating a protective inflammatory response (*1*). The innate immunity consists of immunological effectors that provide robust, immediate and nonspecific immune responses, which include evolutionarily primitive humoral, cellular, and mechanical processes that play a vital role in the protection of the host from pathogenic challenge (*1*).

The pathogen recognition is the first and crucial step in innate immunity, performed by a limited arsenal of pattern recognition receptors (PRRs), like Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), and RIG-like helicases (RLH), that are able to recognize conserved structures of microorganisms called pathogen-associated molecular patterns (PAMPs) (*1-3*).

In addition, the ingestion of extracellular particulate material (such as pathogens) by phagocytosis is one of the most important innate defense mechanisms (*1*). Phagocytosis involves binding of the particle to be phagocytosed and ingestion with consequent activation of the phagocyte

(*1, 4*). This process is performed by specialized cells (the phagocytes), such as neutrophils, dendritic cells and macrophages, that engulf and kill the pathogenic microorganisms (*1, 3*). When bacteria enter into mammalian cells by phagocytosis, the innate immune sensors, like TLRs or NLRs, triggered by bacterial ligands, initiate pro-inflammatory responses and innate immune effector functions (*1, 5*). Phagocytes internalize microorganisms into specialized vesicles known as phagosomes which will acidify and fuse with lysosomes (intracellular vesicles that contain a variety of antimicrobial factors), forming a phagolysosome (*5, 6*). The antimicrobial factors generated within the phagolysosome include reactive oxygen and nitrogen species (ROS and RNS, respectively) (*1, 7*). Pathogens in this stage of infection can be divided in two types: the extracellular pathogens that survive outside host cells because phagocytosis will eliminate them, and intracellular pathogens that invade host cells within which they survive and replicate (*5*).

The phagocytic cells macrophages are the main tools of innate immunity being found in almost every tissue of the host, and are responsible for the initial killing of the majority of the engulfed bacteria. Upon infection, macrophages secrete TNFα (*T*umor *N*ecrosis *F*actor *α*) and IL-12 (*I*nter*l*eukin *12*) that lead NK (*N*atural *K*iller) cells to produce IFNγ (*I*nter*F*ero*N*-*γ*), which will result in the increase of macrophage bactericidal activity (*8-10*). These specialized phagocytes are implicated in other physiological processes, such as development of the haematopoietic system, bone remodeling and wound healing (*11, 12*).

Note that only vertebrates have the adaptive immune system, leaving most eukaryotic organisms to survive with innate immunity alone. Interestingly, new discoveries have been made regarding innate immunity that have discouraged the initial role of this system as the 'non specific'

system but rather as a critical regulator of human inflammatory disease (*2*). Now, it is clear that innate immunity is specific as it is able to discriminate self-molecules from pathogens through the evolutionary conserved receptors TLRs that also act as adjuvant receptors creating a bridge between innate and adaptive immunity (*3*). More important, innate immune responses are crucial for the initiation of adaptive immune responses in vertebrates, conferring a specific and long lasting protection (*1*).

I.1.1 – Oxidative Stress

Reactive oxygen species produced by phagocytes are necessary for efficient host defense against bacterial and fungal infections (*13*). However, the oxygen toxicity is also a problem for non-pathogen aerobic organisms as ROS are inevitable byproducts of aerobic metabolism (*14*). Reactive oxygen species are derivatives of molecular oxygen, that include singlet oxygen, superoxide anion, hydrogen peroxide and hydroxyl radicals (Figure I.1). The generation of all these reactive oxygen species is designated as oxidative burst (*15*).

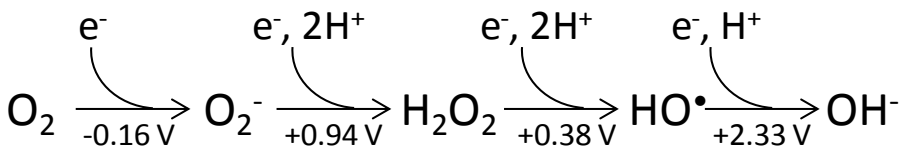


Figure I.1 – The redox states of oxygen with reduction potentials. O_2 – molecular dioxygen; O_2^- - Superoxide anion; H_2O_2 – hydrogen peroxide; $HO^\bullet$ – hydroxyl radical; OH^- - hydroxyl anion; e^- - electrons; and H^+ - protons. Adapted from (*14*).

Upon phagocytosis in macrophages, a membrane-bound oxidase is activated that reduces oxygen to superoxide anion (O_2^-), a reactive oxygen intermediate extremely toxic to ingested microorganisms. This membrane-bound oxidase, designated as Phox (NAD*PH ox*idase), pumps electrons into the phagolysosome catalyzing the following reaction: $2O_2 + NADPH \rightarrow 2O_2^- + NADP^+ + H^+$ (*16*). This enzyme is dormant in the resting phagocyte but becomes activated when the cell receives stimuli, like infectious bacteria and certain inflammatory polypeptides (*17*). Phagocytes produce superoxide in the order of 500 nM/h (*18, 19*). Phox contains haem as prosthetic group and is an important defense mechanism in microbial and parasite killing as mice deficient in Phox have enhanced bacterial burden when compared to wild type (*10, 20, 21*). Further, humans who don't possess a functional Phox suffer from immunodeficiency due to recurrent bacterial and fungal infections (*22*). Superoxide anion is a reactive compound capable of acting as an oxidant or reductant in biological systems, and can diffuse for considerable distances before it exerts toxic effects. Furthermore, extracellularly generated O_2^- can gain access to intracellular targets via cellular channels (*23*). The superoxide anion also generates other powerful oxidizing agents, including hydroxyl radicals ($OH^\bullet$) and hydrogen peroxide (H_2O_2, through superoxide dismutase enzyme) (Figure I.1) (*2*).

Another compound that is involved in microorganism killing is hypochlorous acid (HOCl) (*24-26*). This ROS is produced by myeloperoxidase (MPO), a haem-containing enzyme present in activated macrophages and neutrophils (*26*). Mice deficient in MPO are more susceptible to infection (*27*). In addition, HOCl reacts with ferrous iron to form the hydroxyl radical. Superoxide anion also reacts with ferrous iron to

form hydrogen peroxide which, subsequently, reacts with Fe(II) to form the hydroxyl radical (Fenton reaction: H_2O_2 + Fe(II) → $OH^{\bullet}$ + OH^- + Fe(III)) (Figure I.1) (*28*). All these highly toxic products are capable of killing pathogens (*16, 29*).

Hydrogen peroxide is an uncharged species and readily diffuses across cell membranes (*23*). Therefore, H_2O_2 stress emerges inside cells whenever this species is present in the extracellular space (*15*). During respiratory burst, phagocytic cells produce hydrogen peroxide in the order of 750 nM/h (*18, 19*).

Reactive oxygen species are involved in the killing of pathogens by directly reacting with lipids, proteins, DNA and cell carbohydrates (Figure I.2) (*15, 30*). The lipid peroxidation occurs essentially in biological membranes which serve as impermeable barriers and in cellular transport processes (Figure I.2). The superoxide anion and the hydroxyl radical are the ROS known to initiate the process of autocatalytic lipid peroxidation in eukaryotes, but apparently not in bacteria as most bacterial membranes lack polyunsaturated fatty acids (*18, 30, 31*). This process leads to the conversion of unsaturated lipids into polar lipid hydroperoxides, which causes increased membrane

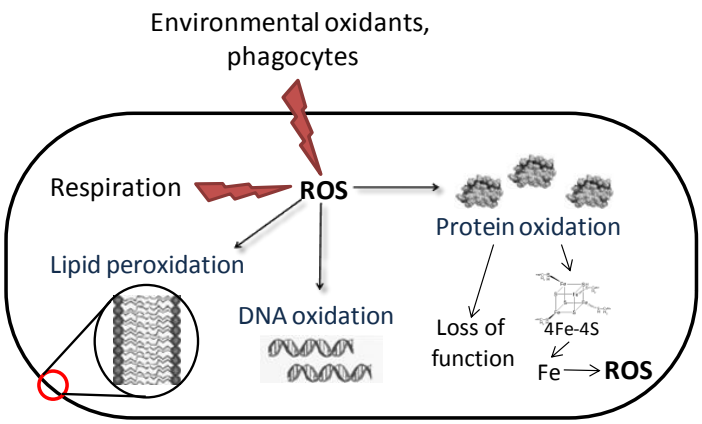


Figure I.2 – Major targets of reactive oxygen species. Adapted from (*30*).

fluidity, efflux of cytosolic solutes and loss of membrane-protein activities. Ultimately, lipid peroxidation results in the damage of the membrane and cell death (*18, 30*). Also, products of oxidized lipids initiate further oxidative damage (*14, 30*).

Although DNA does not react with ROS at physiological pH, the negatively charged phosphodiester backbone of DNA binds to metal ions, especially iron (Figure I.2). Consequently, hydroxyl radical is generated, attacking sugars, purines and pyrimidines of DNA and generating multiple products (*32, 33*). Interestingly, DNA seems to be a more important target of ROS in bacteria as the membrane lipid peroxidation through ROS is unlikely in these organisms (*30*).

Protein susceptibility to oxidative damage depends on several factors, such as the relative content of oxidation-sensitive amino acid residues (methionine, cysteine or tryptophan), the presence of metal-binding sites (e.g. Fe-S clusters, chapter II), protein localization in the cell and solvent exposition that depends upon molecular conformation (Figure I.2). The newly synthesized proteins are the most prone to oxidative damage, indicating that complete folding and incorporation into protein complexes confers protection from oxidative-driven degradation (*30*). Although the oxidative damage affects proteins of important metabolic pathways, the fact is that protein oxidation may not lead to cell death as the importance of a metabolic pathway for cell vitality can depend strongly on the environmental situation, hence some oxidation-sensitive proteins may be dispensable under some conditions, while required in other (*30*). The oxidized amino acid residues can form irreversibly carbonyl products. Carbonylated proteins are degraded by the proteosome, however there is a limit to the cell capacity to process these modified proteins, as the proteosome is also a target for oxidative inactivation. Consequently,

carbonylated proteins that are not degraded form toxic aggregated species leading ultimately to loss of cell viability (*30*).

Pathogenic microorganisms subvert or resist the action of oxidative burst through a range of strategies that also involve the up-regulation of antioxidant proteins that transform ROS into less toxic products. The scavenger enzymes for superoxide anion are SOD (*S*uper*O*xide *D*ismutase) and SOR (*S*uper*O*xide *R*eductase). Superoxide dismutation can occur spontaneously with a constant rate of 10^5 $M^{-1}.s^{-1}$, or under the catalytic influence of SOD with a constant rate of $\sim 10^9$ $M^{-1}.s^{-1}$, that varies between different organisms (*34*). Four types of prokaryotical SODs exist: the copper-zinc (Cu/Zn-SOD), the manganese (Mn-SOD), the iron (Fe-SOD) and the nickel (Ni-SOD) (*17*). Most bacteria possess Fe-SOD and Mn-SOD in their cytoplasm. The Fe-SOD is usually the constitutively expressed enzyme, while Mn-SOD is in general induced by oxygen stress (*35*). The periplasmic bacterial Cu/Zn-SOD is constitutively expressed and confers infection resistance to pathogenic bacteria (*36, 37*). Superoxide reductases are used by anaerobic organisms to scavenge superoxide anion, rather than SODs (*15, 38*). This enzyme reacts with superoxide anion with a rate constant of $\sim 10^9$ $M^{-1}. s^{-1}$, that differs depending on the organism (*39*).

For hydrogen peroxide, the pathogens possess two families of scavenger enzymes (*15, 40*): peroxidases with rate constants of 10^7-10^8 $M^{-1}.s^{-1}$ (*41, 42*), and catalases with rate constants of $\sim 10^6$ $M^{-1}.s^{-1}$ (*43*). Both families of enzymes in general catalyze the reaction: $RH_2 + H_2O_2 \rightarrow R + 2H_2O$, where $R=O_2$ for catalases. One well known peroxidase is AhpCF (*A*lkyl *h*ydro*p*eroxide reductase) an important scavenger of hydrogen peroxide in several bacteria (*44*). Catalases and peroxidases exist in several pathogens such as in *Mycobacterium tuberculosis*, *Staphylococcus aureus*,

Helicobacter pylori, *Streptomyces sp.*, *Leishmania major* and *Escherichia coli* (*15, 45*). Known bacterial species produce more than one catalase and several peroxidases that can scavenge H_2O_2, being able to handle the ROS that results from oxidative burst (*18*).

To resist ROS, bacteria sense these molecules through specific regulators whose regulons include genes encoding proteins involved in the protection against oxidative burst. The OxyR (*Oxy*gen stress *R*egulator) regulon includes genes encoding catalase and AhpC, glutathione reductase and glutaredoxin that restores intracellular thiol/disulphide balance, proteins involved in DNA protection (Dps, chapter II), and important regulators such as *fur* (*f*erric *u*ptake *r*egulator, Chapter II) (*46-48*). PerR (*Per*oxide *R*egulator) is another major regulator of the peroxide stress resistance present in Gram-positive and Gram-negative microorganisms (*49*). Its regulon includes genes encoding catalases and peroxidases, genes involved in haem biosynthesis, regulators as *fur* and the zinc uptake system (*49, 50*). SoxRS (*S*uper*ox*ide *R*egulator and *S*ensor) is a well-distributed bacterial regulator that responds to superoxide stress by inducing the expression of superoxide scavenger proteins and enzymes that repair damaged proteins and DNA (see also I.2) (*15, 51*).

I.1.2 – Nitrosative Stress

At first, nitric oxide (NO) was considered to be merely an atmospheric pollutant. However, in 1992, NO was designated as "the molecule of the year" due to its role as a biological regulator and since then various aspects of its biology have been reviewed extensively.

Nitric oxide is a small soluble radical molecule (30 Da) that can stabilize its unpaired electron by reacting with species containing other unpaired electrons or by interacting with the *d*-orbitals of transition metals (e.g. iron) (*52*). NO is soluble in water (1.6 mM at 37ºC), where it has a short half-life, between 3 and 20 s (*53, 54*). Reactive nitrogen species (RNS) include species derived from nitric oxide (Figure I.3), that arise in physiological environments, and include NO_2^- (nitrite), S-nitrosothiols, peroxynitrite ($ONOO^•$), dinitrosyl-iron complexes (DNIC, chapter II), among others (Figure I.3) (*55*). NO and related nitrogen oxides are endogenous regulators of cell and tissue function and have a role in various body functions, including the vasodilatation of smooth muscle, neurotransmission, regulation of wound healing and non-specific immune responses to infection, host defense and cytotoxicity (*52, 56*). Peroxynitrite, the product of the reaction of NO and superoxide, is involved in inflammation (*57*) and in bacteria killing (*10, 58-60*).

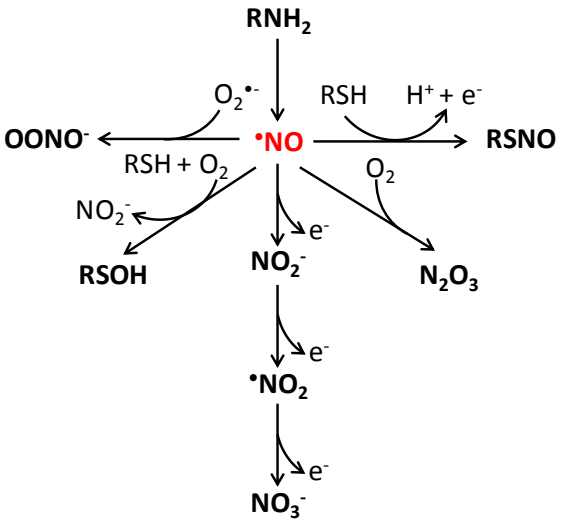


Figure I.3 – Reactive nitrogen species formation. RNH_2 – guanidino nitrogen of L-arginine, RSH - sulphydril, H^+ - proton, e^- - electron, RSNO - nitrosothiol, •NO – nitric oxide, O_2^- - superoxide anion, $ONOO^-$ - peroxinitrite, O_2 - dioxygen, NO_2^- - nitrite, RSOH – sulfenic acid, $•NO_2$ – nitrogen dioxide, NO_3^- - nitrate. Adapted from (*55*).

In mammalian cells, nitric oxide is produced by NOS (*N*itric *O*xide *S*ynthase). This class of enzymes is widely distributed throughout the mammalian tissues. Endothelial (eNOS, constitutive), neuronal (nNOS, constitutive) and inducible (iNOS) isoforms are prompted to convert arginine into nitric oxide (L-arginine + O_2 + NADPH $\rightarrow$ NO + L-citrulline + $NADP^+$) (*57*). The NOS isoforms are soluble and contain flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and haem iron as prosthetic groups, requiring also the cofactor tetrahydrobiopterin (BH_4) (*61*). The constitutive forms are low-activity enzymes that produce small amounts of NO as a signaling molecule (*62*). The inducible form is an enzyme produced by phagocytes when they are stimulated (*63*).

When macrophages are activated with bacterial cell-wall components, such as lipopolysacharide (LPS), together with a T-cell-derived cytokine (IFN-γ), they express high levels of inducible nitric oxide synthase (*64-68*). iNOS is present in other phagocytic cells of the immune system, such as dendritic cells and natural killer cells (*69*). The activity of iNOS affords effective protection against infection (*69*). The NO produced diffuses across membranes and can kill or inhibit a broad range of organisms, such as bacteria, fungi, parasitic worms and protozoa, persisting for a longer period of time, when compared to the short-lived oxidative burst (*31, 55, 70*). The most important targets of nitric oxide and RNS are protein thiols, aromatic amino acid residues, metal centres, lipids and nucleotide bases (DNA), resulting in the blockage of essential microbial physiological processes such as respiration and DNA replication (*71-76*).

Mice deficient in iNOS are more susceptible to infection by many intracellular pathogens like *Mycobacterium tuberculosis*, *Listeria monocytogenes*, *Leishmania spp.*, *Salmonella enterica*, *Candida albicans*,

Porphyromonas gingivalis, *Trypanosoma cruzi* and *Plasmodium falciparum*, when compared to the wild type (*10, 55, 77-85*).

Interestingly, nitric oxide synthase enzymes were also found to be produced by bacteria. As occurs for their homologues in mammals, all the bacterial NOS possess a haem ring and are able to convert L-arginine to nitric oxide, but in contrast they lack the associated NOS_{red} (C-terminal flavoprotein reductase domain) (*86-91*). Moreover, the NO release rates are lower when compared to mammalian NOS (*86*). Homologues were found mostly in Gram-positive bacteria (e.g. *Streptomyces* sp., *Bacillus* sp. and *Sthapylococcus* sp.) and so far in one Gram-negative bacterium (*Sorangium cellulosum*) (*87*). In bacteria, this enzyme is able to provide protection against oxidative and nitrosative stress, as expression of bacterial NOS will result in production of small concentrations of NO, which leads to the activation of sensors that respond to nitric oxide (see I.2). The result is the activation of regulons, which include genes encoding enzymes that participate in protection against oxidative and nitrosative stress (*87, 90, 91*).

I.2 – Bacterial response to nitrosative stress

The ability to scavenge or detoxify NO produced by phagocytic cells is a survival characteristic in several microorganisms that depends on genetic, biochemical and physiological factors. Bacteria possess many mechanisms of defence against nitrosative stress. One of these strategies involves bacterial regulators/sensors that will activate large-scale changes in global gene expression, as well as detoxification systems for nitric oxide

(*92*). Till now several transcriptional regulators that contain different transition metal cofactors had been identified, from Fe-S clusters to haem centres, which control gene expression towards the stimuli nitric oxide.

Fe-S clusters containing-regulators that respond to nitric oxide are SoxR, FNR (*F*umarate and *N*itrate reduction *R*egulator) and IscR (*I*ron-*S*ulphur *C*luster assembly system *R*egulator). SoxR belongs to the MerR (*Mer*cury *R*esistance) family, regulates the stress response to superoxide and nitric oxide and contains a [2Fe-2S] cluster (*93, 94*). SoxR switches on the transcription of the gene encoding the regulator SoxS, which consequently activates the expression of SoxRS regulon members. The SoxRS switches on in response to NO, inducing the expression of genes encoding a superoxide dismutase, a DNA repair enzyme (endonuclease IV), and a nitroreductase, among others (*95, 96*).

The FNR subfamily members belong to a large family of widely distributed regulators that control gene expression in response to nitric oxide (and other ligands such as oxygen) activating the transcription of NO-related genes (*94*). The cofactor in this regulator differs between organisms, as a Fe-S cluster was identified in *E. coli*, whereas in other bacteria this regulator has a haem group (e.g. *Pseudomonas* sp. DNR) (*93, 94*). The most studied regulator of this family is FNR that contains a [4Fe-4S] cluster that forms DNICs (*Di**N**i*trosyl *I*ron *C*omplex*es*, see Chapter II) upon reaction with NO (*93, 94*). FNR controls gene expression during anaerobic growth and one of the genes from the *E. coli* FNR regulon is the important NO-detoxifying protein flavohaemoglobin, whose characteristics will be further discussed (II.3.1/2). Moreover, *Neisseria gonorhoeae* FNR regulates *aniA*, a gene coding a nitrite reductase (*93, 94*). Also, in *Campylobacter jejuni*, the NssR (*N*itro*s*ative *s*tress *R*egulator, an FNR family member) regulon includes two genes encoding globin-like proteins, which

are important for nitric oxide detoxification (*97*). Another important Fe-S cluster-containing regulator that senses NO is IscR whose role and characteristics will be further discussed in Chapter II.

NsrR (*N*itrite *s*ensitive *r*epressor *R*egulator) is a regulator of NO metabolism in gamma- and beta-proteobacteria but also in Gram-positive bacteria like *Bacillus* and *Streptomyces* species (*98*). This transcription factor binds a Fe-S centre through a sequence motif that contains three conserved cysteine residues (*99-101*). NsrR contains in its regulon genes encoding flavohaemoglobin and nitrite reductase proteins (*98, 102*).

The major sensor of nitric oxide in eukaryotes is the soluble guanylate cyclase which contains a haem cofactor that binds NO, resulting in cyclization of GTP to cyclic GMP, which leads to the activation of several pathways. The haem is located in a domain that displays a high sequence identity to a family of sensors designated as H-NOX (*H*aem *N*O/*OX*ygen binding) which are widespread in Bacteria, like *Vibrio chloraea*, *Clostridium botulinum*, *Shewanella oneidensis* and *Thermoanaerobacter tencongensis* (*93, 94*). The physiological role of these regulators is poorly understood in bacteria; nevertheless, two studies carried out in *Legionella pneumophila* and *Vibrio fischeri* showed that H-NOX regulates biofilm formation and iron metabolism, respectively (*103, 104*). *Mycobacterium tuberculosis* contains the DosRST (*Do*rmancy *s*urvival) system. Most DosR-regulated genes are induced in activated macrophages in a NO synthase-dependent manner, and the DosRST regulon includes *glbN* encoding a truncated haemoglobin which has a role in NO detoxification (*93, 94*).

Chapter II will describe the role of the regulatory protein Fur that contains a non-haem iron centre as cofactor and is widely distributed in

bacteria. This regulator is inactivated upon exposure to NO due to the formation of DNIC species leading to derepression of the Fur regulon (*105*). Furthermore, the regulon of PerR (a Fur homologue) is also derepressed upon exposure to nitric oxide *in vivo* (*106*).

The regulatory and biochemical properties of the specific NO regulator, NorR, will be discussed in the flavodiiron proteins subchapter.

I.3 – NO detoxification systems

Among several mechanisms used by bacteria to resist nitrosative stress are the nitric oxide detoxification systems. In enteric bacteria, three proteins were identified as NO detoxificants: the periplasmic cytochrome c *n*itrite *r*eductase *f*ormate-dependent, NrfA; the flavorubredoxin, FlRd and its associated oxidoreductase, NorW; and the flavo*h*ae*m*oglobin *p*rotein, Hmp.

I.3.1 – Flavodiiron family of proteins

Flavodiiron proteins (FDP) represent a large family of enzymes, widespread among Archaea, Bacteria and Protozoa, which contain a conserved two-domain structural core, built by a metallo-β-lactamase-like-domain at the N-terminal region harbouring a non-haem diiron site, and a flavodoxin-like domain, containing a FMN moiety (*107, 108*). FDPs possess NO reductase activity and are involved in microbial resistance to nitric oxide (*107, 108*). Although, the two-domain structural core is conserved in this family of proteins, several members have extra domains fused at the C-terminal (*107, 108*). Therefore, depending on the domain composition, the

proteins were divided into four classes, from A to D: class A in which proteins contain only the two domain core; class B with enzymes that have an extra rubredoxin-like domain containing a $FeCys_4$ binding motif; class C where proteins have an additional module that contains significant similarities to NAD(P)H:flavin oxidoreductases; and class D with proteins containing NAD(P)H:rubredoxin oxidoreductase and rubredoxin modules fused to flavodiiron core (*107-109*).

The first reports of a protein from FDP class A arose with work on *Desulfovibrio gigas*. The protein was named ROO (*R*ubredoxin:*o*xygen *o*xidoreductase) and was shown to accept electrons directly from the rubredoxin partner (*107, 108*). ROO has NO reductase activity, its deletion results in sensitivity to NO and the expression of this enzyme is able to complement the *norV* (encoding a FDP class B enzyme) mutant of *E. coli* under anaerobic conditions (*110*).

The class C enzymes were so far found in cyanobacteria and some eukaryotic oxygenic phototrophic organisms (*107, 111*). In this class of enzymes, the long electron transfer chains are not required, as the fusion of the NAD(P)H:flavin oxidoreductases module to the flavodiiron core allows the protein to accept electrons directly from NAD(P)H and perform several intra-molecular electron transfer steps onto the diiron centre, which reduces the diatomic substrate. This enzymes are proposed to reduce dioxygen to water, avoiding the formation of ROS (*112*). A recent study in *Synechocystis sp.* showed that FDP proteins of class C are induced upon nitrosative stress (*113*).

The class D enzymes were found so far encoded in the genomes of *Clostridiales* and of the pathogen *Trichomonas vaginalis*; further studies are required to understand the role of these protein in NO protection.

Class B enzymes are more widespread when compared to class C and D. The first enzyme of this family of proteins shown to bind NO and to possess a quite considerable NO reductase activity was FlRd from *E. coli* encoded by *norV* (*114, 115*). *norV* is in an operon with *norW* which encodes for the NADH-dependent flavorubredoxin reductase. This gene organisation was thus far observed in all enterobacterial genomes (*107*).

Nitric oxide reductases are enzymes that catalyze the two electron reduction of NO to N_2O: $NADH + 2NO \rightarrow N_2O + H_2O + NAD^+$, and in enterobacteria this activity is performed under microaerobic or anaerobic conditions (*116, 117*). *E. coli* flavorubredoxin is a cytoplasmatic protein that possesses a NO reductase activity with a turnover of 15-20 mol of NO.mol $enzyme^{-1}.s^{-1}$ (*114*). The mechanism studied in this organism, by Vicente et al., revealed that flavorubredoxin reductase rapidly shuttles electrons between NADH and FlRd, which are necessary for the NO reduction (*118*). The electron accepting site in FlRd, the rubredoxin centre, is in very fast redox equilibrium with flavin mononucleotide (*118*). Moreover, structural studies suggest that *E. coli* rubredoxin domain of FlRd acts independently being freely available to participate in redox reactions with other protein partners (*119*).

E. coli and *Salmonella enterica* strains mutated in *norV* were more sensitive to NO donors under anaerobic conditions (*116, 120*). The expression of the *norVW* operon is induced by RNS, anaerobically and aerobically, during nitrate/nitrite respiration under the regulation of FNR and the nitrate/nitrite responsive regulators NarL/NarP (*121, 122*). Moreover, *norV* is upregulated in macrophage-internalized *Salmonella sp.* at a time that corresponds to the NO burst (*123*), while the loss of FlRd did not reduce the ability of bacteria to survive within macrophages, up to this

point (*95*). In enterobacteria, the operon *norVW* is located upstream of the divergently transcribed gene *norR* that encodes for the nitric oxide regulator NorR (Figure I.4). *E. coli norVW* is regulated by NorR (*92, 95, 124, 125*) and studies showed that depletion of *norR* causes a similar phenotype as the deletion of *norV*, and completely abolishes the nitrosative induction of *norVW* (*92, 116, 126-128*).

NorR is a NO sensor in bacteria and a member of the bacterial enhancer-binding protein family (bEBP) (*125, 129, 130*). This regulator is a tri-partite σ^{54}-dependent protein constituted by the following domains: a C-terminal DNA-binding HTH (helix-turn-helix) domain that binds to the conserved sequence 80–150 bp upstream of the bacterial promoter; a central domain belonging to AAA^+ family, responsible for ATPase activity and interaction with σ^{54} subunit of RNA polymerase; and a N-terminal regulatory GAF domain (*G*MP-regulated cyclic nucleotide phosphodiesterase, *A*denyl cyclase and *F*hlA) that is required for the protein activity by binding ATP and which contains a mono-nuclear iron centre (Figure I.4A) (*128, 131*). The NorR non-haem mono-iron centre has a distorted octahedral symmetry and is coordinated by three aspartate residues, an arginine and a cysteine (*132, 133*). The activation of this sensor requires the formation of a mononitrosyl-iron complex in the N-terminal GAF domain and the *Kd* binding of NO to *E. coli* NorR is 50 nM (*129*).

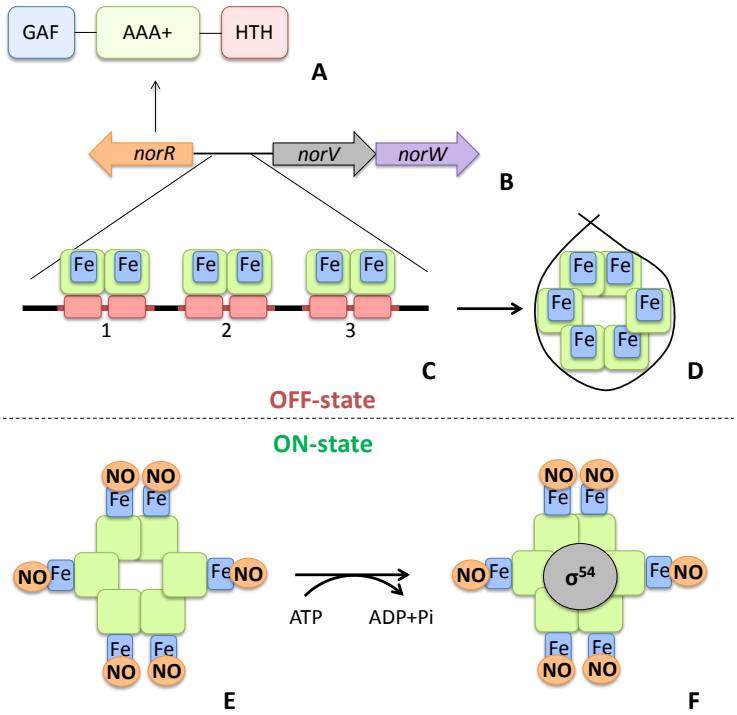


Figure I.4 – Model of NorR-dependent activation of *norVW*. (**A**) NorR contains the regulatory GAF domain (blue), the AAA$^+$ domain (green) and DNA-binding domain (light red). (**B**) *norR* (orange arrow) is located upstream of the *norVW* operon (gray and purple arrows). (**C**) The intergenic region between *norR* and *norVW* contains three NorR binding sites (dark red, 1, 2 and 3). NorR binds to the DNA, through the HTH domain (light red), in the absence of NO; the N-terminal GAF domain (blue) represses the activity of the AAA$^+$ domain (green). (**D**) The binding of NorR to the three binding sites induces the formation of an oligomer. (**E**) In the presence of NO (ON-state), nitric oxide (orange) binds to the iron center in the GAF domain (blue), occurring relieve of the repression of the AAA$^+$ domain (green). (**F**) NorR catalyzes the hydrolysis of ATP necessary for remodeling of σ^{54}-RNA polymerase (grey) that results in transcription initiation. Adapted from (*128, 129, 137*).

Three NorR binding sites were identified in the intergenic region between *norR* and the divergently transcribed genes (Figure I.4C) (*134, 135*). The NorR binding sites domain comprises a GT-(N_7)-AC motif flanking an AT-rich central region. All these binding sites need to be occupied for NO induction of the *norV* genes (*128*) as disruption of any of the three NorR binding sites prevents activation of *norV* expression by NorR (*136*). Furthermore, NorR binds to the three binding sites cooperatively (Figure I.4C) (*135*). Recent work, by Tucker et al., revealed that the three binding sites are required for NorR-dependent catalysis of open complex formation by σ^{54} RNA polymerase holoenzyme (E^{54}) (*137*). In addition, the formation of NorR oligomers is necessary for maximal ATPase activity of NorR, which is necessary to remodel the closed E^{54} and allow melting of the promoter DNA (*137*). A model was proposed in which NorR binds to the DNA sites in the absence of NO and the N-terminal GAF domain negatively regulates the activity of the AAA^+ domains by preventing access to σ^{54} (Figure I.4C/D) (*131*). The binding of NorR to the three DNA sites induces conformational changes that stimulate the formation of a higher-order oligomer (Figure I.4D) (*131*). In the presence of NO, nitric oxide binds to the iron centre in the GAF domain forming a mononitrosyl iron species. The repression of the AAA^+ domain is relieved, enabling ATP hydrolysis by NorR, coupled to conformational changes in the AAA^+ domain (Figure I.4E) (*131*). Then, the interaction and remodeling of σ^{54}-RNA polymerase occurs, leading to the formation of an open complex and transcription initiation (Figure I.4F) (*131*).

I.3.2 – Globin family – Flavohaemoglobin

Globins are an ancient and heterogeneous group of proteins found in all kingdoms of life. These proteins have a highly-conserved α-helical 'globin fold' and contain a *b*-type haem as cofactor. Microbial globin family encloses three classes: the single domain bacterial haemoglobins, the truncated haemoglobins (trHb) and the flavohaemoglobins (Hmp). The classes share high sequence homology and structural similarity in their globin domain, but the physiological role of globins varies among organisms (*138*). Globins can be found in both intracellular and extracellular compartments and encounter widely varying levels of NO and O_2 (*138*).

The first class of globins is typified by *Vitreoscilla* globin (Vgb), the first bacterial globin isolated, that contains two haem b groups per molecule (*139*). Single domain bacterial haemoglobins are also found in *Aquifex aeolicus*, *Campylobacter* and *Clostridium* (*138*). These proteins do not exhibit NO-consuming activity in the presence of NADH when assayed in cell-free extracts, as they do not contain a reductase domain (*138*). Nevertheless, a role for NO protection was shown for the globin CgB of *Campylobacter jejuni* and *Campylobacter coli*, upon experiments showing that gene expression is induced upon exposure to nitrosative stress, and that *C. jejuni cgB* mutant shows higher sensitivity to NO releasing compounds (*97, 140*).

Truncated haemoglobins possess 20-40 less residues than the single-domain haemoglobins. The function of these proteins is not completely understood. However, several trHbs have been implicated in tolerance to nitrosative stress and others appear to be involved in

respiration (*138*). One example of proteins from this class is *Mycobacterium tuberculosis* and *Salmonella* sp. HbN that confer protection against macrophages producing NO (*138*).

Flavohaemoglobins

The main difference between flavohaemoglobin class and the rest of the family is an additional C-terminal flavin-containing oxidoreductase domain. The flavohaemoglobin class is formed by a very homogenous group of proteins that shares highly conserved active sites in both the haem- and flavin-binding domains (*141*). Flavohaemoglobins are widespread in Bacteria where they play a role in virulence, and homologues exist in the protozoa (e.g. *Giardia intestinalis*) and also in fungi (*142-145*).

Hmp acts as NO dioxygenase under aerobic conditions, catalyzing the following reaction: $2^{\bullet}NO + 2O_2 + NADPH \rightarrow 2NO_3^- + NADP^+ + H^+$, where the C-terminal FAD-containing reductase domain uses the reducing power of cellular NAD(P)H to regenerate the ferrous haem (*146-148*). This enzyme was also shown to act as NOR (nitric oxide reductase) under conditions of low partial pressures of oxygen (*148*). In addition, *E. coli* Hmp was able to act as an alkylhydroperoxide reductase interacting with membrane lipids (*149, 150*). Both NO dioxygenase and NO reductase activities were shown to be important for the Hmp NO stress resistance (*92, 147, 151, 152*). In contrast to typical Hmps, *S. aureus* flavohaemoglobin, which has 30% similarity with *E. coli* Hmp, acts rather as NO reductase and is important in a microaerobic environment (*153, 154*).

In *E. coli*, the role of Hmp in resistance to nitrosative stress was demonstrated using viability (*155*), cell respiration (*72*) and macrophage killing assays (*156*). In *Salmonella* sp., *Yersinia pestis* and *S. aureus*, *hmp* is important for bacterial viability and NO detoxification in macrophages (*143-145, 157, 158*). Hmp also protects *Pseudomonas aeruginosa*, *Erwinia crhysanthemi*, *Bacillus subtilis* and *Cryptococcus neoformans* from nitrosative stress generated by different donors (*159-162*). Moreover, in yeast, a strain mutated in *hmp* shows NO accumulation (*163*).

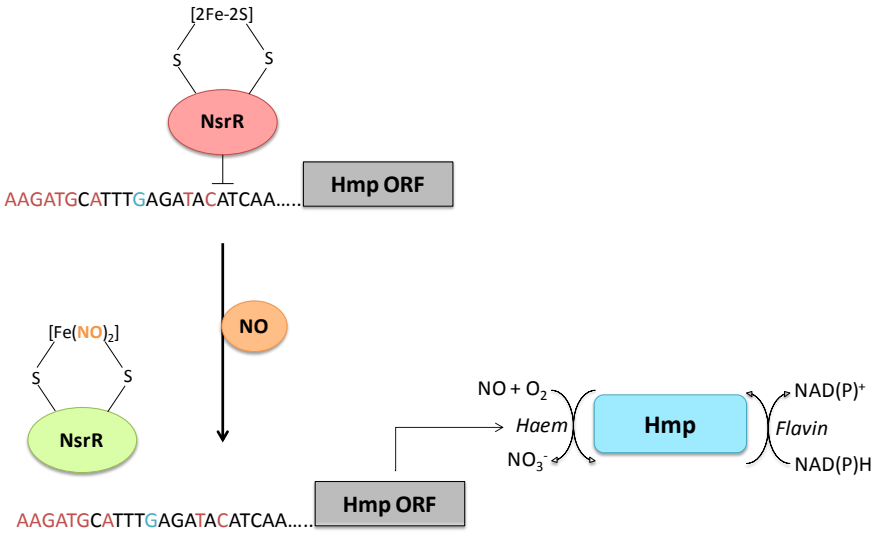


Figure I.5 – The regulation of Hmp by NsrR. The *hmp* transcription start site and consensus NsrR-binding sites are shown in blue and red, respectively. Nitric oxide binds to the Fe-S cluster of NsrR. Binding of NO to the NsrR Fe-S cluster leads to derepression of the Hmp promoter and increased expression of Hmp. Flavohaemoglobin protects microbes from NO attack through its dioxygenase activity under aerobic conditions. Adapted from (*147*).

hmp is induced by nitrosative stress (*106, 159, 164-166*), in stationary cell growth (*167, 168*), with low levels of iron (*164*), in the presence of paraquat (*167*) and upon oxygen limitation (*169, 170*). Its

regulation is complex, as it involves several transcription factors whose nature depends on the microorganism. The best understood and most highly conserved Hmp-regulating transcription factor is the global repressor NsrR. NsrR is the regulator of Hmp response to NO donors, acting as repressor in *Salmonella* sp. (*144, 171*), *B. subtilis* (*106, 161*) and *E. coli* (*172*), under aerobic and anaerobic conditions (Figure I.5).

The promoter region of *hmp* contains a FNR binding site and this regulator represses anaerobically the *hmp* transcription (*164*). Fur is considered a weak repressor of *hmp* in *E. coli* and *Salmonella* sp. with its effects probably indirect (*173*). The role of MetR as a regulator of Hmp is not well defined (*125, 155*). In *B. subtilis*, apart from NsrR, Hmp is regulated by the two-component ResDE (histidine kinase, ResE and response regulator, ResD), a transduction system induced by oxygen limitation and NO (*161, 174*). In *S. aureus*, regulation of *hmp* expression by NO is also dependent on a ResDE homologue, the regulator SrrAB (*S*taphylococcal *r*espiratory *r*esponse) (*158*).

I.3.3 – Nitrite Reductases

In general, nitrite reductases are enzymes that are able to reduce nitrite and belong to the denitrification pathway, an important step of the nitrogen cycle. Besides this primary function as nitrite reductase, some proteins also exhibit nitric oxide reductase activity.

E. coli NrfA was the first nitrite reductase enzyme recognized to perform NO reduction (*175*). This protein possesses a pentahaem cytochrome c and catalyzes the six-electron reduction of nitrite (*176*). NrfA homologues are expressed in the periplasm of a wide range of Gamma,

Delta and Epsilon proteobacteria. The importance of NrfA in the metabolism of NO by *E. coli* was revealed by the higher sensitivity of *nrfA* mutant to NO under anaerobic conditions when compared to the *wt* strain (*177*). The *nrfA* mutant of *Haemophilus influenzae* also showed higher sensitivity, and the double mutant Δ*nrfA* Δ*norV* of *Salmonella enterica* serovar Typhimurium was unable to grow in the presence of NO donors under anaerobic conditions (*178, 179*). The anaerobic nitric oxide detoxification by NrfA involves a five-electron reduction of NO, with rates comparable to bacterial respiratory nitric oxide reductases, like NorBC from *Paracoccus denitrificans* (*180, 181*). The NO reduction by NrfA in *E. coli* has a *Km* of 300 μM (pH=7) which is higher than the one measured for FlRd and Hmp (*175*). Additionally, in *E. coli*, the *nrf* operon is regulated by the NO-sensitive repressor NsrR (*182*), though no regulation with NO was observed (*95, 121*). However, in *H. Influenzae*, the *nrfA* is regulated by FNR upon exposure to NO donors (*178*).

A recent study evaluated the importance of the three NO detoxifying proteins in enterobacteria, FlRd, Hmp and NrfA. Single mutants defective in *norV*, *hmp* and *nrfA* and even the mutant Δ*norV* Δ*hmp* Δ*nrfA* reduced NO at the same rate as the parental strain. Therefore, alternative mechanisms of NO reduction in enterobacteria remain to be discovered (*183*).

The work presented in this dissertation gives an important contribution to the knowledge on flavorubredoxin and its regulator NorR in NO protection upon macrophage infection (Chapter III).

II.4 – References

(1) Goldsby, R. A., Kindt, T. J., Osborne, B. A., and Kuby, J. (2003) *Immunology*, W. H. Freeman and Company, New York.

(2) Turvey, S. E., and Broide, D. H. (2010) Innate immunity. *J Allergy Clin Immunol 125*, S24-32.

(3) Akira, S. (2011) Innate immunity and adjuvants. *Philos Trans R Soc Lond B Biol Sci 366*, 2748-55.

(4) Underhill, D. M., and Ozinsky, A. (2002) Phagocytosis of microbes: complexity in action. *Annu Rev Immunol 20*, 825-52.

(5) Radtke, A. L., and O'Riordan, M. X. (2006) Intracellular innate resistance to bacterial pathogens. *Cell Microbiol 8*, 1720-9.

(6) Stuart, L. M., and Ezekowitz, R. A. (2005) Phagocytosis: elegant complexity. *Immunity 22*, 539-50.

(7) Segal, A. W. (2005) How neutrophils kill microbes. *Annu Rev Immunol 23*, 197-223.

(8) Tripp, C. S., Wolf, S. F., and Unanue, E. R. (1993) Interleukin 12 and tumor necrosis factor alpha are costimulators of interferon gamma production by natural killer cells in severe combined immunodeficiency mice with listeriosis, and interleukin 10 is a physiologic antagonist. *Proc Natl Acad Sci U S A 90*, 3725-9.

(9) Hsieh, C. S., Macatonia, S. E., Tripp, C. S., Wolf, S. F., O'Garra, A., and Murphy, K. M. (1993) Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. *Science 260*, 547-9.

(10) Darrah, P. A., Hondalus, M. K., Chen, Q., Ischiropoulos, H., and Mosser, D. M. (2000) Cooperation between reactive oxygen and nitrogen intermediates in killing of Rhodococcus equi by activated macrophages. *Infect Immun 68*, 3587-93.

(11) Morrissette, N., Gold, E., and Aderem, A. (1999) The macrophage--a cell for all seasons. *Trends Cell Biol 9*, 199-201.

(12) Laskin, D. L., Weinberger, B., and Laskin, J. D. (2001) Functional heterogeneity in liver and lung macrophages. *J Leukoc Biol 70*, 163-70.

(13) Nauseef, W. M. (2007) How human neutrophils kill and degrade microbes: an integrated view. *Immunol Rev 219*, 88-102.

(14) Imlay, J. A. (2003) Pathways of oxidative damage. *Annu Rev Microbiol 57*, 395-418.

(15) Imlay, J. A. (2008) Cellular defenses against superoxide and hydrogen peroxide. *Annu Rev Biochem 77*, 755-76.

(16) Hii, C. S., and Ferrante, A. (2007) Regulation of the NADPH oxidase activity and anti-microbial function of neutrophils by arachidonic acid. *Arch Immunol Ther Exp 55*, 99-110.

(17) Babior, B. M. (2000) Phagocytes and oxidative stress. *Am J Med 109*, 33-44.

(18) Slauch, J. M. (2011) How does the oxidative burst of macrophages kill bacteria? Still an open question. *Mol Microbiol 80*, 580-3.

(19) Fang, F. C. (2011) Antimicrobial actions of reactive oxygen species. *MBio 2*.

(20) Endres, R., Luz, A., Schulze, H., Neubauer, H., Futterer, A., Holland, S. M., Wagner, H., and Pfeffer, K. (1997) Listeriosis in p47(phox-/-) and TRp55-/- mice: protection despite absence of ROI and susceptibility despite presence of RNI. *Immunity 7*, 419-32.
(21) Minakami, R., and Sumimotoa, H. (2006) Phagocytosis-coupled activation of the superoxide-producing phagocyte oxidase, a member of the NADPH oxidase (nox) family. *Int J Hematol 84*, 193-8.
(22) Baehner, R. L., and Nathan, D. G. (1967) Leukocyte oxidase: defective activity in chronic granulomatous disease. *Science 155*, 835-6.
(23) Miller, R. A., and Britigan, B. E. (1997) Role of oxidants in microbial pathophysiology. *Clin Microbiol Rev 10*, 1-18.
(24) Vladimirov, Y. A., and Proskurnina, E. V. (2009) Free radicals and cell chemiluminescence. *Biochemistry (Mosc) 74*, 1545-66.
(25) Aratani, Y., Kura, F., Watanabe, H., Akagawa, H., Takano, Y., Ishida-Okawara, A., Suzuki, K., Maeda, N., and Koyama, H. (2006) Contribution of the myeloperoxidase-dependent oxidative system to host defence against Cryptococcus neoformans. *J Med Microbiol 55*, 1291-9.
(26) Klebanoff, S. J. (2005) Myeloperoxidase: friend and foe. *J Leukoc Biol 77*, 598-625.
(27) Brovkovych, V., Gao, X. P., Ong, E., Brovkovych, S., Brennan, M. L., Su, X., Hazen, S. L., Malik, A. B., and Skidgel, R. A. (2008) Augmented inducible nitric oxide synthase expression and increased NO production reduce sepsis-induced lung injury and mortality in myeloperoxidase-null mice. *Am J Physiol Lung Cell Mol Physiol 295*, L96-103.
(28) Nappi, A. J., and Vass, E. (2002) Interactions of iron with reactive intermediates of oxygen and nitrogen. *Dev Neurosci 24*, 134-42.
(29) von Kockritz-Blickwede, M., and Nizet, V. (2009) Innate immunity turned inside-out: antimicrobial defense by phagocyte extracellular traps. *J Mol Med (Berl) 87*, 775-83.
(30) Avery, S. V. (2011) Molecular targets of oxidative stress. *Biochem J 434*, 201-10.
(31) Fang, F. C. (2004) Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nat Rev Microbiol 2*, 820-32.
(32) Cabiscol, E., Tamarit, J., and Ros, J. (2000) Oxidative stress in bacteria and protein damage by reactive oxygen species. *Int Microbiol 3*, 3-8.
(33) Salmon, T. B., Evert, B. A., Song, B., and Doetsch, P. W. (2004) Biological consequences of oxidative stress-induced DNA damage in Saccharomyces cerevisiae. *Nucleic Acids Res 32*, 3712-23.
(34) Liochev, S. I., and Fridovich, I. (2007) The effects of superoxide dismutase on H2O2 formation. *Free Radic Biol Med 42*, 1465-9.
(35) Geslin, C., Llanos, J., Prieur, D., and Jeanthon, C. (2001) The manganese and iron superoxide dismutases protect Escherichia coli from heavy metal toxicity. *Res Microbiol 152*, 901-5.
(36) De Groote, M. A., Ochsner, U. A., Shiloh, M. U., Nathan, C., McCord, J. M., Dinauer, M. C., Libby, S. J., Vazquez-Torres, A., Xu, Y., and Fang, F. C. (1997)

Periplasmic superoxide dismutase protects Salmonella from products of phagocyte NADPH-oxidase and nitric oxide synthase. *Proc Natl Acad Sci U S A 94*, 13997-4001.

(37) Kim, B., Richards, S. M., Gunn, J. S., and Slauch, J. M. (2010) Protecting against antimicrobial effectors in the phagosome allows SodCII to contribute to virulence in Salmonella enterica serovar Typhimurium. *J Bacteriol 192*, 2140-9.

(38) Lombard, M., Touati, D., Fontecave, M., and Niviere, V. (2000) Superoxide reductase as a unique defense system against superoxide stress in the microaerophile Treponema pallidum. *J Biol Chem 275*, 27021-6.

(39) Pinto, A. F., Rodrigues, J. V., and Teixeira, M. (2010) Reductive elimination of superoxide: Structure and mechanism of superoxide reductases. *Biochim Biophys Acta 1804*, 285-97.

(40) Moore, R. L., Powell, L. J., and Goodwin, D. C. (2008) The kinetic properties producing the perfunctory pH profiles of catalase-peroxidases. *Biochim Biophys Acta 1784*, 900-7.

(41) Hall, A., Karplus, P. A., and Poole, L. B. (2009) Typical 2-Cys peroxiredoxins--structures, mechanisms and functions. *Febs J 276*, 2469-77.

(42) Wood, Z. A., Schroder, E., Robin Harris, J., and Poole, L. B. (2003) Structure, mechanism and regulation of peroxiredoxins. *Trends Biochem Sci 28*, 32-40.

(43) Hillar, A., Peters, B., Pauls, R., Loboda, A., Zhang, H., Mauk, A. G., and Loewen, P. C. (2000) Modulation of the activities of catalase-peroxidase HPI of Escherichia coli by site-directed mutagenesis. *Biochemistry 39*, 5868-75.

(44) Parsonage, D., Youngblood, D. S., Sarma, G. N., Wood, Z. A., Karplus, P. A., and Poole, L. B. (2005) Analysis of the link between enzymatic activity and oligomeric state in AhpC, a bacterial peroxiredoxin. *Biochemistry 44*, 10583-92.

(45) Pal, S., Dolai, S., Yadav, R. K., and Adak, S. (2010) Ascorbate peroxidase from Leishmania major controls the virulence of infective stage of promastigotes by regulating oxidative stress. *PLoS One 5*, e11271.

(46) Zheng, M., and Storz, G. (2000) Redox sensing by prokaryotic transcription factors. *Biochem Pharmacol 59*, 1-6.

(47) Zheng, M., Wang, X., Doan, B., Lewis, K. A., Schneider, T. D., and Storz, G. (2001) Computation-directed identification of OxyR DNA binding sites in Escherichia coli. *J Bacteriol 183*, 4571-9.

(48) Zheng, M., Wang, X., Templeton, L. J., Smulski, D. R., LaRossa, R. A., and Storz, G. (2001) DNA microarray-mediated transcriptional profiling of the Escherichia coli response to hydrogen peroxide. *J Bacteriol 183*, 4562-70.

(49) Fuangthong, M., Herbig, A. F., Bsat, N., and Helmann, J. D. (2002) Regulation of the Bacillus subtilis fur and perR genes by PerR: not all members of the PerR regulon are peroxide inducible. *J Bacteriol 184*, 3276-86.

(50) Gaballa, A., and Helmann, J. D. (2002) A peroxide-induced zinc uptake system plays an important role in protection against oxidative stress in Bacillus subtilis. *Mol Microbiol 45*, 997-1005.
(51) Blanchard, J. L., Wholey, W. Y., Conlon, E. M., and Pomposiello, P. J. (2007) Rapid changes in gene expression dynamics in response to superoxide reveal SoxRS-dependent and independent transcriptional networks. *PLoS One 2*, e1186.
(52) Hill, B. G., Dranka, B. P., Bailey, S. M., Lancaster, J. R., Jr., and Darley-Usmar, V. M. (2010) What part of NO don't you understand? Some answers to the cardinal questions in nitric oxide biology. *J Biol Chem 285*, 19699-704.
(53) Moncada, S., Palmer, R. M., and Higgs, E. A. (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev 43*, 109-42.
(54) Poole, R. K. (2005) Nitric oxide and nitrosative stress tolerance in bacteria. *Biochem Soc Trans 33*, 176-80.
(55) Nathan, C., and Shiloh, M. U. (2000) Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc Natl Acad Sci U S A 97*, 8841-8.
(56) Garcia, X., and Stein, F. (2006) Nitric oxide. *Semin Pediatr Infect Dis 17*, 55-7.
(57) Ferrari, C. K., Franca, E. L., and Honorio-Franca, A. C. (2009) Nitric oxide, health and disease. *J Appl Biomed 7*, 163–173.
(58) De Groote, M. A., Granger, D., Xu, Y., Campbell, G., Prince, R., and Fang, F. C. (1995) Genetic and redox determinants of nitric oxide cytotoxicity in a Salmonella typhimurium model. *Proc Natl Acad Sci U S A 92*, 6399-403.
(59) Zhu, L., Gunn, C., and Beckman, J. S. (1992) Bactericidal activity of peroxynitrite. *Arch Biochem Biophys 298*, 452-7.
(60) Dyet, K., and Moir, J. (2006) Effect of combined oxidative and nitrosative stress on Neisseria meningitidis. *Biochem Soc Trans 34*, 197-9.
(61) Forstermann, U., and Sessa, W. C. (2012) Nitric oxide synthases: regulation and function. *Eur Heart J 33*, 829-37.
(62) Murad, F. (1994) Regulation of cytosolic guanylyl cyclase by nitric oxide: the NO-cyclic GMP signal transduction system. *Adv Pharmacol 26*, 19-33.
(63) Hevel, J. M., White, K. A., and Marletta, M. A. (1991) Purification of the inducible murine macrophage nitric oxide synthase. Identification as a flavoprotein. *J Biol Chem 266*, 22789-91.
(64) Ding, A. H., Nathan, C. F., and Stuehr, D. J. (1988) Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. *J Immunol 141*, 2407-12.
(65) Vazquez-Torres, A., Vallance, B. A., Bergman, M. A., Finlay, B. B., Cookson, B. T., Jones-Carson, J., and Fang, F. C. (2004) Toll-like receptor 4 dependence of innate and adaptive immunity to Salmonella: importance of the Kupffer cell network. *J Immunol 172*, 6202-8.
(66) Vazquez-Torres, A., Jones-Carson, J., Mastroeni, P., Ischiropoulos, H., and Fang, F. C. (2000) Antimicrobial actions of the NADPH phagocyte oxidase

and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages in vitro. *J Exp Med 192*, 227-36.

(67) Griffith, O. W., and Stuehr, D. J. (1995) Nitric oxide synthases: properties and catalytic mechanism. *Annu Rev Physiol 57*, 707-36.

(68) Johnson, E. G., Sparks, J. P., Dzikovski, B., Crane, B. R., Gibson, D. M., and Loria, R. (2008) Plant-pathogenic Streptomyces species produce nitric oxide synthase-derived nitric oxide in response to host signals. *Chem Biol 15*, 43-50.

(69) Ferrari, C. K., Souto, P. C., Franca, E. L., and Honorio-Franca, A. C. (2011) Oxidative and nitrosative stress on phagocytes' function: from effective defense to immunity evasion mechanisms. *Arch Immunol Ther Exp (Warsz) 59*, 441-8.

(70) Brett, P. J., Burtnick, M. N., Su, H., Nair, V., and Gherardini, F. C. (2008) iNOS activity is critical for the clearance of Burkholderia mallei from infected RAW 264.7 murine macrophages. *Cell Microbiol 10*, 487-98.

(71) Stamler, J. S., Lamas, S., and Fang, F. C. (2001) Nitrosylation. the prototypic redox-based signaling mechanism. *Cell 106*, 675-83.

(72) Stevanin, T. M., Ioannidis, N., Mills, C. E., Kim, S. O., Hughes, M. N., and Poole, R. K. (2000) Flavohemoglobin Hmp affords inducible protection for Escherichia coli respiration, catalyzed by cytochromes bo' or bd, from nitric oxide. *J Biol Chem 275*, 35868-75.

(73) Schapiro, J. M., Libby, S. J., and Fang, F. C. (2003) Inhibition of bacterial DNA replication by zinc mobilization during nitrosative stress. *Proc Natl Acad Sci U S A 100*, 8496-501.

(74) Ferrer-Sueta, G., and Radi, R. (2009) Chemical biology of peroxynitrite: kinetics, diffusion, and radicals. *ACS Chem Biol 4*, 161-77.

(75) Pacher, P., Beckman, J. S., and Liaudet, L. (2007) Nitric oxide and peroxynitrite in health and disease. *Physiol Rev 87*, 315-424.

(76) Goldstein, S., and Merenyi, G. (2008) The chemistry of peroxynitrite: implications for biological activity. *Methods Enzymol 436*, 49-61.

(77) MacMicking, J. D., Nathan, C., Hom, G., Chartrain, N., Fletcher, D. S., Trumbauer, M., Stevens, K., Xie, Q. W., Sokol, K., Hutchinson, N., and et al. (1995) Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell 81*, 641-50.

(78) Chakravortty, D., and Hensel, M. (2003) Inducible nitric oxide synthase and control of intracellular bacterial pathogens. *Microbes Infect 5*, 621-7.

(79) Balish, E., Warner, T. F., Nicholas, P. J., Paulling, E. E., Westwater, C., and Schofield, D. A. (2005) Susceptibility of germfree phagocyte oxidase- and nitric oxide synthase 2-deficient mice, defective in the production of reactive metabolites of both oxygen and nitrogen, to mucosal and systemic candidiasis of endogenous origin. *Infect Immun 73*, 1313-20.

(80) Mastroeni, P., Vazquez-Torres, A., Fang, F. C., Xu, Y., Khan, S., Hormaeche, C. E., and Dougan, G. (2000) Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis.

II. Effects on microbial proliferation and host survival in vivo. *J Exp Med 192*, 237-48.

(81) Murray, H. W., Xiang, Z., and Ma, X. (2006) Responses to Leishmania donovani in mice deficient in both phagocyte oxidase and inducible nitric oxide synthase. *Am J Trop Med Hyg 74*, 1013-5.

(82) Gyurko, R., Boustany, G., Huang, P. L., Kantarci, A., Van Dyke, T. E., Genco, C. A., and Gibson, F. C., 3rd. (2003) Mice lacking inducible nitric oxide synthase demonstrate impaired killing of Porphyromonas gingivalis. *Infect Immun 71*, 4917-24.

(83) Holscher, C., Kohler, G., Muller, U., Mossmann, H., Schaub, G. A., and Brombacher, F. (1998) Defective nitric oxide effector functions lead to extreme susceptibility of Trypanosoma cruzi-infected mice deficient in gamma interferon receptor or inducible nitric oxide synthase. *Infect Immun 66*, 1208-15.

(84) Talvani, A., Machado, F. S., Santana, G. C., Klein, A., Barcelos, L., Silva, J. S., and Teixeira, M. M. (2002) Leukotriene B(4) induces nitric oxide synthesis in Trypanosoma cruzi-infected murine macrophages and mediates resistance to infection. *Infect Immun 70*, 4247-53.

(85) Boutlis, C. S., Tjitra, E., Maniboey, H., Misukonis, M. A., Saunders, J. R., Suprianto, S., Weinberg, J. B., and Anstey, N. M. (2003) Nitric oxide production and mononuclear cell nitric oxide synthase activity in malaria-tolerant Papuan adults. *Infect Immun 71*, 3682-9.

(86) Adak, S., Aulak, K. S., and Stuehr, D. J. (2002) Direct evidence for nitric oxide production by a nitric-oxide synthase-like protein from Bacillus subtilis. *J Biol Chem 277*, 16167-71.

(87) Sudhamsu, J., and Crane, B. R. (2009) Bacterial nitric oxide synthases: what are they good for? *Trends Microbiol 17*, 212-8.

(88) Adak, S., Bilwes, A. M., Panda, K., Hosfield, D., Aulak, K. S., McDonald, J. F., Tainer, J. A., Getzoff, E. D., Crane, B. R., and Stuehr, D. J. (2002) Cloning, expression, and characterization of a nitric oxide synthase protein from Deinococcus radiodurans. *Proc Natl Acad Sci U S A 99*, 107-12.

(89) Gusarov, I., Starodubtseva, M., Wang, Z. Q., McQuade, L., Lippard, S. J., Stuehr, D. J., and Nudler, E. (2008) Bacterial nitric-oxide synthases operate without a dedicated redox partner. *J Biol Chem 283*, 13140-7.

(90) Shatalin, K., Gusarov, I., Avetissova, E., Shatalina, Y., McQuade, L. E., Lippard, S. J., and Nudler, E. (2008) Bacillus anthracis-derived nitric oxide is essential for pathogen virulence and survival in macrophages. *Proc Natl Acad Sci U S A 105*, 1009-13.

(91) Gusarov, I., and Nudler, E. (2005) NO-mediated cytoprotection: instant adaptation to oxidative stress in bacteria. *Proc Natl Acad Sci U S A 102*, 13855-60.

(92) Justino, M. C., Vicente, J. B., Teixeira, M., and Saraiva, L. M. (2005) New genes implicated in the protection of anaerobically grown Escherichia coli against nitric oxide. *J Biol Chem 280*, 2636-43.

(93) Spiro, S. (2007) Regulators of bacterial responses to nitric oxide. *FEMS Microbiol Rev 31*, 193-211.
(94) Spiro, S. (2008) Metalloregulatory proteins and nitric oxide signalling in bacteria. *Biochem Soc Trans 36*, 1160-4.
(95) Pullan, S. T., Gidley, M. D., Jones, R. A., Barrett, J., Stevanin, T. M., Read, R. C., Green, J., and Poole, R. K. (2007) Nitric oxide in chemostat-cultured Escherichia coli is sensed by Fnr and other global regulators: unaltered methionine biosynthesis indicates lack of S nitrosation. *J Bacteriol 189*, 1845-55.
(96) Ding, H., and Demple, B. (2000) Direct nitric oxide signal transduction via nitrosylation of iron-sulfur centers in the SoxR transcription activator. *Proc Natl Acad Sci U S A 97*, 5146-50.
(97) Elvers, K. T., Turner, S. M., Wainwright, L. M., Marsden, G., Hinds, J., Cole, J. A., Poole, R. K., Penn, C. W., and Park, S. F. (2005) NssR, a member of the Crp-Fnr superfamily from Campylobacter jejuni, regulates a nitrosative stress-responsive regulon that includes both a single-domain and a truncated haemoglobin. *Mol Microbiol 57*, 735-50.
(98) Rodionov, D. A., Dubchak, I. L., Arkin, A. P., Alm, E. J., and Gelfand, M. S. (2005) Dissimilatory metabolism of nitrogen oxides in bacteria: comparative reconstruction of transcriptional networks. *PLoS Comput Biol 1*, e55.
(99) Tucker, N. P., Hicks, M. G., Clarke, T. A., Crack, J. C., Chandra, G., Le Brun, N. E., Dixon, R., and Hutchings, M. I. (2008) The transcriptional repressor protein NsrR senses nitric oxide directly via a [2Fe-2S] cluster. *PLoS One 3*, e3623.
(100) Isabella, V. M., Lapek, J. D., Jr., Kennedy, E. M., and Clark, V. L. (2009) Functional analysis of NsrR, a nitric oxide-sensing Rrf2 repressor in Neisseria gonorrhoeae. *Mol Microbiol 71*, 227-39.
(101) Yukl, E. T., Elbaz, M. A., Nakano, M. M., and Moenne-Loccoz, P. (2008) Transcription Factor NsrR from Bacillus subtilis Senses Nitric Oxide with a 4Fe-4S Cluster (dagger). *Biochemistry 47*, 13084-92.
(102) Tucker, N. P., Le Brun, N. E., Dixon, R., and Hutchings, M. I. (2010) There's NO stopping NsrR, a global regulator of the bacterial NO stress response. *Trends Microbiol 18*, 149-56.
(103) Wang, Y., Dufour, Y. S., Carlson, H. K., Donohue, T. J., Marletta, M. A., and Ruby, E. G. (2010) H-NOX-mediated nitric oxide sensing modulates symbiotic colonization by Vibrio fischeri. *Proc Natl Acad Sci U S A 107*, 8375-80.
(104) Carlson, H. K., Vance, R. E., and Marletta, M. A. (2010) H-NOX regulation of c-di-GMP metabolism and biofilm formation in Legionella pneumophila. *Mol Microbiol*.
(105) D'Autreaux, B., Horner, O., Oddou, J. L., Jeandey, C., Gambarelli, S., Berthomieu, C., Latour, J. M., and Michaud-Soret, I. (2004) Spectroscopic description of the two nitrosyl-iron complexes responsible for fur inhibition by nitric oxide. *J Am Chem Soc 126*, 6005-16.

(106) Moore, C. M., Nakano, M. M., Wang, T., Ye, R. W., and Helmann, J. D. (2004) Response of Bacillus subtilis to nitric oxide and the nitrosating agent sodium nitroprusside. *J Bacteriol 186*, 4655-64.
(107) Saraiva, L. M., Vicente, J. B., and Teixeira, M. (2004) The role of the flavodiiron proteins in microbial nitric oxide detoxification. *Adv Microb Physiol 49*, 77-129.
(108) Vicente, J. B., Justino, M. C., Goncalves, V. L., Saraiva, L. M., and Teixeira, M. (2008) Biochemical, spectroscopic, and thermodynamic properties of flavodiiron proteins. *Methods Enzymol 437*, 21-45.
(109) Smutna, T., Goncalves, V. L., Saraiva, L. M., Tachezy, J., Teixeira, M., and Hrdy, I. (2009) Flavodiiron protein from Trichomonas vaginalis hydrogenosomes: the terminal oxygen reductase. *Eukaryot Cell 8*, 47-55.
(110) Rodrigues, R., Vicente, J. B., Felix, R., Oliveira, S., Teixeira, M., and Rodrigues-Pousada, C. (2006) Desulfovibrio gigas flavodiiron protein affords protection against nitrosative stress in vivo. *J Bacteriol 188*, 2745-51.
(111) Zhang, P., Allahverdiyeva, Y., Eisenhut, M., and Aro, E. M. (2009) Flavodiiron proteins in oxygenic photosynthetic organisms: photoprotection of photosystem II by Flv2 and Flv4 in Synechocystis sp. PCC 6803. *PLoS One 4*, e5331.
(112) Helman, Y., Tchernov, D., Reinhold, L., Shibata, M., Ogawa, T., Schwarz, R., Ohad, I., and Kaplan, A. (2003) Genes encoding A-type flavoproteins are essential for photoreduction of O2 in cyanobacteria. *Curr Biol 13*, 230-5.
(113) Goncalves, V. L., Saraiva, L. M., and Teixeira, M. (2011) Gene expression study of the flavodi-iron proteins from the cyanobacterium Synechocystis sp. PCC6803. *Biochem Soc Trans 39*, 216-8.
(114) Gomes, C. M., Giuffre, A., Forte, E., Vicente, J. B., Saraiva, L. M., Brunori, M., and Teixeira, M. (2002) A novel type of nitric-oxide reductase. Escherichia coli flavorubredoxin. *J Biol Chem 277*, 25273-6.
(115) Gomes, C. M., Vicente, J. B., Wasserfallen, A., and Teixeira, M. (2000) Spectroscopic studies and characterization of a novel electron-transfer chain from Escherichia coli involving a flavorubredoxin and its flavoprotein reductase partner. *Biochemistry 39*, 16230-7.
(116) Gardner, A. M., Helmick, R. A., and Gardner, P. R. (2002) Flavorubredoxin, an inducible catalyst for nitric oxide reduction and detoxification in Escherichia coli. *J Biol Chem 277*, 8172-7.
(117) Kurtz, D. M. (2007) Flavo-diiron enzymes: nitric oxide or dioxygen reductases? *Dalton Transactions*, 4115-41121.
(118) Vicente, J. B., Scandurra, F. M., Rodrigues, J. V., Brunori, M., Sarti, P., Teixeira, M., and Giuffre, A. (2007) Kinetics of electron transfer from NADH to the Escherichia coli nitric oxide reductase flavorubredoxin. *Febs J 274*, 677-86.
(119) Petoukhov, M. V., Vicente, J. B., Crowley, P. B., Carrondo, M. A., Teixeira, M., and Svergun, D. I. (2008) Quaternary structure of flavorubredoxin as

revealed by synchrotron radiation small-angle X-ray scattering. *Structure 16*, 1428-36.

(120) Mills, P. C., Richardson, D. J., Hinton, J. C., and Spiro, S. (2005) Detoxification of nitric oxide by the flavorubredoxin of Salmonella enterica serovar Typhimurium. *Biochem Soc Trans 33*, 198-9.

(121) da Costa, P. N., Teixeira, M., and Saraiva, L. M. (2003) Regulation of the flavorubredoxin nitric oxide reductase gene in Escherichia coli: nitrate repression, nitrite induction, and possible post-transcription control. *FEMS Microbiol Lett 218*, 385-93.

(122) Constantinidou, C., Hobman, J. L., Griffiths, L., Patel, M. D., Penn, C. W., Cole, J. A., and Overton, T. W. (2006) 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*, 4802-15.

(123) Eriksson, S., Lucchini, S., Thompson, A., Rhen, M., and Hinton, J. C. (2003) Unravelling the biology of macrophage infection by gene expression profiling of intracellular Salmonella enterica. *Mol Microbiol 47*, 103-18.

(124) Flatley, J., Barrett, J., Pullan, S. T., Hughes, M. N., Green, J., and Poole, R. K. (2005) Transcriptional responses of Escherichia coli to S-nitrosoglutathione under defined chemostat conditions reveal major changes in methionine biosynthesis. *J Biol Chem 280*, 10065-72.

(125) Mukhopadhyay, P., Zheng, M., Bedzyk, L. A., LaRossa, R. A., and Storz, G. (2004) Prominent roles of the NorR and Fur regulators in the Escherichia coli transcriptional response to reactive nitrogen species. *Proc Natl Acad Sci U S A 101*, 745-50.

(126) Hutchings, M. I., Mandhana, N., and Spiro, S. (2002) The NorR protein of Escherichia coli activates expression of the flavorubredoxin gene norV in response to reactive nitrogen species. *J Bacteriol 184*, 4640-3.

(127) Gardner, A. M., Gessner, C. R., and Gardner, P. R. (2003) Regulation of the nitric oxide reduction operon (norRVW) in Escherichia coli. Role of NorR and sigma54 in the nitric oxide stress response. *J Biol Chem 278*, 10081-6.

(128) Justino, M. C., Goncalves, V. M., and Saraiva, L. M. (2005) Binding of NorR to three DNA sites is essential for promoter activation of the flavorubredoxin gene, the nitric oxide reductase of Escherichia coli. *Biochem Biophys Res Commun 328*, 540-4.

(129) D'Autreaux, B., Tucker, N. P., Dixon, R., and Spiro, S. (2005) A non-haem iron centre in the transcription factor NorR senses nitric oxide. *Nature 437*, 769-72.

(130) Pohlmann, A., Cramm, R., Schmelz, K., and Friedrich, B. (2000) A novel NO-responding regulator controls the reduction of nitric oxide in Ralstonia eutropha. *Mol Microbiol 38*, 626-38.

(131) Bush, M., Ghosh, T., Tucker, N., Zhang, X., and Dixon, R. (2011) Transcriptional regulation by the dedicated nitric oxide sensor, NorR: a route towards NO detoxification. *Biochem Soc Trans 39*, 289-93.

(132) Tucker, N. P., D'Autreaux, B., Yousafzai, F. K., Fairhurst, S. A., Spiro, S., and Dixon, R. (2008) Analysis of the nitric oxide-sensing non-heme iron center in the NorR regulatory protein. *J Biol Chem 283*, 908-18.
(133) Klink, A., Elsner, B., Strube, K., and Cramm, R. (2007) Characterization of the signaling domain of the NO-responsive regulator NorR from Ralstonia eutropha H16 by site-directed mutagenesis. *J Bacteriol 189*, 2743-9.
(134) Busch, A., Pohlmann, A., Friedrich, B., and Cramm, R. (2004) A DNA region recognized by the nitric oxide-responsive transcriptional activator NorR is conserved in beta- and gamma-proteobacteria. *J Bacteriol 186*, 7980-7.
(135) Tucker, N. P., D'Autreaux, B., Studholme, D. J., Spiro, S., and Dixon, R. (2004) DNA binding activity of the Escherichia coli nitric oxide sensor NorR suggests a conserved target sequence in diverse proteobacteria. *J Bacteriol 186*, 6656-60.
(136) Tucker, N., D'Autreaux, B., Spiro, S., and Dixon, R. (2005) DNA binding properties of the Escherichia coli nitric oxide sensor NorR: towards an understanding of the regulation of flavorubredoxin expression. *Biochem Soc Trans 33*, 181-3.
(137) Bush, M., Ghosh, T., Tucker, N., Zhang, X., and Dixon, R. (2010) Nitric oxide-responsive interdomain regulation targets the sigma54-interaction surface in the enhancer binding protein NorR. *Mol Microbiol 77*, 1278-88.
(138) Angelo, M., Hausladen, A., Singel, D. J., and Stamler, J. S. (2008) Interactions of NO with hemoglobin: from microbes to man. *Methods Enzymol 436*, 131-68.
(139) Wakabayashi, S., Matsubara, H., and Webster, D. A. (1986) Primary sequence of a dimeric bacterial haemoglobin from Vitreoscilla. *Nature 322*, 481-3.
(140) Elvers, K. T., Wu, G., Gilberthorpe, N. J., Poole, R. K., and Park, S. F. (2004) Role of an inducible single-domain hemoglobin in mediating resistance to nitric oxide and nitrosative stress in Campylobacter jejuni and Campylobacter coli. *J Bacteriol 186*, 5332-41.
(141) Bonamore, A., and Boffi, A. (2008) Flavohemoglobin: structure and reactivity. *IUBMB Life 60*, 19-28.
(142) Missall, T. A., Lodge, J. K., and McEwen, J. E. (2004) Mechanisms of resistance to oxidative and nitrosative stress: implications for fungal survival in mammalian hosts. *Eukaryot Cell 3*, 835-46.
(143) Stevanin, T. M., Poole, R. K., Demoncheaux, E. A., and Read, R. C. (2002) Flavohemoglobin Hmp protects Salmonella enterica serovar typhimurium from nitric oxide-related killing by human macrophages. *Infect Immun 70*, 4399-405.
(144) Bang, I. S., Liu, L., Vazquez-Torres, A., Crouch, M. L., Stamler, J. S., and Fang, F. C. (2006) Maintenance of nitric oxide and redox homeostasis by the salmonella flavohemoglobin hmp. *J Biol Chem 281*, 28039-47.
(145) Sebbane, F., Lemaitre, N., Sturdevant, D. E., Rebeil, R., Virtaneva, K., Porcella, S. F., and Hinnebusch, B. J. (2006) Adaptive response of Yersinia

pestis to extracellular effectors of innate immunity during bubonic plague. *Proc Natl Acad Sci U S A 103*, 11766-71.

(146) Gardner, P. R., Gardner, A. M., Martin, L. A., and Salzman, A. L. (1998) Nitric oxide dioxygenase: an enzymic function for flavohemoglobin. *Proc Natl Acad Sci U S A 95*, 10378-83.

(147) Forrester, M. T., and Foster, M. W. (2012) Protection from nitrosative stress: A central role for microbial flavohemoglobin. *Free Radic Biol Med*.

(148) Poole, R. K., and Hughes, M. N. (2000) New functions for the ancient globin family: bacterial responses to nitric oxide and nitrosative stress. *Mol Microbiol 36*, 775-83.

(149) Bonamore, A., Farina, A., Gattoni, M., Schinina, M. E., Bellelli, A., and Boffi, A. (2003) Interaction with membrane lipids and heme ligand binding properties of Escherichia coli flavohemoglobin. *Biochemistry 42*, 5792-801.

(150) Rinaldi, A. C., Bonamore, A., Macone, A., Boffi, A., Bozzi, A., and Di Giulio, A. (2006) Interaction of Vitreoscilla hemoglobin with membrane lipids. *Biochemistry 45*, 4069-76.

(151) Gardner, A. M., and Gardner, P. R. (2002) Flavohemoglobin detoxifies nitric oxide in aerobic, but not anaerobic, Escherichia coli. Evidence for a novel inducible anaerobic nitric oxide-scavenging activity. *J Biol Chem 277*, 8166-71.

(152) Hausladen, A., Gow, A., and Stamler, J. S. (2001) Flavohemoglobin denitrosylase catalyzes the reaction of a nitroxyl equivalent with molecular oxygen. *Proc Natl Acad Sci U S A 98*, 10108-12.

(153) Goncalves, V. L., Nobre, L. S., Vicente, J. B., Teixeira, M., and Saraiva, L. M. (2006) Flavohemoglobin requires microaerophilic conditions for nitrosative protection of Staphylococcus aureus. *FEBS Lett 580*, 1817-21.

(154) Nobre, L. S., Goncalves, V. L., and Saraiva, L. M. (2008) Flavohemoglobin of Staphylococcus aureus. *Methods Enzymol 436*, 203-16.

(155) Membrillo-Hernandez, J., Coopamah, M. D., Channa, A., Hughes, M. N., and Poole, R. K. (1998) A novel mechanism for upregulation of the Escherichia coli K-12 hmp (flavohaemoglobin) gene by the 'NO releaser', S-nitrosoglutathione: nitrosation of homocysteine and modulation of MetR binding to the glyA-hmp intergenic region. *Mol Microbiol 29*, 1101-12.

(156) Stevanin, T. M., Read, R. C., and Poole, R. K. (2007) The hmp gene encoding the NO-inducible flavohaemoglobin in Escherichia coli confers a protective advantage in resisting killing within macrophages, but not in vitro: links with swarming motility. *Gene 398*, 62-8.

(157) McCollister, B. D., Myers, J. T., Jones-Carson, J., Husain, M., Bourret, T. J., and Vazquez-Torres, A. (2007) N(2)O(3) enhances the nitrosative potential of IFNgamma-primed macrophages in response to Salmonella. *Immunobiology 212*, 759-69.

(158) Richardson, A. R., Dunman, P. M., and Fang, F. C. (2006) The nitrosative stress response of Staphylococcus aureus is required for resistance to innate immunity. *Mol Microbiol 61*, 927-39.

(159) Arai, H., Hayashi, M., Kuroi, A., Ishii, M., and Igarashi, Y. (2005) Transcriptional regulation of the flavohemoglobin gene for aerobic nitric oxide detoxification by the second nitric oxide-responsive regulator of Pseudomonas aeruginosa. *J Bacteriol 187*, 3960-8.
(160) Boccara, M., Mills, C. E., Zeier, J., Anzi, C., Lamb, C., Poole, R. K., and Delledonne, M. (2005) Flavohaemoglobin HmpX from Erwinia chrysanthemi confers nitrosative stress tolerance and affects the plant hypersensitive reaction by intercepting nitric oxide produced by the host. *Plant J 43*, 226-37.
(161) Rogstam, A., Larsson, J. T., Kjelgaard, P., and von Wachenfeldt, C. (2007) Mechanisms of adaptation to nitrosative stress in Bacillus subtilis. *J Bacteriol 189*, 3063-71.
(162) de Jesus-Berrios, M., Liu, L., Nussbaum, J. C., Cox, G. M., Stamler, J. S., and Heitman, J. (2003) Enzymes that counteract nitrosative stress promote fungal virulence. *Curr Biol 13*, 1963-8.
(163) Bhattacharjee, A., Majumdar, U., Maity, D., Sarkar, T. S., Goswami, A. M., Sahoo, R., and Ghosh, S. (2009) In vivo protein tyrosine nitration in S. cerevisiae: identification of tyrosine-nitrated proteins in mitochondria. *Biochem Biophys Res Commun 388*, 612-7.
(164) Poole, R. K., Anjum, M. F., Membrillo-Hernandez, J., Kim, S. O., Hughes, M. N., and Stewart, V. (1996) Nitric oxide, nitrite, and Fnr regulation of hmp (flavohemoglobin) gene expression in Escherichia coli K-12. *J Bacteriol 178*, 5487-92.
(165) Hromatka, B. S., Noble, S. M., and Johnson, A. D. (2005) Transcriptional response of Candida albicans to nitric oxide and the role of the YHB1 gene in nitrosative stress and virulence. *Mol Biol Cell 16*, 4814-26.
(166) Hu, Y., Butcher, P. D., Mangan, J. A., Rajandream, M. A., and Coates, A. R. (1999) Regulation of hmp gene transcription in Mycobacterium tuberculosis: effects of oxygen limitation and nitrosative and oxidative stress. *J Bacteriol 181*, 3486-93.
(167) Membrillo-Hernandez, J., Kim, S. O., Cook, G. M., and Poole, R. K. (1997) Paraquat regulation of hmp (flavohemoglobin) gene expression in Escherichia coli K-12 is SoxRS independent but modulated by sigma S. *J Bacteriol 179*, 3164-70.
(168) Membrillo-Hernandez, J., Cook, G. M., and Poole, R. K. (1997) Roles of RpoS (sigmaS), IHF and ppGpp in the expression of the hmp gene encoding the flavohemoglobin (Hmp) of Escherichia coli K-12. *Mol Gen Genet 254*, 599-603.
(169) Bollinger, C. J., Bailey, J. E., and Kallio, P. T. (2001) Novel hemoglobins to enhance microaerobic growth and substrate utilization in Escherichia coli. *Biotechnol Prog 17*, 798-808.
(170) Nakano, M. M. (2006) Essential role of flavohemoglobin in long-term anaerobic survival of Bacillus subtilis. *J Bacteriol 188*, 6415-8.
(171) Gilberthorpe, N. J., Lee, M. E., Stevanin, T. M., Read, R. C., and Poole, R. K. (2007) NsrR: a key regulator circumventing Salmonella enterica serovar

Typhimurium oxidative and nitrosative stress in vitro and in IFN-gamma-stimulated J774.2 macrophages. *Microbiology 153*, 1756-71.

(172) Bodenmiller, D. M., and Spiro, S. (2006) The yjeB (nsrR) gene of Escherichia coli encodes a nitric oxide-sensitive transcriptional regulator. *J Bacteriol 188*, 874-81.

(173) Hernandez-Urzua, E., Zamorano-Sanchez, D. S., Ponce-Coria, J., Morett, E., Grogan, S., Poole, R. K., and Membrillo-Hernandez, J. (2007) Multiple regulators of the Flavohaemoglobin (hmp) gene of Salmonella enterica serovar Typhimurium include RamA, a transcriptional regulator conferring the multidrug resistance phenotype. *Arch Microbiol 187*, 67-77.

(174) Nakano, M. M. (2002) Induction of ResDE-dependent gene expression in Bacillus subtilis in response to nitric oxide and nitrosative stress. *J Bacteriol 184*, 1783-7.

(175) van Wonderen, J. H., Burlat, B., Richardson, D. J., Cheesman, M. R., and Butt, J. N. (2008) The nitric oxide reductase activity of cytochrome c nitrite reductase from Escherichia coli. *J Biol Chem 283*, 9587-94.

(176) Potter, L., Angove, H., Richardson, D., and Cole, J. (2001) Nitrate reduction in the periplasm of gram-negative bacteria. *Adv Microb Physiol 45*, 51-112.

(177) Poock, S. R., Leach, E. R., Moir, J. W., Cole, J. A., and Richardson, D. J. (2002) Respiratory detoxification of nitric oxide by the cytochrome c nitrite reductase of Escherichia coli. *J Biol Chem 277*, 23664-9.

(178) Harrington, J. C., Wong, S. M., Rosadini, C. V., Garifulin, O., Boyartchuk, V., and Akerley, B. J. (2009) Resistance of Haemophilus influenzae to reactive nitrogen donors and gamma interferon-stimulated macrophages requires the formate-dependent nitrite reductase regulator-activated ytfE gene. *Infect Immun 77*, 1945-58.

(179) Mills, P. C., Rowley, G., Spiro, S., Hinton, J. C., and Richardson, D. J. (2008) A combination of cytochrome c nitrite reductase (NrfA) and flavorubredoxin (NorV) protects Salmonella enterica serovar Typhimurium against killing by NO in anoxic environments. *Microbiology 154*, 1218-28.

(180) Stach, P., Einsle, O., Schumacher, W., Kurun, E., and Kroneck, P. M. (2000) Bacterial cytochrome c nitrite reductase: new structural and functional aspects. *J Inorg Biochem 79*, 381-5.

(181) Field, S. J., Thorndycroft, F. H., Matorin, A. D., Richardson, D. J., and Watmough, N. J. (2008) The respiratory nitric oxide reductase (NorBC) from Paracoccus denitrificans. *Methods Enzymol 437*, 79-101.

(182) Filenko, N., Spiro, S., Browning, D. F., Squire, D., Overton, T. W., Cole, J., and Constantinidou, C. (2007) The NsrR regulon of Escherichia coli K-12 includes genes encoding the hybrid cluster protein and the periplasmic, respiratory nitrite reductase. *J Bacteriol 189*, 4410-7.

(183) Vine, C. E., and Cole, J. A. (2011) Nitrosative stress in Escherichia coli: reduction of nitric oxide. *Biochem Soc Trans 39*, 213-5.

Chapter II

Iron,
an essential metal for the living systems

II **Iron**, an essential metal for the living systems

Chapter II

Iron, an essential metal for the living systems

"It is safe to say that, with only a few possible exceptions in the bacterial world, there would be no life without **iron**."

A. Earnsham in Chemistry of the Elements

II.1 – Chemical properties and biological role

Iron is the element 26 in the periodic table, the second most abundant metal and fourth most abundant element in the earth's crust. This metal is the most important transition element involved in biological systems and one of the most versatile, having the capacity to acquire various oxidation states (from –II to +VI). In biological systems, iron prevails in one of two oxidation states, the Fe^{3+} (ferric) or Fe^{2+} (ferrous) form. Moreover, iron can adopt different spin states in both forms, depending on its ligand environment (*1, 2*). Ferrous iron reacts with oxygen resulting in reactive oxygen species (ROS) (*3*):

$$Fe(II) + O_2 \rightarrow Fe(III) + O_2^- \quad (A)$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (B)$$

$$H_2O_2 + Fe(II) \rightarrow OH^\bullet + OH^- + Fe(III) \quad (C)$$

Scheme 1 – Iron reactivity and the resulting reactive oxygen species. (A) Iron oxidation, (B) Superoxide dismutation and (C) Fenton reaction.

Iron is present in living cells performing an essential role in a wide range of biological reactions. Proteins that incorporate iron in their prosthetic groups can profit from its flexible physicochemical properties and use it as a biocatalyst or electron carrier. Hence, iron is present in the catalytic centre of a large variety of proteins that participate in a broad diversity of processes in cells such as photosynthesis, nitrogen fixation, methanogenesis, respiration, gene regulation and DNA biosynthesis (*1, 2*). The abundance of proteins involved in iron uptake emphasises the importance of iron in biological systems.

II.2 – Iron uptake and homeostasis

Upon infection, mammalian cells and pathogens compete for iron since pathogenic microorganisms require this metal for many metabolic pathways and detoxifying systems are iron-dependent enzymes, while the host restricts iron as a way to combat pathogens. The host uses several iron limitation systems like chelation of ferric iron with specific proteins such as lactoferrin, transferrin, lipocalin-1, lipocalin-2 and siderophores, and exportation of iron from the mammalian cells using Fpn1 (ferroportin 1) (*4, 5*). In contrast, pathogens have developed strategies to acquire iron from the host, which will be discussed next.

II.2.1 – Iron uptake systems

While the human body has $\sim 10^{-24}$ M of free iron (*6*), an iron concentration between 10^{-6} and 10^{-7} M is required by most pathogens for various metabolic processes (*7, 8*).

One of the most known strategies of microorganisms to obtain iron from the host is by siderophore iron acquisition. Siderophores are Fe^{3+} chelators with low molecular mass (<1 kDa) whose uptake mechanisms vary between Gram-positive and Gram-negative bacteria (*8*). Free ferric iron is chelated by a siderophore and the complex siderophore-Fe^{3+} binds to a siderophore receptor which transfers the ferrisiderophore to a transporter, directly, in Gram-positive bacteria, or via a periplasmic binding protein, in Gram-negative bacteria, plus the inner membrane TonB protein (a transporter of small molecules, *T-on*e phage) assisted by ExbB and ExbD (encoded by *exbB* and *exbD* genes, comprised in the *exb* operon, *ex*port *b*acteriocin). Once in the cytoplasm, the ferrisiderophore is either degraded by an esterase or releases iron through a reductive process, which leaves the iron chelator intact and available to be used in another process of iron chelation (*8*).

A different strategy used by pathogens to acquire iron, which occurs at the surface of the cell, is the reduction of exogenous Fe(III) to Fe(II), that can be then transported into the cell. This key biological process during cellular iron uptake is used by bacteria and yeast, namely *Lysteria monocytogenes*, *Legionella pneumophila* and *Saccharomyces cerevisiae* (*2*). Other mechanism by which pathogenic bacteria obtain iron involves the use of LIP (*L*abile *I*ron *P*ool) of the host, a pool of redox-active iron that is loosely bound to low molecular weight chelators and is available for metabolic purposes, used by Gram-negative bacteria (*Salmonella* sp.*, Francisella tularensis* and *Chlamydia trachomatis*) and protozoa (*Leishmania donovani*) (*4, 9-12*). In addition, some bacteria can resist the effect of lipocalin-2, an important immunity protein that is able to capture iron-laden bacterial siderophores (*13, 14*). Interestingly, *Salmonella sp.* can even use this protein to directly obtain iron from the host (*15*).

Furthermore, *Neisseria meningitidis* can degrade the mammalian cellular iron storage ferritin and *Listeria monocytogenes* is able to extract iron from this protein (*7, 16-18*). Some pathogens uptake iron by expressing receptors for the host iron binding proteins transferrin and lactoferrin (*19, 20*).

Several microorganisms can acquire iron from the host using transport systems for ferrous iron, such as the Feo transporter (*Fe*rrous iron) (*21*). Other transporters are the metal-type ABC that have specificity for iron but don't require outer membrane receptors (*22*). Examples of these transporters are SitABCD (*Salmonella i*ron *t*ransporter protein found in *Salmonella typhimurium*), FbpABC (*F*erric iron *b*inding *p*rotein found in *Neisseria gonorhoeae*) and FutABC (*F*erric iron *u*ptake and *t*ransport protein in *Synechocystis* PCC 6803) (*8*).

Another source of iron is haem, the biggest body intracellular iron pool (80%), that is found bound to haemoglobin, myoglobin and other haem-containing enzymes (*23*). Pathogens can use haem by extracting it from proteins and, once inside the bacteria's cytoplasm, haem is either broken down via a haem oxygenase with the formation of biliverdin and CO or it can be de-ferrated in a reaction that leaves the tetrapyrrole ring intact (*24, 25*).

II.2.2 – Iron storage proteins

Iron storage is a vital step for survival, as iron needs to be stored in a non-toxic readily available form (*8*). Iron is stored in three types of protein: ferritin (Ftn), bacterioferritin (Bfr) and the DNA-binding protein Dps (*26*). Ferritins and bacterioferritins have a molecular weight (MW) of

~500 KDa and are composed of 24 identical or similar subunits. These subunits form a spherical protein shell that accommodates the iron storage reservoir which has capacity to store 4000 iron atoms. The major difference between Ftns and Bfrs is the presence of haem groups that are non-covalently bound to the latter protein. In bacteria, both proteins have an important role during stress conditions (*27, 28*). Interestingly, in *E. coli*, up to 50% of cellular iron is stored by ferritin alone (*29*).

Dps is a DNA-binding protein that is capable of providing protection to cells during exposure to severe environmental assaults, including oxidative stress, nutritional deprivation, high pressure, ultra-violet and gamma irradiation, thermal and alkaline/acid stress (*30*). This iron storage protein uses H_2O_2 to catalyze the oxidation of Fe^{2+} at its ferroxidase centre generating water rather than ROS. Consequently, this results in the protection of DNA from Fenton-mediated oxidative stress (*26*). In *E. coli*, the gene *dps* is regulated in exponentially growth by OxyR and in stationary phase by IHF (histone-like *I*ntegration *H*ost *F*actor). Moreover, Dps is an iron storage protein with lower capacity when compared to Ftn and Bfr because it is only formed by 12 subunits (~250 kDa), capable of holding ~500 iron atoms (*30*).

These iron-storage proteins obtain iron in the soluble ferrous form but after the ferroxidation step, catalyzed by their ferroxidase centre, the metal is stored in the oxidized ferric form (*8, 31*). When required for intracellular metabolism, iron is mobilized upon reduction of the oxy-hydroxide core of these proteins (*32*), which is performed by ferric reductase proteins (*33*). In the case of *E. coli*, the *yqjH* gene that is part of the Fur (*F*erric-*u*ptake *r*egulator protein) regulon was shown to encode a ferric reductase protein required for iron homeostasis (*34*).

II.2.3 – Regulation of iron homeostasis

A need for a tight control of iron uptake is required as the accumulation of potentially dangerous free iron results ultimately in bacterial death. The regulation of iron levels in the cell involves sensing the cellular concentration of the metal and responding appropriately by modulating the uptake, storage and efflux of iron.

One of the most well-known regulators of iron metabolism is Fur that functions as a global regulator of iron homeostasis controlling both the induction of iron uptake encoding genes (under iron limitation) and the expression of iron storage proteins as well as iron-utilizing enzymes (when iron is sufficient). In *E. coli,* the Fur regulon includes 35 iron acquisition and other 'non-iron' function genes such as the ones encoding proteins that participate in respiration, TCA (*T*ri*C*arboxylic *A*cid) cycle, glycolysis, methionine biosynthesis, DNA synthesis and oxidative stress resistance. In Gram-negative bacteria, Fur is the most important iron regulator (*35, 36*), while in Gram-positives other regulators were described (e.g. DtxR, *Diphtheria T*ox*i*n *R*egulator, family of proteins) along with Fur-like proteins (*37-39*). Fur is abundant in cells during exponential growth and doubles in the stationary phase (*8*). This transcription factor contains two equal subunits of 17 kDa forming a homodimer. Fur is able to bind one ferrous ion per subunit, as well as other similar metals and even haem (*8*). Upon binding of the metal, Fur affinity towards DNA increases by a 1000 fold. Nevertheless, only Fe(II) binds with sufficient affinity to significantly activate Fur at physiological metal concentrations (*40*). The affinity of Fur for Fe^{2+} is ~10 μM, which is compatible with the levels of free ferrous iron in the bacterial cell, and so bacterium is able to respond to the

physiologically relevant fluctuations of iron in the cell (*8*). The protein acts as a transcriptional repressor upon interaction with Fe^{2+} (Figure II.1). The repression occurs because Fur box overlaps the promoter region of the regulated genes (*2, 8*). Hence, two Fur-Fe^{2+} dimers recognize and bind to the Fur box from each DNA strand blocking the access of RNA polymerase to the promoter and therefore preventing the transcription of downstream genes (Figure II.1) (*41, 42*). Fur is also induced by SoxRS and by OxyR in response to oxidative stress (*8*).

Fur can also act indirectly as an inducer due to the existence of the small RNA (sRNA) RyhB, which facilitates the degradation of mRNAs that are regulated by Fur (Figure II.1). The presence of Fur bound to ferrous iron leads to repression of RyhB, such that degradation of the transcripts that are Fur-induced does not proceed, resulting in their expression (Figure II.1). In *E. coli*, Fur-dependent induction of *acnA*, *bfr*, *sdh* and *sodB* genes (coding for aconitase A, bacterioferritin, succinate dehydrogenase and superoxide dismutase B, respectively) was eliminated when *ryhB* was inactivated, which indicates that their Fur dependence is mediated by RyhB (Figure II.1) (*8, 42*). Furthermore, the *E. coli fur* mutant contains 70% less iron content when compared to the wild type, due to the lack of iron storage proteins and to the lower levels of cellular iron-containing proteins. Bacteria using Fur as a regulator of iron metabolism modulate the expression of iron-requiring proteins in response to iron availability, and make use of iron for more important processes. Moreover, previous work showed that when *E. coli* is not able to acquire enough iron using siderophores, the organism suppresses the synthesis of its most abundant Fe-S enzymes by RyhB/Fur regulation, resulting in a less efficient metabolism, and redirects the little iron present to the indispensable iron enzymes of essential biosynthetic pathways (*43*).

Fur is not the only iron regulator. In mammals, the inactive form of IRP (*I*ron-*R*egulatory *P*rotein) is identical to the cytoplasmic isoform of the iron-sulphur protein aconitase, containing a $[4Fe\text{-}4S]^{2+}$ cluster. When iron is scarce, the apo-form (IRP) prevails, blocking ferritin synthesis and protecting transferrin receptor mRNAs. When iron becomes available again, holo-aconitase is reconstituted, the protein loses its function as IRP, but can now perform aconitase activity. Hence, the assembly and disassembly of the iron-sulphur cluster will act as a regulatory step on iron homeostasis (*44*).

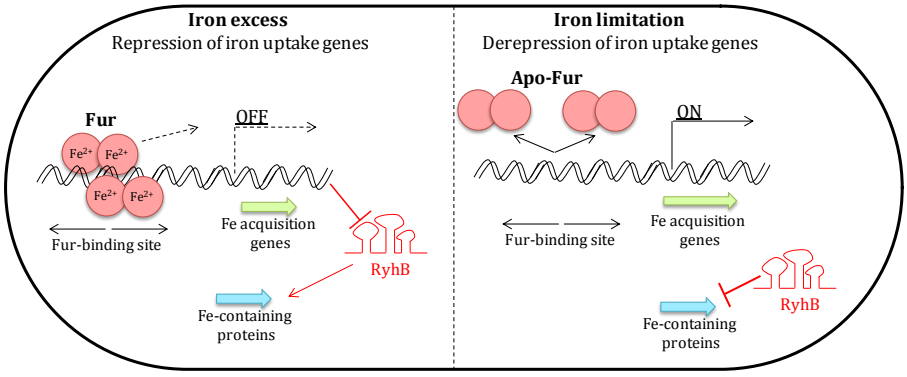


Figure II.1 – Fur regulation mechanisms. When iron is abundant, the Fur-Fe^{2+} dimer blocks the transcription of downstream genes (Fe acquisition genes). Also, the presence of Fur-Fe^{2+} represses the transcription of the small RNA RyhB, leading to enhanced translation of iron-containing proteins. Under iron limiting conditions, Fur loses its iron and apo-Fur has a reduced affinity for the Fur box allowing the transcription of iron acquisition genes. Furthermore, apo-Fur no longer represses RyhB leading to degradation of Fe-containing proteins transcripts. [Adapted from (*8*)].

II.3 – Iron containing proteins

The biological function of iron is almost entirely dependent of its incorporation into proteins, either as mono- and binuclear species, or in more complex forms as part of iron-sulphur (Fe-S) clusters or haem groups. Based on the coordination chemistry of iron, the iron-containing proteins can be divided in two main groups: the haemic and non-haemic proteins.

II.3.1 – Haem proteins

Haem is a prosthetic group that belongs to the tetrapyrrole family and forms a complex macrocycle with four five-membered pyrrole rings attached to one another in a cyclic form via one-carbon bridges. The most common haem structure found in nature is iron-protoporphyrin IX (protohaem or haem *b*), although other haem centres exist like haem *a* or haem *o* (*45-47*). The haem biosynthesis in eukaryotes and prokaryotes is a complex pathway divided in eight enzymic steps, being the first step the formation of aminolevulinic acid and the last step the insertion of ferrous iron into the tetrapyrrole macrocycle of protoporphyrin IX (*48, 49*). Haem containing proteins perform a variety of functions and are present in respiratory and photosynthetic electron transport chains and act as regulatory enzymes to modulate gene expression at transcriptional and translational levels (*46, 48*). A large number of proteins involved in the uptake, trafficking and sensing of haem are present in prokaryotes, showing the importance of this cofactor in evolved systems (*45*).

II.3.2 – Non-haem iron-containing proteins: Mono, dinuclear iron and mixed metal centres

In nature, a wide variety of non-haem iron centres occur, ranging from the simplest one, mononuclear iron centre, to the more complex geometry, the Fe-S clusters. The structure of the centres and the role of the most relevant non-haem iron-containing proteins will be next summarized.

The simplest type of mononuclear iron centre is the $Fe(Cys)_4$ which consists of a tetracoordinated iron bound to cysteines. Nevertheless, these iron centres can be penta or hexacoordinated or have other ligands. A large number of mononuclear iron proteins participate in oxygen insertion into organic substrates and electron transfer reactions (*2, 50, 51*). Furthermore, the mononuclear iron-containing proteins possess a role in regulation as, for example, NorR, the regulator of flavorubredoxin, whose centre binds NO (*52*) (the mechanism and biological role of this regulator was discussed on chapter I).

The family of dinuclear iron proteins has typically a carboxylate-bridged diiron centre. Usually, this prosthetic group holds two irons bound to the protein by four carboxylate and two histidine ligands and the iron ions are bridged by one or two carboxylates. Alternatively, the bridging ligands can be substituted by oxo or hydroxo groups (named diiron-oxo centres). Although this is the characteristic centre, the number and type of ligands and even the oxidation state of the iron changes, occurring in nature different types of dinuclear iron centres (*53*). This family of proteins can be involved in several metabolic processes, like oxygen transport, iron storage, DNA or fatty acid synthesis (*2, 54*). A class of enzymes recently

identified and designated as Ric (*R*epair of *i*ron *c*entres) proteins, also contain a diiron centre, as well as a group of enzymes called *f*lavo*d*iiron *p*roteins that function as nitric oxide and oxygen reductases (*55, 56*) (a subject that will be described in chapter IV and was explored in chapter I, respectively).

Another class of non-haem iron proteins is the mixed metal iron-containing proteins which are not widely distributed in Nature. One example is [Ni-Fe] which is the cofactor of hydrogenases, enzymes that catalyze the production and oxidation of H_2 in microorganisms (*57*). Also, a [Mn-Fe] centre was recently identified in the ribonucleotide reductase of the pathogen *Chlamydia trachomatis* (*58*). Moreover, in plants, a [Zn-Fe] centre was discovered in purple acid phosphatase enzymes in contrast with the di-iron centre identified in mammals (*59*).

II.3.3 – Iron-sulphur containing proteins

Even though Fe-S clusters are one of the most ancient prosthetic groups, it was not until the mid-1960's that Fe-S containing proteins were discovered. These proteins are present in all kingdoms of life. In bacteria, these proteins integrate more than 120 distinct classes of enzymes from which more than 50% of all Fe-S clusters containing proteins are involved in electron transfer systems and 17% are non-redox enzymes (*60*). There are over 30 different Fe-S cluster binding motifs, the most prevalent being $CX_2CX_2CX_3C$ (*60*). Fe-S proteins size range from 6 to 500 kDa and can enclose up to nine centres (*61*). The relative abundance of Fe-S proteins differs between the phylogenetic groups. In mammalian proteomes, although very few Fe-S proteins were identified till now, the lack of Fe-S

clusters leads to severe diseases as Friedreich's ataxia or even X-linked sideroblastic anaemia with cerebellar ataxia (*62*).

II.3.3.1- Types of Fe-S clusters and biological relevance

The most common type of Fe-S clusters are [2Fe-2S], [4Fe-4S] and [3Fe-4S] (Figure II.2) though more complex clusters such as [7Fe-8S] and [8Fe-8S] exist in bacterial nitrogenases. Several spectroscopic methods, namely UV-Visible, EPR, resonance Raman and Mössbauer, revealed the structural framework and the chemical and magnetic properties of Fe-S centres (*61*). Cysteine is the most common protein ligand but others ligands like histidine, aspartate and arginine are also observed (*63*). Another characteristic of Fe-S centres is their possible interconversion of [2Fe-2S], [3Fe-4S] and [4Fe-4S] due to the facile ligand-exchange reactions, coupled to electron transfer from biological reductants (*43, 64*). Due to the protein environment surrounding Fe-S clusters, the redox properties of their ligands is very flexible and this prosthetic group can operate as electron carrier in diverse metabolic processes (*43, 44*).

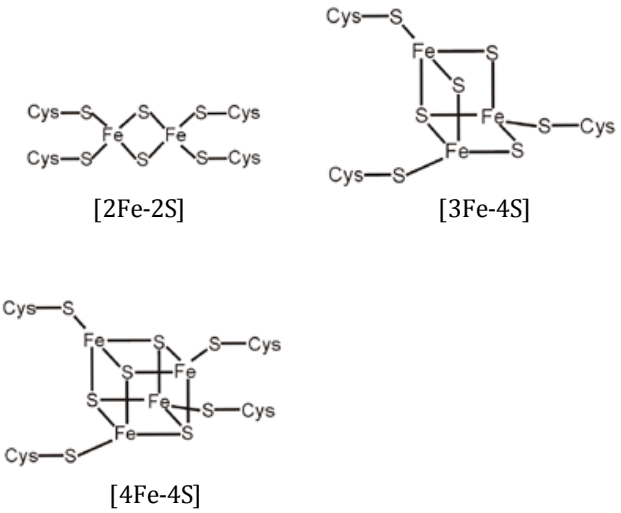


Figure II.2 – Structure of common types of Fe-S clusters. Fe - iron atom; S - sulphur atom; and Cys - cysteine ligands. [Adapted from (*64*)].

The ability of Fe-S clusters to transport electrons makes these cofactors important participants in respiratory and photosynthetic electron transfer chains, as well as in carbon, oxygen, hydrogen, sulphur and nitrogen metabolisms. Furthermore, Fe-S clusters are able to carry electrons through long distances. For example, membrane-bound fumarate reductase (the enzyme that converts fumarate to succinate) possesses three redox Fe-S clusters that transfer electrons from membrane-bound menaquinone to the cytosolic fumarate (*43, 65*).

Moreover, Fe-S proteins are involved in control of gene expression, sensing of iron, dioxygen, superoxide and nitric oxide, and in recognition and repair of DNA damage (*64*). An important family of proteins that contain $[4Fe\text{-}4S]^{2+}$ clusters are the dehydratases, enzymes that catalyze the removal of oxygen and hydrogen from the substrate, and which include fumarase, aconitase, anaerobic ribonucleotide reductase and lipoate synthase, among others (*43*). In this family of proteins, the cluster has three of the four iron atoms with a cysteine thiolate ligand and the fourth iron is solvent-exposed. The binding of substrate will occur via additional coordination of the fourth iron atom by both a carboxylate residue and the hydroxyl group that will be abstracted (*43*).

II.3.3.2- Fe-S clusters assembly mechanisms

Although Fe-S clusters can be formed *in vitro* upon reaction of an apoprotein with ferrous iron and sulphide salts (*66*), *in vivo* assembly machineries are required, as iron and sulphide are toxic to the cells in the concentrations necessary for the assembly of this prosthetic group (*67*).

The prevalence of Fe-S containing proteins in metabolic pathways of most organisms led some scientists to theorize that iron-sulphur

compounds had a significant role in the origin of life in the iron-sulphur world theory (*68*). In this model, in early Earth, the environment was anaerobic and iron and sulphur were present in abundance. Consequently, Fe-S clusters could be assembled spontaneously into polypeptide structures. However, 2.75 billion years ago as the first photosynthetic organisms (cyanobacteria) began to use photosystem II, the product oxygen led to oxidation of iron and, consequently, to the decomposition of Fe-S clusters. As a result, iron could not exist free in the cell due to its high reactivity, and organisms owing proteins specific for assembly of Fe-S clusters along with proteins required for the delivery of iron and sulphur prevailed (*43*).

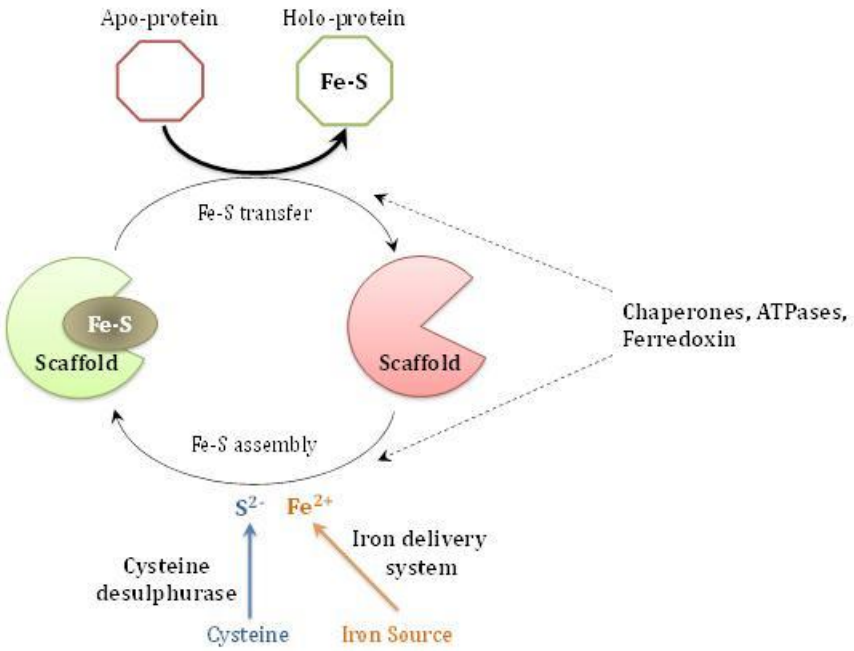


Figure II.3 –General scheme of Fe-S cluster assembly. Fe and S atoms are assembled within a scaffold protein and clusters are then transferred to apoproteins. S^{2-} is derived from cysteine through the action of cysteine desulphurases [Adapted from (*85*)].

The formation of Fe-S centres comprises three main pillars: the existence of a scaffold protein that receives the nascent Fe-S cluster, the sulphur source provided by cysteine desulphurases, and the iron donor. Once the prosthetic group is formed in the scaffold protein, the ultimate step is the delivery to the suitable apo-protein (Figure II.3) (*60*).

Nif system, *Ni*trogen *f*ixation

The Nif system was the first mechanism for Fe-S cluster biogenesis revealed in *Azotobacter vinelandii*, while searching for nitrogenase maturation factors (*69*). Nif discovery started with the identification of two proteins, NifU (the scaffold protein) and NifS (the cysteine desulphurase) (*70*). This assembly mechanism is usually present in nitrogen-fixing organisms. Although catalytically inefficient, nitrogenases are required to fix nitrogen, being necessary to be abundantly produced in nitrogen-fixing cells. Therefore, due to the high demand of the prosthetic group of these enzymes, the need for a specialized biogenesis system of Fe-S clusters of nitrogenases occurs (*63*). Interestingly, NifU/S were identified in *Helicobacter pylori*, indicating that Nif system is not restricted to nitrogen-fixing organisms (*71*).

The cysteine desulphurase NifS is a pyridoxal phosphate (PLP)-containing enzyme (*72*). This cofactor is essential for fold-stability and enzyme activity (*73*). In general, cysteine desulphurases are homodimeric proteins that catalyze the conversion of L-cysteine to L-alanine and an enzyme-bound persulphide that transfers elemental sulphur directly to the scaffold proteins. The trafficking of sulphur in this way avoids the toxicity of this element (*74, 75*).

The NifU protein contains a N-terminal U-type scaffold domain; a central ferredoxin-like domain with a redox-active $[2Fe\text{-}2S]^{2+/1+}$ cluster of

unknown function that participates in a redox process during cluster assembly; and a C-terminal domain with a CXXC motif (*67*). Although the N-terminal and the C-terminal domains can assemble Fe-S clusters and subsequently donate them to apoproteins, the C-terminal domain is the only one required for nitrogenase Fe-S cluster assembly (*70, 76*).

Isc system, *I*ron-*s*ulphur *c*lusters

A second system was discovered in *A. vinelandii* that also participates in Fe-S cluster assembly, Isc. The Isc system is present in several organisms from bacteria, to yeast and humans and is usually considered the housekeeping Fe-S cluster assembly pathway (*63, 70, 77*). The genes of the Isc system are organized in the operon *iscRSUA-hscBA-fdx-iscX* that encodes for the following proteins: a regulatory protein, IscR; a cysteine desulphurase, IscS; a scaffold protein, IscU; an A-type protein, IscA; a DnaJ-like co-chaperone (HscB); a DnaK-like chaperon (HscA); a ferredoxin; and IscX, a protein of unknown function (*63, 70, 77*). However, depending on the organism some genes can be absent from the operon.

IscS is the cysteine desulphurase of Isc but is also able to donate sulphur in other metabolic processes such as thiamine, biotin and thionucleotides synthesis (*63, 70*). The importance of this protein was revealed as *iscS* deletion in *A. vinelandii* is lethal (*70*). Moreover, *iscS* mutation in *E. coli* results in general growth defect, under normal and oxidative stress conditions, due to the deficiency in many biosynthetic pathways and to the lower activity of Fe-S containing enzymes (*78-80*). Crystallographic studies in *E. coli* revealed that an extensive surface area centred on the active site Cys328 is essential for the interaction of IscS with IscU, IscX, IscS and CyaY (whose roles will be described next), forming a quaternary complex (*81*). Cysteine desulphurase activity of IscS is

modulated by iron and sulphide, since desulphurase activity is gradually inhibited as the amount of iron and sulphide bound to IscS increases (*82*).

IscU is a U-type scaffold protein that acts as the primary site for Fe-S cluster assembly in the Isc system. The U-type scaffold proteins, in general, contain three conserved cysteines arranged in the $\underline{C}X_{24\text{-}26}\underline{C}X_{42\text{-}43}\underline{C}$ motif that is required for cluster ligation, but only IscU has the LPVVK motif required for interaction with HscA (*H*eat-*s*hock *c*ognate protein, whose function will be further discussed) (*83-85*). These conserved cysteine residues and one conserved non-cysteinyl ligand (aspartate) are necessary for cluster formation (*86, 87*) and for the transfer of intact Fe-S clusters to apo-proteins as these residues facilitate the release of the Fe-S clusters (*88-90*). *E. coli iscU* deletion strain has growth deficiency when compared to wild type and very low activity of several Fe-S containing enzymes (*79, 91*). ISCU (homologue of IscU) is the main scaffold protein in mammals and mutation of the gene in humans leads to a severe inherited disease called myopathy (*77, 92*).

The formation of Fe-S clusters in U-type scaffolds occurs by the following steps: first, the IscU homodimer assembles a $[2Fe\text{-}2S]^{2+}$ cluster, followed by insertion of another 2Fe-2S cluster; next, a two-electron direct reductive coupling occurs and the two $[2Fe\text{-}2S]^{1+}$ form a $[4Fe\text{-}4S]^{2+}$ (*86, 87*). The first $[2Fe\text{-}2S]^{2+}$ cluster formed in IscU homodimer is stable and resistant to iron chelators, the two $[2Fe\text{-}2S]^{2+}$ clusters are only intermediates in the formation of the $[4Fe\text{-}4S]^{2+}$. Furthermore, if the $[4Fe\text{-}4S]^{2+}$ cluster is exposed to O_2, it converts back to one $[2Fe\text{-}2S]^{2+}$ cluster-bound form of IscU (*67*). Interestingly, Fe-S clusters formed in IscU are degraded in the presence of oxidative stress and consequently the Isc system is inactivated in the presence of ROS (*93*). The transfer of sulphur from the cysteine persulphide on IscS to cysteine residues on IscU is direct,

as a complex between IscS and IscU monomers was observed (*86, 94, 95*), involving a persulphide or polysulphide linkage (*75, 96, 97*).

IscA was previously shown to assemble a Fe-S cluster spectroscopically *in vitro*. Like IscU, IscA has three cysteines conserved in the C-terminal region ($\underline{C}X_{42\text{-}44}DX_{20}\underline{C}G\underline{C}$) required for cluster ligation (*63, 70*). IscA has a tetrameric structure resembling a basket-shape with a central cavity between the two dimers (*63*). IscA accepts iron-sulphur clusters from IscU, but not vice-versa, and transfers them *in vitro* to various apo-proteins (*63*). Hence, at first, IscA was proposed to act as an iron-sulphur scaffold protein either for cluster donation to a subset of Fe-S proteins or as an intermediate in cluster transfer from U-type scaffold to apoproteins (*70*). However, the deletion of IscU in *A. vinelandii* was lethal and IscA could not substitute for IscU as a scaffold protein (*98*).

A new role was proposed for IscA to act as an iron donor for Fe-S cluster assembly in IscU. *E. coli* IscA binds one iron per dimer and is able to donate the iron for cluster assembly on IscU (*99-101*). In addition, the iron in IscA is mobilized, *in vitro*, in the presence of L-cysteine making it available for Fe-S cluster assembly in *E. coli* IscU (*102*).

IscR is a transcriptional regulator that contains, besides a DNA binding domain, a $[2Fe\text{-}2S]^{2+}$ cluster. This protein has three cys residues which are the ligands for the $[2Fe\text{-}2S]^{2+}$ cluster of the holo-protein (*103, 104*). Work in Kiley's laboratory revealed that IscR possesses two distinct types of DNA target sequences. Type-1 sites are preferentially regulated by [2Fe-2S]-IscR under anaerobic conditions when the cluster is presumed to be stable, and type-2 sites are bound by both [2Fe-2S]- and apo-IscR and these promoters are usually regulated under aerobic conditions when apo-IscR becomes more abundant (*105*). The Fe-S cluster of IscR is a sensor for the Fe-S cluster status of the cell. When sufficient Fe-S clusters are present

in the cell to satisfy the metabolic needs, the holo-IscR represses the *isc* operon (type-1 site). However, when these clusters are in low concentrations, IscR loses the centre resulting in the derepression of the *isc* locus (*70, 103*). Apo-IscR is also able to activate the expression of another Fe-S cluster assembly system, the Suf (*Sulf*ur assimilation), in response to oxidative stress (type-2 site). Therefore, under stress conditions, when the [2Fe-2S] cluster of IscR is lost, both Suf and Isc systems can be transcribed (*70, 103*). The IscR regulon is not restricted to Fe-S cluster assembly pathways; for example, in *E. coli*, this transcription factor regulates about 37 genes (type-1 and type-2 sites), among which are *erpA* gene (encoding the *e*ssential *r*espiratory *p*rotein *A* whose role will be discussed next), genes that encode Fe-S proteins and genes related to oxidative stress adaptation, such as *sodA* (encoding the *s*uper*o*xide *d*ismutase *A*) (*106*). As a result, IscR enables the organism to adapt to the varying conditions, from mounting a response to oxidative and nitrosative stress to switch from anaerobic to aerobic respiratory pathways and vice versa.

HscB and HscA (*H*eat *s*hock *c*ognate proteins) are associated with Fe-S protein maturation, as their deletion in *E. coli* perturbs Fe-S cluster assembly *in vivo* (*91, 107*). HscA interacts with the cluster-loaded or apoform of IscU assisted by HscB, which in turn interacts with both IscU and HscA. HscA exhibits a low level of intrinsic ATPase activity that is stimulated by interaction with HscB and highly stimulated upon interaction with both HscB and IscU (*63*). The proposed roles of these proteins are the stabilization of clusters assembled on IscU or facilitating cluster transfer from IscU to acceptor proteins in an ATP-dependent reaction (*67*). In fact, the latter role was demonstrated for the HscA and HscB proteins of *A. vinelandii* because the rate of $[2Fe\text{-}2S]^{2+}$ cluster transfer from IscU to apoferredoxin was shown to be 20-fold enhanced in the presence of both

proteins, in an ATP-dependent manner (*67, 90*). Nonetheless, in *E. coli*, these chaperones were able to bind and stabilize [2Fe-2S] clusters in IscU that could be donated to acceptor proteins such as apoferredoxin (*108*). Although the exact role of both proteins was not yet identified, they are involved in the optimization of the delivery step of Fe-S clusters.

Another gene that belongs to the *isc* operon is *fdx* encoding a ferredoxin. This protein contains a redox-active $[2Fe\text{-}2S]^{2+}$ cluster and was proposed to be the electron donor for the reductive coupling of the two $[2Fe\text{-}2S]^{2+}$ cluster to the $[4Fe\text{-}4S]^{2+}$ cluster in IscU (*109*).

The expression of the *isc* operon is induced upon exposure to oxidative stress and to iron restriction in an IscR-dependent manner (*70, 106*). Isc is also directly regulated in response to cellular iron status via Fur/RyhB regulation (*110*).

Interestingly, *E. coli* Δ*iscS* and Δ*iscU* are viable due to the existence of proteins with similar function involved in the assembly of Fe-S clusters, the most well-known of which are those belonging to the Suf system (*91, 107*).

Suf system

The *sufABCDSE* operon was originally identified in *E. coli* (*111*), but the Suf system is well distributed in bacteria, being found in proteobacteria and cyanobacteria. Usually, Suf is proposed to be the operative system under oxidative stress and iron limited conditions (*70, 112*).

The importance of Suf in assembly of Fe-S clusters was unveiled when the mutation of cysteine desulphurase encoding gene (*sufS*) led to the loss of the Fe-S centre in the ferric iron reductase protein, FhuF (*F*erric *h*ydroxamate *u*ptake) (*111*). Although, IscS and SufS have similar roles, IscS specific activity is 20-fold higher when compared to that of SufS (*113*). This

difference might be related to the active-site orientation of the cysteine desulphurases, as IscS active-site is highly exposed contrary to SufS that isn't solvent accessible (*114*, *115*).

SufS and SufE interact with each other and *E. coli* SufS uses Cys364 to donate the sulphur atom to the active-site Cys51 on the SufE protein (*116, 117*). The formation of the complex SufS-SufE promotes SufS cysteine desulphurase activity to levels comparable to that of IscS and the presence of SufBCD (whose role will be further discussed) results in an even higher activity of SufS (*118, 119*). SufE interacts and transfers sulphur directly to SufB for Fe-S cluster assembly (*120*). Furthermore, a strain mutated in *sufSE* has impaired growth under iron starvation and is lethal in a Δ*iscS* background (*121*).

SufC is a cytoplasmic ABC-ATPase that forms a soluble complex with SufB and SufD and has enhanced ATPase activity in the presence of SufB (*122, 123*). SufB was proposed to act as a primary scaffold protein for [4Fe-4S] clusters in the bacterial SUF system (*67*). In a recent work, $SufBC_2D$ and $SufB_2C_2$ complexes were shown to contain a [4Fe-4S] cluster that was transferred to target apo-proteins (*124*). While SufD is dispensable for sulphur transfer, it is absolutely required for iron acquisition during Fe-S clusters assembly as well as SufC (*125*). In summary, SufS cysteine desulphurase transfers persulphide sulphur from SufE to a $SufBC_2D$ complex, and the $SufBC_2D$ functions as a Fe-S scaffold system to assemble nascent Fe-S clusters. Although the specific mechanism of SufBCD complex action is yet to be defined, the proteins that belong to the complex are very important to Suf system as the deletion of any of the three components (SufB, SufC or SufD) abolishes Suf function *in vivo*, resulting in lower activity of Fe-S containing proteins under stress conditions (*121, 126-128*). Furthermore, *sufC* mutation resulted in

decreased virulence of *Erwinia chrysanthemi* (*126, 127*). As occurs for *sufSE* mutant, a strain mutated in *sufBCD* or in any of these genes has impaired growth under iron starvation and is lethal in a Δ*iscS* background (*121*).

Another gene present in the *suf* operon is *sufA* which encodes a protein that belongs to the A-type scaffold family, SufA. This protein interacts with SufBCD to accept Fe-S clusters formed on the SufBCD complex, acting as a Fe-S shuttle protein to ultimately transfer the cluster to apoproteins (*70, 129, 130*). In *E. coli*, SufA binds iron, and sulphur atoms provided by the SufS–SufE cysteine desulphurase system (*131*). The importance of SufA is not clear as deletion of *sufA* leads to a phenotype much less severe in comparison to the phenotype obtained when other *suf* genes are mutated (*121, 128*). Only the deletion of both *sufA* and *iscA* genes results in a null-growth phenotype in *E. coli* (*132*). Moreover, the fact that *E. coli* survival is recovered upon addition of branched-chain aminoacids and thiamine (products of [4Fe-4S] clusters-containing enzymes), led to the proposal for a role of IscA/SufA in the assembly of [4Fe-4S] clusters rather than [2Fe-2S] (*132*).

SufU is a U-type scaffold protein whose gene is not present in the *suf* operon of enterobacteria and cyanobacteria, but exists in the genome of Gram-positive bacteria, like *Bacillus subtilis* and *S. aureus*. The role of this protein in the Fe-S clusters assembly is not yet known. Work in *B. subtilis* presented a model where the activity of SufS is a 'ping-pong' mechanism leading to successive sulphur loading of the conserved cysteine residues in SufU (Cys41, 66 and 128) upon interaction of SufU with SufS (*133*). In *Enterococcus faecalis*, SufU enhances SufS cysteine desulphurase activity (*134*).

A gene adjacent to and divergently transcribed from *sufBCDS* locus called *sufR* is present in some bacteria being frequently found in

cyanobacteria. The product of this gene contains a Fe-S cluster and was proposed to be the sensor that controls the transcription of *suf* in these organisms (*70*).

In general, the *suf* operon is induced upon exposure to hydrogen peroxide in an OxyR-dependent manner and de-repressed upon iron limitation in a Fur-dependent manner (*121*). OxyR, IHF, apo-IscR and Fur-Fe^{2+} bind directly to the promoter region of this operon activating its expression, except Fur-Fe^{2+} that represses *suf* expression (*135*). Interestingly, an *E. coli* strain mutated in both Isc and Suf systems leads to bacterial death and the *isc* mutant phenotype can be suppressed overexpressing the *suf* operon (*128*). Therefore, both systems are proposed to possess overlapping roles (*135*).

In addition, another Fe-S cluster biogenesis system nominated Cia, after *C*ytosolic *i*ron-sulphur protein *a*ssembly, is present in eukaryotes and is responsible for the maturation of essential cytosolic and nuclear apoproteins (*136*).

Bacterial genome analyses revealed that the number and type of Fe-S clusters biosynthetic machineries varies between microorganisms and depends on their biological needs (*77*). For example, *A. vinelandii* contains the Nif and Isc system, *E. coli* has Isc and Suf, in *H. pylori* only the Nif system was identified so far, cyanobacteria have Suf and Isc, and *E. chrysanthemi* possesses all three systems (*71, 91, 98, 107, 126, 137*). In eukaryotes, the assembly systems are localized in different organelles, since homologues of Isc pathway are found in mitochondria, while the Suf system possess homologues localized in chloroplasts of photosynthetic organisms (*70*).

Additional proteins in Fe-S cluster assembly

The proteins that belong to Isc, Suf and Nif systems are not the only ones that participate in Fe-S assembly. An extra cysteine desulphurase was identified in *E. coli*, the CsdA (*C*ysteine *s*ulphinate *d*esulphinase) protein. CsdA activity is enhanced by CsdE (whose gene is located next to *csdA*) in a SufS-SufE-type manner (*138*). This CsdA-CsdE pair is proposed to be involved in Fe-S cluster assembly as it provides sulphur *in vitro* for reconstitution of a [4Fe-4S] centre and restores cluster assembly of Fe-S enzymes in an *iscS* mutant strain. However, a double *csdA-csdE* mutant strain has no phenotype relative to Fe-S cluster assembly (*70*). Moreover, CsdA participates in two separate sulphur transfer pathways by interacting with SufE-SufBCD (Fe-S biogenesis) or interacting with CsdE and CsdL (involved in the synthesis of a yet unknown compound) (*139*).

ErpA is an A-type scaffold protein, first identified in *E. coli*. An *erpA* mutation leads to an *E. coli* strain unable to respirate in the presence of oxygen or alternative electron acceptors, such as nitrate (*140*). ErpA affects the synthesis of the quinone precursor isopentenyl diphosphate, possibly due to the requirement of [4Fe-4S] clusters by the enzymes that participate in this pathway, IspG and IspH (*Iso*prenoid synthesis). Furthermore, ErpA assembles [2Fe-2S] and [4Fe-4S] clusters and transfers the cluster to apo-IspG (*140*). A recent study showed that ErpA is essential for the maturation of Fe-S clusters-contaning enzymes that participate in the formate-nitrate respiratory pathway in *E. coli* (*141*).

Nfu proteins (*NifU*-like proteins) are found in bacteria, cyanobacteria, plants and other higher eukaryotes, and contain a redox-active CXXC motif which is generally present in the C-terminal domain of NifU (*67*). These proteins assemble $[4Fe\text{-}4S]^{2+}$ clusters *in vitro* and transfer

them to apo-proteins (*67, 87, 142*). In addition, *E. coli nfuA* gene (encoding an Nfu homologue with N-terminal similar to A-type Fe-S scaffold proteins) is essential for growth under oxidative stress and iron starvation conditions (*143*). Upon oxidative stress, *E. coli* NfuA is recruited by the Suf system to secure Fe-S transfer to aconitase, NADH dehydrogenase (respiratory complex I) and Isp (*143*). NfuA receives Fe-S clusters from SufBCD, interacts with ErpA and IspH and transfers Fe-S centres to ErpA and IscA (*143*). Moreover, as IscU is the main Fe-S scaffold protein in several organisms, Nfu proteins could be transporter intermediates of [4Fe-4S] clusters or chaperones for clusters assembled on IscU (*67*). In organisms that use Suf as the major Fe-S assembly system, Nfu-type proteins could function as scaffolds for Fe-S clusters, since usually Suf does not possess U-type scaffold proteins (*67*).

Glutaredoxins (Grxs) are small proteins widespread in eukaryotes and prokaryotes that reduce persulphide bridges or glutathionylated proteins and are involved in Fe-S clusters biosynthesis (*144, 145*). In prokaryotes, several functions were proposed for Grxs, from facilitating Fe-S cluster assembly to storage and delivery of these clusters to apoproteins (*67*). An *E. coli* strain mutated in *grxD* and *isc* genes resulted in cell lethality (*146*). Furthermore, the *E. coli* monothiol glutaredoxin GrxD can form a Fe-S containing heterodimeric complex with BolA (*Bol*us, homologue of Fra_2P in eukaryotes), as occurs in higher eukaryotes, where a role in Fe–S cluster transport was reported for this protein (*147, 148*). In humans *grxD* mutation leads to a disease called sideroblastic anaemia (*149*).

A question that remains open in Fe-S cluster assembly is the process of *in vivo* iron donation. Iron delivery is thought to occur through the existence of metallochaperones that acquire iron and directly donate

this metal to the Fe-S cluster assembly pathway, protecting iron from chelation by other cellular components and limiting this substrate for Fenton reaction (*70*).

Based on *in vivo* and *in vitro* results, frataxin and the bacterial homologue CyaY were shown to be good candidates for iron donation to U-type scaffolds (*92, 150*). Frataxin binds iron and is required for Fe-S cluster assembly and homeostasis; in addition, a mutation in the gene coding for this protein leads to a neurodegenerative disease, Friedrich ataxia (*151*). In *E. coli* and *Salmonella enterica*, deletion of *cyaY* only resulted in the reduction of the levels of Fe-S cluster-containing respiratory complexes (*152, 153*). Nevertheless, bacterial CyaY is able to partially rescue the frataxin (Yfh1) deletion in yeast (*154*). Moreover, *E. coli* CyaY can bind either ferric iron up to 8 Fe^{3+}/polypeptide or ferrous iron up to 2.5 Fe^{2+}/polypeptide (*70*).

A study showed that CyaY participates in iron-sulphur cluster assembly as an iron-dependent inhibitor of cluster formation, through binding to the desulphurase IscS. The authors showed that interaction with IscS involves the iron binding surface of CyaY, which is conserved throughout the frataxin family (*155*). In addition, a more recent work supports the role of CyaY as an inhibitor of the iron sulphur cluster assembly rates by showing that CyaY directly inhibits the enzymatic activity of IscS (*156*). CyaY is able to interact with both IscS and IscU forming a complex. CyaY strengthens the affinity of IscU and IscS, slowing down the Fe-S cluster assembly (*157*). As a result, bacterial CyaY is now considered to be an iron sensor that acts as a regulator of Fe-S cluster assembly.

Furthermore, a protein of unknown function, YfhJ (previously named IscX), whose gene is encoded in the *isc* operon, also binds iron with

low affinity and interacts with IscS. Both YfhJ and CyaY compete for the same site on IscS, so YfhJ acts as a modulator of the inhibitory properties of CyaY. In addition, YfhJ rescues the rate of enzymatic cluster formation which is inhibited by CyaY (*158*).

Interestingly, a recent study showed that frataxin in eukaryotes binds to cysteine desulphurases and stimulates their activity, enhancing the rate of Fe-S cluster assembly (*159*). These results separate the role of frataxin in eukaryotes from that of bacterial CyaY and the difference between eukaryotic and prokaryotic roles could be due to evolutionary differences between the cysteine desulphurases.

In *Salmonella enterica*, the deletion of the *apbC* and *apbE* (*a*lternative *p*yrimidine *b*iosynthetic pathway) genes leads to the reduced activities of dehydratases enzymes, the same phenotype as *Salmonella enterica isc* mutant (*160*). The phenotype was rescued by addition of an inorganic source of iron which led to the proposal that these proteins could act as iron donors for *in vivo* Fe-S cluster assembly (*160*). However, a study in this organism showed that derepression of the *isc* operon or overexpression of *iscU* from a plasmid compensates for the lack of ApbC during growth on tricarballylate (whose metabolism requires [4Fe-4S] containing dehydratase enzymes) proposing a functional redundancy between ApbC and IscU (*161*).

Several enzymes contain more complex clusters that are derived from chemical modifications of simple iron–sulphur clusters. Some examples of these clusters are the cofactors of carbon monoxide dehydrogenase/acetylCoA synthase complexes (contains per dimer two nickel, 11-13 iron, 14 inorganic sulphur and one zinc atoms), nitrogenases (e.g. Fe_7MoS_9), hydrogenases (e.g. [Ni-Fe]S_4), and Hcp (*H*ybrid-*c*luster

*p*rotein with [4Fe-2S-2O]). The assembly process of these type of clusters starts with the synthesis of iron–sulphur precursors through the activity of the Isc or Suf systems or, in the case of nitrogenases, by Nif mechanism. The initial Fe-S clusters are modified by specialized radical S-adenosyl-L-methionine dependent Fe–S enzymes (radical SAM enzymes) that introduce specific modifications to the simpler cluster in the form of unique non-protein ligands. The clusters are assembled on a scaffold and are ultimately inserted into stable forms of the apo-proteins to form active enzymes (*162*).

II.3.3.3- Damage and repair of Fe-S clusters

Prokaryotes and eukaryotes are exposed to oxidative and nitrosative stress prompted by extracellular sources of reactive oxygen species and nitric oxide, like the host organism. One of the main targets of these reactive species are Fe-S containing proteins, whose prosthetic group will be damaged. Although the organisms possess Fe-S cluster assembly systems, it may not be energetically favourable to assemble a Fe-S cluster from scratch, when the cell can simply repair the cluster that was damaged.

Oxidative and nitrosative Fe-S cluster damage

Fe-S containing proteins are sensitive to oxidative damage due to the electrostatic attraction of ROS to the catalytic iron atom. In the presence of ROS, the cluster is oxidized and will degrade, losing the iron atom that sustains the enzymatic activity (*163*):

$$[4Fe\text{-}4S]^{2+} + Ox \rightarrow [4Fe\text{-}4S]^{3+} + Red \quad (1)$$

$$[4Fe\text{-}4S]^{3+} \rightarrow [3Fe\text{-}4S]^{1+} + Fe^{2+} \quad (2)$$

Scheme 2 – Oxidative damage of dehydratase Fe-S clusters. Ox – Oxidant; Red – Reductant; (1) – Oxidation of the cluster; (2) – Loss of the catalytic iron.

Till now several enzymes were shown to be damaged by reactive oxygen species (*163*). The $[4Fe\text{-}4S]^{2+}$ dehydratase clusters are more prone to damage as they are solvent-exposed and small oxidants enter more easily into contact with the active cluster site (*43*). Several ROS, like the superoxide anion, hydrogen peroxide and peroxynitrite, are able to oxidize these clusters (*43*). Inactivation rates were measured for the dehydratase enzymes with superoxide and hydrogen peroxide being in the range of 10^6-10^7 $M^{-1}s^{-1}$ and 10^2-10^3 $M^{-1}s^{-1}$, respectively (*43, 164*). *In vivo*, it was demonstrated that upon continuous exposure to ROS, the damaged $[3Fe\text{-}4S]^{1+}$ can be further degraded causing further iron release (*43*).

Upon reaction of Fe-S clusters with nitric oxide, the immediate formation of DNICs occurs, the dinitrosyl iron complexes, $[(NO)_2Fe(SR)_2]$, that are characterized by a specific electron paramagnetic signal at g=2.03 (Figure II.4) (*165*). Several *in vitro* studies showed that DNIC formation is responsible for the destruction of Fe-S clusters and loss of enzyme function of proteins exposed to NO (*166-171*). The dehydratase enzymes are the major biological targets of nitric oxide due to their Fe-S clusters solvent-exposure

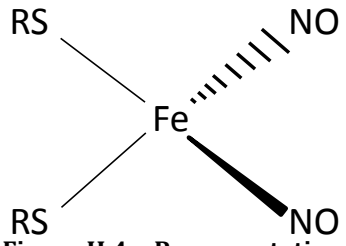


Figure II.4 – Representation of a dinitrosyl iron complex (DNIC). RS – thiolate ligand; NO – nitric oxide; Fe – iron atom.

(*167, 172, 173*). Other Fe-S enzymes known to be damaged *in vivo* by NO are the [4Fe-4S] clusters of *E. coli* endonuclease III and *Mycobacterium tuberculosis* regulatory protein WhiB3 *(174, 175)*.

Even though the enzymatic activity of Fe-S proteins is usually destroyed upon oxidative and nitrosative stress, leading to disruption of multiple catabolic and biosynthetic pathways, some profit can be obtained from the iron reactivity, as Fe-S clusters can act as sensors of ROS and NO.

The dimeric SoxR regulator contains a $[2Fe\text{-}2S]^{2+}$ cluster per subunit that senses both superoxide and nitric oxide. Upon oxidative stress, SoxR activates the expression of the transcription factor SoxS, which results in the stimulation of its regulon, whose gene products help in the removal of superoxide and repair of damaged proteins and DNA, among others (*44*) (see Chapter I).

Another regulator that exploits the Fe-S clusters reactivity is the homodimeric protein FNR that contains an oxygen-labile $[4Fe\text{-}4S]^{2+}$ cluster per monomer. This transcription factor controls the switch from aerobic to anaerobic metabolism, repressing the expression of genes that function in aerobic respiration and activating the expression of genes that will permit the reduction of alternative electron acceptors. FNR is inactivated by NO, via formation of monomeric and dimeric dinitrosyl-iron-dithiol complexes, leading to the expression of its regulon that encode for proteins necessary for a wide number of metabolic pathways (*44, 176*) (see Chapter I).

NsrR contains a $[2Fe\text{-}2S]^{2+}$ cluster and plays an important role in the response of pathogens against reactive nitrogen species, being able to repress a total of 20 genes in *E. coli* that are known to protect bacteria against RNS (*177*). Upon nitrosative damage, NsrR is nytrosilated and consequently this protein derepresses its regulon (see Chapter I).

Repair of oxidatively and nitrosatively damaged Fe-S clusters

Oxidative stress *in vivo* is believed not to get as far as the degradation of clusters to the point that it is necessary to assemble completely new Fe-S clusters. For example, oxidative damage of the [4Fe-4S]$^{2+/1+}$ dehydratase clusters leads to the formation of an inactive [3Fe-4S]$^{+}$ centre; however upon stop of the stress, the enzyme activity is fully recovered with a half time of approximately 3-5 minutes, even when protein synthesis is blocked (*79, 178, 179*). *In vitro*, the oxidatively damaged enzymes can be recovered by addition of iron and the reducing agent, DTT: [3Fe-4S]$^{+}$ + e^{-} → [3Fe-4S]^{0}, followed by [3Fe-4S]^{0} + Fe^{2+} → [4Fe-4S]$^{2+}$ (*180*). *In vivo*, the mechanism of reactivation presumably requires the consecutive donation of an electron donor and ferrous iron, and frataxin was suggested to have a role in the activity of aconitase by donating iron to convert damaged [3Fe-4S]$^{+}$ cluster back to the active [4Fe-4S]$^{2+}$ form (*181*). Moreover, the [4Fe-4S] cluster of *E. coli* endonuclease III was repaired by addition of ferrous ion, IscS and L-cysteine (*174*). A study in *S. enterica* revealed that ferritin B is a major Fe source for the repair of oxidatively damaged Fe-S clusters (*182*).

Recently, it was reported that in a strain accumulating low levels of H_2O_2, the [4Fe–4S] cluster of isopropyl malate isomerase (LeuC, *Leu*cine biosynthesis) is damaged beyond the [3Fe–4S] state, to [2Fe–2S] or even to full degradation (*93*). The cluster is then reconstructed probably by the Suf system which is active under oxidative stress conditions (*93*). In contrast, inactivated apo-FNR seems to depend upon the Isc system to recover its [4Fe–4S] cluster (*183*).

The repair of DNICs is proposed to initially require a substrate to remove the nitrosylated iron with the concomitant release of ferrous iron. L-cysteine can be that substrate as it mediates the destabilization of dinitrosyl iron complexes in proteins, promotes the reassembly of a new iron-sulphur cluster and reacts with molecular thiols or protein thiols to yield S-nitrosothiols (*184-186*). In the repair of nitric oxide-modified [2Fe-2S] cluster of *E. coli* ferredoxin, the dinitrosyl iron complexes are directly transformed back to the ferredoxin [2Fe-2S] cluster using only the cysteine desulphurase IscS and L-cysteine *in vitro*, without the need of addition of iron or any other protein components. The removal of the dinitrosyl iron complex from ferredoxin and the prevention of the reassembly of the [2Fe-2S] cluster showed that the iron in the dinitrosyl iron complex is reused to repair the iron-sulphur clusters (*166, 184, 185*).

Besides frataxin, other proteins were proposed to be iron sources during the repair process of either nitrosylated or oxidized Fe-S clusters, such as the Ric proteins, that were first identified in *E. coli* and whose properties will be elucidated next, and the previously referred *S. enterica* ApbC and ApbE proteins (*187*).

In conclusion, the biochemical properties of the damaged Fe–S-containing protein and/or the degradation status of the cluster direct the choice of the repair pathway. Nevertheless, the oxidatively damaged Fe-S cluster repair always requires a source of iron and a reducing agent, while the nitrosatively damaged Fe-S cluster repair needs a substrate to remove NO from denitrosylated iron.

II.3.3.4- YtfE, a protein that repairs Fe-S clusters

The discovery of *E. coli* YtfE

The transcriptome profile of *E. coli* exposed to NO releasers strongly depends on the experimental conditions used and the short list of genes induced in all assays includes *ytfE* (*10, 188-190*). *ytfE* is induced by nitrite/nitrate, nitric oxide gas and NO releasers, under aerobic and anaerobic conditions, in rich and minimal media (*10, 177, 188-191*). Nonetheless, the *ytfE* induction by nitrate/nitrite only occurs upon nitrate reduction, so *ytfE* is activated probably due to low levels of NO that are produced by *E. coli* as a by-product of nitrate and nitrite respiration (*177, 191*). Moreover, *ytfE* was found to be induced by heat shock, a condition that causes protein denaturation (*192*). In a study with uropathogenic *E. coli*, *ytfE* was upregulated upon infection, contributing to the pathogenicity of this strain (*193*). In addition, in a microarray study where an *E. coli* strain resistant to triclosan was exposed to this germicide, *ytfE* was also induced (*194*).

The regulation of YtfE was first shown to be performed by the nitric oxide-sensitive transcriptional regulator NsrR in a bioinformatic study according to the presence of NsrR-binding motifs in *ytfE* promoter region (*106*), which was confirmed by a later study using reporter fusions (*191*). In order to discover the mechanistic regulation of *ytfE*, more studies have to be carried on.

Role of YtfE in the repair of Fe-S clusters

The first studies revealed a role of YtfE not only for nitrosative stress but also upon oxidative stress as an *E. coli* strain deleted in *ytfE* had increased sensitivity, when compared to wild type, in the presence of nitric

oxide (*189*) or hydrogen peroxide (*195*). In addition, an *E. coli* Δ*ytfE* strain had lower enzymatic activity of Fe-S proteins (e. g. fumarase and aconitase) (*196*) and the role of YtfE in the oxidative and nitrosative damage and repair of Fe-S clusters-containing proteins was investigated (*195*).

Hence, cells overexpressing two *E. coli* dehydratases, aconitase B and fumarase A, were submitted to hydrogen peroxide and nitric oxide and the fate of the $[4Fe\text{-}4S]^{2+/1+}$ cluster was followed by whole-cell EPR. The results showed that the formation of oxidatively damaged Fe-S species and DNICs occurred at a higher rate and to a larger extent in cells lacking an active YtfE protein (*195*). Moreover, the loss of enzymatic activities was more pronounced in Δ*ytfE* when compared to wild type (*195*). Also, in *E. coli ytfE* mutant no repair was detected and only the addition of purified recombinant YtfE protein promoted the regain of activity similar to the one observed in *E. coli* wild type strain, clearly showing that the repair is dependent on YtfE (*195*)

YtfE is a di-iron protein

E. coli YtfE is a homodimeric complex (24 kDa each monomer) and contains two iron atoms per polypeptide chain (*196*). The characterization by UV-visible and EPR spectroscopies have established that the iron atoms form a non-haem binuclear iron centre of the histidine/carboxylate type (*196*). The UV-visible spectra of YtfE exhibits a broad band at c. 360 nm in the oxidized form, which is bleached once the protein is reduced (*196*). The di-iron centre of YtfE has reduction potentials, assessed by an EPR monitored redox titration of E=+260 and +110 mV (*196*).

Moreover, *E. coli* YtfE was analyzed by resonance Raman and XAS/EXAFS (*X*-ray *A*bsorption *S*pectroscopy/*E*xtended *X*-ray *A*bsorption

*F*ine *S*tructure) spectroscopies, which led to a model centre (Figure II.5). In this model, the di-iron centre is proposed to be bridged by a μ-oxo and one or two μ-carboxo bridges, and putatively coordinated by six histidinyl residues and two or three carboxylate ligands (from aspartate and glutamate residues) (*56*).

The binuclear iron centre is the active centre since the *E. coli* apo-YtfE is unable to promote the repair of oxidatively damaged Fe-S clusters (*195*). However, further studies are required to determine the importance of this iron centre in the repair of Fe-S clusters.

The role of YtfE in the repair of Fe-S clusters was studied and chapters IV, V and VI describe the work done under the scope of this thesis.

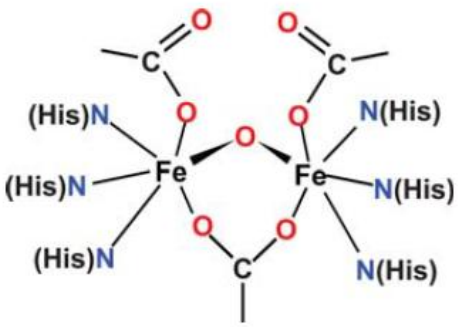


Figure II.5 – Model of *E. coli* YtfE di-iron centre in the oxidized form. The two upper oxygen ligands of the two iron atoms can be also provided by a single carboxylate group, leading to the formation of a second μ-carboxylate bridge. C –carbon atom; O – oxygen atom; Fe – iron atom; N(His) – histidinyl residue. Adapted from (*56*).

II.4 – References

(1) A. Earnshaw, and Greenwood, N. (1997) *Chemistry of the Elements*, Elsevier, School of Chemistry, University of Leeds, UK.

(2) Crichton, R. (2001) *Inorganic Biochemistry of Iron Metabolism: From Molecular Mechanisms to Clinical Consequences*, Wiley, Baffins Lane, Chichester, West Sussex PO19 1UD, England.

(3) Wardman, P., and Candeias, L. P. (1996) Fenton chemistry: an introduction. *Radiat Res 145*, 523-31.

(4) Nairz, M., Theurl, I., Ludwiczek, S., Theurl, M., Mair, S. M., Fritsche, G., and Weiss, G. (2007) The co-ordinated regulation of iron homeostasis in

murine macrophages limits the availability of iron for intracellular Salmonella typhimurium. *Cell Microbiol 9*, 2126-40.

(5) Wang, L., and Cherayil, B. J. (2009) Ironing out the wrinkles in host defense: interactions between iron homeostasis and innate immunity. *J Innate Immun 1*, 455-64.

(6) Doherty, C. P. (2007) Host-pathogen interactions: the role of iron. *J Nutr 137*, 1341-4.

(7) Schaible, U. E., and Kaufmann, S. H. (2004) Iron and microbial infection. *Nat Rev Microbiol 2*, 946-53.

(8) Andrews, S. C., Robinson, A. K., and Rodriguez-Quinones, F. (2003) Bacterial iron homeostasis. *FEMS Microbiol Rev 27*, 215-37.

(9) Kakhlon, O., and Cabantchik, Z. I. (2002) The labile iron pool: characterization, measurement, and participation in cellular processes(1). *Free Radic Biol Med 33*, 1037-46.

(10) Das, N. K., Biswas, S., Solanki, S., and Mukhopadhyay, C. K. (2009) Leishmania donovani depletes labile iron pool to exploit iron uptake capacity of macrophage for its intracellular growth. *Cell Microbiol 11*, 83-94.

(11) Pan, X., Tamilselvam, B., Hansen, E. J., and Daefler, S. (2010) Modulation of iron homeostasis in macrophages by bacterial intracellular pathogens. *BMC Microbiol 10*, 64.

(12) Paradkar, P. N., De Domenico, I., Durchfort, N., Zohn, I., Kaplan, J., and Ward, D. M. (2008) Iron depletion limits intracellular bacterial growth in macrophages. *Blood 112*, 866-74.

(13) Flo, T. H., Smith, K. D., Sato, S., Rodriguez, D. J., Holmes, M. A., Strong, R. K., Akira, S., and Aderem, A. (2004) Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. *Nature 432*, 917-21.

(14) Holmes, M. A., Paulsene, W., Jide, X., Ratledge, C., and Strong, R. K. (2005) Siderocalin (Lcn 2) also binds carboxymycobactins, potentially defending against mycobacterial infections through iron sequestration. *Structure 13*, 29-41.

(15) Nairz, M., Theurl, I., Schroll, A., Theurl, M., Fritsche, G., Lindner, E., Seifert, M., Crouch, M. L., Hantke, K., Akira, S., Fang, F. C., and Weiss, G. (2009) Absence of functional Hfe protects mice from invasive Salmonella enterica serovar Typhimurium infection via induction of lipocalin-2. *Blood 114*, 3642-51.

(16) Larson, J. A., Howie, H. L., and So, M. (2004) Neisseria meningitidis accelerates ferritin degradation in host epithelial cells to yield an essential iron source. *Mol Microbiol 53*, 807-20.

(17) Torti, F. M., and Torti, S. V. (2002) Regulation of ferritin genes and protein. *Blood 99*, 3505-16.

(18) Deneer, H. G., Healey, V., and Boychuk, I. (1995) Reduction of exogenous ferric iron by a surface-associated ferric reductase of Listeria spp. *Microbiology 141 (Pt 8)*, 1985-92.

(19) Wilson, M. E., Vorhies, R. W., Andersen, K. A., and Britigan, B. E. (1994) Acquisition of iron from transferrin and lactoferrin by the protozoan Leishmania chagasi. *Infect Immun 62*, 3262-9.
(20) Marx, J. J. (2002) Iron and infection: competition between host and microbes for a precious element. *Best Pract Res Clin Haematol 15*, 411-26.
(21) Cartron, M. L., Maddocks, S., Gillingham, P., Craven, C. J., and Andrews, S. C. (2006) Feo--transport of ferrous iron into bacteria. *Biometals 19*, 143-57.
(22) Koster, W. (2001) ABC transporter-mediated uptake of iron, siderophores, heme and vitamin B12. *Res Microbiol 152*, 291-301.
(23) Bridges, K., Seligman, PA. (1995) *Disorders of iron metabolism.*
(24) Wandersman, C., and Delepelaire, P. (2004) Bacterial iron sources: from siderophores to hemophores. *Annu Rev Microbiol 58*, 611-47.
(25) Letoffe, S., Heuck, G., Delepelaire, P., Lange, N., and Wandersman, C. (2009) Bacteria capture iron from heme by keeping tetrapyrrol skeleton intact. *Proc Natl Acad Sci U S A 106*, 11719-24.
(26) Cornelis, P., Wei, Q., Andrews, S. C., and Vinckx, T. (2011) Iron homeostasis and management of oxidative stress response in bacteria. *Metallomics 3*, 540-9.
(27) Nandal, A., Huggins, C. C., Woodhall, M. R., McHugh, J., Rodriguez-Quinones, F., Quail, M. A., Guest, J. R., and Andrews, S. C. (2010) Induction of the ferritin gene (ftnA) of Escherichia coli by Fe(2+)-Fur is mediated by reversal of H-NS silencing and is RyhB independent. *Mol Microbiol 75*, 637-57.
(28) Bou-Abdallah, F., Lewin, A. C., Le Brun, N. E., Moore, G. R., and Chasteen, N. D. (2002) Iron detoxification properties of Escherichia coli bacterioferritin. Attenuation of oxyradical chemistry. *J Biol Chem 277*, 37064-9.
(29) Abdul-Tehrani, H., Hudson, A. J., Chang, Y. S., Timms, A. R., Hawkins, C., Williams, J. M., Harrison, P. M., Guest, J. R., and Andrews, S. C. (1999) Ferritin mutants of Escherichia coli are iron deficient and growth impaired, and fur mutants are iron deficient. *J Bacteriol 181*, 1415-28.
(30) Calhoun, L. N., and Kwon, Y. M. (2010) Structure, function and regulation of the DNA-binding protein Dps and its role in acid and oxidative stress resistance in Escherichia coli: a review. *J Appl Microbiol 110*, 375-86.
(31) Arosio, P., Ingrassia, R., and Cavadini, P. (2009) Ferritins: a family of molecules for iron storage, antioxidation and more. *Biochim Biophys Acta 1790*, 589-99.
(32) Chiancone, E., Ceci, P., Ilari, A., Ribacchi, F., and Stefanini, S. (2004) Iron and proteins for iron storage and detoxification. *Biometals 17*, 197-202.
(33) Schroder, I., Johnson, E., and de Vries, S. (2003) Microbial ferric iron reductases. *FEMS Microbiol Rev 27*, 427-47.
(34) Wang, S., Wu, Y., and Outten, F. W. (2011) Fur and the novel regulator YqjI control transcription of the ferric reductase gene yqjH in Escherichia coli. *J Bacteriol 193*, 563-74.

(35) Braun, V., Hantke, K., and Koster, W. (1998) Bacterial iron transport: mechanisms, genetics, and regulation. *Met Ions Biol Syst 35*, 67-145.
(36) Escolar, L., Perez-Martin, J., and de Lorenzo, V. (1999) Opening the iron box: transcriptional metalloregulation by the Fur protein. *J Bacteriol 181*, 6223-9.
(37) Boyd, J., Oza, M. N., and Murphy, J. R. (1990) Molecular cloning and DNA sequence analysis of a diphtheria tox iron-dependent regulatory element (dtxR) from Corynebacterium diphtheriae. *Proc Natl Acad Sci U S A 87*, 5968-72.
(38) Schmitt, M. P., Twiddy, E. M., and Holmes, R. K. (1992) Purification and characterization of the diphtheria toxin repressor. *Proc Natl Acad Sci U S A 89*, 7576-80.
(39) Bsat, N., Herbig, A., Casillas-Martinez, L., Setlow, P., and Helmann, J. D. (1998) Bacillus subtilis contains multiple Fur homologues: identification of the iron uptake (Fur) and peroxide regulon (PerR) repressors. *Mol Microbiol 29*, 189-98.
(40) Mills, S. A., and Marletta, M. A. (2005) Metal binding characteristics and role of iron oxidation in the ferric uptake regulator from Escherichia coli. *Biochemistry 44*, 13553-9.
(41) Ahmad, R., Brandsdal, B. O., Michaud-Soret, I., and Willassen, N. P. (2009) Ferric uptake regulator protein: binding free energy calculations and per-residue free energy decomposition. *Proteins 75*, 373-86.
(42) Lee, J. W., and Helmann, J. D. (2007) Functional specialization within the Fur family of metalloregulators. *Biometals 20*, 485-99.
(43) Imlay, J. A. (2006) Iron-sulphur clusters and the problem with oxygen. *Mol Microbiol 59*, 1073-82.
(44) Kiley, P. J., and Beinert, H. (2003) The role of Fe-S proteins in sensing and regulation in bacteria. *Curr Opin Microbiol 6*, 181-5.
(45) Mayfield, J. A., Dehner, C. A., and DuBois, J. L. (2011) Recent advances in bacterial heme protein biochemistry. *Curr Opin Chem Biol 15*, 260-6.
(46) Cheek, J., and Dawson, J. H. (2001) *Coenzymes: Haem*, John Wiley & Sons.
(47) Vicente, M. G. H. (2001) *Haem structure and function*, John Wiley & Sons.
(48) Frankenberg, N., Moser, J., and Jahn, D. (2003) Bacterial heme biosynthesis and its biotechnological application. *Appl Microbiol Biotechnol 63*, 115-27.
(49) Layer, G., Reichelt, J., Jahn, D., and Heinz, D. W. (2010) Structure and function of enzymes in heme biosynthesis. *Protein Sci 19*, 1137-61.
(50) Solomon, E. I., Brunold, T. C., Davis, M. I., Kemsley, J. N., Lee, S. K., Lehnert, N., Neese, F., Skulan, A. J., Yang, Y. S., and Zhou, J. (2000) Geometric and electronic structure/function correlations in non-heme iron enzymes. *Chem Rev 100*, 235-350.
(51) Costas, M., Mehn, M. P., Jensen, M. P., and Que, L., Jr. (2004) Dioxygen activation at mononuclear nonheme iron active sites: enzymes, models, and intermediates. *Chem Rev 104*, 939-86.

(52) D'Autreaux, B., Tucker, N. P., Dixon, R., and Spiro, S. (2005) A non-haem iron centre in the transcription factor NorR senses nitric oxide. *Nature 437*, 769-72.
(53) Maglioa, O., Nastria, F., Rosalesb, R. F. M., Faiellaa, M., Pavonea, V., DeGradoc, W. F., and Lombardi, A. (2007) Diiron-containing metalloproteins: Developing functional models. *Comptes Rendus Chimie 10*, 703–720.
(54) Albrecht Messerschmidt, R. H., Karl Wieghardt, Thomas Poulos (2001) *Handbook of Metalloproteins*, Vol. 2, Wiley, West Sussex, England.
(55) Vicente, J. B., Justino, M. C., Goncalves, V. L., Saraiva, L. M., and Teixeira, M. (2008) Biochemical, spectroscopic, and thermodynamic properties of flavodiiron proteins. *Methods Enzymol 437*, 21-45.
(56) Todorovic, S., Justino, M. C., Wellenreuther, G., Hildebrandt, P., Murgida, D. H., Meyer-Klaucke, W., and Saraiva, L. M. (2008) Iron-sulfur repair YtfE protein from Escherichia coli: structural characterization of the di-iron center. *J Biol Inorg Chem 13*, 765-70.
(57) Armstrong, F. A., and Albracht, S. P. (2005) [NiFe]-hydrogenases: spectroscopic and electrochemical definition of reactions and intermediates. *Philos Transact A Math Phys Eng Sci 363*, 937-54; discussion 1035-40.
(58) Roos, K., and Siegbahn, P. E. (2011) Oxygen cleavage with manganese and iron in ribonucleotide reductase from Chlamydia trachomatis. *J Biol Inorg Chem 16*, 553-65.
(59) Schenk, G., Elliott, T. W., Leung, E., Carrington, L. E., Mitic, N., Gahan, L. R., and Guddat, L. W. (2008) Crystal structures of a purple acid phosphatase, representing different steps of this enzyme's catalytic cycle. *BMC Struct Biol 8*, 6.
(60) Fontecave, M. (2006) Iron-sulfur clusters: ever-expanding roles. *Nat Chem Biol 2*, 171-4.
(61) Meyer, J. (2008) Iron-sulfur protein folds, iron-sulfur chemistry, and evolution. *J Biol Inorg Chem 13*, 157-70.
(62) Sheftel, A., Stehling, O., and Lill, R. (2009) Iron-sulfur proteins in health and disease. *Trends Endocrinol Metab 21*, 302-14.
(63) Johnson, D. C., Dean, D. R., Smith, A. D., and Johnson, M. K. (2005) Structure, function, and formation of biological iron-sulfur clusters. *Annu Rev Biochem 74*, 247-81.
(64) Brzoska, K., Meczynska, S., and Kruszewski, M. (2006) Iron-sulfur cluster proteins: electron transfer and beyond. *Acta Biochim Pol 53*, 685-91.
(65) Johnson, M. K. (1998) Iron-sulfur proteins: new roles for old clusters. *Curr Opin Chem Biol 2*, 173-81.
(66) Malkin, R., and Rabinowitz, J. C. (1966) The reconstitution of clostridial ferredoxin. *Biochem Biophys Res Commun 23*, 822-7.
(67) Bandyopadhyay, S., Chandramouli, K., and Johnson, M. K. (2008) Iron-sulfur cluster biosynthesis. *Biochem Soc Trans 36*, 1112-9.

(68) Wachtershauser, G. (1992) Groundworks for an evolutionary biochemistry: the iron-sulphur world. *Prog Biophys Mol Biol 58*, 85-201.
(69) Jacobson, M. R., Cash, V. L., Weiss, M. C., Laird, N. F., Newton, W. E., and Dean, D. R. (1989) Biochemical and genetic analysis of the nifUSVWZM cluster from Azotobacter vinelandii. *Mol Gen Genet 219*, 49-57.
(70) Ayala-Castro, C., Saini, A., and Outten, F. W. (2008) Fe-S cluster assembly pathways in bacteria. *Microbiol Mol Biol Rev 72*, 110-25, table of contents.
(71) Olson, J. W., Agar, J. N., Johnson, M. K., and Maier, R. J. (2000) Characterization of the NifU and NifS Fe-S cluster formation proteins essential for viability in Helicobacter pylori. *Biochemistry 39*, 16213-9.
(72) Yuvaniyama, P., Agar, J. N., Cash, V. L., Johnson, M. K., and Dean, D. R. (2000) NifS-directed assembly of a transient [2Fe-2S] cluster within the NifU protein. *Proc Natl Acad Sci U S A 97*, 599-604.
(73) Prischi, F., Pastore, C., Carroni, M., Iannuzzi, C., Adinolfi, S., Temussi, P., and Pastore, A. (2010) Of the vulnerability of orphan complex proteins: the case study of the E. coli IscU and IscS proteins. *Protein Expr Purif 73*, 161-6.
(74) Mihara, H., and Esaki, N. (2002) Bacterial cysteine desulfurases: their function and mechanisms. *Appl Microbiol Biotechnol 60*, 12-23.
(75) Smith, A. D., Agar, J. N., Johnson, K. A., Frazzon, J., Amster, I. J., Dean, D. R., and Johnson, M. K. (2001) Sulfur transfer from IscS to IscU: the first step in iron-sulfur cluster biosynthesis. *J Am Chem Soc 123*, 11103-4.
(76) Dos Santos, P. C., Smith, A. D., Frazzon, J., Cash, V. L., Johnson, M. K., and Dean, D. R. (2004) Iron-sulfur cluster assembly: NifU-directed activation of the nitrogenase Fe protein. *J Biol Chem 279*, 19705-11.
(77) Py, B., and Barras, F. (2010) Building Fe-S proteins: bacterial strategies. *Nat Rev Microbiol 8*, 436-46.
(78) Schwartz, C. J., Djaman, O., Imlay, J. A., and Kiley, P. J. (2000) The cysteine desulfurase, IscS, has a major role in in vivo Fe-S cluster formation in Escherichia coli. *Proc Natl Acad Sci U S A 97*, 9009-14.
(79) Djaman, O., Outten, F. W., and Imlay, J. A. (2004) Repair of oxidized iron-sulfur clusters in Escherichia coli. *J Biol Chem 279*, 44590-9.
(80) Lauhon, C. T. (2002) Requirement for IscS in biosynthesis of all thionucleosides in Escherichia coli. *J Bacteriol 184*, 6820-9.
(81) Shi, R., Proteau, A., Villarroya, M., Moukadiri, I., Zhang, L., Trempe, J. F., Matte, A., Armengod, M. E., and Cygler, M. (2010) Structural basis for Fe-S cluster assembly and tRNA thiolation mediated by IscS protein-protein interactions. *PLoS Biol 8*, e1000354.
(82) Wu, G., Li, P., and Wu, X. (2008) Regulation of Escherichia coli IscS desulfurase activity by ferrous iron and cysteine. *Biochem Biophys Res Commun 374*, 399-404.
(83) Fontecave, M., and Ollagnier-de-Choudens, S. (2008) Iron-sulfur cluster biosynthesis in bacteria: Mechanisms of cluster assembly and transfer. *Arch Biochem Biophys 474*, 226-37.

(84) Hoff, K. G., Cupp-Vickery, J. R., and Vickery, L. E. (2003) Contributions of the LPPVK motif of the iron-sulfur template protein IscU to interactions with the Hsc66-Hsc20 chaperone system. *J Biol Chem 278*, 37582-9.

(85) Hoff, K. G., Ta, D. T., Tapley, T. L., Silberg, J. J., and Vickery, L. E. (2002) Hsc66 substrate specificity is directed toward a discrete region of the iron-sulfur cluster template protein IscU. *J Biol Chem 277*, 27353-9.

(86) Agar, J. N., Krebs, C., Frazzon, J., Huynh, B. H., Dean, D. R., and Johnson, M. K. (2000) IscU as a scaffold for iron-sulfur cluster biosynthesis: sequential assembly of [2Fe-2S] and [4Fe-4S] clusters in IscU. *Biochemistry 39*, 7856-62.

(87) Smith, A. D., Jameson, G. N., Dos Santos, P. C., Agar, J. N., Naik, S., Krebs, C., Frazzon, J., Dean, D. R., Huynh, B. H., and Johnson, M. K. (2005) NifS-mediated assembly of [4Fe-4S] clusters in the N- and C-terminal domains of the NifU scaffold protein. *Biochemistry 44*, 12955-69.

(88) Wu, S. P., Wu, G., Surerus, K. K., and Cowan, J. A. (2002) Iron-sulfur cluster biosynthesis. Kinetic analysis of [2Fe-2S] cluster transfer from holo ISU to apo Fd: role of redox chemistry and a conserved aspartate. *Biochemistry 41*, 8876-85.

(89) Unciuleac, M. C., Chandramouli, K., Naik, S., Mayer, S., Huynh, B. H., Johnson, M. K., and Dean, D. R. (2007) In vitro activation of apo-aconitase using a [4Fe-4S] cluster-loaded form of the IscU [Fe-S] cluster scaffolding protein. *Biochemistry 46*, 6812-21.

(90) Chandramouli, K., and Johnson, M. K. (2006) HscA and HscB stimulate [2Fe-2S] cluster transfer from IscU to apoferredoxin in an ATP-dependent reaction. *Biochemistry 45*, 11087-95.

(91) Tokumoto, U., and Takahashi, Y. (2001) Genetic analysis of the isc operon in Escherichia coli involved in the biogenesis of cellular iron-sulfur proteins. *J Biochem 130*, 63-71.

(92) Lill, R., and Muhlenhoff, U. (2006) Iron-sulfur protein biogenesis in eukaryotes: components and mechanisms. *Annu Rev Cell Dev Biol 22*, 457-86.

(93) Jang, S., and Imlay, J. A. (2010) Hydrogen peroxide inactivates the Escherichia coli Isc iron-sulphur assembly system, and OxyR induces the Suf system to compensate. *Mol Microbiol 78*, 1448-67.

(94) Tokumoto, U., Nomura, S., Minami, Y., Mihara, H., Kato, S., Kurihara, T., Esaki, N., Kanazawa, H., Matsubara, H., and Takahashi, Y. (2002) Network of protein-protein interactions among iron-sulfur cluster assembly proteins in Escherichia coli. *J Biochem 131*, 713-9.

(95) Agar, J. N., Zheng, L., Cash, V. L., Dean, D. R., and Johnson M. K. (2000) Role of the IscU Protein in Iron−Sulfur Cluster Biosynthesis: IscS-mediated Assembly of a [Fe2S2] Cluster in IscU. *J. Am. Chem. Soc. 122*, 2136–2137.

(96) Urbina, H. D., Silberg, J. J., Hoff, K. G., and Vickery, L. E. (2001) Transfer of sulfur from IscS to IscU during Fe/S cluster assembly. *J Biol Chem 276*, 44521-6.

(97) Kato, S., Mihara, H., Kurihara, T., Takahashi, Y., Tokumoto, U., Yoshimura, T., and Esaki, N. (2002) Cys-328 of IscS and Cys-63 of IscU are the sites of disulfide bridge formation in a covalently bound IscS/IscU complex: implications for the mechanism of iron-sulfur cluster assembly. *Proc Natl Acad Sci U S A 99*, 5948-52.

(98) Johnson, D. C., Unciuleac, M. C., and Dean, D. R. (2006) Controlled expression and functional analysis of iron-sulfur cluster biosynthetic components within Azotobacter vinelandii. *J Bacteriol 188*, 7551-61.

(99) Ding, H., Harrison, K., and Lu, J. (2005) Thioredoxin reductase system mediates iron binding in IscA and iron delivery for the iron-sulfur cluster assembly in IscU. *J Biol Chem 280*, 30432-7.

(100) Ding, H., and Clark, R. J. (2004) Characterization of iron binding in IscA, an ancient iron-sulphur cluster assembly protein. *Biochem J 379*, 433-40.

(101) Ding, H., Clark, R. J., and Ding, B. (2004) IscA mediates iron delivery for assembly of iron-sulfur clusters in IscU under the limited accessible free iron conditions. *J Biol Chem 279*, 37499-504.

(102) Ding, B., Smith, E. S., and Ding, H. (2005) Mobilization of the iron centre in IscA for the iron-sulphur cluster assembly in IscU. *Biochem J 389*, 797-802.

(103) Schwartz, C. J., Giel, J. L., Patschkowski, T., Luther, C., Ruzicka, F. J., Beinert, H., and Kiley, P. J. (2001) IscR, an Fe-S cluster-containing transcription factor, represses expression of Escherichia coli genes encoding Fe-S cluster assembly proteins. *Proc Natl Acad Sci U S A 98*, 14895-900.

(104) Yeo, W. S., Lee, J. H., Lee, K. C., and Roe, J. H. (2006) IscR acts as an activator in response to oxidative stress for the suf operon encoding Fe-S assembly proteins. *Mol Microbiol 61*, 206-18.

(105) Nesbit, A. D., Giel, J. L., Rose, J. C., and Kiley, P. J. (2009) Sequence-specific binding to a subset of IscR-regulated promoters does not require IscR Fe-S cluster ligation. *J Mol Biol 387*, 28-41.

(106) Giel, J. L., Rodionov, D., Liu, M., Blattner, F. R., and Kiley, P. J. (2006) IscR-dependent gene expression links iron-sulphur cluster assembly to the control of O2-regulated genes in Escherichia coli. *Mol Microbiol 60*, 1058-75.

(107) Takahashi, Y., and Nakamura, M. (1999) Functional assignment of the ORF2-iscS-iscU-iscA-hscB-hscA-fdx-ORF3 gene cluster involved in the assembly of Fe-S clusters in Escherichia coli. *J Biochem 126*, 917-26.

(108) Bonomi, F., Iametti, S., Morleo, A., Ta, D., and Vickery, L. E. (2011) Facilitated transfer of IscU-[2Fe2S] clusters by chaperone-mediated ligand exchange. *Biochemistry 50*, 9641-50.

(109) Chandramouli, K., Unciuleac, M. C., Naik, S., Dean, D. R., Huynh, B. H., and Johnson, M. K. (2007) Formation and properties of [4Fe-4S] clusters on the IscU scaffold protein. *Biochemistry 46*, 6804-11.

(110) Masse, E., Vanderpool, C. K., and Gottesman, S. (2005) Effect of RyhB small RNA on global iron use in Escherichia coli. *J Bacteriol 187*, 6962-71.

(111) Patzer, S. I., and Hantke, K. (1999) SufS is a NifS-like protein, and SufD is necessary for stability of the [2Fe-2S] FhuF protein in Escherichia coli. *J Bacteriol 181*, 3307-9.
(112) Xu, X. M., and Moller, S. G. (2011) Iron-sulfur clusters: biogenesis, molecular mechanisms, and their functional significance. *Antioxid Redox Signal 15*, 271-307.
(113) Mihara, H., Kurihara, T., Yoshimura, T., and Esaki, N. (2000) Kinetic and mutational studies of three NifS homologs from Escherichia coli: mechanistic difference between L-cysteine desulfurase and L-selenocysteine lyase reactions. *J Biochem 127*, 559-67.
(114) Cupp-Vickery, J. R., Urbina, H., and Vickery, L. E. (2003) Crystal structure of IscS, a cysteine desulfurase from Escherichia coli. *J Mol Biol 330*, 1049-59.
(115) Lima, C. D. (2002) Analysis of the E. coli NifS CsdB protein at 2.0 A reveals the structural basis for perselenide and persulfide intermediate formation. *J Mol Biol 315*, 1199-208.
(116) Mihara, H., Maeda, M., Fujii, T., Kurihara, T., Hata, Y., and Esaki, N. (1999) A nifS-like gene, csdB, encodes an Escherichia coli counterpart of mammalian selenocysteine lyase. Gene cloning, purification, characterization and preliminary x-ray crystallographic studies. *J Biol Chem 274*, 14768-72.
(117) Ollagnier-de-Choudens, S., Lascoux, D., Loiseau, L., Barras, F., Forest, E., and Fontecave, M. (2003) Mechanistic studies of the SufS-SufE cysteine desulfurase: evidence for sulfur transfer from SufS to SufE. *FEBS Lett 555*, 263-7.
(118) Loiseau, L., Ollagnier-de-Choudens, S., Nachin, L., Fontecave, M., and Barras, F. (2003) Biogenesis of Fe-S cluster by the bacterial Suf system: SufS and SufE form a new type of cysteine desulfurase. *J Biol Chem 278*, 38352-9.
(119) Outten, F. W., Wood, M. J., Munoz, F. M., and Storz, G. (2003) The SufE protein and the SufBCD complex enhance SufS cysteine desulfurase activity as part of a sulfur transfer pathway for Fe-S cluster assembly in Escherichia coli. *J Biol Chem 278*, 45713-9.
(120) Layer, G., Gaddam, S. A., Ayala-Castro, C. N., Ollagnier-de Choudens, S., Lascoux, D., Fontecave, M., and Outten, F. W. (2007) SufE transfers sulfur from SufS to SufB for iron-sulfur cluster assembly. *J Biol Chem 282*, 13342-50.
(121) Outten, F. W., Djaman, O., and Storz, G. (2004) A suf operon requirement for Fe-S cluster assembly during iron starvation in Escherichia coli. *Mol Microbiol 52*, 861-72.
(122) Kitaoka, S., Wada, K., Hasegawa, Y., Minami, Y., Fukuyama, K., and Takahashi, Y. (2006) Crystal structure of Escherichia coli SufC, an ABC-type ATPase component of the SUF iron-sulfur cluster assembly machinery. *FEBS Lett 580*, 137-43.

(123) Eccleston, J. F., Petrovic, A., Davis, C. T., Rangachari, K., and Wilson, R. J. (2006) The kinetic mechanism of the SufC ATPase: the cleavage step is accelerated by SufB. *J Biol Chem 281*, 8371-8.
(124) Wollers, S., Layer, G., Garcia-Serres, R., Signor, L., Clemancey, M., Latour, J. M., Fontecave, M., and Ollagnier de Choudens, S. (2010) Iron-sulfur (Fe-S) cluster assembly: the SufBCD complex is a new type of Fe-S scaffold with a flavin redox cofactor. *J Biol Chem 285*, 23331-41.
(125) Saini, A., Mapolelo, D. T., Chahal, H. K., Johnson, M. K., and Outten, F. W. (2010) SufD and SufC ATPase activity are required for iron acquisition during in vivo Fe-S cluster formation on SufB. *Biochemistry 49*, 9402-12.
(126) Nachin, L., El Hassouni, M., Loiseau, L., Expert, D., and Barras, F. (2001) SoxR-dependent response to oxidative stress and virulence of Erwinia chrysanthemi: the key role of SufC, an orphan ABC ATPase. *Mol Microbiol 39*, 960-72.
(127) Nachin, L., Loiseau, L., Expert, D., and Barras, F. (2003) SufC: an unorthodox cytoplasmic ABC/ATPase required for [Fe-S] biogenesis under oxidative stress. *Embo J 22*, 427-37.
(128) Takahashi, Y., and Tokumoto, U. (2002) A third bacterial system for the assembly of iron-sulfur clusters with homologs in archaea and plastids. *J Biol Chem 277*, 28380-3.
(129) Gupta, V., Sendra, M., Naik, S. G., Chahal, H. K., Huynh, B. H., Outten, F. W., Fontecave, M., and Ollagnier de Choudens, S. (2009) Native Escherichia coli SufA, coexpressed with SufBCDSE, purifies as a [2Fe-2S] protein and acts as an Fe-S transporter to Fe-S target enzymes. *J Am Chem Soc 131*, 6149-53.
(130) Chahal, H. K., Dai, Y., Saini, A., Ayala-Castro, C., and Outten, F. W. (2009) The SufBCD Fe-S scaffold complex interacts with SufA for Fe-S cluster transfer. *Biochemistry 48*, 10644-53.
(131) Sendra, M., Ollagnier de Choudens, S., Lascoux, D., Sanakis, Y., and Fontecave, M. (2007) The SUF iron-sulfur cluster biosynthetic machinery: sulfur transfer from the SUFS-SUFE complex to SUFA. *FEBS Lett 581*, 1362-8.
(132) Tan, G., Lu, J., Bitoun, J. P., Huang, H., and Ding, H. (2009) IscA/SufA paralogues are required for the [4Fe-4S] cluster assembly in enzymes of multiple physiological pathways in Escherichia coli under aerobic growth conditions. *Biochem J 420*, 463-72.
(133) Albrecht, A. G., Peuckert, F., Landmann, H., Miethke, M., Seubert, A., and Marahiel, M. A. (2011) Mechanistic characterization of sulfur transfer from cysteine desulfurase SufS to the iron-sulfur scaffold SufU in Bacillus subtilis. *FEBS Lett 585*, 465-70.
(134) Riboldi, G. P., de Oliveira, J. S., and Frazzon, J. (2011) Enterococcus faecalis SufU scaffold protein enhances SufS desulfurase activity by acquiring sulfur from its cysteine-153. *Biochim Biophys Acta 1814*, 1910-8.
(135) Xu, X. M., and Moller, S. G. (2008) Iron-sulfur cluster biogenesis systems and their crosstalk. *Chembiochem 9*, 2355-62.

(136) Sharma, A. K., Pallesen, L. J., Spang, R. J., and Walden, W. E. (2010) Cytosolic iron-sulfur cluster assembly (CIA) system: factors, mechanism, and relevance to cellular iron regulation. *J Biol Chem 285*, 26745-51.
(137) Rincon-Enriquez, G., Crete, P., Barras, F., and Py, B. (2008) Biogenesis of Fe/S proteins and pathogenicity: IscR plays a key role in allowing Erwinia chrysanthemi to adapt to hostile conditions. *Mol Microbiol 67*, 1257-73.
(138) Loiseau, L., Ollagnier-de Choudens, S., Lascoux, D., Forest, E., Fontecave, M., and Barras, F. (2005) Analysis of the heteromeric CsdA-CsdE cysteine desulfurase, assisting Fe-S cluster biogenesis in Escherichia coli. *J Biol Chem 280*, 26760-9.
(139) Trotter, V., Vinella, D., Loiseau, L., Ollagnier de Choudens, S., Fontecave, M., and Barras, F. (2009) The CsdA cysteine desulphurase promotes Fe/S biogenesis by recruiting Suf components and participates to a new sulphur transfer pathway by recruiting CsdL (ex-YgdL), a ubiquitin-modifying-like protein. *Mol Microbiol 74*, 1527-42.
(140) Loiseau, L., Gerez, C., Bekker, M., Ollagnier-de Choudens, S., Py, B., Sanakis, Y., Teixeira de Mattos, J., Fontecave, M., and Barras, F. (2007) ErpA, an iron sulfur (Fe S) protein of the A-type essential for respiratory metabolism in Escherichia coli. *Proc Natl Acad Sci U S A 104*, 13626-31.
(141) Pinske, C., and Sawers, R. G. (2012) A-type carrier protein ErpA is essential for formation of an active formate-nitrate respiratory pathway in Escherichia coli K-12. *J Bacteriol 194*, 346-53.
(142) Tong, W. H., Jameson, G. N., Huynh, B. H., and Rouault, T. A. (2003) Subcellular compartmentalization of human Nfu, an iron-sulfur cluster scaffold protein, and its ability to assemble a [4Fe-4S] cluster. *Proc Natl Acad Sci U S A 100*, 9762-7.
(143) Angelini, S., Gerez, C., Ollagnier-de Choudens, S., Sanakis, Y., Fontecave, M., Barras, F., and Py, B. (2008) NfuA, a new factor required for maturing Fe/S proteins in Escherichia coli under oxidative stress and iron starvation conditions. *J Biol Chem 283*, 14084-91.
(144) Muhlenhoff, U., Gerber, J., Richhardt, N., and Lill, R. (2003) Components involved in assembly and dislocation of iron-sulfur clusters on the scaffold protein Isu1p. *Embo J 22*, 4815-25.
(145) Rodriguez-Manzaneque, M. T., Tamarit, J., Belli, G., Ros, J., and Herrero, E. (2002) Grx5 is a mitochondrial glutaredoxin required for the activity of iron/sulfur enzymes. *Mol Biol Cell 13*, 1109-21.
(146) Butland, G., Babu, M., Diaz-Mejia, J. J., Bohdana, F., Phanse, S., Gold, B., Yang, W., Li, J., Gagarinova, A. G., Pogoutse, O., Mori, H., Wanner, B. L., Lo, H., Wasniewski, J., Christopolous, C., Ali, M., Venn, P., Safavi-Naini, A., Sourour, N., Caron, S., Choi, J. Y., Laigle, L., Nazarians-Armavil, A., Deshpande, A., Joe, S., Datsenko, K. A., Yamamoto, N., Andrews, B. J., Boone, C., Ding, H., Sheikh, B., Moreno-Hagelseib, G., Greenblatt, J. F., and Emili, A. (2008) eSGA: E. coli synthetic genetic array analysis. *Nat Methods 5*, 789-95.
(147) Yeung, N., Gold, B., Liu, N. L., Prathapam, R., Sterling, H. J., Willams, E. R., and Butland, G. (2011) The E. coli monothiol glutaredoxin GrxD forms

homodimeric and heterodimeric FeS cluster containing complexes. *Biochemistry 50*, 8957-69.
(148) Py, B., Moreau, P. L., and Barras, F. (2011) Fe-S clusters, fragile sentinels of the cell. *Curr Opin Microbiol 14*, 218-23.
(149) Ye, H., and Rouault, T. A. (2010) Human iron-sulfur cluster assembly, cellular iron homeostasis, and disease. *Biochemistry 49*, 4945-56.
(150) Layer, G., Ollagnier-de Choudens, S., Sanakis, Y., and Fontecave, M. (2006) Iron-sulfur cluster biosynthesis: characterization of Escherichia coli CYaY as an iron donor for the assembly of [2Fe-2S] clusters in the scaffold IscU. *J Biol Chem 281*, 16256-63.
(151) Bencze, K. Z., Kondapalli, K. C., Cook, J. D., McMahon, S., Millan-Pacheco, C., Pastor, N., and Stemmler, T. L. (2006) The structure and function of frataxin. *Crit Rev Biochem Mol Biol 41*, 269-91.
(152) Pohl, T., Walter, J., Stolpe, S., Soufo, J. H., Grauman, P. L., and Friedrich, T. (2007) Effects of the deletion of the Escherichia coli frataxin homologue CyaY on the respiratory NADH:ubiquinone oxidoreductase. *BMC Biochem 8*, 13.
(153) Vivas, E., Skovran, E., and Downs, D. M. (2006) Salmonella enterica strains lacking the frataxin homolog CyaY show defects in Fe-S cluster metabolism in vivo. *J Bacteriol 188*, 1175-9.
(154) Bedekovics, T., Gajdos, G. B., Kispal, G., and Isaya, G. (2007) Partial conservation of functions between eukaryotic frataxin and the Escherichia coli frataxin homolog CyaY. *FEMS Yeast Res 7*, 1276-84.
(155) Adinolfi, S., Iannuzzi, C., Prischi, F., Pastore, C., Iametti, S., Martin, S. R., Bonomi, F., and Pastore, A. (2009) Bacterial frataxin CyaY is the gatekeeper of iron-sulfur cluster formation catalyzed by IscS. *Nat Struct Mol Biol 16*, 390-6.
(156) Iannuzzi, C., Adinolfi, S., Howes, B. D., Garcia-Serres, R., Clemancey, M., Latour, J. M., Smulevich, G., and Pastore, A. (2011) The role of CyaY in iron sulfur cluster assembly on the E. coli IscU scaffold protein. *PLoS One 6*, e21992.
(157) Prischi, F., Konarev, P. V., Iannuzzi, C., Pastore, C., Adinolfi, S., Martin, S. R., Svergun, D. I., and Pastore, A. (2010) Structural bases for the interaction of frataxin with the central components of iron-sulphur cluster assembly. *Nat Commun 1*, 95.
(158) Pastore, C., Adinolfi, S., Huynen, M. A., Rybin, V., Martin, S., Mayer, M., Bukau, B., and Pastore, A. (2006) YfhJ, a molecular adaptor in iron-sulfur cluster formation or a frataxin-like protein? *Structure 14*, 857-67.
(159) Tsai, C. L., and Barondeau, D. P. (2010) Human frataxin is an allosteric switch that activates the Fe-S cluster biosynthetic complex. *Biochemistry 49*, 9132-9.
(160) Skovran, E., and Downs, D. M. (2003) Lack of the ApbC or ApbE protein results in a defect in Fe-S cluster metabolism in Salmonella enterica serovar Typhimurium. *J Bacteriol 185*, 98-106.

(161) Boyd, J. M., Lewis, J. A., Escalante-Semerena, J. C., and Downs, D. M. (2008) Salmonella enterica requires ApbC function for growth on tricarballylate: evidence of functional redundancy between ApbC and IscU. *J Bacteriol 190*, 4596-602.
(162) Shepard, E. M., Boyd, E. S., Broderick, J. B., and Peters, J. W. (2011) Biosynthesis of complex iron-sulfur enzymes. *Curr Opin Chem Biol 15*, 319-27.
(163) Imlay, J. A. (2003) Pathways of oxidative damage. *Annu Rev Microbiol 57*, 395-418.
(164) Jang, S., and Imlay, J. A. (2007) Micromolar intracellular hydrogen peroxide disrupts metabolism by damaging iron-sulfur enzymes. *J Biol Chem 282*, 929-37.
(165) Tsou, C. C., Lin, Z. S., Lu, T. T., and Liaw, W. F. (2008) Transformation of dinitrosyl iron complexes [(NO)2Fe(SR)2]- (R = Et, Ph) into [4Fe-4S] Clusters [Fe4S4(SPh)4]2-: relevance to the repair of the nitric oxide-modified ferredoxin [4Fe-4S] clusters. *J Am Chem Soc 130*, 17154-60.
(166) Ding, H., and Demple, B. (2000) Direct nitric oxide signal transduction via nitrosylation of iron-sulfur centers in the SoxR transcription activator. *Proc Natl Acad Sci U S A 97*, 5146-50.
(167) Drapier, J. C. (1997) Interplay between NO and [Fe-S] clusters: relevance to biological systems. *Methods 11*, 319-29.
(168) Welter, R., Yu, L., and Yu, C. A. (1996) The effects of nitric oxide on electron transport complexes. *Arch Biochem Biophys 331*, 9-14.
(169) Sellers, V. M., Johnson, M. K., and Dailey, H. A. (1996) Function of the [2FE-2S] cluster in mammalian ferrochelatase: a possible role as a nitric oxide sensor. *Biochemistry 35*, 2699-704.
(170) Meyer, J. (1981) Comparison of carbon monoxide, nitric oxide, and nitrite as inhibitors of the nitrogenase from Clostridium pasteurianum. *Arch Biochem Biophys 210*, 246-56.
(171) Kennedy, M. C., Antholine, W. E., and Beinert, H. (1997) An EPR investigation of the products of the reaction of cytosolic and mitochondrial aconitases with nitric oxide. *J Biol Chem 272*, 20340-7.
(172) Cooper, C. E. (1999) Nitric oxide and iron proteins. *Biochim Biophys Acta 1411*, 290-309.
(173) Hughes, M. N. (2008) Chemistry of nitric oxide and related species. *Methods Enzymol 436*, 3-19.
(174) Rogers, P. A., Eide, L., Klungland, A., and Ding, H. (2003) Reversible inactivation of E. coli endonuclease III via modification of its [4Fe-4S] cluster by nitric oxide. *DNA Repair (Amst) 2*, 809-17.
(175) Singh, A., Guidry, L., Narasimhulu, K. V., Mai, D., Trombley, J., Redding, K. E., Giles, G. I., Lancaster, J. R., Jr., and Steyn, A. J. (2007) Mycobacterium tuberculosis WhiB3 responds to O2 and nitric oxide via its [4Fe-4S] cluster and is essential for nutrient starvation survival. *Proc Natl Acad Sci U S A 104*, 11562-7.

(176) Constantinidou, C., Hobman, J. L., Griffiths, L., Patel, M. D., Penn, C. W., Cole, J. A., and Overton, T. W. (2006) 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*, 4802-15.

(177) Filenko, N., Spiro, S., Browning, D. F., Squire, D., Overton, T. W., Cole, J., and Constantinidou, C. (2007) The NsrR regulon of Escherichia coli K-12 includes genes encoding the hybrid cluster protein and the periplasmic, respiratory nitrite reductase. *J Bacteriol 189*, 4410-7.

(178) Keyer, K., and Imlay, J. A. (1997) Inactivation of dehydratase [4Fe-4S] clusters and disruption of iron homeostasis upon cell exposure to peroxynitrite. *J Biol Chem 272*, 27652-9.

(179) Gardner, P. R., and Fridovich, I. (1992) Inactivation-reactivation of aconitase in Escherichia coli. A sensitive measure of superoxide radical. *J Biol Chem 267*, 8757-63.

(180) Kennedy, M. C., Emptage, M. H., Dreyer, J. L., and Beinert, H. (1983) The role of iron in the activation-inactivation of aconitase. *J Biol Chem 258*, 11098-105.

(181) Bulteau, A. L., O'Neill, H. A., Kennedy, M. C., Ikeda-Saito, M., Isaya, G., and Szweda, L. I. (2004) Frataxin acts as an iron chaperone protein to modulate mitochondrial aconitase activity. *Science 305*, 242-5.

(182) Velayudhan, J., Castor, M., Richardson, A., Main-Hester, K. L., and Fang, F. C. (2007) The role of ferritins in the physiology of Salmonella enterica sv. Typhimurium: a unique role for ferritin B in iron-sulphur cluster repair and virulence. *Mol Microbiol 63*, 1495-507.

(183) Mettert, E. L., Outten, F. W., Wanta, B., and Kiley, P. J. (2008) The impact of O(2) on the Fe-S cluster biogenesis requirements of Escherichia coli FNR. *J Mol Biol 384*, 798-811.

(184) Rogers, P. A., and Ding, H. (2001) L-cysteine-mediated destabilization of dinitrosyl iron complexes in proteins. *J Biol Chem 276*, 30980-6.

(185) Yang, W., Rogers, P. A., and Ding, H. (2002) Repair of nitric oxide-modified ferredoxin [2Fe-2S] cluster by cysteine desulfurase (IscS). *J Biol Chem 277*, 12868-73.

(186) Tsai, M. L., Chen, C. C., Hsu, I. J., Ke, S. C., Hsieh, C. H., Chiang, K. A., Lee, G. H., Wang, Y., Chen, J. M., Lee, J. F., and Liaw, W. F. (2004) Photochemistry of the dinitrosyl iron complex [S5Fe(NO)2]- leading to reversible formation of [S5Fe(mu-S)2FeS5]2-: spectroscopic characterization of species relevant to the nitric oxide modification and repair of [2Fe-2S] ferredoxins. *Inorg Chem 43*, 5159-67.

(187) Rouhier, N., Unno, H., Bandyopadhyay, S., Masip, L., Kim, S. K., Hirasawa, M., Gualberto, J. M., Lattard, V., Kusunoki, M., Knaff, D. B., Georgiou, G., Hase, T., Johnson, M. K., and Jacquot, J. P. (2007) Functional, structural, and spectroscopic characterization of a glutathione-ligated [2Fe-2S] cluster in poplar glutaredoxin C1. *Proc Natl Acad Sci U S A 104*, 7379-84.

(188) Flatley, J., Barrett, J., Pullan, S. T., Hughes, M. N., Green, J., and Poole, R. K. (2005) Transcriptional responses of Escherichia coli to S-nitrosoglutathione under defined chemostat conditions reveal major changes in methionine biosynthesis. *J Biol Chem 280*, 10065-72.

(189) Justino, M. C., Vicente, J. B., Teixeira, M., and Saraiva, L. M. (2005) New genes implicated in the protection of anaerobically grown Escherichia coli against nitric oxide. *J Biol Chem 280*, 2636-43.

(190) Pullan, S. T., Gidley, M. D., Jones, R. A., Barrett, J., Stevanin, T. M., Read, R. C., Green, J., and Poole, R. K. (2007) Nitric oxide in chemostat-cultured Escherichia coli is sensed by Fnr and other global regulators: unaltered methionine biosynthesis indicates lack of S nitrosation. *J Bacteriol 189*, 1845-55.

(191) Bodenmiller, D. M., and Spiro, S. (2006) The yjeB (nsrR) gene of Escherichia coli encodes a nitric oxide-sensitive transcriptional regulator. *J Bacteriol 188*, 874-81.

(192) Richmond, C. S., Glasner, J. D., Mau, R., Jin, H., and Blattner, F. R. (1999) Genome-wide expression profiling in Escherichia coli K-12. *Nucleic Acids Res 27*, 3821-35.

(193) Lowenstein, C. J., Dinerman, J. L., and Snyder, S. H. (1994) Nitric oxide: a physiologic messenger. *Ann Intern Med 120*, 227-37.

(194) Yu, B. J., Kim, J. A., and Pan, J. G. (2010) Signature gene expression profile of triclosan-resistant Escherichia coli. *J Antimicrob Chemother 65*, 1171-7.

(195) Justino, M. C., Almeida, C. C., Teixeira, M., and Saraiva, L. M. (2007) Escherichia coli di-iron YtfE protein is necessary for the repair of stress-damaged iron-sulfur clusters. *J Biol Chem 282*, 10352-9.

(196) Justino, M. C., Almeida, C. C., Goncalves, V. L., Teixeira, M., and Saraiva, L. M. (2006) Escherichia coli YtfE is a di-iron protein with an important function in assembly of iron-sulphur clusters. *FEMS Microbiol Lett 257*, 278-84.

Results

Chapter III

Oxidative stress modulates the nitric oxide defense promoted by *E. coli*

This Chapter was published in:

Baptista, J. M., Justino, M. C., Melo, A. M., Teixeira, M., Saraiva, L. M. (2012) "Oxidative stress modulates the nitric oxide defense promoted by *E. coli* flavorubredoxin ", Journal of Bacteriology, 194 (14): 3611-3617

Joana M. Baptista performed the construction of the plasmids, the expression, purification and all the experiments involving the NorR protein, and the immunoblotting, β-galactosidase and macrophage assays. Joana M. Baptista also contributed to the manuscript writing.

III Oxidative stress modulates the nitric oxide defense promoted by *E. coli*

Summary

Mammalian cells of innate immunity respond to pathogen invasion by activating proteins that generate a burst of oxidative and nitrosative stress. Pathogens defend themselves from the toxic compounds by triggering a variety of detoxifying enzymes. *Escherichia coli* flavorubredoxin is a nitric oxide reductase that is expressed under nitrosative stress conditions. We report that, in contrast to nitrosative stress alone, exposure to both nitrosative and oxidative stress abolishes the expression of flavorubredoxin. EPR experiments showed that under these conditions, the iron centre of the flavorubredoxin transcription activator NorR loses the ability to bind nitric oxide. Accordingly, triggering of the NorR ATPase activity, a requisite for flavorubredoxin activation, was impaired by treatment of the protein with the double stress.

Studies on macrophages revealed that the contribution of flavorubredoxin to the survival of *E. coli* depends on the stage of macrophage infection and the lack of protection observed at the early phase is related to inhibition of the NorR activity by the oxidative burst. We propose that the time dependent activation of flavorubredoxin contributes to the adaptation of *E. coli* to different fluxes of hydrogen peroxide and nitric oxide to which bacterium is submitted during the course of macrophage infection.

III.1 – Introduction

In order to control infection, mammalian phagocytes express NADPH oxidase (Phox) which produces superoxide that spontaneously dismutates to hydrogen peroxide, and the inducible nitric oxide synthase (iNOS) that generates nitric oxide (NO) (*1*). However, microorganisms possess a diverse range of defense mechanisms for sensing and responding to these stresses that are crucial for survival and virulence. To detoxify reactive oxygen species (ROS), microbes utilize an array of enzymes that include scavengers of superoxide and hydrogen peroxide such as superoxide dismutases or reductases, peroxidases and catalases (*2-4*). Nitric oxide detoxification is achieved by NO dioxygenases and reductases which are widespread in denitrifying bacteria, nitrate-dissimilating fungi, pathogenic bacteria and protozoa (*5-7*).

E. coli contains three NO detoxifying enzymes, namely the cytochrome c nitrite reductase (NrfA), flavohaemoglobin (Hmp) and a flavodiiron protein known as flavorubredoxin (FlRd encoded by the *norV* gene) (*8-10*). NrfA is a periplasmic enzyme with high NO reductase activity (*11*) but its role *in vivo* is still under debate (*12*). Hmp acts as a NO dioxygenase or reductase but the latter activity is low (*10, 13*). On the contrary, FlRd seems to be dedicated to scavenge NO under anaerobic conditions with a significant activity (*14*). While most studies have focused on non-pathogenic *E. coli* strains, the two encoding genes *hmp* and *norV* are also present in uropathogenic, enteropathogenic and enterohemorrhagic *E. coli* strains, as well as in closely related pathogens like *Salmonella* or *Shigella* genus. In *E. coli*, strains deleted in *hmp* or *norV* genes have higher sensitivity to NO under aerobic and anaerobic conditions, respectively (*15, 16*). Although the NO reduction rate of single mutants defective in *hmp* or

norV is similar to that of parental strains, the double *hmp-norV* mutant exhibits a clear defect in the ability to anaerobically metabolize NO (*15, 17*).

The expression of *hmp* is highly induced by NO, under aerobic and anaerobic conditions, through a complex regulation that involves at least three regulators, namely FNR, MetR and the NO-sensitive repressor NsrR (*18-20*). The transcription of the *norV* gene is strongly up-regulated in cells cultured anaerobically and exposed to NO through the activation of the nitric oxide sensor, NorR (*21, 22*). The *norR* gene is divergently transcribed from the *norVW* operon that encodes FlRd and its redox partner, the NADH-flavorubredoxin reductase (NorW) (*21, 23*). Induction of *norVW* occurs upon ligation of NO to NorR and binding of the regulator to three motifs present in the promoter region of *norVW* (*24*). NorR is a σ^{54}-dependent transcription factor formed by three domains: a N-terminal regulatory GAF domain harboring a mononuclear iron site that binds NO, a central AAA^{+} domain responsible for ATPase activity and interaction with σ^{54} subunit of RNA polymerase and a C-terminal DNA binding domain that interacts with enhancer sequences (*21, 24, 25*). The binding of NO to the ferrous iron centre stimulates the ATPase activity of NorR and enables NorR to activate the transcription of *norV* (*25*).

In this work, we addressed the behavior of *E. coli* FlRd in the presence of the combined effects of NO and hydrogen peroxide, having analyzed the *norV* gene transcription and the protein expression profile. Furthermore, the survival of *E. coli norV* mutant strain in activated macrophages was also studied.

III.2 – Materials and Methods

Reagents and bacterial strains.

Hydrogen peroxide (Carl-Roth), spermine NONOate (Cayman Chemical) prepared in 0.01 M NaOH (herein named NO donor) and pure NO-saturated anaerobic water solution (~ 2mM) (*26*) were used as stress inducers.

The strains and plasmids utilized in this study are described in Table V.1.

Immunoblotting assays.

E. coli K12 ATCC 23716 cells were grown anaerobically in Luria Bertani (LB) medium at 37ºC and 150 rpm to the early exponential phase (OD_{600}~0.3) and collected after treatment with NO (50 μM), hydrogen peroxide (3 mM) or (NO, 50 μM + H_2O_2, 3 mM) for 45 min. Cells were disrupted in a French pressure cell (Thermo Electron Corporation), the cell extracts were cleared by centrifugation (30 min 12000 *g* at 4ºC) and their protein concentration determined by the bicinchoninic acid (BCA) method (*27*). Protein samples (75 μg) were separated by SDS-PAGE, transferred onto nitrocellulose membranes and detected with polyclonal antibodies raised against *E. coli* FlRd and Hmp as previously described (*15*).

β-galactosidase activity assays.

Cultures of *E. coli* RK4353 carrying pAA182-P*norV* (*24*) were grown anaerobically in LB, at 37ºC and 150 rpm, to an OD_{600}~0.3. At this point, they were treated for 25 min with: NO (50 μM) or spermine NONOate (25 μM), hydrogen peroxide (3 mM or 25 μM), NO (50 μM) plus H_2O_2 (3mM), spermine NONOate (25 μM) plus H_2O_2 (25 μM). Double treatments were

also performed sequentially, in which the second chemical was added 10 min after the first, and cells incubated for further 15 min, to a total of 25 min. Cells were lysed and assayed for β-galactosidase activity, as previously described (*24*). At least three independent cultures were analyzed in duplicate.

Table III.1 – Bacterial strains and plasmids used in this work

Name	Description/Genotype	Source
***E. coli* strains**		
XL2-Blue	F´ *pro*AB *lac*IqZΔM15 *Tn*10 (Tet^r) *end*A1, *sup*E44, *thi*-1, *recA*1, *gyrA*96, *relA*1, *lac*	Lab stock
BL21Gold(DE3)	F$^-$, *ompT*, *hsdS*(r_B^- m_B^-) *dcm*$^+$ Tet^r *gal* λ(DE3) *endA* Hte	Stratagene
Wild type	*E. coli* K-12 ATCC 23716	ATCC
RK4353	*lacZ* mutant strain, [(*argF-lac*)U169]	(*28*)
Δ*norV*	LMS2710, K-12 *norV* mutant [Δ*norV*::Cm^R]	(*15*)
Δ*hmp*	LMS2552, K-12 *hmp* mutant [Δ*hmp*::Km^R]	(*15*)
Δ*norV*Δ*hmp*	LMS5262, K-12 *hmp* and *norV* double mutant [Δ*hmp*::Km^R Δ*norV*::Cm^R]	(*15*)
Plasmids		
pAA182-P*norV*	Plasmid pAA182 carrying the entire *norV* promoter fused to *lacZ*, Amp^r	(*24*)
pET24a	T7-based expression vector, Km^R	Novagen
pME2337	pET24a carrying the *norV* coding region	(*29*)
pET28a	T7-based expression vector, Km^R	Novagen
pET28a-*norR*	pET28a with the *norR* coding region cloned *Nde*I/*Eco*RI, bearing an N-terminal His_6-tag fusion.	This work
pFLAG-CTC	Vector for protein expression under the influence of the *tac* promoter, Amp^R	Sigma
pFLAG-*norV*	pFLAG-CTC carrying *norV* coding region subcloned *Nde*I/*Hind*III from pME2337	This work

Macrophage assays.

Murine macrophages J774A.1 (LGC Promochem) were maintained at 37ºC in a 5% CO_2/air atmosphere in Dulbecco's Modified Eagle medium (DMEM) supplemented as previously described (*30*). For infection studies, macrophages were seeded $5x10^5$ cells/well and incubated for 24 h prior to being activated for 16 h with 0.3 μg/ml interferon-γ (Sigma) and 1.6 μg/ml lipopolysaccharide (Sigma). The medium was then changed to DMEM without antibiotics and macrophages were infected with bacterial suspensions at a multiplicity of infection (MOI) of 20, for 30 min at 37ºC. Bacterial suspensions were prepared from cells of *E. coli* K-12 *wt*, Δ*norV*, and Δ*hmp* grown aerobically in LB to an OD_{600}~0.3, harvested, washed three times with PBS and resuspended in DMEM. Non-internalized bacteria were killed upon incubation in DMEM supplemented with penicillin-streptomycin antibiotics (Gibco-Invitrogen) and internalized bacteria were further incubated in macrophages up to 48 h. At the indicated times, macrophages were lysed with 2% saponin and intracellular bacterial content assessed by CFU counting of viable cells.

For the complementation studies, *norV* gene was excised from plasmid pME2337 (*29*) and cloned into *Nde*I-*Hind*III digested pFLAG-CTC, yielding pFLAG-*norV* (Table III.1). *E. coli* K-12 wild type and Δ*norV*Δ*hmp* cells carrying the empty pFLAG and pFLAG-*norV* were grown aerobically for 16 h in LB medium with 100 μg/mL ampicillin and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), harvested, washed thrice with PBS and resuspended in DMEM.

***E. coli* NorR: gene expression, production of recombinant protein, ATPase activity assays and EPR studies.**

For the production of recombinant *E. coli* NorR, the DNA coding region was cloned into *Nde*I-*EcoR*I digested pET28a and expressed in *E. coli* BL21Gold (DE3) cells as previously reported (*25*). NorR was isolated from the soluble fraction of *E. coli* cells and purified under anaerobic conditions in a Coy model A-2463 anaerobic chamber. Cell extracts were incubated with 1 mM MgATP and $Fe(NH_4)_2(SO_4)$ at room temperature for 1 h (*31*), and then loaded into a HisTrap HP column (GE Healthcare), equilibrated in 20 mM Tris-HCl pH 7.4, 500 mM NaCl and 20% glycerol (buffer A). The fraction containing NorR was eluted with 250 mM imidazole, concentrated in an Amicon ultrafiltration cell (Millipore) and desalted in a Superdex 30 column (GE Healthcare) equilibrated with buffer A. The protein purity was evaluated by SDS-PAGE, and the concentration and iron content determined by BCA and 2,4,6-tripyridyl-1,2,3-triazine (TPTZ) methods, respectively (*27, 32*). The purified NorR contained ~0.9 Fe atoms per monomer.

For the ATPase activity assays (*33*), the reaction mixtures contained 30 mM ATP (Sigma), 1 mM phosphoenolpyruvate (PEP, Sigma), 5 nM 320 bp DNA fragment that spans the entire *norV* promoter (*24*), 7 U pyruvate kinase (Roche Applied Science), 23 U lactate dehydrogenase (Roche Applied Science), 2 mM $MgCl_2$ and 300 nM *E. coli* NorR in 50 mM Tris-HCl, pH 8.0 plus 100 mM KCl. Activities were evaluated at 37º C for 20 min, upon addition of 0.3 mM NADH, following its oxidation at 340 nm (ε=6.22 x 10^3 $M^{-1}cm^{-1}$). For these assays, NorR was left untreated or incubated for 5 min with 20 μM NO, 100 μM hydrogen peroxide, and with mixtures of both chemicals.

EPR spectra were acquired on a Bruker EMX spectrometer equipped with an Oxford Instrument continuous flow helium cryostat and recorded at 9.38 MHz at a temperature of 6-7 K. For whole-cell EPR, *E. coli* BL21(DE3)Gold cells carrying pT7.7-NorR (*24*) were grown for 16 h at 28ºC in LB supplemented with 50 μM IPTG. The cells were washed twice and resuspended in 1/100 of the culture volume in Tris-HCl 20 mM pH 7.5. Aliquots (300 μL) were treated at room temperature with 150 μM NO, 4 mM H_2O_2, both added simultaneously or sequentially with an interval of 5 min.

The purified NorR (75 μM) was incubated for 5 min at room temperature with 2 equivalents of H_2O_2, potassium ferricyanide and NO before being transferred to EPR tubes and frozen in liquid nitrogen. All manipulations and incubations were made in an anaerobic chamber.

III.3 – Results

Expression of *E. coli* FlRd is reduced under the combined effect of oxidative and nitrosative stress.

In the present work, we have compared the expression of FlRd in cells exposed to nitric oxide, H_2O_2 and a combination of the two.

Immunoblotting assays revealed that *E. coli* NO-treated cell extracts contained increased amounts of FlRd, which is consistent with the reported induction of *norV* by NO (*21, 22, 34*). On the contrary, cells exposed to hydrogen peroxide and to nitric oxide plus hydrogen peroxide displayed a very low level of FlRd (Figure III.1A). Although hydrogen peroxide was not expected to induce expression of FlRd, in cells treated nitric oxide plus hydrogen peroxide the marked decreased amount of FlRd was surprisingly. One possible explanation was that compounds resultant from the chemical

reaction between H_2O_2 and NO that have no ability to trigger *norV* expression were being generated. However, this seems not to be the case since the expression of the NO detoxifier Hmp was similar in *E. coli* cells treated only with NO and exposed to NO plus H_2O_2 (Figure III.1A).

To assess whether the lower amount of FlRd was due to transcriptional alterations, the activation of the reporter fusion containing the *norV* promoter was evaluated. While the *norV* promoter was activated by NO, no activation occurred in cells treated with hydrogen peroxide and also in cells exposed to NO plus H_2O_2 (Figure III.1B). When *E. coli* was first treated with H_2O_2 (10 min) and then with NO (15 min), the promoter activation was very low and almost comparable to that observed upon simultaneous exposure to the two stresses for 25 min (Figure III.1B). Initial exposure to NO (10 min) followed by H_2O_2 (15 min) resulted in approximately 3-fold less activation of the promoter than exposure to NO alone for 25 min, consistent with an abolishment/impairment of NO induction upon introduction of the oxidative stress.

We next performed a set of experiments using lower concentrations of NO and H_2O_2 (25 μM) that are within the range of physiological concentrations described to be produced by macrophages (*35*-*37*). Under these conditions, the activation of the promoter in cells sequentially and simultaneously exposed to both stresses was higher than in non-exposed cells but considerably lower than that caused by NO alone (Figure III.1C).

Hence, we concluded that, in cells submitted to both nitric oxide and hydrogen peroxide, the expression of FlRd is essentially impaired.

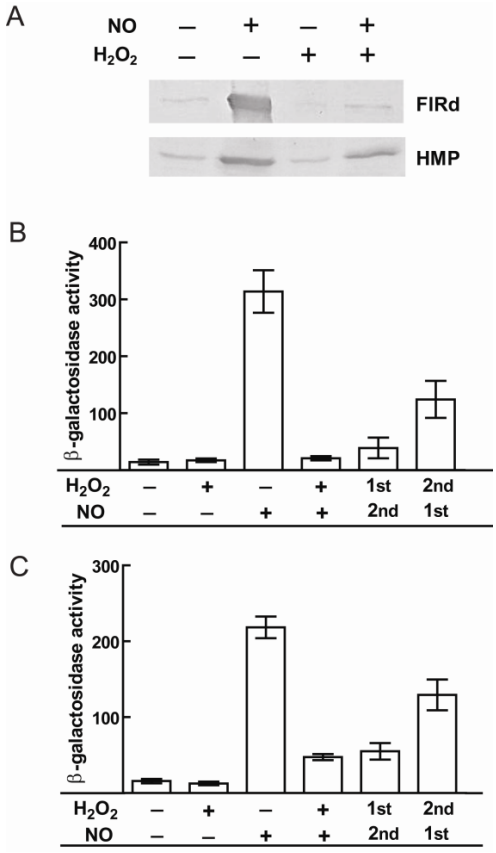


Figure III.1 – Analysis of FlRd expression in cells exposed to combined nitrosative and oxidative stress.
(**A**) Immunoblotting analysis of *E. coli* K-12 cells grown anaerobically and exposed, for 45 min, to 50 μM NO and 3 mM H_2O_2, using antibodies against flavorubredoxin (FlRd) and flavohaemoglobin (Hmp). (**B, C**) β-galactosidase activities of *E. coli* RK4353 cells carrying plasmid pAA182-P*norV* that contains the *norV* promoter-*lacZ* fusion. Cells cultured anaerobically in LB were treated, at an OD_{600}=0.3, with (B) 50 μM NO and/or 3 mM H_2O_2; (C) 25 μM spermine NONOate and/or 25 μM H_2O_2. All cultures were submitted to the stresses for a total of 25 min. Sequential exposures were done by a first 10 min exposure to H_2O_2 followed by 15 min treatment with NO/NO donor and by exposure for 10 min to NO/NO donor followed by 15 min treatment with H_2O_2. Results are means ± SE of three independent cultures assayed in duplicate. Activities are expressed in nmol of *o*-nitrophenol/min.mg of bacterial dry mass.

Effect of hydrogen peroxide on the NorR properties.

It is well established that the transcription of *E. coli norR* is not altered upon exposure to nitrosative stress (*21*). However, the lack of *norV* induction by NO in the presence of H_2O_2 led to the analysis of hydrogen peroxide effect in *norR* transcription levels. We observed that hydrogen peroxide didn't cause changes in the levels of *norR* mRNA (data not shown).

Given that the compromised expression of FlRd in cells treated with NO plus H_2O_2 could not be attributed to lowered NorR levels, we

hypothesized that the ligation of NO to the mononuclear iron site located in the GAF domain of NorR, which triggers the NorR activation of the *norV* promoter (*25*), was impaired in the presence of hydrogen peroxide, a study that was addressed by EPR spectroscopy.

To this end, *E. coli* cells over-expressing NorR protein were exposed to NO, H_2O_2 and a combination of both, and the EPR spectra recorded (Figure III.2A). The spectrum of cells treated with 150 μM NO displayed an intense signal with *g* values at 4.1 and 3.93 attributed to a high spin ferrous-NO complex with a total spin S=3/2 and a rhombicity of E/D ~0.03, as previously observed (*25*), compatible with the formation of a mononitrosyl-iron complex. Cells treated with 4 mM H_2O_2 exhibited a *g*=4.3 signal that is characteristic of high-spin (S=5/2) ferric ions. The EPR spectrum of cells exposed simultaneously to NO and H_2O_2 revealed in the *g*=4 region only the resonance at *g*=4.3 due to the ferric ions, *i.e.,* the signal corresponding to the Fe-NO species was absent.

Cells were also treated with NO and H_2O_2 but in a sequential mode. For cells treated first with NO (5 min) and further exposed to H_2O_2 (5 min), the EPR spectrum contained only one signal, at *g*=4.3, suggesting that H_2O_2 destroys the already formed iron-NO complex. The EPR spectrum of cells that were exposed first to H_2O_2 (5 min) and then to NO (5 min), exhibited an overlay of signals at *g*=4.3, 4.1 and 3.93 that could be attributed to the superimposition of the resonances due to the high-spin ferric iron and the Fe-NO species.

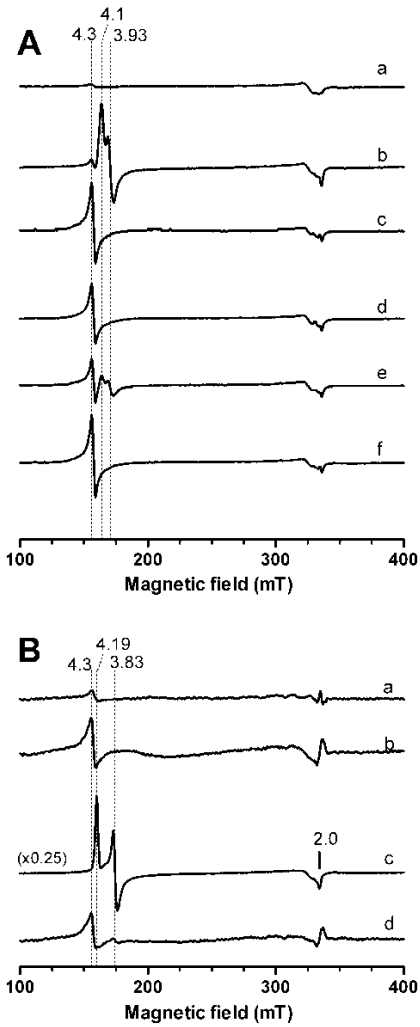


Figure III.2 – EPR analysis of the influence of H_2O_2 on the binding of NO to the mononuclear iron centre of NorR. (A) Whole cell EPR analysis of *E. coli* overexpressing NorR. *E. coli* cells carrying pT7.7-NorR were recorded in the absence of stress (a), after 5 min treatment with 150 µM NO (b), 4 mM H_2O_2 (c), after sequential treatment with 150 µM NO (5 min) followed by 4 mM H_2O_2 (5 min) (d), 4 mM H_2O_2 (5 min) followed by 150 µM NO (5 min) (e), and after simultaneous 5 min treatment with 150 µM NO and 4 mM H_2O_2 (f). **(B)** EPR spectra of the purified NorR protein (75 µM) as isolated (a), treated for 5 min with 150 µM H_2O_2 (b), 150 µM NO (c), with NO and H_2O_2 (150 µM each) (d). With the exception of spectrum (c), all spectra have the same intensity scale. EPR spectra were recorded at 9.39 MHz microwave frequency, 2.4 mW microwave power and at 6-7 K.

A similar study was conducted using the purified recombinant *E. coli* NorR (Figure III.2B). The as-isolated NorR is essentially EPR silent, suggesting that it is purified in the ferrous form. Upon reaction with hydrogen peroxide, a resonance at g=4.3 develops that is consistent with the oxidation of the mononuclear iron centre. NorR incubation with NO resulted in the formation of a high-spin ferrous-NO complex (Figure III.2B),

with *g*-values at 4.19, 3.83, and 2.0, that were slightly different from those of the protein in whole cells; upon treatment with H_2O_2 plus NO the spectrum displayed a single signal at g=4.3, as also observed in whole cells under the same conditions. Moreover, the intensity of this signal is similar to that of H_2O_2 treated protein, indicating that formation of a ferric-NO species didn't occur. To confirm that lack of formation of the Fe-NO complex was due to iron oxidation, identical experiments were performed with NorR oxidized with potassium ferricyanide, which revealed that there was no binding of NO (data not shown).

Altogether, it was concluded that the presence of H_2O_2 inhibits the binding of NO to the iron centre of NorR, due to oxidation of the mononuclear iron centre, impairing the formation of nitrosylated ferrous site.

The ATPase activity of NorR is induced by conformational changes triggered by the formation of the mononitrosyl-iron complex (*25*). Since our results suggest that hydrogen peroxide prevents binding of NO, the ATPase activity of NorR under these stress conditions was examined (Table IIIV.2). The basal level of the ATPase activity of NorR remained essentially unchanged when the protein was exposed to H_2O_2; in contrast, when treated with NO, the activity increased approximately 7-fold. However, in the presence of both NO and H_2O_2, no enhancement of the activity was seen, thus confirming that hydrogen peroxide hinders the NO-dependent ATPase activity of NorR.

Table III.2 – ATPase activity of NorR upon treatment with NO and H_2O_2

NO	H_2O_2	ATPase activity (μmol ATP/min.mg protein)
–	–	0.4 ± 0.1
–	+	0.4 ± 0.1
+	–	2.9 ± 0.4
+	+	1.1 ± 0.1

NorR was treated for 5 min with 100 μM H_2O_2, 20 μM NO and with a mixture of the two. The results represent the average of three independent samples ± SE.

Role of FlRd in protection of *E. coli* within macrophages.

The contribution of FlRd to the survival of *E. coli* was investigated in macrophages and compared with the other soluble NO detoxifier, Hmp. To this purpose, macrophages were infected with *E. coli* wild type and strains mutated in *norV* and *hmp* genes. The results showed that up to 6 h macrophage infection, Δ*norV* cell counts had no significant difference when compared to wild type cells while the survival of Δ*hmp* was lower (Figure III.3A). However, for longer incubation times survival of the Δ*norV* mutant strain within macrophages decreased and became similar to that of the Δ*hmp* strain (Figure III.3B), indicating that FlRd enhances the long-term survival of *E. coli* within macrophages.

No differences in the survival of the wild type and the Δ*norV* mutant upon incubation in macrophages were observed within the first 6 h of macrophage infection, which in light of our earlier findings that *norV* induction in response to NO is compromised when H_2O_2 is present, may be due to the NorR inhibition caused by the macrophage oxidative burst. However, if regulation by NorR was lifted and flavorubredoxin was expressed, it should be able to detoxify nitric oxide and afford protection to *E. coli*. To test this hypothesis, we designed a complementation experiment

in which macrophages were incubated for 6 h with a strain expressing the *norV* gene from a NorR-independent, IPTG controlled plasmid. To avoid scavenging of NO by Hmp, it was necessary to use the double *ΔnorVΔhmp* mutant strain whose phenotype within macrophages, in this early range of time, is due to the absence of *hmp*. Under these conditions, the behavior of the *E. coli* wild type strain was rescued (Figure III.3B) therefore showing that when expressed independently of its own promoter, *i.e.,* when not regulated by NorR, FlRd confers protection to *E. coli* against macrophages.

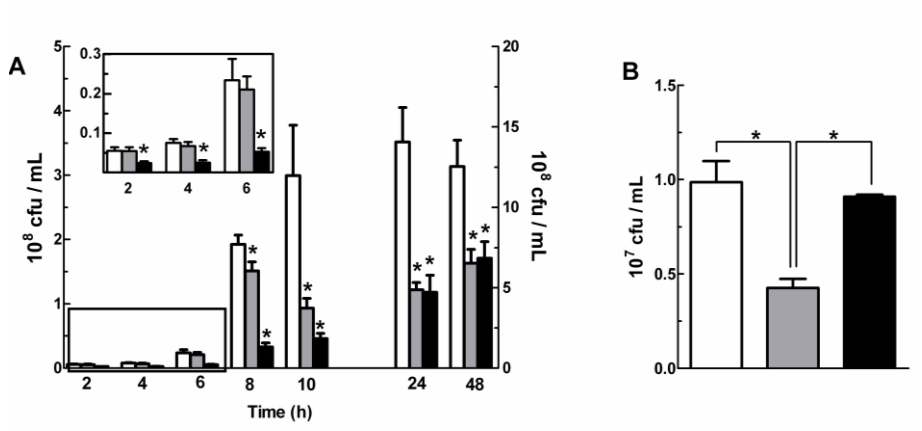


Figure III.3 – Survival of *E. coli* wild type and *norV* mutant upon macrophage infection. (A) Murine macrophages J774A.1 were infected with *E. coli* K-12 wild type (white), Δ*norV* (gray) and Δ*hmp* (black). Viable counts *E. coli* cells were determined at the indicated times. Data from 24 h and 48 h infection are depicted in the right Y-axes scale, and data from early times are expanded in the inset. **(B)** Intracellular survival of *E. coli* wild type carrying the empty vector pFLAG (white), Δ*hmp*Δ*norV* carrying pFLAG (gray) and carrying pFLAG-*norV* plasmid that expresses *norV* from a NorR-independent promoter (black), after 6 h infection in macrophage. Values are means ± SE from at least 6 independent experiments. *, $P < 0.05$ (ANOVA One-way and Bonferroni multiple comparison test).

III.4 – Discussion

In this study we present evidence that combination of NO and H_2O_2 impairs induction of FlRd, the flavodiiron NO reductase of *E. coli*. The cell growth was performed under anaerobic conditions to avoid the interference of oxygen and the possible formation of peroxynitrite that is generated under aerated environments when superoxide, derived from molecular oxygen, reacts with NO.

It was previously shown that the Fe(II)-GAF domain of NorR inhibits its ATPase activity and that binding of NO to the iron centre is required to stimulate the ATPase activity (*25*). Our EPR studies suggest that upon incubation of NorR with NO plus H_2O_2, the oxidation of the iron centre blocks the NO ligation. Consequently, the ATPase activity of NorR was not triggered. Hence, it might be concluded that NorR needs to be in the reduced state in order to bind NO and to promote the transcription of *norV*, *i.e.,* the iron oxidation state influences the activation of NorR (Figure III.4).

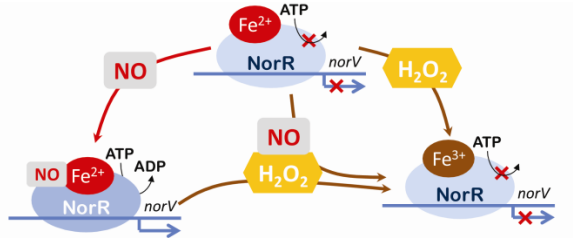


Figure III.4 – Schematic representation of FlRd expression under stress conditions. The ferrous mononitrosyl–iron complex formed in NorR upon exposure to NO promotes transcription of the *norV* gene. In the presence of hydrogen peroxide, the iron center is oxidized and no longer able to bind NO resulting in lack of *norV* expression.

We observed that *hmp* accounts for the successful *E. coli* infection in macrophages demonstrating the importance of the protein for bacterial stress resistance, which is in agreement with previous reports (*38, 39*). Moreover we provide evidence that Hmp protects *E. coli* at all stages of

macrophage infection was provided by us. On the contrary, the contribution of *norV* to survival of *E. coli* within macrophages exhibited a time dependent profile, as protection only occurred for incubations in mammalian cells longer than 8 h. Therefore, the previous failures to demonstrate a role for FlRd in the protection against macrophage killing were most probably due to the macrophage infection times studied, which ranged from 15 to 120 min (*40*). Interestingly, a study of the gene expression profile of *Salmonella enterica* following macrophages infection indicated that induction of *norV* transcription is higher at times correspondent to the generation of the NO burst (*41*). An apparent oscillation in *norV* mRNA levels was also detected in *E. coli* exposed to acidified nitrite and grown under aerobic conditions while no variation was observed under anaerobic conditions, also suggesting that oxygen is required for the oscillatory expression pattern of *norV* (*34*).

The expression of NADPH oxidase and nitric oxide synthase is induced in macrophages upon phagocytosis of bacteria and the subsequent production of superoxide and nitric oxide is used to suppress bacterial growth (*1, 42*). Studies on the production of chemical species by macrophages during the first hours after bacteria invasion revealed that H_2O_2, resultant from spontaneous dismutation of superoxide via SODs, is the most abundant specie (*43, 44*). The ROS level is considered to abate within 6-10 h with increasingly abundant generation of nitrosative species (*35*). In spite of the toxicity of the species generated, macrophages do not provide complete protection against infection partially because several bacterial proteins have the ability to detoxify oxygen and nitrogen reactive species. Moreover, some bacteria contain more than one enzyme that apparently detoxifies the same chemical species. This is the case of *E. coli* that has, at least, two soluble NO scavenging enzymes. So far, the need for

two systems remained largely unclear. The present data revealed, to our best knowledge, for the first time that FlRd has a time-differentiated action within macrophages. The blockage of *norV* expression that is expected to occur during the first stage of macrophage infection due to the predominance of hydrogen peroxide decreases the ability of bacteria to scavenge NO produced by macrophages. Consequently, the remaining NO will stimulate the expression of Hmp as its encoded gene is regulated by iron-sulphur containing transcription factors, such as NsrR, that only upon binding to NO lift their repression and trigger the Hmp NO activity. Over time, macrophages decrease the oxidative burst while the NO production increases, which will allow binding of NO to NorR and subsequent triggering of *norV* expression.

It is recognized that, at low concentrations, NO has a protective role to bacteria as it activates transcription factors such as SoxRS, OxyR and Fur, whose regulons encode antioxidant enzymes (*45*). *Bacillus subtilis* and *Staphylococcus aureus* express a NO-synthase enzyme that is proposed to play a critical role in adaptation to oxidative stress (*46, 47*). While a gene encoding NO-synthase is apparently absent in *E. coli*, the existence of two NO detoxifying systems may go beyond the need for functional redundancy to represent an alternative way of tuning the intracellular NO concentration. Moreover, it endows bacteria to take profit from the fact that mammalian Phox and iNOS activities peak at different times after phagocytosis providing a metabolic flexibility that helps protecting *E. coli* from the variety of environmental conditions experienced during the course of macrophage infection. Indeed, survival within macrophages is an important mechanism of infection of pathogens (*48, 49*).

Flavodiiron-like proteins are found widespread in nature including in several commensal and pathogenic Gram-negative and Gram-positive

bacteria that colonize the human oro-gastric tract (*8, 9*). These include members of the genera *Bacillus*, *Bacteroides*, *Clostridium*, *Fusobacterium*, *Ruminococcus*, *Porphyromonas*, *Prevotella*, *Vibrio*, *Salmonella*, *Shigella*, *Yersinia* and uropathogenic, enteropathogenic and enterohemorrhagic *E. coli* strains. Consistent with the significant *in vitro* activity of this enzyme, in this work we succeeded in demonstrating the contribution of FlRd to microbial survival against the iNOS-mediated host immune defenses.

III.5 – References

(1) Nathan, C., and Shiloh, M. U. (2000) Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc. Natl. Acad. Sci. U. S. A. 97*, 8841-8.

(2) Imlay, J. A. (2008) Cellular defenses against superoxide and hydrogen peroxide. *Annu. Rev. Biochem. 77*, 755-76.

(3) Pinto, A. F., Rodrigues, J. V., and Teixeira, M. (2010) Reductive elimination of superoxide: Structure and mechanism of superoxide reductases. *Biochim. Biophys. Acta. 1804*, 285-97.

(4) Kurtz, D. M., Jr. (2006) Avoiding high-valent iron intermediates: superoxide reductase and rubrerythrin. *J. Inorg. Biochem. 100*, 679-93.

(5) Wasser, I. M., de Vries, S., Moenne-Loccoz, P., Schroder, I., and Karlin, K. D. (2002) Nitric oxide in biological denitrification: Fe/Cu metalloenzyme and metal complex NO(x) redox chemistry. *Chem. Rev. 102*, 1201-34.

(6) Poole, R. K. (2005) Nitric oxide and nitrosative stress tolerance in bacteria. *Biochem. Soc. Trans. 33*, 176-80.

(7) Bowman, L. A., McLean, S., Poole, R. K., and Fukuto, J. M. (2011) The diversity of microbial responses to nitric oxide and agents of nitrosative stress close cousins but not identical twins. *Adv. Microb. Physiol. 59*, 135-219.

(8) Saraiva, L. M., Vicente, J. B., and Teixeira, M. (2004) The role of the flavodiiron proteins in microbial nitric oxide detoxification. *Adv. Microb. Physiol. 49*, 77-129.

(9) Vicente, J. B., Justino, M. C., Goncalves, V. L., Saraiva, L. M., and Teixeira, M. (2008) Biochemical, spectroscopic, and thermodynamic properties of flavodiiron proteins. *Methods Enzymol. 437*, 21-45.

(10) Bonamore, A., and Boffi, A. (2008) Flavohemoglobin: structure and reactivity. *IUBMB Life. 60*, 19-28.

(11) van Wonderen, J. H., Burlat, B., Richardson, D. J., Cheesman, M. R., and Butt, J. N. (2008) The nitric oxide reductase activity of cytochrome c nitrite reductase from Escherichia coli. *J. Biol. Chem. 283*, 9587-94.

(12) Vine, C. E., and Cole, J. A. (2011) Unresolved sources, sinks, and pathways for the recovery of enteric bacteria from nitrosative stress. *FEMS Microbiol. Lett. 325*, 99-107.
(13) Angelo, M., Hausladen, A., Singel, D. J., and Stamler, J. S. (2008) Interactions of NO with hemoglobin: from microbes to man. *Methods Enzymol. 436*, 131-68.
(14) Gomes, C. M., Giuffre, A., Forte, E., Vicente, J. B., Saraiva, L. M., Brunori, M., and Teixeira, M. (2002) A novel type of nitric-oxide reductase. Escherichia coli flavorubredoxin. *J. Biol. Chem. 277*, 25273-6.
(15) Justino, M. C., Vicente, J. B., Teixeira, M., and Saraiva, L. M. (2005) New genes implicated in the protection of anaerobically grown Escherichia coli against nitric oxide. *J. Biol. Chem. 280*, 2636-43.
(16) Membrillo-Hernandez, J., Coopamah, M. D., Anjum, M. F., Stevanin, T. M., Kelly, A., Hughes, M. N., and Poole, R. K. (1999) The flavohemoglobin of Escherichia coli confers resistance to a nitrosating agent, a "Nitric oxide Releaser," and paraquat and is essential for transcriptional responses to oxidative stress. *J. Biol. Chem. 274*, 748-54.
(17) Vine, C. E., and Cole, J. A. (2011) Nitrosative stress in Escherichia coli: reduction of nitric oxide. *Biochem Soc Trans 39*, 213-5.
(18) Poole, R. K. (2005) Nitric oxide and nitrosative stress tolerance in bacteria. *Biochem Soc Trans 33*, 176-80.
(19) Filenko, N., Spiro, S., Browning, D. F., Squire, D., Overton, T. W., Cole, J., and Constantinidou, C. (2007) The NsrR regulon of Escherichia coli K-12 includes genes encoding the hybrid cluster protein and the periplasmic, respiratory nitrite reductase. *J. Bacteriol. 189*, 4410-7.
(20) Constantinidou, C., Hobman, J. L., Griffiths, L., Patel, M. D., Penn, C. W., Cole, J. A., and Overton, T. W. (2006) 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*, 4802-15.
(21) Gardner, A. M., Gessner, C. R., and Gardner, P. R. (2003) Regulation of the nitric oxide reduction operon (norRVW) in Escherichia coli. Role of NorR and sigma54 in the nitric oxide stress response. *J. Biol. Chem. 278*, 10081-6.
(22) Justino, M. C., Vicente, J. B., Teixeira, M., and Saraiva, L. M. (2005) New genes implicated in the protection of anaerobically grown Escherichia coli against nitric oxide. *J Biol Chem 280*, 2636-43.
(23) da Costa, P. N., Teixeira, M., and Saraiva, L. M. (2003) Regulation of the flavorubredoxin nitric oxide reductase gene in Escherichia coli: nitrate repression, nitrite induction, and possible post-transcription control. *FEMS Microbiol. Lett. 218*, 385-93.
(24) Justino, M. C., Goncalves, V. M., and Saraiva, L. M. (2005) Binding of NorR to three DNA sites is essential for promoter activation of the flavorubredoxin gene, the nitric oxide reductase of Escherichia coli. *Biochem. Biophys. Res. Commun. 328*, 540-4.

(25) D'Autreaux, B., Tucker, N. P., Dixon, R., and Spiro, S. (2005) A non-haem iron centre in the transcription factor NorR senses nitric oxide. *Nature. 437*, 769-72.
(26) Beckman, J. S., Wink, D. A., and Crow, J. P. (1996) Methods in Nitric Oxide Research, (Feelisch, M., and Stamler, J. S., Ed.) pp 61-70, Chichester, UK.
(27) Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem. 150*, 76-85.
(28) Stewart, V., and MacGregor, C. H. (1982) Nitrate reductase in Escherichia coli K-12: involvement of chlC, chlE, and chlG loci. *J. Bacteriol. 151*, 788-99.
(29) Wasserfallen, A., Ragettli, S., Jouanneau, Y., and Leisinger, T. (1998) A family of flavoproteins in the domains Archaea and Bacteria. *Eur. J. Biochem. 254*, 325-32.
(30) Nobre, L. S., Todorovic, S., Tavares, A. F., Oldfield, E., Hildebrandt, P., Teixeira, M., and Saraiva, L. M. (2010) Binding of azole antibiotics to Staphylococcus aureus flavohemoglobin increases intracellular oxidative stress. *J. Bacteriol. 192*, 1527-33.
(31) D'Autreaux, B., Tucker, N., Spiro, S., and Dixon, R. (2008) Characterization of the nitric oxide-reactive transcriptional activator NorR. *Methods Enzymol. 437*, 235-51.
(32) Fischer, D. S., and Price, D. C. (1964) A Simple Serum Iron Method Using the New Sensitive Chromogen Tripyridyl-S-Triazine. *Clin. Chem. 10*, 21-31.
(33) Norby, J. G. (1988) Coupled assay of Na+,K+-ATPase activity. *Methods Enzymol. 156*, 116-9.
(34) Mukhopadhyay, P., Zheng, M., Bedzyk, L. A., LaRossa, R. A., and Storz, G. (2004) Prominent roles of the NorR and Fur regulators in the Escherichia coli transcriptional response to reactive nitrogen species. *Proc. Natl. Acad. Sci. U. S. A. 101*, 745-50.
(35) Vazquez-Torres, A., Jones-Carson, J., Mastroeni, P., Ischiropoulos, H., and Fang, F. C. (2000) Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages in vitro. *J. Exp. Med. 192*, 227-36.
(36) Fang, F. C. (2011) Antimicrobial actions of reactive oxygen species. *MBio. 2*.
(37) Slauch, J. M. (2011) How does the oxidative burst of macrophages kill bacteria? Still an open question. *Mol. Microbiol. 80*, 580-3.
(38) Stevanin, T. M., Poole, R. K., Demoncheaux, E. A., and Read, R. C. (2002) Flavohemoglobin Hmp protects Salmonella enterica serovar typhimurium from nitric oxide-related killing by human macrophages. *Infect. Immun. 70*, 4399-405.
(39) Stevanin, T. M., Read, R. C., and Poole, R. K. (2007) The hmp gene encoding the NO-inducible flavohaemoglobin in Escherichia coli confers a protective advantage in resisting killing within macrophages, but not in vitro: links with swarming motility. *Gene 398*, 62-8.

(40) Pullan, S. T., Gidley, M. D., Jones, R. A., Barrett, J., Stevanin, T. M., Read, R. C., Green, J., and Poole, R. K. (2007) Nitric oxide in chemostat-cultured Escherichia coli is sensed by Fnr and other global regulators: unaltered methionine biosynthesis indicates lack of S nitrosation. *J. Bacteriol. 189*, 1845-55.

(41) Eriksson, S., Lucchini, S., Thompson, A., Rhen, M., and Hinton, J. C. (2003) Unravelling the biology of macrophage infection by gene expression profiling of intracellular Salmonella enterica. *Mol. Microbiol. 47*, 103-18.

(42) Shiloh, M. U., MacMicking, J. D., Nicholson, S., Brause, J. E., Potter, S., Marino, M., Fang, F., Dinauer, M., and Nathan, C. (1999) Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase. *Immunity. 10*, 29-38.

(43) Paulsen, C. E., and Carroll, K. S. (2010) Orchestrating redox signaling networks through regulatory cysteine switches. *ACS Chem. Biol. 5*, 47-62.

(44) Giorgio, M., Trinei, M., Migliaccio, E., and Pelicci, P. G. (2007) Hydrogen peroxide: a metabolic by-product or a common mediator of ageing signals? *Nat. Rev. Mol. Cell. Biol. 8*, 722-8.

(45) Gusarov, I., and Nudler, E. (2005) NO-mediated cytoprotection: instant adaptation to oxidative stress in bacteria. *Proc. Natl. Acad. Sci. U. S. A. 102*, 13855-60.

(46) Shatalin, K., Gusarov, I., Avetissova, E., Shatalina, Y., McQuade, L. E., Lippard, S. J., and Nudler, E. (2008) Bacillus anthracis-derived nitric oxide is essential for pathogen virulence and survival in macrophages. *Proc. Natl. Acad. Sci. U. S. A. 105*, 1009-13.

(47) Hong, I. S., Kim, Y. K., Choi, W. S., Seo, D. W., Yoon, J. W., Han, J. W., Lee, H. Y., and Lee, H. W. (2003) Purification and characterization of nitric oxide synthase from Staphylococcus aureus. *FEMS Microbiol. Lett. 222*, 177-82.

(48) Linehan, S. A., and Holden, D. W. (2003) The interplay between Salmonella typhimurium and its macrophage host--what can it teach us about innate immunity? *Immunol Lett 85*, 183-92.

(49) Svensson, L., Poljakovic, M., Save, S., Gilberthorpe, N., Schon, T., Strid, S., Corker, H., Poole, R. K., and Persson, K. (2010) Role of flavohemoglobin in combating nitrosative stress in uropathogenic Escherichia coli--implications for urinary tract infection. *Microb Pathog 49*, 59-66.

III.6 – Acknowledgments

We are grateful to Prof. Robert Poole for providing the antibody against *E. coli* Hmp and to Prof. Miguel Teixeira (ITQB) for the EPR experiments and helpful discussions.

Chapter IV

Widespread distribution in pathogenic bacteria of di-iron proteins that repair oxidative and nitrosative damage to iron-sulphur centres

This Chapter was published in:

Overton, T. W., Justino, M. C., Li, Y., **Baptista, J. M.**, Melo, A. M., Cole, J. A., Saraiva, L. M. (2008) "Widespread distribution in pathogenic bacteria of di-iron proteins that repair oxidative and nitrosative damage to iron-sulphur centres" Journal of Bacteriology, 190 (6): 2004-2013

Joana M. Baptista constructed the *S. aureus ric* mutant, did the growth and sensitivity assays, and performed the complementation studies. Expression, purification and the assays carried out with *S. aureus* Ric protein was done by Joana M. Baptista. Joana M. Baptista participated in the writing of the manuscript.

IV Widespread distribution in pathogenic bacteria of di-iron proteins that repair oxidative and nitrosative damage to iron-sulphur centres

Summary

Expression of two genes of unknown function, *Staphylococcus aureus scdA* and *Neisseria gonorrhoeae dnrN*, is induced by exposure to oxidative or nitrosative stress. We show that DnrN and ScdA are di-iron proteins that protect their hosts from damage caused by exposure to nitric oxide and to hydrogen peroxide. Loss of FNR-dependent activation of *aniA* expression and NsrR-dependent repression of *norB* and *dnrN* expression on exposure to NO was restored in the gonococcal parent strain but not in a *dnrN* mutant, suggesting that DnrN is necessary for the repair of NO damage to the gonococcal transcription factors, FNR and NsrR. Restoration of aconitase activity destroyed by exposure of *S. aureus* to NO or H_2O_2 required a functional *scdA* gene. Electron paramagnetic resonance spectra of recombinant ScdA purified from *Escherichia coli* confirmed the presence of a di-iron centre. The recombinant *scdA* plasmid, but not recombinant plasmids encoding the complete *Escherichia coli sufABCDSE* or *iscRSUAhscBAfdx* operons, complemented repair defects of an *E. coli ytfE* mutant. Analysis of the protein sequence database revealed the importance of the two proteins based on the widespread distribution of highly conserved homologues in both gram-positive and gram-negative bacteria that are human pathogens. We provide *in vivo* and *in vitro* evidence that Fe-S clusters damaged by exposure to NO and H_2O_2 can be repaired by this new protein family, for which we propose the name *r*epair of *i*ron *c*entres, or RIC, proteins.

IV.1 – Introduction

Neutrophils and macrophages of the mammalian immune system produce reactive oxygen and reactive nitrogen species that have important roles in killing pathogenic bacteria by damaging such cellular components as DNA, lipids, and proteins. Particularly vulnerable to inactivation are iron-sulphur (Fe-S) proteins, which were among the first catalysts used by nature (*1*). They participate in numerous cellular processes in virtually all organisms where they fulfill crucial redox, catalytic, and regulatory functions (*2-4*). Specialized systems have evolved that facilitate the assembly and insertion of Fe-S clusters into proteins, namely, the products of *isc*, *suf*, and *csd* operons (*3, 5, 6*). Analysis of bacterial genomes shows that one or more of these systems can be present in any organism for the *in vivo* maturation of Fe-S proteins. The *isc* operon encodes several proteins that are necessary for *de novo* synthesis, and at least one of them, IscS, is proposed to be required for cluster repair (*7*). The Suf system sustains Fe-S cluster biogenesis during iron starvation and oxidative stress (*7-9*), and CSD is proposed to act as a sulphur-generating system (*10*). Despite their established roles in pathogen survival, little is known about how oxidative and nitrosative damage to Fe-S clusters is repaired since so far only IscS is proposed to have such a function (*7, 11, 12*).

Transcriptomic studies have shown that nitrosative stress conditions elicit increased expression of not only the *isc* and *suf* operons but also various genes of known and unknown function (*13-18*). The products of some of these genes are required to detoxify the reactive nitrogen species and are under the control of iron-sulphur regulators. For example, the *hmpA* gene present in various bacteria encodes an enzyme that catalyzes the oxidation of NO to nitrate in aerobic cultures or the

reduction to nitrous oxide during anaerobic growth (*19-22*). In *Escherichia coli*, expression of *hmpA* is repressed by FNR, the regulator of fumarate and nitrate reduction, which contains an $[4Fe\text{-}4S]^{2+/1+}$ iron-sulphur centre that is essential for the binding of FNR to its DNA binding site. FNR, originally identified as an oxygen-sensitive transcription regulator, is also inactivated on exposure to nitric oxide, providing a mechanism by which FNR-repressed genes respond to nitrosative stress (*23, 24*). Similarly, the repressor activity of NsrR, which from sequence analysis is assumed to contain an [2Fe-2S] iron-sulphur centre, is inactivated on exposure to nitric oxide (*25-27*). There is overlap between the biological responses to oxidative stress caused by exposure to hydrogen peroxide and to nitrosative stress (*28-32*). This overlap includes various iron-sulphur-containing enzymes and the transcription factors that regulate their synthesis, which is a reflection of the fact that iron-sulphur centres are damaged by both reactive oxygen and nitrogen species. In addition, the perturbation of iron homeostasis that occurs under stress conditions causes changes in the transcriptional regulation of a large number of genes involved in iron metabolism, many of which code for iron-containing proteins (*4, 33*).

Analysis of the data available for the gram-positive pathogen *Staphylococcus aureus* and for the gram-negative pathogens *Neisseria meningitidis* and *Neisseria gonorrhoeae*, organisms that have serious impacts on human health, revealed that exposure to nitric oxide and hydrogen peroxide causes the induction of genes encoding putative iron-containing proteins. Examples include the *S. aureus scdA*, whose expression was reported to be induced by both NO and hydrogen peroxide (*17, 28*), and the gonococcal *dnrN*, which is induced when the NsrR repressor protein is inactivated by NO (*27*). We therefore investigated whether either

of these proteins is implicated in protection against nitric oxide or hydrogen peroxide, reactive nitrogen and oxygen species generated by the human body as part of its defenses against infection by pathogenic bacteria, and in iron metabolism. The results of *in vivo* and *in vitro* experiments revealed a role for these proteins in the repair of iron-sulphur centres of both transcription factors and housekeeping enzymes damaged by oxidative and nitrosative stress. Furthermore, the analysis of protein databases emphasizes their importance since related proteins were found in a wide range of prokaryotic and eukaryotic pathogens.

IV.2 – Materials and Methods

Strains, plasmids, and primers.

Bacterial strains and plasmids used in this work are listed in Table IV.1 and oligonucleotides are listed in Table IV.2. To disrupt the *scdA* gene (SAOUHSC_00229) of *S. aureus* NCTC 8325, an 820-bp fragment spanning the upstream region and 5' end of the gene was amplified by PCR using the primers ScdAmutEco and ScdAmutBam, and the fragment was cloned into pSP64D-E (*34*). The resulting plasmid, pSPScdA, was electroporated into *S. aureus* RN4220, and transformants were selected on tryptic soy agar (TSA; Difco) plates containing erythromycin (10 μg/ml). The correct integration of pSPScdA into the chromosome of RN4220 in the strain obtained, LMSA0229 (*scdA*::Erm^r), was confirmed by single-colony PCR analysis.

The *dnrN* gene of *N. gonorrhoeae* (open reading frame NGO0653) was interrupted with a kanamycin resistance cassette using crossover PCR (*35*). Primers DnrNA plus DnrNB and DnrNC plus DnrND were used to generate DNA fragments upstream and downstream of the *dnrN* gene. The

flanking fragments were cleaned and combined in a crossover PCR with primers DnrNA and DnrND, yielding a single fragment with an AgeI restriction site between the upstream and downstream sequences. The crossover PCR product was cloned into pGEM T-Easy (Promega, Madison, WI), yielding pGEMDnrN. A kanamycin resistance cassette was amplified from pSUB11 by PCR using primers KanAgeIFwd and KanAgeIRev, which introduced AgeI sites at each end of the resultant fragment, and was ligated into AgeI-digested pGEMDnrN, yielding pGEMDnrN-KO. The *dnrN*::*kan* fragment was generated by digestion of pGEMDnrN-KO with EcoRI and was transformed, as previously described (*36*), into piliated *N. gonorrhoeae* strain F62, yielding strain JCGC704.

Growth of *S. aureus* and sensitivity assays.

S. aureus RN4220 and LMSA0229 strains were streaked onto TSA plates and incubated for 16 h at 37°C. Isolated colonies were cultivated aerobically in tryptic soy broth medium (Difco) for 16 h at 37°C and 150 rpm. These were used to inoculate, in duplicate, 20 ml of fresh tryptic soy broth, adjusting the starting optical density at 600 nm (OD_{600}) to 0.1. The cultures, grown aerobically at 37°C, were treated with 10 mM H_2O_2 or left untreated. After 4 h of growth, 5 µl of serial dilutions of the cultures was spread onto TSA plates and incubated overnight.

Table IV.1 – Strains and plasmids used in this study

Name	Description	Source
***E. coli* strains**		
BL21(DE3)Gold	Protein expression cells	Stratagene
K-12 ATCC 23716	Parental strain	Laboratory stocks
LMS4209	K-12 (ATCC 23716) *ytfE*::Cm^R	(*14*)
***S. aureus* strains**		
NCTC 8325	Parental strain	Laboratory stocks
RN4220	Restriction negative derivative of NCTC 8325, transformable by electroporation	Laboratory stocks
LMSA0229	RN4220 *scdA::*Erm^R	This study
***N. gonorrhoeae* strains**		
F62	Parental strain	Laboratory stocks
JCGC704	*dnrN::*kan^R	This study
JCGC212	*kat::ermC*	(*37*)
JCGC705	*kat::ermC dnrN::*kan^R	This study
Plasmids		
pSP64D-E	Cloning vector carrying an erythromycin resistance cassette	(*38*)
pSPScdA	Upstream region and 5'-end of *scdA* cloned into pSP64D-E, next to the erythromycin resistance cassette	This study
pSUB11	Epitope tagging plasmid carrying 3xFLAG tag and kanamycin resistance cassette	(*39*)
pGEM-T Easy	Cloning vector	Promega
pGEMDnrN	Sequences upstream and downstream of the *dnrN* gene (NGO0653) cloned into pGEM-T Easy	This study
pGEMDnrN-KO	Sequences upstream and downstream of the *dnrN* gene (NGO0653) flanking a kanamycin resistance cassette cloned into pGEM-T Easy	This study
pET28a(+)	T7 based expression vector that inserts a sequence encoding a $(His)_6$-tag at the N-terminus	Novagen
pET-ScdA	*S. aureus scdA* gene cloned into pET28a(+)	This study
pGS57	Fumarase A expressing plasmid	(*40*)
pUC18	Cloning vector	Laboratory stocks
pScdA	pUC18 carrying the *scdA* gene of *S. aureus* and its promoter region	This study
pYtfE	pUC18 carrying the *ytfE* gene of *E. coli* and its promoter region	(*41*)
pRKISC	Plasmid for expression of the *E. coli isc* operon	(*42*)
pRKSUF	Plasmid for expression of the *E. coli suf* operon	(*43*)

Table IV.2 – Oligonucleotide primers used in this study

Name	Description	Sequence (5' → 3')
DnrNA	Deletion of gonococcal *dnrN*	TTTTTGAACACCATCAGGGTCGG
DnrNB	Deletion of gonococcal *dnrN*	GGCTGAGGTGGCCAAGGAAAATATCCCAAAGCCC
DnrNC	Deletion of gonococcal *dnrN*	TAAAAGGAACCGGTGGAGTCGGATATTTCAATGC
DnrND	Deletion of gonococcal *dnrN*	GCCGTAAACGGTTAGTCGGCG
KanAgeIFwd	Amplification of Kan^R cassette	CGTAACCGGTAAAGCCAGTCCGCAG
KanAgeIRev	Amplification of Kan^R cassette	CGATACCGGTGGAGGATCATCCAGC
ScdANheI	Expression of *S. aureus* ScdA	AGGAGGTTAGCTAGCATGATAAAT
ScdAEcoRI	Expression of *S. aureus* ScdA	ACTGCTGGAATTCTTTTTGCCAA
ScdAHindIII	Complementation of *S. aureus scdA*	GAAGTGCAAAGCTTACTTAGCAA
ScdAmutEco	Deletion of *S. aureus scdA*	TTGGCGAATTCCATTATGATGTC
ScdAmutBam	Deletion of *S. aureus scdA*	AACAAGGATCCATTCCCACACTT
aniA_RT_539F	Detection of *aniA* transcript in qRT-PCR	TCGGTATGCACATCGCCAA
aniA_RT_705R	Detection of *aniA* transcript in qRT-PCR	GGCAACGGCTTTGTCCATATC
dnrN_RT_33F	Detection of *dnrN* transcript in qRT-PCR	CTTTGGCGCAACCGTTGAT
dnrN_RT_160R	Detection of *dnrN* transcript in qRT-PCR	CAATTTCCGCCGGAAAGGT
norB_RT_133F	Detection of *norB* transcript in qRT-PCR	TCCGCCTGGTTGGATTTGA
norB_RT_251R	Detection of *norB* transcript in qRT-PCR	CGGCTTTGATTGCGGTATTCA
polA_RT_441F	Detection of *polA* transcript in qRT-PCR	CGTTACGCTGGTGAACACGAT
polA_RT_546R	Detection of *polA* transcript in qRT-PCR	GATCAGCGCGAGATAATCACG

Growth of *N. gonorrhoeae* and sensitivity to hydrogen peroxide.

N. gonorrhoeae was grown on gonococcal agar plates and in gonococcal broth (GCB; BD, Oxford, United Kingdom). Solid and liquid media were supplemented with 1% (v/v) Kellogg's supplement (*44*). For liquid cultures, 2 µl of a stock of *N. gonorrhoeae* was plated onto a gonococcal agar plate and incubated in a candle jar at 37°C for 24 h. Bacteria from this plate were swabbed onto a second plate and incubated in the same way for a further 16 h. The entire bacterial growth from this second plate was swabbed into 10 ml of GCB and incubated at 37°C in an orbital shaker at 100 rpm for 1 h. This 10-ml preculture was then

transferred into 50 ml of GCB in a 100-ml conical flask and incubated in the same way. For growth in the presence of nitrite, 1 mM $NaNO_2$ was added after 1 h, and 4 mM $NaNO_2$ was added 1 h later.

A modified disk diffusion assay was used to compare areas of growth inhibition of various gonococcal strains (*37*). For growth experiments, different concentrations of H_2O_2 were added to 60 ml of oxygen-limited cultures of the gonococcal *kat* mutant and the *kat dnrN* double mutant in 100-ml conical flasks, and growth was monitored for the following 5 h. Greatest differences between the two strains were observed when the H_2O_2 concentration added was 0.5 mM.

Complementation assays in *E. coli*.

A DNA fragment of 955 bp comprising the promoter and coding regions of *scdA* was amplified by PCR from *S. aureus* NCTC 8325 genomic DNA, using the primers ScdAHindIII and ScdAEcoRI, and cloned into pUC18 digested with HindIII and EcoRI, generating the plasmid pScdA. The *E. coli ytfE* mutant strain LMS4209 was transformed with the plasmids pYtfE, pScdA, pRKISC, and pRKSUF that express, respectively, the *E. coli ytfE* gene, *S. aureus scdA* gene, *E. coli isc* operon, and the *suf* operon from their own promoters. *E. coli* strains were grown in LB medium under anaerobic conditions (i.e., closed flasks completely filled), from a starting OD_{600} of 0.1. When cultures reached an OD_{600} of 0.3, they were treated with 4 mM hydrogen peroxide (Sigma) or left untreated, and the growth was followed for ~3 h.

Production of the *S. aureus* recombinant ScdA protein.

The coding region of the *scdA* gene was amplified by PCR from genomic DNA of *S. aureus* NCTC 8325 using the primers ScdANheI and

ScdAEcoRI and cloned into pET-28a (Novagen) that allows insertion of a nucleotide sequence that encodes a His_6 tail at the N terminus. The resulting plasmid, pET-ScdA, was sequenced to ensure the integrity of the cloned sequence. The recombinant protein was overproduced in cells of *E. coli* BL21 Gold(DE3) (Stratagene) grown aerobically in M9 minimal medium, which was supplemented with 10 mM glucose, 100 μM $Fe(NH_4)_2(SO_4)_2$, and 30 μg/ml kanamycin; cells were cultured at 37°C and 150 rpm. At an OD_{600} of 0.3, the cultures were induced with 400 μM isopropyl-1-thio-β-D-galactopyranoside (IPTG, Sigma). After the temperature was lowered to 30°C, cultures were grown for 6 h at 130 rpm and harvested by centrifugation. Cells were resuspended in ice-cold buffer A (20 mM Tris-HCl, pH 7.6), disrupted in a French press (Thermo Electron Corporation), and ultracentrifuged at 100,000 x *g* for 2 h at 4°C. The soluble extract was loaded onto an immobilized metal affinity chromatography Sepharose Fast Flow column (GE Healthcare) and ScdA was eluted at 300 mM imidazole and immediately dialyzed against buffer A. The protein was found to be pure, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and this sample was used for further characterization.

Protein concentration was determined by a bicinchoninic acid protein assay (Pierce) (*45*), and the iron content was determined by the TPTZ (2,4,6-tripyridyl-1,2,3-triazine) method (*46*). Molecular mass determination was performed in a Superdex 200 (10/300) GL column (GE Healthcare) using standard proteins. The electron paramagnetic resonance (EPR) spectrum was obtained in a Bruker EMX spectrometer equipped with an Oxford Instruments continuous-flow helium cryostat and was recorded at a 9.39-MHz microwave frequency with 2.4 mW of microwave power at 10 K.

Repair of the damaged [4Fe-4S] cluster of *E. coli* fumarase A.

Cells of the *E. coli ytfE* mutant strain transformed with pGS57 were grown aerobically with 1 mM IPTG to an OD600 of ~0.5, collected by centrifugation, resuspended (1/100) in fumarase assay buffer (*47*), and lysed by four freeze-thaw cycles. Two minutes before the stresses were imposed, 100 µg/ml tetracycline was added to the cell extracts to inhibit *de novo* protein synthesis. After 1 min of incubation with 4 mM H_2O_2 or 5 min with 150 µM NO, 400 U/ml of catalase or 40 µM hemoglobin (Sigma) was added, respectively, and the fumarase activity was determined at fixed time points. Purified ScdA protein was added at a final concentration of 20 µM immediately after the stresses were removed.

Fumarase activity was determined spectrophotometrically by following the disappearance of fumarate as described by Massey (*47*). The cell samples were quickly thawed at room temperature, cleared by the addition of 0.5% (wt/vol) sodium deoxycholate and then diluted in 50 mM sodium phosphate buffer, pH 7.3. The reactions were started by the addition of 10 mM fumarate and followed (at 295 nm, ε=0.07 mM^{-1} cm^{-1}). Enzyme activities were determined at 25°C and are defined as units (µmol of fumarate consumed per min) per mg of total protein. The enzyme activities were determined in duplicate from two independent cultures and are presented as averaged values, with error bars representing one standard deviation.

Determination of the aconitase activity in *S. aureus*.

Aconitase activity was determined in cell lysates of *S. aureus* RN4220 and *scdA* mutant that had been grown aerobically in LB medium at 37°C to an OD_{600} of 0.5. The cells were collected by centrifugation, resuspended (1/200) in assay buffer (50 mM Tris-HCl, pH 7.7, 0.6 mM

$MnCl_2$), and lysed by a 10-min incubation at 37°C with 75 μg/ml lysostaphin (Sigma). Cell lysates were exposed to 100 μM NO (prepared as previously described in (*48*)) or 3 mM H_2O_2 and at specific times aliquots were frozen in liquid nitrogen and later assayed. To monitor the repair of the damaged enzyme, the lysates were treated with tetracycline (100 μg/ml, Sigma) prior to exposure to H_2O_2 for 5 min or NO for 15 min. Upon addition of catalase (400 U/ml, Sigma) or hemoglobin (40 μM, Sigma), the aliquots were collected and frozen. Aconitase activity was determined by following the formation of NADPH through the indirect method described by Gardner (*49*). Samples were quickly thawed at room temperature, cleared by the addition of 0.5% (wt/vol) sodium deoxycholate, and immediately inserted into suba-sealed cuvettes with deaerated assay buffer that contained 0.2 mM $NADP^+$ and 1 U of isocitrate dehydrogenase (Sigma). The reaction was initiated with 50 mM sodium citrate. Aconitase activities determined at 25°C in duplicate from two independent cultures are defined as units (μmol of NADPH formed per min) per mg of total protein and are presented as averaged values, with error bars representing one standard deviation.

Quantitative real-time PCR analysis of gene expression.

Relative gene expression was measured using quantitative reverse transcription-PCR (qRT-PCR) as described previously (*27*). RNA was stabilized by mixing 500 μl of bacterial culture with 900 μl of RNAlater solution (Ambion). After a 5-min incubation at room temperature, the bacteria were harvested by centrifugation at 3,000 x *g* for 10 min. RNA was isolated from the pellet using an RNeasy mini kit (Qiagen) using the manufacturer's protocol. Genomic DNA was removed from the purified RNA using Turbo DNase (Ambion). The RNA was reverse transcribed to

cDNA using a Superscript first-strand synthesis kit (Invitrogen). For each sample, a control to check for DNA contamination in the RNA preparation was included from which reverse transcriptase was omitted. Transcript levels were measured by quantitative real-time PCR using SensiMix with Sybr green detection (Quantace) and an ABI 7000 sequence analyzer (Applied Biosystems). Primers designed using PrimerExpress (Applied Biosystems) are described in Table III.2. Transcript levels were quantified using the $\Delta\Delta C_T$ (where C_T is threshold cycle) method (*50*) relative to expression of the *polA* gene. Expression levels were normalized for each strain prior to shock with nitrite. For each experiment, quantitative real-time PCR was used to determine transcript levels on three independent cDNA samples derived from three independent cultures.

Determination of rates of NO reduction by washed bacterial suspensions.

N. gonorrhoeae strain F62 and its *dnrN* mutant were grown as described above in oxygen-limited cultures supplemented with nitrite and harvested by centrifugation, and the rates of NO reduction were assayed using a Hansatech Instruments oxygen electrode adapted for increased sensitivity to NO (*51*). All solutions used for these assays were purged of oxygen for at least 10 min using oxygen-free nitrogen gas. The concentration of NO at the start of the assay was 200 μM, and the bacterial density assayed was in the range of 1 to 2 mg of dry mass ml^{-1}.

IV.3 – Results and Discussion

Effect of a mutation in *S. aureus scdA* and *Neisseria gonorrhoeae dnrN* on recovery from oxidative and nitrosative stress.

To assess the function of ScdA in *S. aureus*, the effects of NO and H_2O_2 on growth of *scdA* mutant and its parent were compared. The *scdA* mutant strain showed no morphological defects, contrary to what had been previously described (*52*). Although no differences could be detected between the wild type and mutant strain in response to exposure to NO (data not shown), the *scdA* mutant was more sensitive to oxidative conditions than its parent (Figure IV.1). Hence, ScdA constitutes an efficient protection system against hydrogen peroxide.

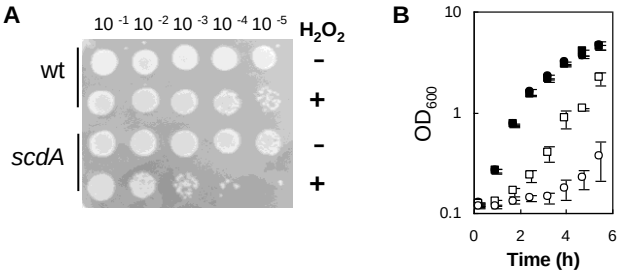


Figure IV.1 – The sensitivity of *S. aureus* to hydrogen peroxide increases in the absence of *scdA*. (A) Serial dilutions of cultures of *S. aureus* RN4220 (wt) and the *scdA* mutant strain after 4 h of growth in the presence (+) or absence (–) of 10 mM H_2O_2. A representative plate of independent experiments performed in duplicate is shown. **(B)** Growth of *S. aureus* RN4220 (squares) and the *scdA* mutant (circles) monitored by the OD_{600} in cultures untreated (filled symbols) or treated with 10 mM H_2O_2 (open symbols). Mean values of two independent cultures are given, with error bars showing the standard deviations.

In *N. gonorrhoeae*, binding sites for the NO-sensitive transcription factor NsrR, a member of the Rrf2 family of transcription factors, were identified at the promoters of *aniA* that controls the expression of the gene encoding a copper-containing nitrite reductase similar to NirK in other

bacteria; *norB* encoding the single subunit nitric oxide reductase; and a gene of unknown function, *dnrN*. All of these genes are known to be induced upon exposure to nitric oxide (*27, 53*). The gonococcal DnrN protein is 16% identical and 31% similar in amino acid sequence to *S. aureus* ScdA, suggesting that it might be a functional homologue of ScdA. A *dnrN* deletion mutant was constructed and the effects of the mutation on recovery from exposure to NO were assessed. As the gonococcal *dnrN* gene is monocistronic, the possibility of secondary effects of the mutation on downstream genes was discounted. Since gonococci generate NO as the product of nitrite reduction during oxygen-limited growth, it was predicted that the *dnrN* mutant might be more sensitive to sudden exposure to nitrite, which will be converted rapidly to NO, than its *dnrN*$^{+}$ parent. Sudden addition of nitrite to a culture in which AniA has accumulated but NorB synthesis has not been induced will lead to the sudden generation of NO, which would cause damage from which only the parent strain can recover. In contrast, if *aniA* and *norB* transcription are gradually induced sequentially because nitrite is available during adaptation to oxygen-limited growth, both the mutant and the parent are able to adapt.

To test these predictions, the mutant and parent strains were first grown in oxygen-limited medium supplemented with 5 mM nitrite. FNR, the regulator of fumarate and nitrate reduction during anaerobic growth, is essential in gonococci for expression of several genes including the nitrite reductase *aniA*. As the culture became oxygen-limited, FNR gradually became activated inducing the transcription of *aniA*. The consequent production of NO, generated during nitrite reduction, induced synthesis of the gonococcal nitric oxide reductase, NorB, which scavenges the NO present in the cell. We propose that, under these conditions, the concentration of NO available is low; therefore, the mutant grew only

slightly more slowly than the parent and growth of both strains stopped at similar cell densities (Figure IV.2A).

Parallel cultures were also grown in the absence of nitrite, in which case expression of the nitrite reductase *aniA* would still occur (*36, 54*), but, as NO would not be formed, *norB* would remain repressed by NsrR. When the cultures became oxygen-limited, nitrite was added, resulting in a sudden pulse of NO generation. As predicted, after an initial inhibition of growth the parent strain, F62, recovered, but growth inhibition of the *dnrN* mutant persisted (Figure IV.2B). It was concluded that the gonococcal *dnrN* mutant is more sensitive than its parent to damage induced by a sudden exposure to nitric oxide generated from nitrite.

A NO-sensitive electrode was used to eliminate the alternative possibility that the *dnrN* mutant is defective in its ability to reduce NO compared with the parent strain (*55*). The rates of NO reduction by bacteria harvested from these cultures were measured using an NO-sensitive electrode. The average values for the two strains were indistinguishable, 162 (±18) nmol of NO reduced min^{-1}.mg of bacterial dry $mass^{-1}$ for the mutant compared with 164 (±46) nmol of NO reduced min^{-1}. mg of bacterial dry $mass^{-1}$ for the parental strain. Furthermore, these rates of NO reduction were sufficiently high to exclude the possibility that NO accumulates to a higher concentration in cultures of the mutant, causing more severe or even different types of damage (*55*).

Pathogenic *Neisseria* synthesize an extremely active catalase that masks any protective functions of other proteins that protect the bacteria from exposure to hydrogen peroxide (*56*). To reveal whether DnrN plays any role in protection against oxidative stress, the *dnrN* deletion mutation was transferred into *N. gonorrhoeae* strain JCGC212, from which the *kat* gene has been deleted. The effects of exposure to hydrogen peroxide on

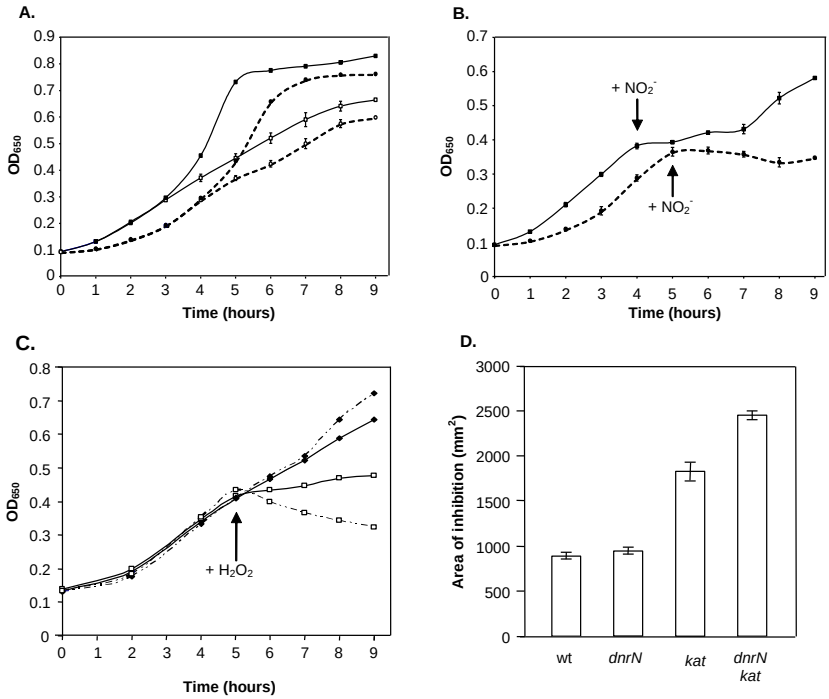


Figure IV.2 – Effect of a *dnrN* mutation on the recovery of *N. gonorrhoeae* from damage induced by sudden exposure to nitric oxide or hydrogen peroxide. A & B. Deletion of *dnrN* results in a growth phenotype in *N. gonorrhoeae*. The optical density at 650 nm of oxygen-limited cultures of *N. gonorrhoeae* strains F62 (*dnrN*$^+$; solid lines) and JCGC704 (*dnrN*; dotted lines) was measured at hourly intervals. **A.** Growth in the presence (filled symbols) or absence (open symbols) of 5 mM nitrite. **B.** Growth in the absence of nitrite until an OD_{650} of around 0.4 (about 0.16 mg dry mass ml^{-1}), followed by shock with 0.5 mM $NaNO_2$ (arrows). Error bars show standard deviation of duplicate cultures. **C & D.** Effect of hydrogen peroxide on the growth of *kat* and *dnrN kat* mutants. **C.** Oxygen-limited cultures of *N. gonorrhoeae* JCGC212 (*kat*; solid lines) and JCGC807 (*dnrN kat*; dotted lines) were grown in the absence of nitrite. Half of the cultures were shocked with 0.5 mM hydrogen peroxide at an OD_{650} of around 0.4 (shown by arrow; open squares) while the remaining cultures were not treated (filled diamonds). The growth experiment was repeated twice. **D.** *N. gonorrhoeae* strains F62 (wild-type), JCGC704 (*dnrN*), JCGC212 (*kat*) and JCGC807 (*dnrN kat*) were first grown on GC agar plates for one day at 37°C. A lawn of each strain was spread onto a fresh GC agar plate supplemented with 1 mM sodium nitrite. A 12 mm filter paper disc was seeded in the centre of the plate with 10 µl of 30 % hydrogen peroxide, and plates were incubated for 4 days at 37°C in an anaerobic jar. The area of growth inhibition was calculated. Error bars are standard deviations of triplicate samples.

growth of both the *dnrN kat* double mutant and its isogenic *dnrN*$^+$ strain in liquid medium were then compared. Despite the absence of catalase activity, at very low concentrations of H_2O_2 (<0.5 mM), growth of neither the mutant nor the parental strain was significantly inhibited. Conversely, growth of both strains was completely inhibited at high concentration of H_2O_2. However, the *dnrN* mutant was more sensitive than its parent at an intermediate concentration of H_2O_2 (Figure IV.2C). Disk diffusion assays confirmed that the *kat dnrN* double mutant was also more sensitive than the *kat* single mutant to growth in the presence of hydrogen peroxide on solid medium (Figure IV.2D). These results implicated DnrN in protection not only against nitrosative stress, but also in oxidative stress.

Increased sensitivity of the gonococcal *dnrN* mutant to damage to iron-sulphur centres of the transcription factors, FNR and NsrR.

The results presented above established that strains mutated in the gonococcal *dnrN* and in *S. aureus scdA* have increased susceptibility to exogenous hydrogen peroxide. This phenotype is frequently correlated with elevated levels of intracellular free iron to which the degradation of iron-sulphur centres contributes (*57*). In addition, one possible explanation for the sensitivity of the gonococcal *dnrN* mutant to nitrosative stress is that sudden exposure to NO damaged the iron-sulphur centres of FNR, NsrR, and also many other iron-sulphur proteins. As there is currently no system for over-expressing proteins in the gonococcus, the strategy devised to demonstrate the role for DnrN in repair of nitrosative damage was to monitor by quantitative real-time PCR the accumulation of mRNA synthesized under the control of the two transcription factors, FNR and NsrR, in which iron-sulphur centres are critical for function. First, we

exploited the NO-induced damage to the oxygen-sensing $[4Fe\text{-}4S]^{2+/1+}$ iron-sulphur centre of FNR that results in loss of DNA-binding and transcription activation and consequent loss of *aniA* expression. The qRT-PCR experiments showed that the loss of *aniA* expression immediately after exposure to NO was followed by restoration of the accumulation of *aniA* mRNA in the parental strain, but not in the mutant (Figure IV.3A). This result confirmed that the damage was repaired more rapidly in a parental strain, F62, than in a *dnrN* mutant.

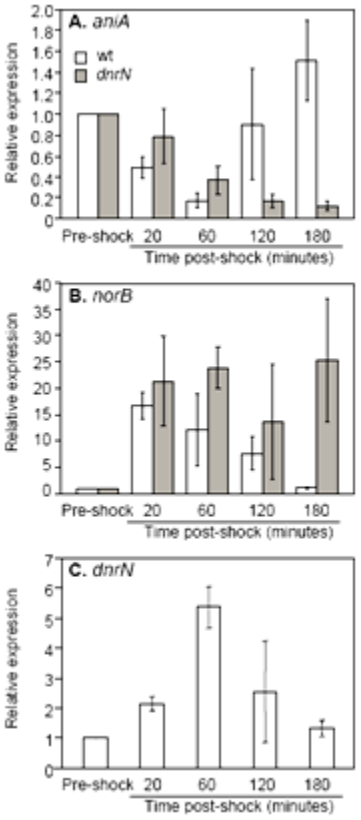


Figure IV.3. Quantitative RT-PCR analysis of gene expression before and after shock with nitrite. *N. gonorrhoeae* strains F62 (parent) and JCGC704 (*dnrN*) were grown in oxygen-limited conditions in the absence of nitrite to an OD_{650} of ~0.4, then shocked with 0.5 mM $NaNO_2$. RNA was isolated pre-shock and 20, 60, 120 and 180 minutes after the shock and qPCR was used to quantify *aniA* **(A)**, *norB* **(B)** and *dnrN* **(C)** transcript. Quantities are normalized against the pre-shock transcript level for each strain.

The NsrR protein, which is also predicted to contain a Fe-S centre (*25*), represses the expression of the *norB* gene, but repression is lifted on exposure to low concentrations of NO (*27, 58*). If the interpretation of the effects of a *dnrN* mutation on *aniA* expression is correct, it can be predicted that exposure to NO would result in a rapid increase in *norB* expression. Repression would be restored rapidly in the parental *dnrN*$^+$ strain, but not

in a *dnrN* mutant. This prediction was confirmed (Figure IV.3B). Furthermore, *dnrN* mRNA also accumulated rapidly in the parental strain following NO exposure, but the level of this transcript also decreased rapidly as NsrR repression was restored (Figure IV.3C).

In the absence of *S. aureus scdA* the activity of the iron-sulphur enzyme aconitase is decreased.

S. aureus synthesizes a single aconitase, a dehydratase of the tricarboxylic acid cycle, that contains a $[4Fe\text{-}4S]^{2+/1+}$ cluster that is susceptible to damage by NO and oxidants such as hydrogen peroxide. In the absence of *scdA*, the activity of aconitase was found to be 33% lower than in the *S. aureus* parent strain. Furthermore, when cell lysates of *S. aureus* were exposed to NO or to hydrogen peroxide, a faster decrease of the aconitase activity was observed in the *scdA* mutant than in its parent (Figure IV.4A and C). We also tested the influence of ScdA in the recovery of aconitase activity upon damage caused by oxidative or nitrosative stress. The aconitase activities of cell lysates prepared from each culture during subsequent incubation in the absence of NO or H_2O_2 were then assayed. Tetracycline was added to cultures of each strain to inhibit *de novo* protein synthesis and, after a brief exposure to NO or H_2O_2, haemoglobin or catalase was added to scavenge excess NO or H_2O_2. Aconitase activity was restored rapidly only in the parental strain (Figure IV.4B and D).

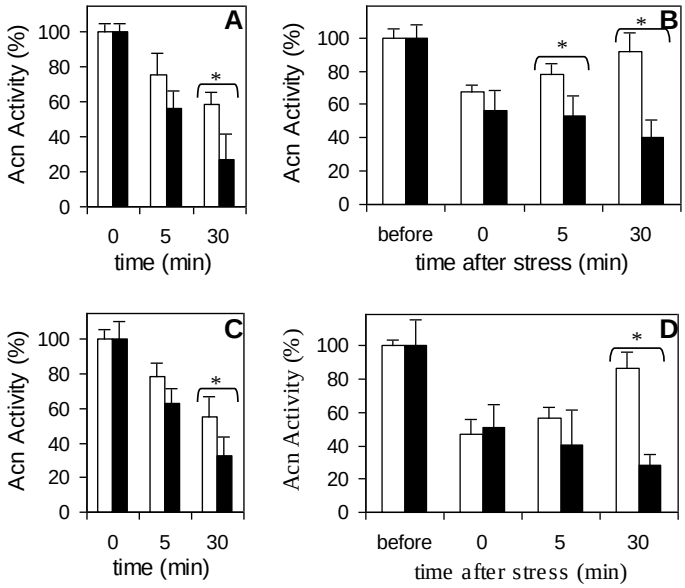


Figure IV.4 – Nitric oxide and hydrogen peroxide-induced damage to aconitase is more pronounced and the repair of the damage is severely impaired in the absence of *scdA*. Cell lysates of *S. aureus* RN4220 parent strain (*white bars*) and the *scdA* mutant (*black bars*) were subjected to 3 mM H_2O_2 (A,B) or 100 μM NO (C,D). For the time course of damage (A and C) the aconitase activity was monitored for 30 min. To follow the repair of aconitase, after 2 min with H_2O_2 (B) or 15 min with NO (D), catalase and haemoglobin were added to interrupt the exposures (time zero) and the activity was then monitored. The values are averages of duplicate determinations from two (B and D) or four (A and C) independent experiments with error bars representing one standard deviation unit. The asterisk (*) represents statistical significance ($p < 0.05$) using a Student's *t*-test. The values are normalized for the initial activity of each strain (wild type: 17.1 mU/mg protein and *scdA*: 11.5 mU/mg protein).

Major contribution of *Staphylococcus aureus* di-iron ScdA to repair of stress-induced damage to the iron-sulphur centre of fumarase.

The phenotype of the *S. aureus scdA* mutant resembles the one recently described for *E. coli ytfE* mutant. In both cases, the activities of

iron-sulphur containing enzymes are lower in the mutant (*41*). To determine whether ScdA and YtfE also showed similar biochemical properties, the recombinant *S. aureus* ScdA was produced in *E. coli* and characterized. The purified ScdA protein was isolated as a dimer with a molecular mass of 57 kDa and contained two iron atoms per monomer. The visible spectrum exhibited a broad band at 350 nm, characteristic of iron-containing proteins (data not shown). *S. aureus* ScdA exhibited an EPR spectrum with *g*-values of 1.96, 1.92 and 1.86 (Figure IV.5), which are within the range of values usually observed for proteins containing di-iron centres, including the *E. coli* YtfE (*41, 59*).

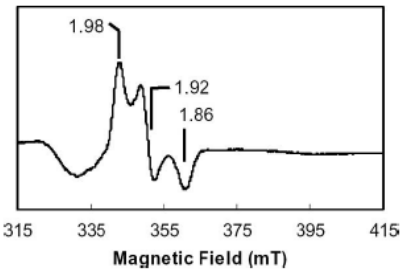


Figure IV.5 – ScdA protein of *S. aureus* has a di-iron centre. EPR spectrum of the as prepared ScdA protein, recorded at 8 K with 9.4 GHz microwave frequency and 2.4 mW microwave power.

The similarity between the *E. coli* YtfE and *S. aureus* ScdA proteins led us to investigate whether the recombinant ScdA could support the *in vitro* repair of a damaged [4Fe-4S] cluster, as shown for *E. coli* YtfE (*60*). Indeed, addition of purified ScdA protein to cell lysates of *E. coli* Δ*ytfE* expressing fumarase A and exposed to hydrogen peroxide (Figure IV.6A) or nitric oxide (Figure IV.6B) demonstrated that ScdA promotes restoration of the fumarase activity to the levels observed before damage. These results show that *S. aureus* ScdA is essential for the repair of a $[4Fe\text{-}4S]^{2+/1+}$ protein whose cluster is damaged by oxidative or nitrosative compounds.

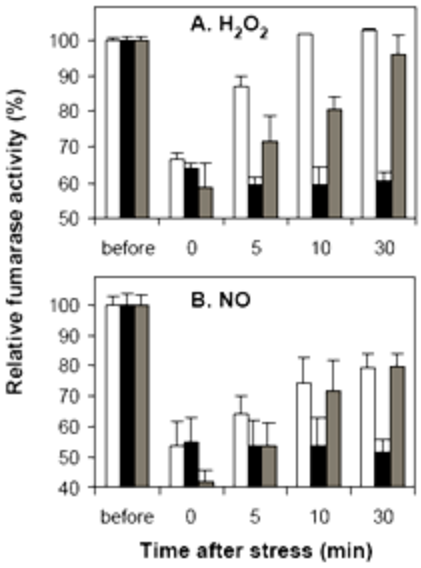


Figure IV.6 – The ScdA protein of *S. aureus* repairs the [4Fe-4S] cluster of fumarase A after damage by nitric oxide and hydrogen peroxide. Fumarase activity was monitored in lysates of *E. coli* K-12 cells (*white bars*) and *E. coli ytfE* mutant cells (*black bars*) expressing fumarase A after treatment with tetracycline and subjection to (A) 4 mM H_2O_2 for 1 min, or to (B) 150 μM NO for 10 min. Immediately after terminating the stresses by the addition of catalase or haemoglobin, purified ScdA protein was added to *ytfE* mutant cell lysates (*grey bars*) and the activity was measured (time zero) and monitored for 30 min. The values are normalized for the initial activity ("before") of each strain (wild type: 3.7 U/mg protein and *ytfE*: 2.9 U/mg protein), and are mean values of two experiments analysed in duplicate. Error bars represent 1 standard deviation unit.

***S. aureus scdA*, but not *suf* or *isc* operons, complements the hydrogen peroxide sensitivity of the *E. coli ytfE* mutant.**

Next, we addressed the question of whether *S. aureus scdA* could replace the function of *ytfE*. To this end, *E. coli ytfE* mutant strain was transformed with plasmids encoding either the *E. coli ytfE* or the *S. aureus scdA* genes, and sensitivity of the strains was measured under oxidative stress conditions generated by hydrogen peroxide. The *ytfE* mutant was more sensitive to hydrogen peroxide than the parent and hypersensitivity was suppressed by expression *in trans* of either the *E. coli ytfE* or the *S. aureus scdA* genes (Figure IV.7).

The *E. coli isc* and *suf* operons are proposed to encode proteins that may also be involved in the repair of Fe-S clusters. However, the resistance of the *ytfE* mutant to hydrogen peroxide was not restored by the plasmid

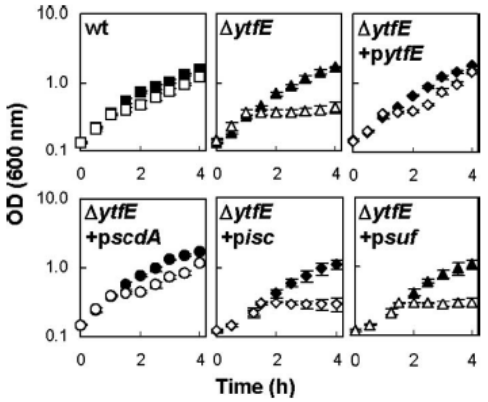


Figure IV.7 – *S. aureus scdA*, but not the *suf* or *isc* operons of *E. coli*, complement the sensitivity to hydrogen peroxide of the *E. coli ytfE* mutant. *E. coli* K-12 parent strain (wt), *ytfE* mutant strain (Δ*ytfE*), *ytfE* strain expressing *E. coli ytfE* in trans (Δ*ytfE*+p*ytfE*), *ytfE* strain expressing *S. aureus scdA* in *trans* (Δ*ytfE*+p*scdA*), *ytfE* strain expressing the *E. coli isc* operon in trans (Δ*ytfE*+p*isc*) and *ytfE* strain expressing the *E. coli suf* operon in trans (Δ*ytfE*+p*suf*) were grown in LB under anaerobic conditions. Cultures were left untreated (filled symbols), or treated with 2 mM H_2O_2, at an OD_{600} ~0.3 (open symbols). Mean values of two independent experiments with error bars representing the standard deviation are shown.

pRKSUF neither by pRKISC (Figure IV.7), containing the full *sufABCDSE* and *iscRSUAhscBAfdx* operons of *E. coli*, respectively (*42, 43*). Hence, the ISC and SUF system cannot replace YtfE, even though SUF was reported to operate under stress conditions as oxidative stress and iron starvation (*8, 9*). Note that, however, the plasmid containing the complete set of *suf* genes could complement most defects of the Δ*iscRSUAhscBAfdx* strain (*43*). We conclude that *S. aureus* ScdA and *E. coli* YtfE have similar biochemical roles.

Phylogenetic analysis of ScdA and DnrN homologues.

The amino acid sequences of *S. aureus* ScdA and gonococcal DnrN share 25 and 31% identity and 46 and 41% similarity to *E. coli* YtfE, respectively. Moreover, a comprehensive search of the amino acid sequence database revealed that DnrN, ScdA and *E. coli* YtfE are members of a large family of proteins that occur widely in the bacterial phyla

Proteobacteria, *Bacteroidetes*, *Firmicutes*, *Actinobacteria* and *Acidobacteria*. In particular, homologues of these proteins are encoded in the genomes of a significant number of human pathogens, such as *Bacillus anthracis*, *Haemophilus influenzae* and species of the genera *Salmonella*, *Shewanella*, *Yersinia* or *Clostridium*. Interestingly, two orthologue sequences were found in the eukaryotic organism (*Trichomonas vaginalis*), which is also a human pathogen.

Since a recent study in the pathogenic yeast *Cryptococcus neoformans* suggests that this eukaryote contains a homologue of the *E. coli* YtfE (*61*), this protein (CNA2870) and other putative fungal homologues were included in the analysis, in spite of the low sequence similarity of CNA2870 with the ScdA, DnrN and YtfE proteins (7-8% identity and 16-17% similarity). Using all the above-mentioned amino acid sequences, a dendrogram was constructed (Figure IV.8) showing two main groups, one that includes the ScdA/DnrN/YtfE-like proteins and the other with the CNA2870-like proteins, in agreement with the low identity (3-11%) and similarity (9-22%) values between the sequences from both groups. The group of the ScdA/DnrN/YtfE-like proteins is apparently divided into two other groups, one comprising the majority of the proteobacteria and another containing the sequences of several taxa. The ScdA protein of *S. aureus* and the DnrN protein of *N. gonorrhoeae* are clustered separately due to the low amino acid sequence identity between the two proteins (16%).

The alignment of amino acid sequences of the proteins (Figure IV.S1 in section IV.5) that produced the dendrogram in Figure IV.8 revealed conservation of some regions (particularly within the ScdA/DnrN/YtfE-like sequences) and a high degree of conservation of the residues His^{84}, His^{105}, His^{129}, Glu^{133}, His^{160} and His^{204} (numbering refer to residues in *E. coli* YtfE). Exceptions are observed for three yeast-like sequences in which Glu^{133} was

substituted by an Asp. Based upon studies with *E. coli* YtfE, these residues are proposed to constitute the ligand sphere for the di-iron centre (our unpublished results). In particular, they are located in conserved α-helix regions of a predicted secondary structure (Figure IV.S1), corroborating the importance for the function of the four-helix-bundle protein fold that is predicted for ScdA/DnrN/YtfE and characterizes many other di-iron proteins.

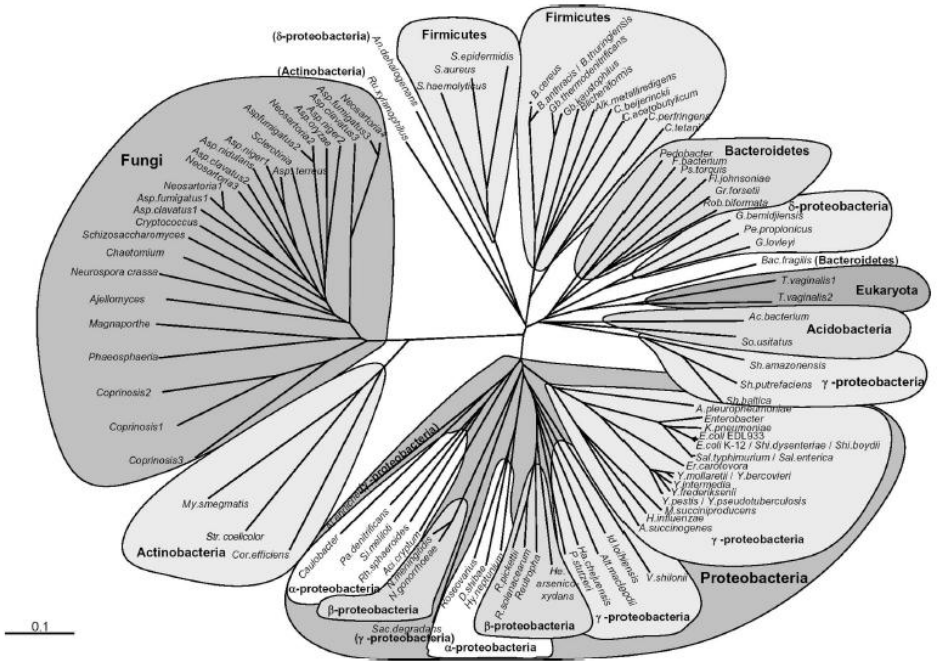


Figure IV.8 – Unrooted dendrogram of ScdA/DnrN family of proteins. The dendrogram was generated with Clustal X and manipulated in TreeView. A total of 73 sequences from *S. aureus* ScdA and *N. gonorrhoeae* DnrN homologues were aligned and the dendrogram was bootstrapped by exclusion gap positions and correcting for multiple substitutions. Shaded boxes distinguish the different taxonomic groups. Abbreviations for the organisms are described in the legend of the annexed Figure III.S1 (see Section III-5).

RIC, a new family of proteins involved in the *r*epair of *i*ron *centre*s.

The work presented above has revealed the presence in a wide range of human, animal and plant pathogens of a family of di-iron proteins that have similar functions. Based upon *in vivo* and *in vitro* evidence, we have shown that these proteins are present in both gram-positive and gram-negative bacteria, and that the two main branches of this protein family can repair Fe-S clusters damaged by exposure to NO and H_2O_2. Our work corroborates and significantly extends the proposal of Rodionov *et al*. (*53*), based on the bioinformatic analysis of complete genome sequences, that DnrN in pathogenic *Neisseria* is involved in the response to nitrosative stress. Future research must focus on the exact chemical reactions catalysed by this protein family during the repair process, for example, removal of the nitrosated iron atoms or reinsertion of iron once the primary damage has been removed by other proteins. As it is not known whether the substrates on which these protein work are limited to those with iron-sulphur centres, we propose the name RIC, for *r*epair of *i*ron *centre*s, for this new and widely distributed protein family.

IV.4 – References

(1) Huber, C., and Wachtershauser, G. (1998) Peptides by activation of amino acids with CO on (Ni,Fe)S surfaces: implications for the origin of life. *Science 281*, 670-2.

(2) Beinert, H., Holm, R. H., and Munck, E. (1997) Iron-sulfur clusters: nature's modular, multipurpose structures. *Science 277*, 653-9.

(3) Johnson, D. C., Dean, D. R., Smith, A. D., and Johnson, M. K. (2005) Structure, function, and formation of biological iron-sulfur clusters. *Annu Rev Biochem 74*, 247-81.

(4) Kiley, P. J., and Beinert, H. (2003) The role of Fe-S proteins in sensing and regulation in bacteria. *Curr Opin Microbiol 6*, 181-5.

(5) Fontecave, M., Choudens, S. O., Py, B., and Barras, F. (2005) Mechanisms of iron-sulfur cluster assembly: the SUF machinery. *J Biol Inorg Chem 10*, 713-21.

(6) Frazzon, J., and Dean, D. R. (2003) Formation of iron-sulfur clusters in bacteria: an emerging field in bioinorganic chemistry. *Curr Opin Chem Biol 7*, 166-73.

(7) Djaman, O., Outten, F. W., and Imlay, J. A. (2004) Repair of oxidized iron-sulfur clusters in Escherichia coli. *J Biol Chem 279*, 44590-9.

(8) Nachin, L., Loiseau, L., Expert, D., and Barras, F. (2003) SufC: an unorthodox cytoplasmic ABC/ATPase required for [Fe-S] biogenesis under oxidative stress. *Embo J 22*, 427-37.

(9) Outten, F. W., Djaman, O., and Storz, G. (2004) A suf operon requirement for Fe-S cluster assembly during iron starvation in Escherichia coli. *Mol Microbiol 52*, 861-72.

(10) Loiseau, L., Ollagnier-de Choudens, S., Lascoux, D., Forest, E., Fontecave, M., and Barras, F. (2005) Analysis of the heteromeric CsdA-CsdE cysteine desulfurase, assisting Fe-S cluster biogenesis in Escherichia coli. *J Biol Chem 280*, 26760-9.

(11) Yang, W., Rogers, P. A., and Ding, H. (2002) Repair of nitric oxide-modified ferredoxin [2Fe-2S] cluster by cysteine desulfurase (IscS). *J Biol Chem 277*, 12868-73.

(12) Rogers, P. A., Eide, L., Klungland, A., and Ding, H. (2003) Reversible inactivation of E. coli endonuclease III via modification of its [4Fe-4S] cluster by nitric oxide. *DNA Repair (Amst) 2*, 809-17.

(13) Constantinidou, C., Hobman, J. L., Griffiths, L., Patel, M. D., Penn, C. W., Cole, J. A., and Overton, T. W. (2006) 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*, 4802-15.

(14) Justino, M. C., Vicente, J. B., Teixeira, M., and Saraiva, L. M. (2005) New genes implicated in the protection of anaerobically grown Escherichia coli against nitric oxide. *J Biol Chem 280*, 2636-43.

(15) Mukhopadhyay, P., Zheng, M., Bedzyk, L. A., LaRossa, R. A., and Storz, G. (2004) Prominent roles of the NorR and Fur regulators in the Escherichia coli transcriptional response to reactive nitrogen species. *Proc Natl Acad Sci U S A 101*, 745-50.

(16) Pullan, S. T., Gidley, M. D., Jones, R. A., Barrett, J., Stevanin, T. M., Read, R. C., Green, J., and Poole, R. K. (2007) Nitric oxide in chemostat-cultured Escherichia coli is sensed by Fnr and other global regulators: unaltered methionine biosynthesis indicates lack of S nitrosation. *J Bacteriol 189*, 1845-55.

(17) Richardson, A. R., Dunman, P. M., and Fang, F. C. (2006) The nitrosative stress response of Staphylococcus aureus is required for resistance to innate immunity. *Mol Microbiol 61*, 927-39.

(18) Sebbane, F., Lemaitre, N., Sturdevant, D. E., Rebeil, R., Virtaneva, K., Porcella, S. F., and Hinnebusch, B. J. (2006) Adaptive response of Yersinia pestis to extracellular effectors of innate immunity during bubonic plague. *Proc Natl Acad Sci U S A 103*, 11766-71.

(19) Gardner, A. M., and Gardner, P. R. (2002) Flavohemoglobin detoxifies nitric oxide in aerobic, but not anaerobic, Escherichia coli. Evidence for a novel inducible anaerobic nitric oxide-scavenging activity. *J Biol Chem 277*, 8166-71.

(20) Gardner, A. M., Helmick, R. A., and Gardner, P. R. (2002) Flavorubredoxin, an inducible catalyst for nitric oxide reduction and detoxification in Escherichia coli. *J Biol Chem 277*, 8172-7.

(21) Kim, S. O., Orii, Y., Lloyd, D., Hughes, M. N., and Poole, R. K. (1999) Anoxic function for the Escherichia coli flavohaemoglobin (Hmp): reversible binding of nitric oxide and reduction to nitrous oxide. *FEBS Lett 445*, 389-94.

(22) Poole, R. K., and Hughes, M. N. (2000) New functions for the ancient globin family: bacterial responses to nitric oxide and nitrosative stress. *Mol Microbiol 36*, 775-83.

(23) Cruz-Ramos, H., Crack, J., Wu, G., Hughes, M. N., Scott, C., Thomson, A. J., Green, J., and Poole, R. K. (2002) NO sensing by FNR: regulation of the Escherichia coli NO-detoxifying flavohaemoglobin, Hmp. *Embo J 21*, 3235-44.

(24) Nakano, M. M., Geng, H., Nakano, S., and Kobayashi, K. (2006) The nitric oxide-responsive regulator NsrR controls ResDE-dependent gene expression. *J Bacteriol 188*, 5878-87.

(25) Bodenmiller, D. M., and Spiro, S. (2006) The yjeB (nsrR) gene of Escherichia coli encodes a nitric oxide-sensitive transcriptional regulator. *J Bacteriol 188*, 874-81.

(26) Filenko, N., Spiro, S., Browning, D. F., Squire, D., Overton, T. W., Cole, J., and Constantinidou, C. (2007) The NsrR regulon of Escherichia coli K-12 includes genes encoding the hybrid cluster protein and the periplasmic, respiratory nitrite reductase. *J Bacteriol 189*, 4410-7.

(27) Overton, T. W., Whitehead, R., Li, Y., Snyder, L. A., Saunders, N. J., Smith, H., and Cole, J. A. (2006) Coordinated regulation of the Neisseria gonorrhoeae-truncated denitrification pathway by the nitric oxide-sensitive repressor, NsrR, and nitrite-insensitive NarQ-NarP. *J Biol Chem 281*, 33115-26.

(28) Chang, W., Small, D. A., Toghrol, F., and Bentley, W. E. (2006) Global transcriptome analysis of Staphylococcus aureus response to hydrogen peroxide. *J Bacteriol 188*, 1648-59.

(29) Hausladen, A., Privalle, C. T., Keng, T., DeAngelo, J., and Stamler, J. S. (1996) Nitrosative stress: activation of the transcription factor OxyR. *Cell 86*, 719-29.

(30) Seib, K. L., Wu, H. J., Kidd, S. P., Apicella, M. A., Jennings, M. P., and McEwan, A. G. (2006) Defenses against oxidative stress in Neisseria gonorrhoeae: a

system tailored for a challenging environment. *Microbiol Mol Biol Rev 70*, 344-61.

(31) Yeo, W. S., Lee, J. H., Lee, K. C., and Roe, J. H. (2006) IscR acts as an activator in response to oxidative stress for the suf operon encoding Fe-S assembly proteins. *Mol Microbiol 61*, 206-18.

(32) Zheng, M., Wang, X., Templeton, L. J., Smulski, D. R., LaRossa, R. A., and Storz, G. (2001) DNA microarray-mediated transcriptional profiling of the Escherichia coli response to hydrogen peroxide. *J Bacteriol 183*, 4562-70.

(33) McHugh, J. P., Rodriguez-Quinones, F., Abdul-Tehrani, H., Svistunenko, D. A., Poole, R. K., Cooper, C. E., and Andrews, S. C. (2003) Global iron-dependent gene regulation in Escherichia coli. A new mechanism for iron homeostasis. *J Biol Chem 278*, 29478-86.

(34) Goncalves, V. L., Nobre, L. S., Vicente, J. B., Teixeira, M., and Saraiva, L. M. (2006) Flavohemoglobin requires microaerophilic conditions for nitrosative protection of Staphylococcus aureus. *FEBS Lett 580*, 1817-21.

(35) Link, A. J., Phillips, D., and Church, G. M. (1997) Methods for generating precise deletions and insertions in the genome of wild-type Escherichia coli: application to open reading frame characterization. *J Bacteriol 179*, 6228-37.

(36) Lissenden, S., Mohan, S., Overton, T., Regan, T., Crooke, H., Cardinale, J. A., Householder, T. C., Adams, P., O'Conner, C. D., Clark, V. L., Smith, H., and Cole, J. A. (2000) Identification of transcription activators that regulate gonococcal adaptation from aerobic to anaerobic or oxygen-limited growth. *Mol Microbiol 37*, 839-55.

(37) Turner, S., Reid, E., Smith, H., and Cole, J. (2003) A novel cytochrome c peroxidase from Neisseria gonorrhoeae: a lipoprotein from a Gram-negative bacterium. *Biochem J 373*, 865-73.

(38) de Lencastre, H., Couto, I., Santos, I., Melo-Cristino, J., Torres-Pereira, A., and Tomasz, A. (1994) Methicillin-resistant Staphylococcus aureus disease in a Portuguese hospital: characterization of clonal types by a combination of DNA typing methods. *Eur J Clin Microbiol Infect Dis 13*, 64-73.

(39) Uzzau, S., Figueroa-Bossi, N., Rubino, S., and Bossi, L. (2001) Epitope tagging of chromosomal genes in Salmonella. *Proc Natl Acad Sci U S A 98*, 15264-9.

(40) Woods, S. A., Schwartzbach, S. D., and Guest, J. R. (1988) Two biochemically distinct classes of fumarase in Escherichia coli. *Biochim Biophys Acta 954*, 14-26.

(41) Justino, M. C., Almeida, C. C., Goncalves, V. L., Teixeira, M., and Saraiva, L. M. (2006) Escherichia coli YtfE is a di-iron protein with an important function in assembly of iron-sulphur clusters. *FEMS Microbiol Lett 257*, 278-84.

(42) Takahashi, Y., and Nakamura, M. (1999) Functional assignment of the ORF2-iscS-iscU-iscA-hscB-hscA-fdx-ORF3 gene cluster involved in the assembly of Fe-S clusters in Escherichia coli. *J Biochem 126*, 917-26.

(43) Takahashi, Y., and Tokumoto, U. (2002) A third bacterial system for the assembly of iron-sulfur clusters with homologs in archaea and plastids. *J Biol Chem 277*, 28380-3.

(44) Kellogg, D. S., Jr., Peacock, W. L., Jr., Deacon, W. E., Brown, L., and Pirkle, D. I. (1963) Neisseria Gonorrhoeae. I. Virulence Genetically Linked to Clonal Variation. *J Bacteriol 85*, 1274-9.

(45) Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. *Anal Biochem 150*, 76-85.

(46) Fischer, D. S., and Price, D. C. (1964) A Simple Serum Iron Method Using the New Sensitive Chromogen Tripyridyl-S-Triazine. *Clin Chem 10*, 21-31.

(47) Massey, V. (1953) Studies on fumarase. III. The effect of temperature. *Biochem J 53*, 72-9.

(48) Beckman, J. S., Wink, D. A., and Crow, J. P. (1996) *Methods in Nitric Oxide Research*, John Wiley & Sons Ltd. ed., Chichester, UK.

(49) Gardner, P. R. (2002) Aconitase: sensitive target and measure of superoxide. *Methods Enzymol 349*, 9-23.

(50) Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods 25*, 402-8.

(51) Thorndycroft, F. H., Butland, G., Richardson, D. J., and Watmough, N. J. (2007) A new assay for nitric oxide reductase reveals two conserved glutamate residues form the entrance to a proton-conducting channel in the bacterial enzyme. *Biochem J 401*, 111-9.

(52) Brunskill, E. W., de Jonge, B. L., and Bayles, K. W. (1997) The Staphylococcus aureus scdA gene: a novel locus that affects cell division and morphogenesis. *Microbiology 143 (Pt 9)*, 2877-82.

(53) Rodionov, D. A., Dubchak, I. L., Arkin, A. P., Alm, E. J., and Gelfand, M. S. (2005) Dissimilatory metabolism of nitrogen oxides in bacteria: comparative reconstruction of transcriptional networks. *PLoS Comput Biol 1*, e55.

(54) Whitehead, R. N., Overton, T. W., Snyder, L. A., McGowan, S. J., Smith, H., Cole, J. A., and Saunders, N. J. (2007) The small FNR regulon of Neisseria gonorrhoeae: comparison with the larger Escherichia coli FNR regulon and interaction with the NarQ-NarP regulon. *BMC Genomics 8*, 35.

(55) Strube, K., de Vries, S., and Cramm, R. (2007) Formation of a dinitrosyl iron complex by NorA, a nitric oxide-binding di-iron protein from Ralstonia eutropha H16. *J Biol Chem 282*, 20292-300.

(56) Johnson, S. R., Steiner, B. M., Cruce, D. D., Perkins, G. H., and Arko, R. J. (1993) Characterization of a catalase-deficient strain of Neisseria gonorrhoeae: evidence for the significance of catalase in the biology of N. gonorrhoeae. *Infect Immun 61*, 1232-8.

(57) Keyer, K., and Imlay, J. A. (1996) Superoxide accelerates DNA damage by elevating free-iron levels. *Proc Natl Acad Sci U S A 93*, 13635-40.

(58) Isabella, V., Wright, L. F., Barth, K., Spence, J. M., Grogan, S., Genco, C. A., and Clark, V. L. (2008) cis- and trans-acting elements involved in regulation of norB (norZ), the gene encoding nitric oxide reductase in Neisseria gonorrhoeae. *Microbiology 154*, 226-39.
(59) Kurtz, D. M., Jr. (1997) Structural similarity and functional diversity in diironoxo proteins. *J. Biol. Inorg. Chem. 2*, 159–167.
(60) Justino, M. C., Almeida, C. C., Teixeira, M., and Saraiva, L. M. (2007) Escherichia coli di-iron YtfE protein is necessary for the repair of stress-damaged iron-sulfur clusters. *J Biol Chem 282*, 10352-9.
(61) Chow, E. D., Liu, O. W., O'Brien, S., and Madhani, H. D. (2007) Exploration of whole-genome responses of the human AIDS-associated yeast pathogen Cryptococcus neoformans var grubii: nitric oxide stress and body temperature. *Curr Genet 52*, 137-48.

IV.5 – Acknowledgements

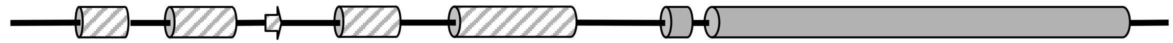
We are grateful to Prof. Miguel Teixeira (ITQB) for the EPR analysis and we thank Prof. Yasuhiro Takahashi for providing the complementation vectors pRKISC and pRKSUF.

IV.6 – Annex

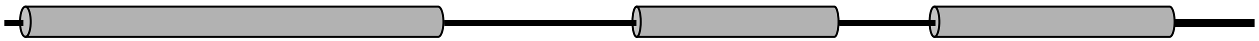
Figure III.S1 – Amino acid sequence analysis of the ScdA/DnrN/YtfE family of proteins. Sequence alignment of ScdA of *S. aureus*, DnrN of *N. gonorrhoeae* and YtfE of *E. coli* with homologues selected from the database and produced with Clustal X, version 1.81. Residues that are strictly conserved in all sequences are highlighted in black boxes, and dark grey boxes highlight residues conserved in more than 80% of the sequences. Light grey boxes highlight residues that are conserved in more than 90% of the sequences that contain the N-terminus domain. Represented at the top of the alignment is a consensus of the predicted secondary structures obtained from PSIPRED server for the sequences aligned. Full lines represent coil regions, arrows represent β-sheet and cylinders represent α-helices. The grey filled cylinders represent the 4 α-helices regions that are conserved for all sequences, except those belonging to the subgroup of sequences that include those from Fungi. Stripped symbols represent conserved secondary structure present in the sequences that contain the N-terminus domain. Organism and protein sequence gi number corresponding to each abbreviation: Acicryptum (148260835) *Acidiphilium cryptum* JF-5; Acbacterium (94967396) *Acidobacteria bacterium* Ellin345; Apleuropneumoniae (126097862) *Actinobacillus pleuropneumoniae* L20;

Asuccinogenes (150840754) *Actinobacillus succinogenes* 130Z; Ajellomyces (154277908) *Ajellomyces capsulatus* NAm1; Alehrlichei (114226155) *Alkalilimnicola ehrlichei* MLHE1; Alkmetalliredigens (149951947) *Alkaliphilus metalliredigens* QYMF; Altmacleodii (88775658) *Alteromonas macleodii* 'Deep ecotype'; Andehalogenans (85775066) *Anaeromyxobacter dehalogenans* 2CP-C; Aspclavatus1 (119398655), Aspclavatus2 (119403875) and Aspclavatus3 (121706066) *Aspergillus clavatus* NRRL1; Aspfumigatus1 (70981708), Aspfumigatus2 (70983051) and Aspfumigatus3 (66851946) *Aspergillus fumigatus* Af293; Aspnidulans (67903302) *Aspergillus nidulans* FGSC A4; Aspniger1 (134054653) and Aspniger2 (134080860) *Aspergillus niger*; Asporyzae (83773074) *Aspergillus oryzae*; Aspterreus (115383664) *Aspergillus terreus* NIH2624; Banthracis (47502589) *Bacillus anthracis* str. 'Ames Ancestor'; Bcereus (30020272) *Bacillus cereus* ATCC 14579; Blicheniformis (52080458) *Bacillus licheniformis* ATCC 14580; Bthuringiensis (118477580) *Bacillus thuringiensis* str. Al Hakam; Bacfragilis (60682784) *Bacteroides fragilis* NCTC 9343; Caulobacter (113935146) *Caulobacter* sp. K31; Chaetomium (116178806) *Chaetomium globosum* CBS 148.51; Cacetobutylicum (15893368) *Clostridium acetobutylicum* ATCC 824; Cbeijerinckii (150016894) *Clostridium beijerinckii* NCIMB 8052; Cperfrigens (18309757) *Clostridium perfringens* str. 13; Ctetani (28211509) *Clostridium tetani* E88; Coprinosis1 (116503767),Coprinosis2 (116506575) and Coprinosis3 (116503768) *Coprinosis cinerea* okayama7#130; Corefficiens (25029316) *Corynebacterium efficiens* YS-314; Cryptococcus (134106553) *Cryptococcus neoformans* var. *neoformans* B-3501A*;* Dshibae (118673578) *Dinoroseobacter shibae* DFL 12; Enterobacter (145316920) *Enterobacter* sp. 638; Ercarovotora (50122527) *Erwinia carotovora* subsp. *atroseptica* SCRI1043; Ecoli K-12 (16132031) *Escherichia coli* K-12; Ecoli EDL933 (15804800) *Escherichia coli* O157:H7 EDL933; Fbacterium (88712189) *Flavobacteriales bacterium* HTCC2170; Fljohnsoniae (146154587) *Flavobacterium johnsoniae* UW101; Gbkaustiophilus (56419306) *Geobacillus kaustophilus* HTA426; Gbthermodenitrificans (138894332) *Geobacillus thermodenitrificans* NG80-2; Gbemidjiensis (144942836) *Geobacter bemidjiensis Bem;* Glovleyi (18745009) *Geobacter lovleyi* SZ; Grforsetti (120435756) *Gramella forsetii* KT0803; Hinfluenzae (148827101) *Haemophilus influenzae* PittGG; Hachejuensis (83643602) *Hahella chejuensis* KCTC 2396; Hearsenicodyxans (133738491) *Herminiimonas arsenicoxydans*; Hyneptunium(114800024) *Hyphomonas neptunium* ATCC 15444; Idloihiensis (56459308) *Idiomarina loihiensis* L2TR; Kpneumoniae (150957923) *Klebsiella pneumoniae* subsp. *pneumoniae* MGH 78578; Magnaporthe (39940942) *Magnaporthe grisea* 70-15; Msucciniciproducens (52307350) *Mannheimia succiniciproducens* MBEL55E; Mysmegmatis (118473167) *Mycobacterium smegmatis* str. MC2 155; Neosartorya1 (119501631), Neosartorya2 (119483756),Neosartorya3 (119481719) and Neosartorya4 (119490985) *Neosartorya fischeri* NRRL 184; Neurosporacrassa (28916965) *Neurospora crassa*; Ngonorrhoeae (59717975) *Neisseria gonorrhoeae* FA 1090; Nmeningiditis (15677230) *Neisseria meningitidis* MC58; Padenitrificans (69938253) *Paracoccus denitrificans* PD1222; Pedobacter (149276683) *Pedobacter* sp. BAL39;

Pepropionicus (118504809) *Pelobacter propionicus* DSM 2379; Phaeosphaeria (111068832) *Phaeosphaeria nodorum*; Pstutzeri (11071577) *Pseudomonas stutzeri*; Pstorquis (91216610) *Psychroflexus torquis* ATCC 700755; Reutropha (72122218) *Ralstonia eutropha* JMP134; Rpickettii (121531262) *Ralstonia pickettii* 12J; Rsolanacearum (17549179) *Ralstonia solanacearum* GMI1000; Rhsphaeroides (145556675) *Rhodobacter sphaeroides* ATCC 17025; Robbiformata (88806027) *Robiginitalea biformata* HTCC2501; Roseovarius (85705585) *Roseovarius* sp. 217; Ruxylanophilus (108765272) *Rubrobacter xylanophilus* DSM 9941; Sacdegradans (90020223) *Saccharophagus degradans* 2-40; Salenterica (29144697) *Salmonella enterica* subsp. *enterica serovar Typhi* Ty2; Saltyphimurium (16767645) *Salmonella typhimurium* LT2; Schizosaccharomyces (6224597) *Schizosaccharomyces pombe*; Sclerotinia (154693903) *Sclerotinia sclerotiorum* 1980; Shamazonensis (119776368) *Shewanella amazonensis* SB2B; Shbaltica (146865931) *Shewanella baltica* OS223; Shputrefaciens(124546375) *Shewanella putrefaciens* 200; Shboydii (81247951) *Shigella boydii* Sb227; Shdysenteriae (83569422) *Shigella dysenteriae* 1012; Simeliloti (14523766) *Sinorhizobium meliloti* 1021; Sousitatus (116622425) *Solibacter usitatus* Ellin6076; Saureus (88194036) *Staphylococcus aureus* NCTC8325; Sepidermis (27467357) *Staphylococcus epidermidis* ATCC 12228; Shaemolyticus (70725629) *Staphylococcus haemolyticus* JCSC1435; Strcoelicor (8052384) *Streptomyces coelicor* A3(2); Tvaginalis1 (121909109) and Tvaginalis2 (121888849) *Trichomonas vaginalis* G3; Vshiloni (148836556) *Vibrio shilonii* AK1; Ybercovieri (77958756) *Yersinia bercovieri* ATCC 43970; Yfrederiksenii (77973982) *Yersinia frederiksenii* ATCC 33641; Yintermedia (77977615) *Yersinia intermedia* ATCC 29909; Ymollaretii (77961285) *Yersinia mollaretii* ATCC 43969; Ypestis (16123677) *Yersinia pestis* CO92; Ypseudotuberculosis (51588079) *Yersinia pseudotuberculosis* IP 32953.



```
Saureus               : -----------------------------------------MINKNDIVADVVTDYPKAADIFRSVGIDFCCGGQVSIEAALEKKNVDLNELLQRLNDVEQT---NTPGSLNPKFLNVSSLIQYIQSAYHEPLREEFKNLTPYVTKLSK-------------VHGPNH- : 112
Sepidermidis          : -----------------------------------------MITKEDIVADVVTDYPKSADIFRNAGIDFCCGGQESIASAVNHKPNIDLNSLLNKLNHIDNT---EGNSTINPKFLNVESLIQYIQSAYHETLKEEFKNLTPYMTKLAK-------------VHGPSH- : 112
Shaemolyticus         : -----------------------------------------MITNESIVAEIVTDIPLSADIFRKYGIDFCCGGNISINEAVKNKKVDAETLIDEINELPNH-----DQGNINVKYLDAPSLIQYIQSRYHETMREEFKNLSPYVTKVAK-------------VHGPNH- : 110
EcoliK-12.            : -----------------------------------------MAYRDQPLGELALSIPRASALFRKYDMDYCCGGKQTLARAAARKELDVEVIEAELAKLAEQ------PIEKDWRSAPLAEIIDHIIVRYHDRHREQLPELILQATKVER-------------VHADKP- : 109
EcoliEDL933           : -----------------------------------------MAYRDQPLGELALSIPRASALFRKYDMDYCCGGKQTLARAAARKELDVEVIEAELAKLAEQ------PIEKDWRSAPLAEIIDHIIVRYHARHREQLPELILQATKVER-------------VHADKP- : 109
Shiboydii             : -----------------------------------------MAYRDQPLGELALSIPRASALFRKYNMDYCCGGKQTLARAAARKELDVEVIEAELAKLAEQ------PIEKDWRSAPLAEIIDHIIVRYHDRHREQLPELILQATKVER-------------VHADKP- : 109
Salenterica           : -----------------------------------------MAYRDQPLGELALSIPRASALFRQYDMDYCCGGKQTLARAAARHDVDIDIIEAQLAQLAEQ------PIEKDWRAVPLADIIDHIVVRYHDRHREQLPELILQATKVER-------------VHADKP- : 109
Saltyphimurium        : -----------------------------------------MAYRDQPLGELALSIPRASALFRQYDMDYCCGGKQTLARAAARHDVDIDIIEAQLAQLAEQ------PIEKDWRAVPLADIIDHIVVRYHDRHREQLPELILQATKVER-------------VHADKP- : 109
Shidysenteriae        : -----------------------------------------MAYRDQPLGELALSIPHASALFRKYDMDYCCGGKQTLARAAARKELDVEVIEAELAKLAEQ------PIEKDWRSAPLAEIIDHIIVRYHDRHREQLPELILQATKVER-------------VHADKP- : 109
Ercarotovora          : -----------------------------------------MAYRDQSLGELAIAIPRATKLFRELNLDFCCGGKQTLSRAAGKKDLNIDELEAQLEKLAAQ-----PSDARDWREAPLADIIAYIIPRFHDRHREQLPELILMAKKVER-------------VHHDKA- : 110
Kpneumoniae           : -----------------------------------------MAFRDQPLGELALTIPRASALFRQYDMDYCCGGKQTLARAASRKALDVAVIEAELAKLAEQ------PLSRDWRAAPLAEIIDHIIVRYHDRHREQLPELILQATKVER-------------VHADKP- : 109
Enterobacter          : -----------------------------------------MAFRDQPLGELVLSIPRASALFRKYDMDYCCGGKQTLERAALRKELDVDAIEAELAQLAEQ------PIDKDWRTVALGEIIDHIIVRYHDRHREQLPELILQATKVER-------------VHADKA- : 109
Ymollaretii           : -----------------------------------------MDYRNQSLGALAIAIPRATKLFRHYQLDFCCGGKQTLLRAANKLELDIDELEAQLSALQAE-----PPSSEDWQRQPLTNLIDFIISRYHNRHREQLPELILMAEKVER-------------VHGEKP- : 110
Ybercovieri           : -----------------------------------------MDYRNQSLGALAIAIPRATKLFRHYQLDFCCGGKQTLLRAANKLELDIDELEAQLSALQAE-----PQSAEDWQQQPLTNLIDFIISRYHNRHREQLPELILMAEKVER-------------VHGEKP- : 110
Yintermedia           : -----------------------------------------MDYRNQSLGALAIAIPRATKLFRHHQLDFCCGGKQTLLRAANKLNLDIDELEAQLNALQSE-----PQSSEDWQQQPLANIIDFIISRYHNRHREQLPELILMAEKVER-------------VHGEKP- : 110
Yfrederiksenii        : -----------------------------------------MDYRNQSLGALAIAIPRATKLFRQHQLDFCCGGKQTLLRAANKLNLDIDELEAQLSALHIE-----PQSSEDWQQRSLTDIINFIISRYHNRHREQLPELILMAEKVER-------------VHGEKP- : 110
Ypestis               : -----------------------------------------MDYRNQSLGALAIAIPRATKLFRQHQLDFCCGGKQTLLRAANKLNLDIDALEAQLSALQTE-----PHSSEDWQQQPLTNLISFIISRYHDRHREQLPELVLMAEKVER-------------VHGEKP- : 110
Ypseudotuberculosis   : -----------------------------------------MDYRNQSLGALAIAIPRATKLFRQHQLDFCCGGKQTLLRAANKLNLDIDALEAQLSALQTE-----PHSSEDWQQQPLTNLISFIISRYHDRHREQLPELVLMAEKVER-------------VHGDKP- : 110
Hinfluenzae           : -----------------------------------------MSFAQQKLSELAVSIPGATKIFREYDLDFCCGGSVLLEVAAQQKNLNLAEIEKRLTDLQQSKA---ENNDKDWTSASYAEMIDHIITRFHNRHREQLPELITLAEKVEN-------------VHGDRD- : 112
Msucciniproducens     : -----------------------------------------MSFANQKLSDIALTVPGAIQLFREYDLDYCCGGAVELAVAVQEKNLDINEINARLTELQNNPV---NAEERDWTSASFDELIDYIVPRFHDGHRSQLPELITLAEKVEQ-------------VHGDRP- : 112
Asuccinogenes         : -----------------------------------------MSFANQKLGEIAATIPGATQLFREYDLDFCCGGAEELSAAAQQKNINLAEIEAKLIKLQQNP----TTPEKDWTTANYDEFTQFIVTRFHDRHREQLPELIRLAETVER-------------VHAERD- : 111
Rsolanacearum         : -----------------------------------------MPLLDQPLGHLARNIPGATGIFHEYQLDFCCGGQHSLRDAAQAKGIDAAPIAARLQALQAEA---TPDGAVDWSAVSPSMLIDHILERFHERHREQLPELIRLARRVEH-------------VHGDRP- : 112
Rpickettii            : -----------------------------------------MALLDQPLGHLARNIPGATGIFHEYQLDFCCGGQHSLREAAQARGIDAGPIAERLEALNAA---VTPDQVVDWTTVSPSMLIDHIVERFHDRHREQLPELIRLARRVEH-------------VHGDRE- : 112
Reutropha             : -----------------------------------------MRLQDQPLGQLARRIPGATGIFHGYDLDFCCGGKQTLRDAAVAKGLDADAIESRLQTLQRD----SEPVQTDWGAVPPASLIAHILTRFHERHREQLPELIRLARRVEH-------------VHGDRP- : 111
Hearsenicoxydans      : -----------------------------------------MDILDQSLGQLACSVAGATRIFNDYQLDFCCGGKYSLRNAAQEKGIDAEEVARRLASLQGA------TDVTDWNAESSETLIVHILLRFHDRHRQQLPELIRLARKVER-------------THGNRK- : 109
Pstutzeri             : ---------------------------------------MTTDLIDHTLGHLACSVPGATRVFRQFRLDFCCGGDLSLREAAQLIDVDPQVIAGALLALEPE------DSEKDWRAAPAGELIEYILARFHERHRDQFPELIRLASRVEH-------------VHGGRP- : 111
Hachejuensis          : -----------------------------------------MTLLDRPVGWIARNLPGSTRLFREYQLDFCCGGAKKLRDALTEKELEPDVVLERLNALQE-------EPDDNWSNAGRPELIDHILTRYHARHREQLPELIHLARRVEH-------------VHGERD- : 108
Fbacterium            : ----------------------------------------MIITENKTVGETVTENIKTAHIFKKYGIDFCCGGGISINKACEKKNVDFSKLVEELQLVDKTQ------KAYDYDKWELGFLIDHIVNIHHAYVTESIPLILQYSARVAE-------------VHGHHY- : 110
Pstorquis             : ----------------------------------------MVSIKDKTVAELVSENINTAHVFKKHGIDFCCGGGISISRACKKNQVKLDVLLNDLQNLEDKG------RTYDYKKWNLHFLAQHIQNVHHSYVEDSIPLLIQYSNKVAS-------------VHSKTN- : 110
Pedobacter            : ------------------------------------METIGILKKEETIGEIVTSDFRKAQVFKSFGIDFCCGGKKTVAEVCEKKGINPDTVINALNQLNAQDS----TTENEHSKWNIAFLAEYIVNTHHEYVKENIPFMTELAEKVAR-------------VHGAEH- : 116
Grforsetii            : ----------------------------------------MENLIEKPIGQIVAEDYRTAQIFKNHKIDFCCQGNRSIQDAAEKSKIDAQLVIDEINAVQQAEQ----EGTIDFKSWPLDLLADYIEKTHHRYVEEKISVLKPYLEKLCR-------------VHGERH- : 112
Robbiformata          : ----------------------------------------MEQTLQQPIGQWVADDYRTAQVFKKHKIDFCCKGNRTVAEVALKKGLDPDNLLQELTAVLRSEG-----PREDCTSWPLDLLADYIEKIHHRYVEATLPTLKQYLDKLCK-------------VHGERH- : 111
Fljohnsoniae          : ----------------------------------------MENLKNKTIGSFVAQDYRTAAVFSKYRIDFCCKGNRTVDEVCEKQNIDADVLLQNIHEVIQSEN----NGSIDFNSWPLDLLADYIEKTHHRYVEDKTHTLLAFLDKLCK-------------VHGANY- : 112
Tvaginali1            : ----------------------------------------MESFGERTVGSIVAESFKSASTFAKFGIDFCCHGYNKLNVACKEAGVSLEDVYNELNKPAVSG-----SAHAEFSTWPLDLLVDYVLKIHHRGIRKNAPNTQRLLSKVDG-------------VHGEHH- : 111
Tvaginali2            : --------------------------------------MDKASLAQQSVGSIVANSFSTANVFAKFGIDFCCHGYVKLLDACKTAKVPLDTVYDALMENEKSQK----SNGIEFSKWPLDLLIDYVLKTHHRNIRKYGPETLKLISKVVK-------------VHGEKH- : 114
Bacfragilis           : -------------------------------------MKVMKDYKLMAVGQIVADCFDYAKVFNKYGIDFCCNGDVSLADACGKMGIDADCLLEELKQIKSEQ-----SLTLDFKSWPIDLLVDYILKFHHRNIRYQGPQILQLLDRVCE-------------AHAGKH- : 114
Pepropionicus         : ----------------------------------------MKVTSNSSVGEIVAADFRTAKVFEKHGIDFCCGGQVSLAASCTEKGIDQKIIEEELESIKNQPV----ERSQNYTAWALPFLADYIVNTHHAYLNENMEQIAAYANKIAG-------------VHGGHH- : 112
Glovleyi              : ---------------------------------------MQEITGNKTIGEIVATDFRTATVFEKHGIDFCCGGKVALAATCTEKRLDLNTITSELEAVQNEPV----ERDQNYSSWSLPFLADYIVNTHHVYLKENDEQIAAYARKIAG-------------VHGAHH- : 113
Gbemidjiensis         : --------------------------------MEKTAKIPVEATDSETVGAVVARDYRAAGVLEKYGIDFCCGGNVPLDAACRDKGVDPQAVRRELEEAAARPL----DRSQNYDAWELPFLADYIVNTHHVYLKESIPTIAAYSQKIAE-------------VHGANH- : 120
Acbacterium           : -----------------------------------------MVDTNKTVRDIAVELPYAPKVFEKLGIDFCCGGKRPLSEACHASGIAVGEVVNHLESAEKAIQEGRESAAKNWSISTVSEVLDQILSRHHVYVREESPRIQQLFAKVAS-------------KHGENH- : 115
Sousitatus            : ---------------------------------------MTTLTPERTVGQIAAALPASVRVFEKHGIDFCCGGSVPITDACRRAGVDAALLLHEIDQAGQAP----AADATDWLTAPLPALMDHILDTHHVYMKAQLPRLEAMLAKVLE-------------AHGDRHG : 114
Shputrefaciens        : ------------MSATHQSNAVLATADLSSERLDHQEASASSPWLAKKVGELVAEDFRRAHVFSQFGIDFCCGGGKSLAVACERANVEPAKVVAALNAATLTG-----CKEDQLNQLPLDQLIEYIESTHHQYVREKAPLLIEYSEKMVR-------------AHGEHY- : 139
Shbaltica             : --MSATSIPQDVQTSVHGVSSQPRHAAQLSQGSELSESAQDSHWLARKVGELVAEDFRRAHVFSQFGIDFCCGGGKPLAVACERADIDPAKVVAALNAITLTG-----SKEDELNQLPLDQLVEYIESTHHQYVREKAPLLVEYSEKMVR-------------AHGEHY- : 149
Shamazonensis         : --------------------------------------MYSQALLECSVGELVAQDYRRAHVFSRFGIDFCCGGGRPLKEACDRADADPSEVMQALLHSAATG-----AAEDQLDKLPLAELIDYIEATHHTYVREKAPLLLEYAAKMVR-------------AHGENY- : 113
Bthuringiensis        : --------------------------------------MEHTFTETSIVGEIVTQFPKASDLFKSYRIDFCCGGNKPLIDAIHERNLSATEVITELNTLYHNTKRLNESEIDWKNASYRELIDYVI-HKHHRYLNEELPQLSPYVTKVLR-------------VHGANH- : 117
Banthracis            : --------------------------------------MEHTFTETSIVGEIVTQFPKASDLFKSYRIDFCCGGNKPLIDAIHERNLSATEVITELNTLYHNTKRLNESEIDWKNASYRELIDYVI-HKHHRYLNEELPQLSPYVTKVLR-------------VHGANQ- : 117
Bcereus               : --------------------------------------MEHTFTETSIVGEIVTQFPKASNLFKSYRIDFCCGGNKPLIDAIHERNLSATEVLTELNTLYHNTKRLNESEIDWKNASYRELIDYVI-NKHHRYLNEELPQLSPYVTKVLR-------------VHGASQ- : 117
Gbthermodenitrificans : --------------------------------------MEQRFTEQSLIGDIVTQFPKAADLFKARRIDFCCGGQRPLKEAIEERGLDGEALLRELNALYAEAQ---NKPTGNWSEAPLTDLVDHIVSTHHRYLNEELPQLSPYVTKVLR-------------VHGMHH- : 115
Gbkaustophilus        : --------------------------------------MEQRFTEQSLIGDIVAEIPKAADLLKAHRIDFCCGGQRPLKEAIEELGLDGEALLRELNTLYAEAQ---NKPAGNWGEAPLADLVDHIINTHHRYLNEELPQLSPYVTKVLR-------------VHGMHH- : 115
Cbeijerinckii         : ---------------------------------------MNTFNSNQKIGEIVTKFPNAADIFKEYKIDFCCGGDRPLITAINEQGVNEAEILEKINASYEKLQNKLYTNNKNWVEAPFDELVDHIVNVHHGYLYENLPKISELTTKILR-------------VHGGNH- : 117
Cacetobutylicum       : --------------------------------------MQNVFNVNQKIGDIVVKFPRAIEILKRYKIDFCCGGDRVLEEAIKEQGLNGQEIIDEINEDYNKYIMEEVKDRDWATEPYSKLIEHVI-NTHHAYLNETLPRLSELTTKILR-------------VHGAKH- : 117
Alkmetalliredigens    : --------------------------------------MKNIFSGEDKVGEIVAKCPKASEVFQKYQIDFCCGGDRSLRVVLQEQNLNEERVLSALTEAHNKVMSLQDKGIDWQMAPFSDLIDHVI-STHHTYLVRELPKLSDLTTKIYR-------------VHGVDHG : 118
Blicheniformis        : --------------------------------------MEAVFDQNTKTGDIVTRFPRASRLLKEYRIDFCCGGNRPIGEAIKEQDLNEEEILARINTLYQETKALNAKETNWSEAPYSQLIDHVI-HTHHAYLYEVLPELSGFVTKVYR-------------VHGIHH- : 117
Cperfringens          : --------------------------------------MEKLIRKDYSLGEVVTVYPAVVKKFNDMELDYCCGGNKSLELALKEKGVDVDKFVEDLNKEFKEFK-FENSQYVDWREKSSEELISHIVETHHGETFRLLKEIDPLMVKVFR-------------VHFSHDP : 118
Ctetani               : --------------------------------------MKNIININQKLGEVVSIFPGSSRIFNDAKIDYCCGGHDTLGEALKGKGMNLDEFIQKLNEEYEKFI-SSNEEHIDWRKEKPVVLMRHIVDTHHDYTKKELKEIDGLLSKILK-------------VHFEHHG : 118
Ruxylanophilus        : -----------------------------------------MISAERRVDELVVERPARSRVFERFGIDYCCGGGVPLREAC-EVAGVEPEVVISELERLDFGP----EEGPAVAEMGVEEMVDHIVRVHHDYLREELPRLGAMVEKVAR-------------VHGGSH- : 110
Andehalogenans        : ---------------------------------------MPAIDRNATVAEIVTAHAVTARVFQKHRIDFCCHGNVSVAEAC-RPRALDPDAVFAELDAALPQA----DGDEDPRALSTFALVARIVDRHHAYLRRTLPFLEPLAHKVAS-------------VHGDHN- : 112
Rhsphaeroides         : -------------------------------------MSDISLTLDSEVGEVAARLPGAAGLFRRHGISFCCGGGQSLAEAA-GNRNLAPEALLSELQA-------LATAAQAEAPAETAALIEHILARYHETHRRELADLIPLAEKVEA-------------VHGDHE- : 111
Simeliloti            : MPFSCTNISVQGRGFCSEWLQRVLSQGHLWQATPDKEQIMTGIRLDNTVAAIAAELPGAAELFRGHDISFCCGGNVQLSEAAVKAGVAPSALLAELQA--------LVVAARRDAPAETSDLIGHILDRYHQTHRAELAWLIPLAQKVER-------------VHGDHP- : 148
Padenitrificans       : ---------------------------------MEEINMTDITLAPDSIVGDIARLPGAADVFRRAGISFCCGGNATLADAAAKAGMDLPALTAELQS--------LIDRAGRDAPAETPALIDHILTRYHDTHREELGWLIGLAERVEM-------------VHGDHD- : 116
Caulobacter           : ----------------------------------------------MRLGARVPRVRGGGTPMTEHVRLAVLDRATVPQDAAVDFALDPADTRAIEGY------LVLNGPDDPRAAQQATALIDHILTRYHQVHMEDFPQAIALARKVEA-------------VHAADA- : 104
Acicryptum            : -------------------------------MSGQIETPADAGLLNAPVGQIAASRAGAASVFQTFGIDFCCQGKRTLAEALAGQGIAPSPVLAALM-------SAAAEPLDDWRDAPTPALIDHIKTRYHAVHLAELPNLRDLAAKVEA-------------THRKHP- : 118
Vshilonii             : -----------------------------------------MNINNTSLCDIIKNVPGSINVLKSYNINVYINLRKTLNEVLVIDDIFKYSNMIDELNELSRDN----KELLKFCDIPHQELIKYIIANFHIKHRDQLNRAIELAKRVET-------------VHYDHP- : 111
Sacdegradans          : ------------------------------------------------MDILHSTLSEFLAIAPSAKQYLTSLGIEMQDDTQLLSALQKVNKQDCIPRCEFLYR--RAMAERDWSAISNKDLVRYIIRNYHDVHRQQIATLIDLARKVEA-------------IHFGEP- : 106
Altmacleodii          : ------------------------------------------MLLTQPLGQLATRIAGATAVFHQFKLDFCCGGQQSLGEAIAKRGLNQQEVLQALENLQHQK-----DPAINWHAEPAPKLIDYILVNFHQKHRQQLPELIRLAKRVET-------------VHGDSP- : 109
Ngonorrhoeae          : -----------------------------------------------------------------------------------------------------------MTDFSVWEAAPFGATVDHILQRYHNVHRAQFEELVPLAQKVAQ-------------VHADTF- :  49
Nmeningitidis         : -----------------------------------------------------------------------------------------------------------MTDFSVWETAPFGATVDHILQRYHNVHRAQFEELVPLAQKVAQ-------------VHADTF- :  49
Apleuropneumoniae     : ---------------------------------------------------------------------------------------------------------MSVLTDKDWRTAPLGEIIDFIIPRFHDTHRNQLPTLIELAEKVES-------------VHADSA- :  51
Dshibae               : -------------------------------------------------------------------------------------------------------------MTDQPPLHDPAELTRYIETRYHARHRDQLPLLAELSAMVEA-------------VHIDDP- :  47
Roseovarius           : -------------------------------------------------------------------------------------------------------------MTEAAPIHDTAALTRYIEVRYHARHRAQLPALVKLAEMVED-------------LHCDDT- :  47
Hyneptunium           : -------------------------------------------------------------------------------------------------------------MTDAAPIQNASDLTRYIETRYHQTHREQLPVLAELAAKVER-------------VHAGHE- :  47
Alehrlichei           : -----------------------------------------------------------------------------------------------------MTPSTQCPTDSSELPEATGALIDYILTTFHDAHRRELPPLIELARKVEA-------------VHAGHP- :  55
Idloihiensis          : ---------------------------------------------------------------------------------------------------MSTFDDRLQTAEQQWQDASPKHLIEHIYLRFHQRHREQLPELKQLAMRVEA-------------VHGDHP- :  57
Strcoelicor           : --------------------------------------------------------------------------------------------------------------------MGHGGNVIDELTTDHREVEELFGKIEALPPGHKDRKLYADQATIELVRHSVAEE :  54
Mysmegmatis           : ------------------------------------------------------------------------------------------------------------------MAGSRGNIIDDI-IADHREFESVFVEIESSDD-PRTQPELVEHVISGIVRHAVAEE :  54
Corefficiens          : ---------------------------------------------------------------------------------MAASCDHGYTRGPQEKSPGNISTAERIPTMAHADQPASSTDVTAILTADHKDMRDLLAQVHLTTD-PEQRRNLVDTVTAEVMRHSVAEE :  88
Coprinosis1           : ----------------------------------------------------------------------------------------------------------------MTSNPSAQHTLLQIITSDHRDIEAFYNEYLKNAGNKDGQTRWANQLTWTIARHSISEE :  58
Coprinosis2           : ----------------------------------------------------------------------------------------MMMQTRLLRRTVLPTVRYPLARTLATTAPGHVRLLQAI-TDDHREIEAFYDQYLKNGGNTDAQQRWANQLTWEIARHSISEE :  81
Coprinosis3           : -------------------------------------------------------------------------------------------------------------------MASSSNPLSAAVAGDHQEMYEYYDQYLKNRGDKAAQQRWVNQLIWEIARHAVGEE :  55
Phaeosphaeria         : ------------------------------------------------------------------------------------MFSARTFITPVARAAFRSQAVHAPVNSRLASIRAASTISEAI-TKDHRELKEYYNEIVNNPDNIDHQTRYGNQFTWELARHSVAEE :  85
Magnaporthe           : -----------------------------------------------------------------------MLLSRSVALASAQLRAVARPQALLLNTSRPLFGQRQFASASASAGSGFVKISDAI-KKDHQELEQYYNEVINSTDQEHQQKFGNQ-FTWELARHSVAEE :  97
Ajellomyces           : ---------------------------------------------------------------------------------------------MPELVISCIVLTLQFIYLSHTTPVAMVSISDSV-TKDHKELEAFHNQIVNADNDIIKTRFQNQ-FVWELARHSAAEE :  75
Neurosporacrassa      : ------------------------------------------------------------------------MYRLATHTGLRQVPRSIANSISRPALATPRFIQPVAIRTMMDSPYSAVKVSDRV-KHDHAELEKQYRNILAAEDEDTKVRWQNQ-FIWELARHSIAEE :  96
Chaetomium            : -----------------------------------------------------------------------------------------------------------------MAASAATRVSERI-KHDHDELREYYNNIKNAQRDDDKVKWQNQ-FVWELARHSVAEE :  55
Schizosaccharomyces   : ------------------------------------------------------------------------------------------------MLQYSKKKVSLNFFPVRLLSYKMTRISDAI-FKDHRKLQSDYQNIKSANDYDTATRWQNQ-FVWELARHSVGEE :  72
Aspfumigatus1         : ---------------------------------------------------------------------------------MAVPLRLLRPIPLSVPIFRFQFLPIAARSVSTSPIQMIGRITDTV-KQDHREIEACYQRIINAKDRDEQTRYQNL-FTWELARHSIGEE :  87
Neosartorya1          : ---------------------------------------------------------------------------------MAVPVRLLRPIPLSIPISRFQSFPIAARSVSTSPIQMIGRITDTV-KQDHREIEACYQRIINAKDRDEQTRYQNL-FTWELARHSIGEE :  87
Aspclavatus1          : -------------------------------------------------------------------------------------MASIRVLRPIHTHLHPYSYSLPIARAVSTTPIMTGRITDTV-KQDHRQLEACYDKIMHATDRDDQIRFQNL-FTWELARHSIGEE :  83
Neosartorya3          : ----------------------------------------------------------------------------------------------------------------------MARLMDAI-KKDHRELEEYYKIIVSSRDTDEQIRFQNK-FTWELARHAIGEE :  50
Aspclavatus2          : ---------------------------------------------------------------------------------------------------------------------MSTRISDVI-KRDHRELEAYYNTITSSNDPDEQTRFQNK-FSWELARHAVGEE :  51
Aspfumigatus3         : -------------------------------------------------------------------------------MAATHGRKSTADSNVSEPHVLRGNVSHQVQAFTDWSQARRFRILDTI-KHDHSEIKSFYELIVSSPGPEEQTKYQNQ-FTWELARHTVGEE :  89
Neosartorya4          : -------------------------------------------------------------------------------MAATHGRKSTADSNVSEPHVLRGNISHQVQAFTDWSQARRFRILDTI-KHDHREIKSFYELIVSSTDPEEQTKYQNQ-FTWELARHAVGEE :  89
Aspclavatus3          : -------------------------------------------------------------------------------MAATHGRKPTTDDSIFEPHILRGNLTDQVQAFSDWAAAPKFRILDTI-KHDHREITSFYELLLKATEPDDATKCQNQ-FVWELARHAVAEE :  89
Cryptococcus          : --------------------------------------------------------------------------------MLSSRAFSPRTIFNRAALSPLAISTRFFASPTTSSMAGNTSVADAV-KHDHRELEEYYDQIIKATDADTKTRYQNL-FTWELARHSVGEE :  88
Aspfumigatus2         : --------------------------------------------------------------------------------MFPRCFASRATLTSRPSTLTPFPSTLIQNRTFAITSPTMTRLADAI-KDDHRELEEAYNKILSAKTSDEKTRWQNQ-FTWELARHSIGEE :  88
Neosartorya2          : --------------------------------------------------------------------------------MFPRCLASRTTLTLRPSTVTPFTSKLIQNRTFAITSPTMTRLADAI-KDDHRELEEAYNKILSAKTSDEKTRWQNQ-FTWELARHSIGEE :  88
Asporyzae             : ----------------------------------------------------------------------------------MYSSRYLFKPFRAPTHYSLARTTLGASRGFRTTAPAATRVSEVI-KNDHRELEDQYNRILSAKTKDEKEQWQNQ-FTWELARHSIGEE :  86
Aspniger2             : ----------------------------------------------------------------------------------MSFALCARRSFRGPEASLFSKAVRTPKRDFRITAPAAIRVSELI-KNDHQELRGAYHQILSAQTNDDRVRWQNQ-FTWELARHSIGEE :  86
Aspniger1             : ------------------------------------------------------------------------------------MTSRYILNSSRPILRPLLSIPKQHRSISQSPIIMAPRISEAI-KTDHREIEDYYNKILNSATEKEKIEWQNQ-FTWELARHSIAEE :  84
Aspterreus            : ---------------------------------------------------------------------------------------------------------------------MATRIIDTI-KTDHREIEDYYNKVLGSATDKEKVEWQNQ-FTWELARHSIAEE :  51
Aspnidulans           : ---------------------------------------------------------------------------------MSIRFLNPTRPLRQLSITAPRYITPLQQRFISQSPIAMTRIIDAI-KQDHREIEDYYNKILSATTEKEKIQWQNQ-FTWELARHSIGEE :  87
Sclerotinia           : ----------------------------------------------------------------------------------------------------------------------MTTITDAI-LKDHQEIREYADKVRQATDDDTRGRWQNQ-FTWELARHSIGEE :  50
```



```
Saureus               : PYLVELKETYDTFK------NGMLEHMQ-----KEDDVDF----PKLIKYEQG---------EVVDDINTVIDDLVSDHIATGELLVKMSELTSSYEPPIEACGTWRLVYQRLKALEVLTHEHVHLENHVLFKKVS---------------------------------- : 224
Sepidermidis          : PYLLKLQDLYREFR------DSMLDHIR-----KEDEEDF----PKLIQYSQG---------QDVQNIKIILEDLINDHEDTGQLLNVMNQLTSDYQTPEEACGTWKLVYQRLQNIERQTHQHVHLENHVLFKKVS---------------------------------- : 224
Shaemolyticus         : PFLIQLQDLYRQYR------DGMLEHMA-----QEDEHDF----PALIKLSRG---------EQVDHSSDIIQSLVDDHTQTGQLLEDMRELTSQYQPPSEACQTWRLVYHRLMNLERETHEHVHLENHVLFNKFS---------------------------------- : 222
EcoliK-12.            : SVPKGLTKYLTMLH------EELSSHMM-----KEEQILF----PMIKQGMGS-------------QAMGPISVMESEHDEAGELLEVIKHTTNNVTPPPEACTTWKAMYNGINELIDDLMDHISLENNVLFPRALAGE------------------------------- : 220
EcoliEDL933           : SVPKGLTKYLTMLH------EELSSHMM-----KEEQILF----PMIKQGMGS-------------QAMGPISVMESEHDEAGELLEVIKHNTNNVTPPPEACTTWKAMYNGINELIDDLMDHISLENNVLFPRALAGE------------------------------- : 220
Shiboydii             : SVPKGLTKYLTMLH------EELSSHMM-----KEEQILF----PMIKQGMGS-------------QAMGPISVMESEHDEAGELLEVIKHTTNNVTPPPEACTTWKAMYNGINELIDDLMDHISLENNVLFPRALAGE------------------------------- : 220
Salenterica           : NVPRGLTKYLTALH------EELSSHMM-----KEEQILF----PMIKQGMGR-------------QATGPISVMESEHDEAGELVDVIKHVTQNVTPPPEACTTWKAMYNGINEMIDALMEHISLENNVLFPRALAGE------------------------------- : 220
Saltyphimurium        : NVPRGLTKYLTALH------EELSSHMM-----KEEQILF----PMIKQGMGR-------------QATGPISVMESEHDEAGELVDVIKHVTKNVTPPPEACTTWKAMYNGINEMIDDLMEHISLENNVLFPRALAGE------------------------------- : 220
Shidysenteriae        : SVPKGLTKYLTMLH------EELSSHMM-----KEEQILF----PMIKQGMGS-------------QAMGPISVMESEHDEAGELLEVIKHTTNNVTPPPEACTTWKAMYNGINELIDDLMDHISLENNVLFPHALAGE------------------------------- : 220
Ercarotovora          : DCPHGLANQLTAIY------NELSQHMM-----KEERILF----PMIGQGMGA-------------NAAAPISVMEHEHDDAGRDVEVVKELTNGVVPPEGACNTWRALYSGINEFITDLMEHIHLENNLLFPRALRGE------------------------------- : 221
Kpneumoniae           : NVPKGLTKYLTMLH------QELSSHMM-----KEEQILF----PMIKQGMGA-------------QAGGPISVMESEHDEAGELLEVIKHITHNVTPPPEACTTWKAMYNGINEMIDDLMEHISLENNVLFPRALGGK------------------------------- : 220
Enterobacter          : SVPRGLAKYLTMLH------EELFSHMM-----KEEQILF----PMIKQGMGS-------------QAMGPISVMESEHDDAGELLEVIKHTTDNVTPPADACTTWKAMYNGINELIDDLMEHISLENNVLFPRALAGE------------------------------- : 220
Ymollaretii           : ACPRGLAAELTAIL------EELTQHMY-----KEEQILF----PMIQRGMGS-------------QAGGPIFVMEAEHDAVGQQLEVVKQLTQNMTPPEGACNTWRALYTGINEFMTDLMEHIHLENNLLFPRALKGE------------------------------- : 221
Ybercovieri           : ACPRGLAAELTAIL------EELTQHMY-----KEEQILF----PMIQRGMGS-------------QAGGPIFVMEAEHDAVGLQLEVVKQLTQNVTPPDGACNTWRALYTGINEFMTDLMEHIHLENNLLFPRALKGE------------------------------- : 221
Yintermedia           : ACPRGLAVELTAIL------EELTQHMY-----KEEQILF----PMITRGMGS-------------QASGPIFVMEAEHDAVGQQLEVVKQLTQNVTPPEGACNTWRALYTGINEFITDLMEHIHLENNLLFPRALKGE------------------------------- : 221
Yfrederiksenii        : ACPRGLAAELTAIL------EELTQHMY-----KEEQILF----PMIKRGMGS-------------QASGPIFVMEAEHDAVGQQLEVVKQLTQNMTPPEGACNTWQALYNGINEFITDLMEHIHLENNLLFPRALKGE------------------------------- : 221
Ypestis               : TCPRGLAAELSAIL------EELTQHMY-----KEEQILF----PMIQRGMGS-------------QASGPIFVMEAEHDAVGQQLDVVKQLTQNVTPPEGACNTWRALYTGINEFITDLMEHIHLENNLLFPRALRGE------------------------------- : 221
Ypseudotuberculosis   : TCPRGLAAELSAIL------EELTQHMY-----KEEQILF----PMIQRGMGS-------------QASGPIFVMEAEHDAVGQQLDVVKQLTQNVTPPEGACNTWRALYTGINEFITDLMEHIHLENNLLFPRALRGE------------------------------- : 221
Hinfluenzae           : DCPIGVTAQLEKIY------AELSQHLM-----KEEQILF----PMIKMGNYA-------------MASMPIRVMEMEHDEAGQDVEVIKSLTNNCTPPADACFSWKALYSGINEFIDDLMHHIHLENNILFPRVLNEK------------------------------- : 223
Msucciniproducens     : DCPTGVAAELRNML------TDLTQHMM-----KEEQILF----PMIKAGNYM-------------MARMPIQVMEMEHAEMGDQLEVLKSLTDNLTPPADACTSWLALYSGIEHFIDELMLHTHTENNILFPRVRNAA------------------------------- : 223
Asuccinogenes         : DCPAGLTTELQTLY------DHLSQHFI-----KEEKVLF----PMIRAGHYA-------------MAVMPIRVMEMEHAEAGEQLETLQSLTNNVTVPADACATWRALYAGISTFIDDLMEHTHLENNILFPRVRAEA------------------------------- : 222
Rsolanacearum         : ECPVGLSELLEAMW------QELESHMQ-----KEEQILF----PMLARGHGL-------------RAGGPIAVMRMEHDQHGEALQRLMTLTNDITPPRAACTTWRALYLGLSVFREDLMEHIHLENNILFEGAVAAD------------------------------- : 223
Rpickettii            : DCPVGLADLLDGMQ------QELESHMQ-----KEEQILF----PMLSRGHGS-------------RAGGPIAVMRMEHDQHGDALQQLMTLTNDITLPRGACTTWRALYLGLRAFREDLMEHIHLENNVLFEGAAGAG------------------------------- : 223
Reutropha             : DCPVGLADHLVAMR------SELEAHMQ-----KEEQILF----PMLARGLQA-------------AATAPITVMRMEHDDHGEALQRLAALTNDITLPRAACNTWRALYLGLRTLREDLMEHIHLENNILFEPAAVVAAND---------------------------- : 225
Hearsenicoxydans      : DCPVGLTDHLSEMQ------QELESHMK-----KEEQILF----PLLLCGQAA-------------LVGGPITIMRMEHEQHGDALQHLLALTNDINLPSAACNTWRALYFALRVFREDLMEHIHLENNILFENAALMRSAHA--------------------------- : 224
Pstutzeri             : ECPNGLAEHLWNMQ------QELESHML-----KEEQILF----PMLQRGMRF-P-----------QAQGPISVMRYEHQEHGNALEQLAALTDDITPPANACNTWRALYRGLEEMRSDLMQHIHLENNVLFRNAEASMPVQSAQVVEPVVEIRP--------------- : 239
Hachejuensis          : ECPNGLAQHLTEMF------QELESHMM-----KEENILF----PILLAGMSG-M-----------QAQGPISVMRMEHDHHGEALAQLEALTNNITPPKGACNTWRALYAGLAQLRDDLMQHIHLENNILFTDVVEDAHG----------------------------- : 222
Fbacterium            : KEVIEINTLFGEVS------IELKAHLK-----DEETILF----PYVKQMVKAEKEETRIGLADFGSVSNPIKTMFEEHEGAGDAFKKIAQLTNNYTPPQDACNTFRALYAKLEEFEQDLHQHIHLENNILFPKAIQLEKSL---------------------------- : 237
Pstorquis             : PELIQIKKLFAEVA------NELSQHLR-----KEELILF----PFIAKMEAAFKKGEKVERPHFGTIENPIAMMEDEHEAAGDKFKEIADLTNNYTLPPHACNTYKALYHKLEEFENDLHLHIHLENNILFPKALAMEKETLS-------------------------- : 239
Pedobacter            : PELVKVAELFGIVA------NELISHLM-----KEERMLF----PFIKELHAAQINGTQIPPTVFGDVSNPIQMMESEHEQAGDILRSIREITNNFALPQEACNSYTILFKKLEEFENDLHRHVHLENNILFPKTLAVKKELESA------------------------- : 246
Grforsetii            : PELFEITEHFNKSA------GELASHMK-----KEEIILF----PAVRKMVQAKQTGIKLEKPHFGTVENPIQMMMMEHETEGERFRLIEKLSNNYTPPQDACNTYRVTFALLQEFENDLHRHIHLENNILFPKSAELEKELNGQV------------------------ : 243
Robbiformata          : PELHEIREHLYASA------GELAMHMK-----KEELVLF----PWIRKMENARQSGTSPGRPKFKTVKNPIAAMMDEHDNEGERFRKIAALSDDYQAPSDACATYRVAYALLQEFEADLHRHIHLENNILFPKAQELERVFTAPSGNRG-------------------- : 246
Fljohnsoniae          : PELFKINELFIGCA------GELSQHMK-----KEELILF----PFVKRMTKTKESDGILSQPSFGSVSNPIAMMMHEHDNEGERFREIAALTDHYTPPADACTTYKVTFAMLKEFEEDLHKHIHLENNILFPKAVLLEKDFVEVE------------------------ : 243
Tvaginali1            : PELHEVKKLFNQSI------DELTKHLA-----KEEEMLF----PYIVELFNASETGTQIAPNKYGSVQSIIDSLKTEHEAEGSRYFHLAKITNNYTCPPDGCNSFRLVYQQIHDFVDALFEHIHIENNIIFPLAIELEKKYVKKEE----------------------- : 243
Tvaginali2            : PELHEVKKNFLNSL------NDLENHLQ-----KEEQVLF----PFVYQLCEADAKGLTMGRMHCMTVDNPIRVMMEEHENEGARYFHMAKLTNNYTAPADGCNSYKLAYSQIKQFNEALFEHIHIENNLIFPQASALEKKIVFGAE----------------------- : 246
Bacfragilis           : PELYEVRELFQESW------IDLNNHLT-----KEEMVLF----PYIYDLFDAVAQHRPIPAFHCGSVSSPISVMMSEHDAEGERFRRISGLTHGYLVPGDACSSYRLLLEMLRTFEDNLHHHIHLENNIVFPKAIELQENCE--------------------------- : 242
Pepropionicus         : PELYEIASIFGKIV------TDMVPHLK-----EEEEILF----PAIKRIEAGKIAGLTKETKDLELIRLSLEKLQKEHEAIGDAVHAIRHLAKGYAIPDDVCNTFRITYQKLEEFEDDLHKHVHLENNILFLKAAQL-------------------------------- : 235
Glovleyi              : PEVIRIATIFEKIA------TDMVGHLK-----EEEEVFF----PALKRADAARTAGSTPEAKDRETIRVSLLRLHREHEEIGDAIHEIRHLANDYVIPDDVCNTFMLTYKKLSEFEDDLHKHVHLENNILFLKATQL-------------------------------- : 236
Gbemidjiensis         : PEAVEIAAIFRKVG------EDMMLHLQ-----KEEEKLF----PAIKRLAELKAKGTSADAQEVAGLKEVLADLSHEHDEVGAAVHEIRRLANDYTVPGDVCNTFMVTYEKLKEFEDDLHKHVHLENNILFPKAARV-------------------------------- : 243
Acbacterium           : PELVQAKELFDALA------GELMVHLM-----KEEQILF----PYVRRMEESQVSGEPLPPSCFGTVRNPIQMMFMEHDSAGELLKEIRKQTNDLKAPPDACISFQSLYRDLLAFEADLHQHIHLENNVLFPKVLEMEGAE---------------------------- : 242
Sousitatus            : ETLRALAAVYGPMK------AELDAHLL-----KEERILF----PMIRTGQ-----------SNCGGVQNPIRVMLFEHDSAGDALAGMRRLTAGYTPPPDACNTFRALYYELSEMERDLHRHIHLENNILFPRALAE-------------------------------- : 226
Shputrefaciens        : AEIIPFAGWVRALI------EDLMPHLM-----KEEKILF----PAIRALS-----QGEHVEGCFGHIGNPINAMQHEHEEAGAILQKLHELTNDFTPPEHACTTWRVCYATLAEFEADLHQHIHLENNILFPKSLDLAQ------------------------------ : 259
Shbaltica             : TEIIPFAGWVRALV------EDLMPHLM-----KEEKILF----PAIRGLS-----QGEQVETCFGHMGNPINAMQHEHEEVGLILQKLRELTNDFTPPEHACTTWRVCYATLAEFEADLHQHIHLENNILFPKALGLAQ------------------------------ : 269
Shamazonensis         : SEIKPLAGWIRALV------DDLMPHLL-----KEEQILF----PAIRALA-----AGEQVNGCFGHIGNPVRAMEYEHEDAGNVLKKLRELTGDFTPPPHACTTWRICYHTLAEFEADLHRHIHLENNVLFPKTLKLTQG----------------------------- : 234
Bthuringiensis        : PHLAKIHKLFHELK------MELEQHLI-----KEETEDF----PLILEFE------KNPTDENYAKLRKVVDELENEHNHAGNIIKELRKVTNDFTPPEGACGTYRLVYNRLEALESDLFEHIHLENNILFPRAITRA------------------------------- : 235
Banthracis            : PHLAKIHKLFHELK------MELEQHLI-----KEETEDF----PLILEFE------KNPTDENYAKLRKVVDELENEHNHAGNIIKELRKVTNDFTPPEGACGTYRLVYNRLEALESDLFEHIHLENNILFPRAITRA------------------------------- : 235
Bcereus               : PHLAQIHKLFHELK------MELEQHLI-----KEETEDF----PLILEFE------QNPIDENYVKLRKIVDELENEHNHAGNIIKELRKVTNDFTPPEGACGTYRLVYQRLEALESDLFEHIHLENNILFPRAITRA------------------------------- : 235
Gbthermodenitrificans : PHLSRVHKLFHELK------TELEQHLI-----KEETGAF----PLILQFA------DNPSPENREAVERAVRELVNEHDAAGDLIKEIREITNDFTPPEDACGTYRLVYSRLAALEEDLFTHIHLENNVLFPRVLAAVKA----------------------------- : 235
Gbkaustophilus        : PHLSHVHKLFHELK------TELEQHLI-----KEETGAF----PLILQFA------DNPSPENREAVERAVRELVNEHDAAGDLMKEIREITNDFTPPEDACGTYRLVYSRLAALEDDLFTHIHLENNILFPRVLAAVKN----------------------------- : 235
Cbeijerinckii         : SELSKVHKLFHTVK------MELEAHLI-----EEETIQY----PAIKEYL------RSNSEVDLDKAINIINQLQDEHTGAGDILKELRKVTNDYKAPSDGCNTYKLTYAKLEEMESDIFQHIHLENNILFPRLS-ELKK----------------------------- : 236
Cacetobutylicum       : SELAKVHKLLHSLK------MELEQHLI-----KEEEIVF----PLIQEYE------RTGNNETLSNAVEKIIELEEEHEGAGSILKELREVTKNYELPEDACNSYKAAYKLLEDVEDDTFRHIHLENNIMFPRLVSESKKHK--------------------------- : 239
Alkmetalliredigens    : KVLSKVHKLFHSLK------MELDQHLI-----KEEEILF----PLIKAYE------AQPSNELLVKAINVIDELESEHEGAGDILKELRHITDQYQVPSGGCHSYELTFKGLEMLESDLFQHIHLENNILFPRLE-ALKK----------------------------- : 237
Blicheniformis        : PELAQVHQLFHQLK------TELEHHLF-----QEEEHIF----PKVMAYE------ETRSMDRLAEAVQTIDVLEQEHEAAGSILKELRRVTNDYTLPEGACTTYTLTYLKLDELETDLFQHIHLENNILFPRLEAEANQ----------------------------- : 237
Cperfringens          : ELLMKVHSLFGKLK------CELEEHLL-----KEENILF----PLMIKYD------KAKNEKEKKEIEEDIRIIVNEHEAAGDILKELAEVTDDYKVPEWGCISFKLLYDYLHDLEKDLFIHIHKENNILFARY----------------------------------- : 232
Ctetani               : EELLKVHRLFGLLK------IELEEHLI-----KEEENLF----PLIEEYE------LTKNKNVKEEIDKFIKETENEHDEAGDILKELEKITRDFTVPEGVCTTFKLTYDKIHALEKDLFIHIYKENSVLFNMF----------------------------------- : 232
Ruxylanophilus        : PELYELCEVFAELR------GELEEHTE-----KEERVLF----PACIELA------SGRRPAALGYVRALVSSLVSEHVESGEGLRSIREITRGYRVPEDACNTYRAMLDGLAELERDTHEHVFKENSLLFPRVVAAEEALERR------------------------- : 234
Andehalogenans        : PRLFGVRDAYLELA------AALEPHLD-----HEEAVLF----PALLSPA-----------PDREILRKELEGMMSDHLQVGALLERIRALSEGFTVPEWGCRSYRVLMSELETLETDVLRHVHLENHVLMPRFAGRATAEARG------------------------- : 231
Rhsphaeroides         : EAPHGLMLLLDDMR------DEMEDHMQ-----KEEQILF----PMMRMGG-------------APMIFQPIAVMRAEHDSHGEQLRRLEHLTRGFAPPEGACRSWHALYAGVRKFADDLVAHMHLENEVLFPRFERQATA----------------------------- : 224
Simeliloti            : SAPIGLSQVLERLR------DDLESHMM-----KEEQVLF----PIMRRGG-------------SAVIAHPITQMRDEHEEEAEHLRTVEHVTHGLSLPPGACGSWTALYTGLRKFTDDLVTHMHLENAVLFPRFETQAQAAV--------------------------- : 263
Padenitrificans       : EAPLGLTEALVALR------DDLELHMS-----KEETVLF----PAILQGA-------------GAMLAGPIRVMQAEHEDTGQLLRRIEHVTHGLTLPVGACGSWTALYTGLRKLCDDVVAHIHLEEEVLFPRVLAA-------------------------------- : 226
Caulobacter           : ECPDGLADHLALMA------DELAGHQR-----KEETALF----PMMRQGG-------------GPMVRIAIARMQVEHRDVVEQLTRLAVITKDFTPPQEACRTWRALNQLCRKLDRELREHMRLEDAILFPHFAGAV------------------------------- : 215
Acicryptum            : AVPDGLAETLSRMI------AELRLHQQ-----REEVVLF----PLLAQGG-------------GEMARAAIQVMRAEHEDHGAVLAGIEAITGGRVAPQGACATWRALYLGLGKFHDDLMMHVHLENNVLFPRFEAEEEAGEGNPASGANH------------------ : 242
Vshilonii             : KCPLGLSECLEGIE------NDLCSHME-----KEEQILF----PMISQGI---------------YPDGPIFVMEKDHDDHIQALSELMTLVNYLEVHEDACQSWKKLYLLLEQFESDILTHIALENNILFKN------------------------------------ : 215
Sacdegradans          : DCPTGITKALKKIY------SDLDIHML-----KEERTVF----PHFINSPR-------------TNYDEQIKNAQHDHEDHLTAIHSIKALTKTFSPPAKASHYWLDLYEQLEMLYLDLTDHTYLEDSVLFMRS----------------------------------- : 213
Altmacleodii          : DCPHGLAAHLHDMQ------QELESHML-----KEEQILF----PMLRQGI---------------YPYGPISVMLEEHQEHGDALKKLDELSFEQQLPLGACNTWTALYLGLKELKEDLMQHILLENEILFVEPTKPGHGEQHCCGSCQ-------------------- : 229
Ngonorrhoeae          : P--AEIAGLLADMR------DELLMHMM-----KEERMLF----PMINQGVGR-------------GAAMPISVMMHEHEEHDRAIARLKELTGNFHAPEGACGSWTRLYALAKEMADDLNDHIHLENDILFARVLDS-------------------------------- : 157
Nmeningitidis         : P--AEIAELLAYMQ------NELLMHMM-----KEERMLF----PMINQGVGR-------------GAAMPIGVMMHEHEEHDRAIARLKELTDNFQPPEGACGSWTRLYALAKEMVEDLNDHIHLENDILFARVLDS-------------------------------- : 157
Apleuropneumoniae     : DCPKGLAELIRKVY------ADLVNHMM-----KEEQILF----PLIKAGRGK-------------MAAAPISVMEAEHDEAGNDVEEIQKLTNNFTLPEGACTSWRNLYQGLQEFAADLDAHVDLENNNLFPRALAGDQP----------------------------- : 164
Dshibae               : RAPAGLAAVLDRMI------GDMEVHMK-----KEELILF----PAIRNGG-------------GPGIENPIAAMRADHDNHAFELSEIRRITGGPDLPDDACGSWTRLYRGLEEFMDDLQEHIRLENDVLFPQFERETRLEA--------------------------- : 162
Roseovarius           : GVPKGLSDLLAGMV------GEMEVHMK-----KEELILF----PAIRKGG-------------GPGLEHPIAVMRADHDSHGHEIDQIRRLTHDMTTPAGACTSWATLYDGLEEFVGDLTEHIRLENDVLFPKFEPAR------------------------------- : 158
Hyneptunium           : RAPKGLRELLRQMI------GELEVHMK-----KEELILF----PAIRAGG-------------TPGLAHPIAVMRADHDDHGRAIAAIAQLTYNLELPEGACGTWTRLYAGLAEFITDLTSHIRLENEVLFPQFDQVNHTDT--------------------------- : 162
Alehrlichei           : QVPAGLADALEATR------IDLEDHML-----KEENVLF----PFMRQGM--------------SPLSAPIGRMRHEHADHEESLLRLQALAGGFEPPEDACDSWRTLYVGVRHLTGELRTHMRVENELLFPRFE---------------------------------- : 162
Idloihiensis          : EAPVGLTEHLDNLM------QELESHMT-----KEEQILF----PLLSRGV---------------YPGGPISVMESEHVQHEGELNRIGELTNNLTLPEGACGTWTALYKGLKELQDDLHEHIHLENNLLFVEKKASAPEHGKDFCCGSCQ------------------ : 179
Strcoelicor           : AYLYPAVREHVPGGGALA-DKELEDHAG-----AEQIMKDLEGCAAD--------------DAEFDRLIGKLMSEIREHVADEEGNLFPRLREACPAD----ALDELGEKVRRAKKTAPTRPHPAAPDKPPMDKLLAPGAGMVDRVRDALSGRGRSG------------- : 187
Mysmegmatis           : QYVYPAARSVLPDGDEVA-DHELEEHAE----AEEVMKAIEKTDPED---------------SRYGELVRRLIDDIRHHIEEEESGMLSKLRDACPPE----RLHELSEEFQRAKSLAPTRPHPLAPDHPPANKILGPGAGLVDRLRDILSGRNS--------------- : 185
Corefficiens          : MYVYPVMEEHLPNG------AEEVEHDK-----KEHDELVTVMKELE---------GVDAAHPDFIELVKKFEEMLAHHAKDEEEDQFPKLRAHLPRE----QLEELGEKVEQAKKLAPTRPHPSAPHSELFHKTVGAGVGMVDRLRDKLTGRAT--------------- : 219
Coprinosis1           : LVVYPQFEKHLGDKGRAMAEQDRQDHQF----VKDRLKLLEDMEVGS---------------SQYDATLKEIMTELKKHMASEETEDLPLLEQKLGFE----NSISVAKSFERTKNFVPTRSHPNAPAKPPFETLAGLLAAPIDKLKDAFSKFPSDQAVENVSQSATTTA : 205
Coprinosis2           : LVVYPLLEKVMADQGHVLAESDRDDHQF----VKDRLKKLESIEVGT---------------TAYNAILKDIMDHLKEHMRKEETEQFPQLEAKLGED----QSISTARSFERTKAFVPTRSHPNAPNKPPYETLAGLMAAPMDKLMDAFSKFPSKDERESVSNAATSRI : 228
Coprinosis3           : IVIYPLMEQHLGAKGVELADNDRRDHQF----VKENLYKLENMEVGT---------------ENYDALLKTVIDHLRVHNDSEEQTDLPMLYEKIGAE----GAQQAAASFKRTKKFAPTHPHPSAPDKPPFETVVGLMTAPIDKLRDMFEQFPSTEEKKQASQN----- : 197
Phaeosphaeria         : LLVYPALEKYLGDK-----GKEHADHDR-----AEHHKVKVLLKEFQNM----SAKD-----TGYVPKLKEIWSLLEHHIEDEESKDLPALEKALSSSDNRGSSESLAKNFGRTKAFVPSRSHPSAGENPYFEGPMGMLAAPIDHIADIFRKFPDGTVSPNPSKK----- : 231
Magnaporthe           : LLVYPAFETHLGEEGRQMAEDDRKQHHEVKILLKEFQNMRSNDSEYV-------------------PKLKELWAKLSLHIQEEESHDMPALEKAIELD--AEASETMARNFHRTKMFVPSRSHPSAGENPYFESAMGLMAAPMDKLADMFRKFPDAAEKEGKK------- : 239
Ajellomyces           : LLLYPALEKHLADGKERV-EKDRERHQK----TKEQLMEFQSMKPTD---------------ANFEPTVNSLMDNLSAHMRDEEGDNLPALENAISNE----DSEELSKSFKRTKMFVPTRLHPAAPNQPAFESVIGFMMAPIDKLADTFRRFP---------------- : 205
Neurosporacrassa      : IIVYPAFEKFVPNGLVMA-EKDRAEHQRVKNLLYEFEKMTPSS-PDFL------------------PTLDKLWDALSDHITEEERDDLPALEENIDVE----YSSKLASSFNTTKHFVPTHSHPSAPDKPPYETAIGLLTTPMDKLIDMFRKFPKEDKKA---------- : 232
Chaetomium            : IVVYPAFEKHIPNGMVMA-EKDRSEHQQ----VKEKLHTFQSLAPTD---------------PTFHSTIDSLWEALAQHMQEEERDDLPALEQALDDE----ASGQLARSFDRTKHFVPTRSHPSAPDRPPYETAVGMLATPLDKLRDMFRRFPEGKPGANYLKAF---- : 197
Schizosaccharomyces   : IVVYPKFEKYLGEEGKEMAEKDRHEHQL----VKEMLYKFQSMKANQ---------------SNFIPALDELMESLQKHIDEEEQHDIPFLEKHLSEE----ESLHMASSFERTKKFVPTHSHPSAPNKPPFETVAGLFAAPIDKLRDMMEKWP---------------- : 203
Aspfumigatus1         : LVVYPAFEKHLPDGISIA-EKDRREHQT----VKEKLKKFQNLDPSN---------------ADFIPTIKSLMADLAEHIKEEETNDLVKLDQALSHD----DSVSLSKSFDRTKIFVPTRSHPSAPNKPPYETAVGLLTAPIDQLADLFRKWPHEGQQSPMGHERPMGH : 233
Neosartorya1          : LVVYPAFEKHLPDGISMA-EKDRREHQT----VKEKLKKFQNLDPSN---------------ADFIPTLKSLMGDLAEHIREEETNDLVKLDQALSHD----DSVSLSKSFDRTKMFVPTRSHPSAPNRPPYETAVGLLTAPIDQLADLFRKWPHEGQESPMGHQRPMGH : 233
Aspclavatus1          : LVVYPAFEKHLPDGVSMA-DKDRREHQT----VKEKLKKFQNLDPSN---------------PEFMSTIKSLMVDLAQHIKEEETNDLVKLDQALSHG----DSVGLSKSFERTKMFVPTRSHPSAPSKPPFETAVGLLTAPIDQLADLFRKWPHEGQASR--------- : 220
Neosartorya3          : LVVYPALEKYLPGGAETA-DKDREEHQT----IKEKLQKFQDMHPSD---------------ASFMPILKSMMADLEKHIEEEETIDFVKLEDAITPK----ESESLSKSFGRTKMFVPTRSHPNTPNKPPFETVVGLLAAPIDHLADMFRKWPDDVINPNPSSE----- : 191
Aspclavatus2          : LVVYPAMEKYLRDGKETA-DKDRREHQV----VKDKLEKFQNMHATE---------------PAFLPTIKSLMTDLFQHIDEEETIDFVKLESAITPK----ESDRLARSFGRTKMFVPTRSHPSAPNKPPFETVVGLLTAPMDHLADLFRKWPEEITPNPSTG------ : 191
Aspfumigatus3         : LVIYPALEKYLDDGKELA-RKDRAEHQT----VKEKLKAFQDMKSTD---------------PRFIPTLQSLWDDLQEHIRHEETEDIQLLEDVLSEQ----ESLGLSQSLNRTKLFVPSHAHPGAPSTPPFETAIGLLTAPIDRLSDLFRKWPAT-------------- : 221
Neosartorya4          : LVVYPALEKYLDDGKELA-RKDRAEHQT----VKEKLKAFQDMKSTD---------------PRFIPTLQSLWDDLQEHIHHEETEDIQLLEDALSEQ----ESLGLSQSLNRTKLFVPSHAHPGAPSTPPFETAVGLLTAPIDRLSDLFRKWPAM-------------- : 221
Aspclavatus3          : LVVYPAMEKYLDGGKEMA-DRDRAEHQT----VKERLKLFQDLKSTD---------------PRFLPTLTSLMADLKEHMMSEEAEDIPRLDSALSEE----ESVGLSQSLNRTKIFVPSRSHPSAPSAPPFETAVGLLTAPLDHIADVFRKWPS--------------- : 220
Cryptococcus          : LVVYPALEKYVDGGKALA-DRDREEHQK----VKEMLYKFQQLSPSD---------------SEFEPTIKKLMSDLAEHIKEEETEDLVKLEQAVPSS----ESSSLAASFKRTKMFAPTRSHPSAPDKPPFETVAGLLAAPLDKLGDVFRKFPNE-------------- : 220
Aspfumigatus2         : LIVYPLMEKNLPNGKEMA-DKDRAEHQI----VKEHLYKFQSLSSED---------------AEFEPTLKSLWSNLGQHIKEEESDDLPSLEKTLDQD----DSEKHTKKFMRTKKFIPTRSHPSAPDKPPFETVAGLMAAPIDHLADLFRKFPEESKSQLPP------- : 227
Neosartorya2          : LIVYPLMEKNLPNGKEMA-DKDRAEHQI----VKEHLYKFQGLSPED---------------PEFEPTLKSLWNNLGQHIKEEESEDLPSLEKTLDQD----DSEKHTKKFMRTKKFIPTRSHPSAPDKPPFETVAGLMAAPIDHLADLFRKFPEESKSQLPP------- : 227
Asporyzae             : LVVYPRMEKVLDNGKTMA-DHDRHEHQI----VKEDLYKFQGLQPDD---------------PEFIPTLKTLWANLAQHIKEEETQDLPALEHALSDS----DSDGMARSFGRTKKFIPTRSHPAAPDKPPYETVAGLMSAPMDRLGDLLRKFPDEARS----------- : 221
Aspniger2             : LVVYPEMELKLNNGKAMA-QHDREEHQV----IKELLYTFQGLQPDD---------------TEFIPTLKSLWANLEQHIKEEESQDLPALESALGDS----ASESMARSFGRTKNFIPTRSHPAAPDQPPYETVVGLMSAPIDRLGDMLRKFPDQKA------------ : 220
Aspniger1             : LVVYPQFEKSIPDGRAMA-DKDRKEHQS----VKEQLKKFQNMKPAD---------------PEFESTIRALMKDLSEHIKEEESQDLPKLEDAVSAE----ESEKLSKSFGRTKMFVPSRSHPSAPDKPPFETAIGLMTAPIDHLADLFRKWPHTSGMPNPSTK----- : 225
Aspterreus            : LVVYPQFEKKLPDGRSLA-DKDRKEHQS----VKEQLKKFQNMKPSD---------------PEFESTIKALMQDLSEHIKEEETQDLPKLEDALTTE----ESEGLSKSFGRTKIFVPSRSHPSAPDKPPFETAIGLMTAPIDHLADLFRRWPHTSGMPNPSTK----- : 192
Aspnidulans           : LVVYPVFEKNLPDGRAMA-DKDRHEHNT----VKEKLKQFQNMKPSD---------------PNFEPTLKSLMSTLSEHIQEEESQDLPKLEDAISSE----ESEKLSKSFGRTKMFVPSRSHPSAPDKPPFETVVGLLTAPIDHISDLFRSWPHTKGMPNPSTK----- : 228
Sclerotinia           : LVVYPAFTKHLGMQGQKMADEDRAEHQT----VKEALYKFQNLKPSD---------------ADLIPTLDALMKNLNEHIKGEEADDLPALESALESH----ESESMARSFGRTKAFVPSRSHPSAPSKPPFETVVGLMTAPIDHLGDMLRKFPDKTISPNPSTK----- : 192
```

Chapter V

Escherichia coli YtfE is able to promote the formation of iron-sulphur clusters

The results presented in this chapter will be submitted for publication.

Joana M. Baptista performed the expression and purification of YtfE, IscS and IscU, the assembly assays of Fe-S clusters in IscU, and the preparation of the samples for resonance Raman measurements.

V *Escherichia coli* YtfE is able to promote the formation of iron-sulphur clusters

Summary

Escherichia coli di-iron YtfE was shown to be involved in the repair of iron-sulphur proteins, as deletion of *ytfE* generates a strain with lower activity of iron-sulphur enzymes and compromised in repairing damaged iron-sulphur centres. Additionally, YtfE promotes the recovery of iron-sulphur enzymes inactivated by oxidative and nitrosative stresses.

In the present work, using UV-visible and resonance Raman experiments, we show that YtfE promotes the assembly of iron-sulphur clusters in proteins that contain this type of centre, namely apo-ferredoxin and the scaffold protein IscU.

V.1 – Introduction

Iron-sulphur (Fe-S) clusters are among the most ancient and functionally versatile prosthetic groups in nature that underpin the action of multiple proteins involved in key metabolic pathways. In bacteria, two major systems assist the assembly of iron-sulphur clusters, namely the house-keeping Isc (*I*ron-*s*ulphur *c*luster) and the stress dedicated Suf (*Sulf*ur assimilation) systems. Both machineries require the action of pyridoxal-phosphate-dependent cysteine desulphurases (IscS, SufS), scaffold proteins (IscU, SufU) and A-type carriers (IscA, SufA) to build and transfer the nascent iron-sulphur cluster to the target apo-protein. A-type carriers IscA/SufA were also reported to bind iron and are proposed to act as iron donors for assembly of Fe-S clusters in IscU/SufU (*1*). Apart from Isc and Suf systems, *E. coli* encodes YtfE which was previously demonstrated to promote the recovery of iron-sulphur enzymes (*2*). Loss of *ytfE* generates an *E. coli* strain with a wide range of growth–defective phenotypes, like slower growth of anaerobic cultures when using fumarate or nitrite as the terminal acceptors, and lower aconitase, fumarase and 6-phosphogluconate dehydratase activity (*3*). Moreover, YtfE is also required to restore the activity of fumarase and aconitase upon exposure to oxidative and nitrosative damage (*2*).

Highly conserved homologues of the *E. coli* YtfE protein have a widespread distribution particularly among pathogens, forming the Ric (*R*epair of *i*ron *c*entres) family of proteins ((*4*) and chapter IV). The importance of Rics *in vivo* has also been proved for *Neisseria gonorrhoeae* and *Staphylococcus aureus* (chapter IV) and for survival of *Haemophilus influenza* in nitric oxide producing macrophages (*5*).

The study of *E. coli* and *S. aureus* Rics revealed that the proteins contain a di-iron centre of the histidine/carboxylate type ((*6*) and chapter IV). The UV-visible spectra of oxidized Rics exhibit a broad band at 350 nm and the EPR spectra display all the principal g-values below 2 (g=1.96, 1.92 and 1.88), characteristic of the S=½ state of mixed valence and anti-ferromagnetically coupled Fe(III)-Fe(II) state of the binuclear iron centre. The resonance Raman (RR) spectrum of the oxidized *E. coli* YtfE displays a band at 490 cm^{-1}, attributed to a symmetric Fe–O–Fe stretching mode of the μ-oxo-bridged di-iron centre, which is proposed to be bridged by one or two μ-carboxylate bridges and coordinated by six histidines, aspartate or glutamate residues (*6*).

To further understand the functional mechanism of Rics, we tested whether *E. coli* YtfE is able to participate in the *in vitro* assembly of an iron-sulphur cluster.

V.2 – Materials and Methods

Protein production

E. coli YtfE was produced in *E. coli* BL21(DE3) Gold (Stratagene) transformed with pET-YtfE (*3*). Cells were grown aerobically in a 10-L fermenter, at 30 ºC, in M9 minimal medium with 20 mM glucose (Merck), 30 $\mu g.mL^{-1}$ kanamycin (Sigma) and 100 μM $FeSO_4$ (Merck). When cells reached an OD_{600}~0.3, protein expression was induced with 200 μM isopropyl-1-thio-β-D-galactopyranoside (IPTG, Sigma) and the growth continued for 7 h. Cells were disrupted in a French Pressure cell (Thermo Electron Corporation), the protein was purified by first separating the soluble extract over a Q-Sepharose High-Performance column (GE Healthcare) using a linear gradient (0-1 M NaCl) at 2.5 mL/min, and YtfE

eluted at ~200 mM NaCl. Protein fractions were subsequently loaded onto a Superdex S-75 gel filtration column (GE Healthcare), equilibrated in 20 mM Tris-HCl plus 150 mM NaCl, pH 7.5 (buffer A).

E. coli IscS and IscU proteins, fused to an His-tag at the N- and C-terminal respectively, were produced in *E. coli* M15:pREP4 cells harbouring pQE30-$(His)_6$-IscS and pQE60-IscU-$(His)_6$, and proteins were purified as previously described (*7*). Spinach ferredoxin was acquired from Sigma.

The purity of all proteins was confirmed by SDS-PAGE, their concentration assayed by the bicinchoninic acid method (*8*) and the iron content determined by the TPTZ (2,4,6-tripyridyl-1,2,3-triazine) method (*9*).

Assembly of iron-sulphur clusters

The $[2Fe\text{-}2S]^{2+/1+}$ cluster of spinach ferredoxin (Sigma) was removed by incubation with trichloroacetic acid (TCA, 10%) on ice for 30 min, the apo-protein was then washed with TCA (1%) and resuspended in buffer A. The reconstitution of the Fe-S centre was achieved by anaerobic incubation of the apo-ferredoxin (25 μM) with IscS (2.5 μM), L-cysteine (2 mM), DTT (10 mM) and $Fe(SO_4)_2(NH_4)_2$ (500 μM) or di-iron YtfE (50 μM). After an overnight incubation, reconstituted ferredoxin was separated from the other components using a Superdex S-75 column and the amount of iron-sulphur cluster in ferredoxin was quantified by measuring the absorbance of the visible spectrum at 415 and 459 nm. The amount of iron-sulphur cluster in ferredoxin was quantified using the ratio Abs_{280}/Abs_{415} and Abs_{280}/Abs_{459}.

The cluster formation in *E. coli* IscU was monitored by UV-visible spectroscopy and performed, anaerobically, in two ways. First, *E. coli* IscU (50 μM) was mixed in buffer A, with IscS (4 μM), DTT (4 mM), holo-YtfE

(150 μM), and the reactions initiated by addition of L-cysteine (2.5 mM). Second, IscU (50 μM) was incubated in 50 mM Tris-HCl pH 7.5, with Na_2S (2.4 mM) and DTT (4 mM). The reaction was initiated by addition of holo-YtfE (150 μM). The amount of iron-sulphur cluster in IscU was determined at 456 nm (*10*), after subtracting the intensity of the same band of a control sample that contained all components, except the apo-protein.

Resonance Raman spectroscopy

For RR studies, anaerobic reactions, containing IscU (70 μM), IscS (3.5 μM), DTT (4 mM), L-cysteine (3 mM), with or without holo-YtfE (200 μM), after 150 min, were concentrated in an ultrafiltration cell (Vivaspin 500, Vivascience Sartorius) to ~2 mM IscU and introduced in a cryostat (Linkam) mounted on a microscope stage. Spectra were recorded at -190 ºC from droplets of frozen samples in backscattering geometry by using a confocal Raman microscope (Jobin Yvon, XY), equipped with 1200 l/mm grating and a liquid-nitrogen-cooled back-illuminated CCD detector. The 457-nm line from an Argon ion laser (Coherent Innova 70) was used for excitation, with the laser power at the sample set to 13 mW and accumulation time of 40 seconds.

V.3 – Results

In the present work, we analyzed whether the presence of holo-YtfE could promote the formation of Fe-S clusters in the recipient proteins such as the apo-form of spinach ferredoxin and *E. coli* IscU, monitored by means of UV-visible and resonance Raman (RR) spectroscopies.

Reconstitution of ferredoxin by YtfE

After incubation of apo-ferredoxin with di-iron YtfE, in the presence of L-cysteine and IscS, the visible spectrum exhibited bands at 415 and 459 nm (Figure V.1A) that increased in intensity over time, reaching a maximum after 75 min. These bands are typical of a $[2Fe\text{-}2S]^{2+/1+}$ containing ferredoxin (Figure V.1B). After overnight incubation, we estimated that ~70% of a single $[2Fe\text{-}2S]^{2+}$ cluster was formed in ferredoxin (Figure V.1B).

Assembly of iron-sulphur centre in IscU

The visible spectrum of the sample containing holo-YtfE, IscU, IscS and L-cysteine exhibited a band at ~456 nm (Figure V.1C), typical of the formation of $[2Fe\text{-}2S]^{2+}$ cluster in IscU (*10, 11*). The intensity of this band after subtraction from that of the control (overall reaction without the scaffold protein IscU), showed that ~100% of a single $[2Fe\text{-}2S]^{2+}$ centre was formed per IscU/homodimer. Moreover, the percentage of reconstitution of $[2Fe\text{-}2S]^{2+}$ centre in IscU varied with the concentration of YtfE used in the assay (Table V.1).

Table V.1 – Assembly of $[2Fe\text{-}2S]^{2+}$ cluster in IscU.

[YtfE] (μM)	**% $[2Fe\text{-}2S]^{2+}$ IscU***
50	40
150	100

*Percentage of cluster formed after 75 min

To infer if the cluster formation in IscU promoted by YtfE was influenced by the presence of IscS, similar experiments were performed but replacing IscS by an excess of Na_2S. The visible spectrum of IscU in these conditions differed from that obtained when using IscS and the percentage

of $[2Fe\text{-}2S]^{2+}$ centre formed reached only ~50% after 75 min (Figure V.1D). No further changes were observed even after an overnight incubation.

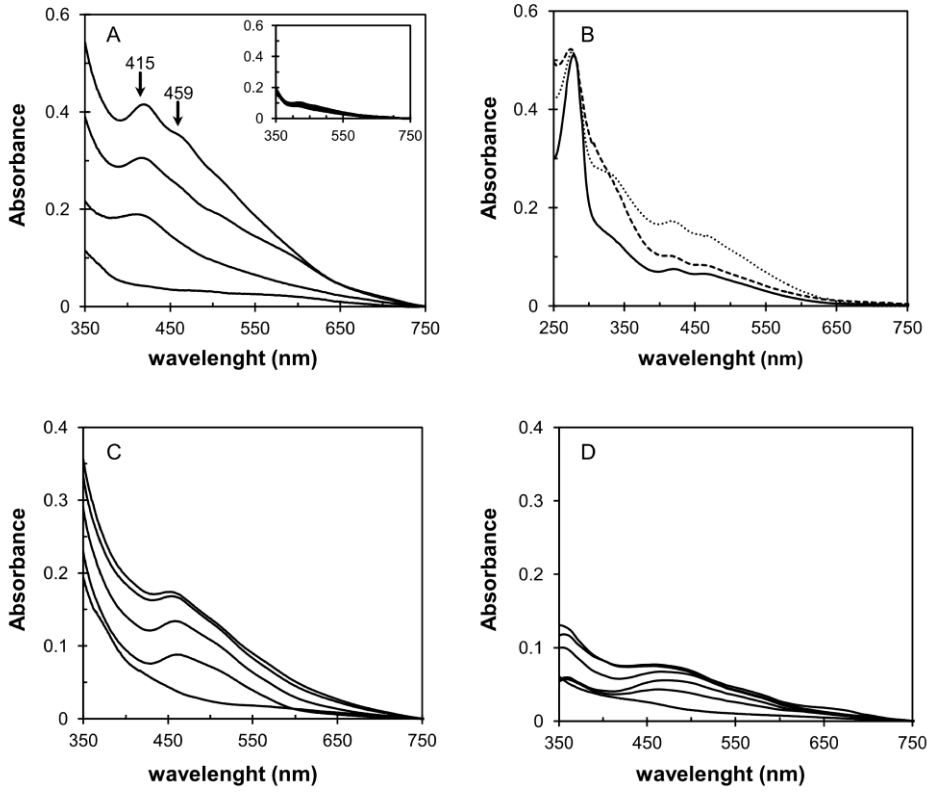


Figure V.1 – Formation of $[2Fe\text{-}2S]^{2+}$ clusters mediated by YtfE. (A, B) Visible spectra of apo-ferredoxin (25 µM) upon incubation with IscS (2.5 µM), L-Cys (2 mM) and holo-YtfE (50 µM) (A). Spectra are represented at 0, 15, 30 and 75 min (from bottom to top) after subtracting the contribution of YtfE. (B) Visible spectra of holo-ferredoxin (25 µM, dotted line), and apo-ferredoxin (25 µM) after an overnight incubation with holo-YtfE (50 µM) or $Fe(SO_4)_2(NH_4)_2$ (500 µM, black line and traced line, respectively). **(C, D)** Visible spectra of IscU (50 µM) depicting the Fe-S cluster formed upon incubation with holo-YtfE (150 µM), IscS (4 µM) and L-cysteine (2.5 mM) (C), or Na_2S (2.4 mM) (D). Spectra were subtracted from that of the control for 0, 15, 30, 60 and 75 min (C) and 0, 1, 5, 15, 60 and 75 min (D) (bottom to top).

Resonance Raman studies of Fe-S cluster assembly in IscU

The presence of Fe-S clusters in *E. coli* IscU was also analyzed by RR spectroscopy, after incubation of IscU, L-Cys, IscS, in the presence and in the absence of di-iron YtfE, and using an excitation laser line at 457 nm. Note that RR bands originating from YtfE do not contribute to the spectrum due to the lack of sufficient resonance enhancement under the experimental conditions employed (*6*). The spectrum reveals several bands around 340 cm^{-1} (Figure V.2, spectrum a), which are absent from the spectrum of the sample lacking holo-YtfE (Figure V.2, spectrum b). The bands are broad and asymmetric indicating the presence of several populations. Vibrational modes at 295, 345, 395 and 420 cm^{-1} fall into the range of frequencies characteristic of $[2Fe\text{-}2S]^{2+}$ clusters (*6, 10, 12-14*), and are attributed to terminal (t) modes B_{3u} (295 cm^{-1}) and A_g (345 cm^{-1}) and bridging (b) modes A_g (396 cm^{-1}) and B_{2u} (420 cm^{-1}) of the $Fe_2S_2{}^bS_4{}^t$ cluster (*15*). The shoulders at 336 and 356 cm^{-1} are indicative of bridging and terminal modes of $[4Fe\text{-}4S]^{2+}$ clusters, and these bands were also observed in the spectrum of IscU from *E. coli* (*12*). The

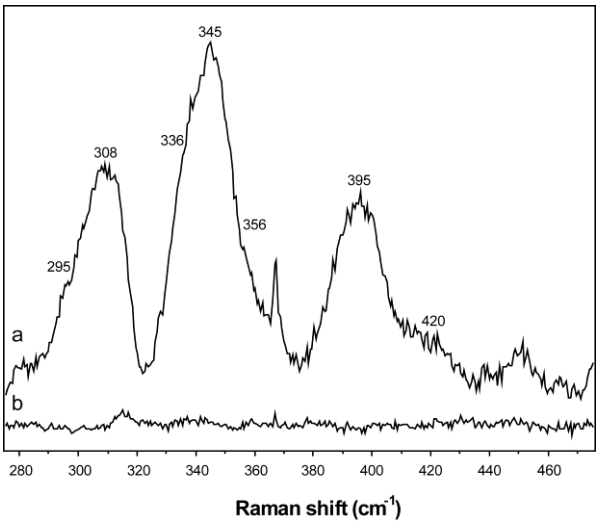


Figure V.2 – Resonance Raman spectrum of the Fe-S cluster formed in *E. coli* IscU via YtfE. RR spectra of a reaction of IscU, L-Cys, IscS and DTT in the presence **(a)** and in the absence of holo-YtfE **(b)**. Spectra were acquired at -190 ºC with 457 nm excitation, a laser power of 13 mW, and accumulation time of 40 s.

modes assigned to the $[2Fe\text{-}2S]^{2+}$ centre show slight downshifts in comparison with those previously reported (*12*), but are consistent with the energies of vibrational modes observed in other $[2Fe\text{-}2S]^{2+}$ clusters (*6, 10, 13-15*). The origin of the intense band at 308 cm^{-1} is not clear at this point; nevertheless, it might be due to contributions from non-resolved ice lattice mode (~312 cm^{-1}) and, possibly, from another adventitious iron-thiolate species (*16*).

V.4 – Discussion

Organisms have developed multicomponent systems that promote the biogenesis of Fe-S proteins while protecting the cellular surroundings from the potentially deleterious effects of free iron and sulphur (*1, 17*). The Isc and Suf systems are the mechanisms for Fe-S clusters assembly in *E. coli* encoded by the operons *iscRSUAhscBAfdx* and *sufABCDSE*, respectively. IscS and SufS are the cysteine desulphurases that provide the sulphur required for the assembly process and at the same time protect the cell from free sulphur (*1, 17*). Although, several iron-binding proteins like IscA, SufA, frataxin (CyaY) and YggX were shown to give iron for the *in vitro* maturation of Fe-S clusters, the iron donor for iron-sulphur clusters assembly was not yet identified (*11, 18, 19*).

The data here obtained reveal that the holo-YtfE promotes the formation of iron-sulphur clusters in apo-ferredoxin and IscU, which suggests that YtfE could function as an iron donor in the assembly of these clusters. The process seems to involve the di-iron centre of YtfE. This agrees with previous results showing that the cluster was required for the recovery of the aconitase and fumarase activities (*2*).

In summary, the present work helped to clarify the role of YtfE in the repair of Fe-S centres.

V.5 – References

(1) Py, B., and Barras, F. (2010) Building Fe-S proteins: bacterial strategies. *Nat Rev Microbiol 8*, 436-46.

(2) Justino, M. C., Almeida, C. C., Teixeira, M., and Saraiva, L. M. (2007) Escherichia coli di-iron YtfE protein is necessary for the repair of stress-damaged iron-sulfur clusters. *J Biol Chem 282*, 10352-9.

(3) Justino, M. C., Almeida, C. C., Goncalves, V. L., Teixeira, M., and Saraiva, L. M. (2006) Escherichia coli YtfE is a di-iron protein with an important function in assembly of iron-sulphur clusters. *FEMS Microbiol Lett 257*, 278-84.

(4) Justino, M. C., Baptista, J. M., and Saraiva, L. M. (2009) Di-iron proteins of the Ric family are involved in iron-sulfur cluster repair. *Biometals 22*, 99-108.

(5) Harrington, J. C., Wong, S. M., Rosadini, C. V., Garifulin, O., Boyartchuk, V., and Akerley, B. J. (2009) Resistance of Haemophilus influenzae to reactive nitrogen donors and gamma interferon-stimulated macrophages requires the formate-dependent nitrite reductase regulator-activated ytfE gene. *Infect Immun 77*, 1945-58.

(6) Todorovic, S., Justino, M. C., Wellenreuther, G., Hildebrandt, P., Murgida, D. H., Meyer-Klaucke, W., and Saraiva, L. M. (2008) Iron-sulfur repair YtfE protein from Escherichia coli: structural characterization of the di-iron center. *J Biol Inorg Chem 13*, 765-70.

(7) Tokumoto, U., Nomura, S., Minami, Y., Mihara, H., Kato, S., Kurihara, T., Esaki, N., Kanazawa, H., Matsubara, H., and Takahashi, Y. (2002) Network of protein-protein interactions among iron-sulfur cluster assembly proteins in Escherichia coli. *J Biochem 131*, 713-9.

(8) Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. *Anal Biochem 150*, 76-85.

(9) Fischer, D. S., and Price, D. C. (1964) A Simple Serum Iron Method Using the New Sensitive Chromogen Tripyridyl-S-Triazine. *Clin Chem 10*, 21-31.

(10) Agar, J. N., Krebs, C., Frazzon, J., Huynh, B. H., Dean, D. R., and Johnson, M. K. (2000) IscU as a scaffold for iron-sulfur cluster biosynthesis: sequential assembly of [2Fe-2S] and [4Fe-4S] clusters in IscU. *Biochemistry 39*, 7856-62.

(11) Layer, G., Ollagnier-de Choudens, S., Sanakis, Y., and Fontecave, M. (2006) Iron-sulfur cluster biosynthesis: characterization of Escherichia coli CYaY as an iron donor for the assembly of [2Fe-2S] clusters in the scaffold IscU. *J Biol Chem 281*, 16256-63.

(12) Iannuzzi, C., Adinolfi, S., Howes, B. D., Garcia-Serres, R., Clemancey, M., Latour, J. M., Smulevich, G., and Pastore, A. (2011) The role of CyaY in iron sulfur cluster assembly on the E. coli IscU scaffold protein. *PLoS One 6*, e21992.
(13) Crouse, B. R., Sellers, V. M., Finnegan, M. G., Dailey, H. A., and Johnson, M. K. (1996) Site-directed mutagenesis and spectroscopic characterization of human ferrochelatase: identification of residues coordinating the [2Fe-2S] cluster. *Biochemistry 35*, 16222-9.
(14) Fu, W., Drozdzewski, P. M., Davies, M. D., Sligar, S. G., and Johnson, M. K. (1992) Resonance Raman and magnetic circular dichroism studies of reduced [2Fe-2S] proteins. *J Biol Chem 267*, 15502-10.
(15) Fu, W. G., Morgan, T. V., Mortenson, L. E., and Johnson, M. K. (1991) Resonance Raman studies of the [4Fe-4S] to [2Fe-2S] cluster conversion in the iron protein of nitrogenase. *FEBS Lett 284*, 165-8.
(16) Czernuszewicz, R. S., Kilpatrick, L. K., Koch, S. A., and Spiro, T. G. (1994) Resonance Raman Spectroscopy of Iron(III) Tetrathiolate Complexes: Implications for the Conformation and Force Field of Rubredoxin. *J. Am. Chem. Soc. 116*, 7134–41.
(17) Ayala-Castro, C., Saini, A., and Outten, F. W. (2008) Fe-S cluster assembly pathways in bacteria. *Microbiol Mol Biol Rev 72*, 110-25, table of contents.
(18) Ding, H., and Clark, R. J. (2004) Characterization of iron binding in IscA, an ancient iron-sulphur cluster assembly protein. *Biochem J 379*, 433-40.
(19) Lu, J., Yang, J., Tan, G., and Ding, H. (2008) Complementary roles of SufA and IscA in the biogenesis of iron-sulfur clusters in Escherichia coli. *Biochem J 409*, 535-43.

V.6 – Acknowledgments

Joana M. Baptista is grateful to André Fernandes, Dr. Lígia Nobre and Dr. Marta Justino for their contribution to this work.

The author is thankful to Dr. Smilja Todorovic and Dr. Peter Hildebrandt for the resonance Raman analysis, to Prof. Yasuhiro Takahashi for plasmids pQE60-IscU-$(His)_6$ and pQE30-$(His)_6$-IscS, and to Prof. Miguel Teixeira (ITQB) for helpful discussions.

Chapter VI

Searching for YtfE interactions in the *Escherichia coli* proteome

The results presented in this chapter will be submitted for publication.

Joana M. Baptista cloned *iscS*, *sufS*, *iscU* and *ytfE* in all the BACTH plasmids and performed the BACTH assays using these plasmids. The screening of the two libraries used in this study was also carried out by Joana M. Baptista.

VI Searching for YtfE interactions in the *Escherichia coli* proteome

Summary

Escherichia coli YtfE is required for the repair of oxidatively and nitrosatively damaged Fe-S clusters, a requisite for pathogens to survive inside the host. Our previous data revealed that *E. coli* YtfE promotes the assembly of Fe-S centres in the scaffold protein IscU and in the apo-form of spinach ferredoxin.

In this work, we searched for *E. coli* YtfE interactants *in vivo* using the bacterial adenylate cyclase two-hybrid system (BACTH). To this end, we first performed the analysis of YtfE interaction with the cysteine desulphurases IscS and SufS, and with IscU. Our results show that YtfE is able to interact with IscS and SufS. In the second part, two libraries covering for part of *E. coli* genome were used to find the protein interactants of YtfE. The full sequence of the positive interactants was cloned into BACTH system plasmids to confirm the interactions with YtfE.

Overall, the BACTH system allowed identifying proteins that interact with *E. coli* YtfE.

VI.1 – Introduction

Proteins that contain iron-sulphur clusters are the most ubiquitous metalloproteins in nature, performing a wide range of biological processes that are essential to the metabolism of the cell (*1, 2*). Fe-S clusters are formed, in bacteria, by two major systems: the house-keeping Isc (*I*ron-*s*ulphur *c*luster) and the stress dedicated Suf (*Sulf*ur assimilation). The assembly of Fe-S centres involves three main proteins: the scaffold protein that receives the nascent Fe-S cluster, the cysteine desulphurase that provides the sulphur source using cysteine, and the iron donor (*3*). When Fe-S clusters are damaged by oxidative and nitrosative stress, a family of proteins named Ric (*R*epair of *i*ron *c*entres) is required for the repair of these clusters ((*4, 5*) and chapter IV). Ric proteins are widespread in nature and are present in human, animal and plant pathogens (chapter IV). The genes encoding these proteins are induced by nitrosative stress and their deletion leads to strains of *Escherichia coli* or *Staphylococcus aureus* or *Neisseria gonorrhoeae* with a wide-range of growth defective phenotypes including lower activity of Fe-S containing enzymes (*(4-6)* and chapter IV). Previous work in our laboratory showed that addition of holo-YtfE (Ric homologue in *E. coli*) is able to recover the activity of the $[4Fe\text{-}4S]^{2+/1+}$ clusters of aconitase and fumarase after damage by oxidative and nitrosative stresses (*5*). Furthermore, the assembly of Fe-S clusters in IscU and apo-ferredoxin is promoted by YtfE (chapter V).

The bacterial adenylate cyclase two hybrid system (BACTH) is used to detect protein-protein interactions *in vivo*. This method is based on interaction-mediated reconstitution of the adenylate cyclase activity. The catalytic domain of adenylate cyclase from the bacteria *Bordetella pertussis* consists of two fragments, T18 and T25 (Figure VI.1A), that are not active

when physically separated (Figure VI.1B). Here, the proteins of interest were genetically fused to the two fragments and co-expressed in an *E. coli cya* strain (*i.e.* a strain deficient in endogenous adenylate cyclase). Interaction of the two hybrid proteins results in a functional complementation between the T25 and T18 fragment (Figure VI.C), leading to cAMP synthesis that binds to the catabolite activator protein, CAP, and will consequently activate several operons, such as lactose operon and maltose regulon (Figure VI.D) (*7-9*).

The goal of the current study was to search for proteins that interact with YtfE and therefore may be involved in the repair of Fe-S clusters. For this purpose, using the BACTH system, members of Isc and Suf systems were tested for interactions with *E. coli* YtfE. Furthermore, the *E. coli* proteome was screened for YtfE interactants that were then cloned in the BACTH plasmids for confirmation purposes.

VI.2 – Materials and Methods

Bacterial strains

The non-reverting adenylate cyclase deficient *(cya) E. coli* reporter strain, DHM1 (genotype: F-, *cya-854*, *recA1, endA1, gyrA96 (Nalr)*, *thi1, hsdR17*, *spoT1*, *rfbD1, glnV44(AS)*), was used for detection of protein-protein interactions and grown in Luria-Bertani (LB) broth or on LB agar. *E. coli* XL2Blue (genotype: F′ *pro*AB *lac*IqZΔM15 *Tn*10 (Tet^{r}) *end*A1, *sup*E44, *thi*-1, *recA*1, *gyrA*96, *relA*1, *lac*) was used as the cloning host.

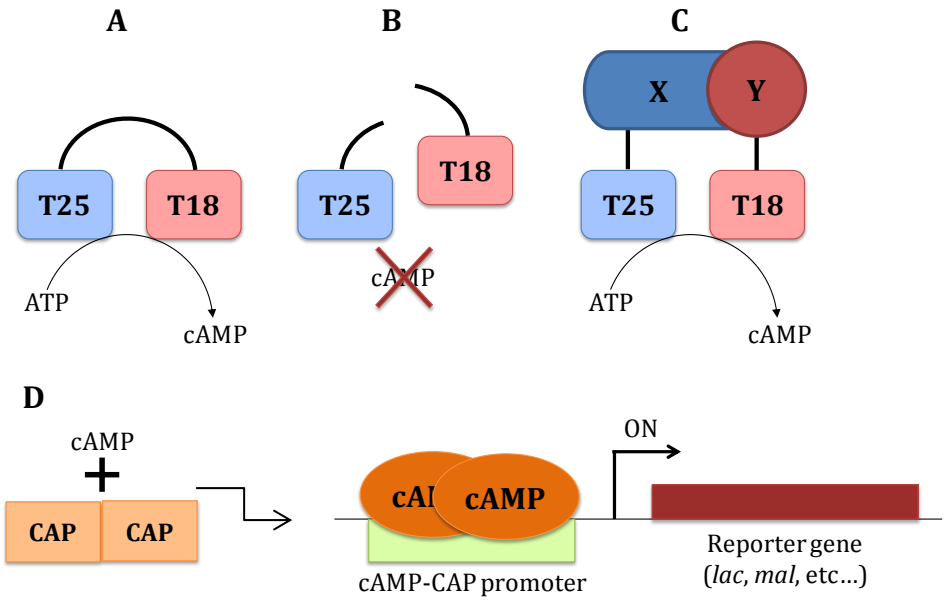


Figure VI.1 – Principle of BACTH system. The catalytic domain of adenylate cyclase (CyaA) is formed by two fragments, T25 and T18 **(A)**, that are inactive when physically separated **(B)**. When the two fragments are fused to interacting polypeptides (X and Y), the complementation between T25 and T18 fragments occurs and then cAMP is synthesized **(C)**. Cyclic AMP binds to CAP and turns on the expression of several genes, like *lac* and *mal* operons involved in lactose and maltose catabolism **(D)**. ATP – adenosine tryphosphate; AMP – adenosine monophosphate; cAMP – cyclic AMP ; CAP – catabolite activator protein. Adapted from [8].

Plasmids construction

The plasmids used in this work are shown in Table VI.1. The construction of the plasmids was carried out with the primers described in Table VI.2. Standard protocols were used for molecular cloning, PCR, DNA analysis and transformation. Briefly, *ytfE*, *iscS*, *sufS*, *iscU* and *dps* genes were individually amplified by PCR using *E. coli* K12 ATCC23716 genomic DNA as template. The amplified DNA fragments were digested with BamHI/SalI (*ytfE*) and BamHI/KpnI (*iscS*, *sufS*, *iscU* and *dps*) and subcloned into the corresponding sites of pUT18, pUT18C, pKT25 and pKNT25 to check for

possible dependence of the interactions on the N- or C-terminal location of the protein. The resulting recombinant plasmids expressed hybrid proteins in which the complete amino acid sequence of YtfE, IscS, IscU, SufS and Dps were fused to the C- or N-terminal of the T25 and T18 fragments of adenylate cyclase of *B. pertussis* (Figure VI.2). All recombinant clones were sequenced to ensure that no undesired mismatches had been introduced during the PCR amplification procedure.

Table VI.1 – List of plasmids used in this study.

Plasmids

Name	Description	Source/ Reference
pUT18/ pUT18C	Vector that allows construction of in-frame fusions at the N-terminus/C-terminus of T18 fragment (amino acids 225–399 of CyaA)	(*7*)
pKT25/ pKNT25	Vector that allows construction of in-frame fusions at the C-terminus/N-terminus of T25 fragment (amino acids 1–224 of CyaA)	(*7*)
pUT18/ pUT18C-YtfE	*ytfE* fused to T18 fragment in the N/C-terminal	This study
pKT25/ pKNT25-YtfE	*ytfE* fused to T25 fragment in the C/N-terminal	This study
pUT18/ pUT18C-IscS	*iscS* fused to T18 fragment in the N/C-terminal	This study
pKT25/ pKNT25-IscS	*iscS* fused to T25 fragment in the C/N-terminal	This study
pUT18/ pUT18C-SufS	*sufS* fused to T18 fragment in the N/C-terminal	This study
pKT25/ pKNT25-SufS	*sufS* fused to T25 fragment in the C/N-terminal	This study
pUT18/ pUT18C-IscU	*iscU* fused to T18 fragment in the N/C-terminal	This study

Cont. **Table VI.1 – List of plasmids used in this study.**		
Name	**Description**	**Source/ Reference**
pKT25/ pKNT25-IscU	*iscU* fused to T25 fragment in the C/N-terminal	This study
pUT18/ pUT18C-Dps	*dps* fused to T18 fragment in the N/C-terminal	This study
pKT25/ pKNT25-Dps	*dps* fused to T25 fragment in the C/N-terminal	This study
pUT18-Zip	Leucine zipper fused to T18 fragment in the N-terminal	(*7*)
pKT25-Zip	Leucine zipper fused to T25 fragment in the C-terminal	(*7*)
pUT18-TorD	*torD* fused to T18 fragment in the N-terminal	Simon Andrews lab
pKT25-TorD	*torD* fused to T25 fragment in the C-terminal	Simon Andrews lab
BamHI	pUT18 plasmid that contains chromosomal fragments obtained via partial digest of the MC4100 chromosomal DNA with Sau3A1 and cloned into BamHI site	Simon Andrews lab (Tracy Palmer, Dundee, UK)
BamHI+1	pUT18 plasmid with a +1 frameshift in the polylinker that contains chromosomal fragments obtained via partial digest of the MC4100 chromosomal DNA with Sau3A1 and cloned into	Simon Andrews lab (Tracy Palmer, Dundee, UK)

BACTH complementation assays

For BACTH complementation assays, DHM1 cells were co-transformed with the plasmids carrying *ytfE* in various combinations with the complementary plasmids harbouring *iscS*, *sufS*, *iscU* and *dps*. Co-transformants were selected on LB-agar plates containing 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside), 100 μg/mL ampicillin, 30 μg/mL

kanamycin and 40 μg/mL of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and incubated for 36 h at 30º C.

Table VI.2 – List of primers used in this study.

Primers

Name	Sequence (5'-3')
$YtfE_{Fw/rev}$	GAGGTGTCGACTATGGCTTATC CTTTTAGGATCCTCACCCGCC
$Dps_{Fw/Rev}$	GTTAATTACTGGGATCCAACATCAAGAGG TCCTGTCAGGTACCCGCTTTTATC
$IscS_{Fw/rev}$	GAGTGATGGATCCAGTTTATAGAG GGCTCATCAGGTACCCGGTATCG
$SufS_{Fw/Rev}$	CAGCAGGTGCGGATCCGAATCG GTTTGCTGGGGGTACCGGGAGG
$IscU_{Fw/Rev}$	GAATCAGGGGATCCTATAATGGC GAAGCAAAGGTACCGTTGAGGTTT
$T25_{Fw/Rev}$	ATGCCGCCGGTATTCCACTG CGGGCCTCTTCGCTATTACG
$NT25_{Fw/Rev}$	CACCCCAGGCTTTACACTTTATGC CAATGTGGCGTTTTTTTCCTTCG
$T18_{Fw/Rev}$	CATTAGGCACCCCAGGCTTTAC GAGCGATTTTCCACAACAAGTC
$T18C_{Fw/rev}$	CATACGGCGTGGCGGGGAAAAG AGCGGGTGTTGGCGGGTGTCG

To search the *E. coli* proteome for possible interactants of YtfE, about 1 μg of plasmid DNA library (BamHI or BamHI+1) was used to co-transform *E. coli* DHM1 electrocompetent cells harbouring pKT25-YtfE. The DNA libraries, a kind gift of Dr. Simon Andrews, were obtained by partial digestion of the *E. coli* MC4100 chromosomal DNA with Sau3A1 and cloned into the BamHI site of the pUT18 plasmid (BamHI) and pUT18 plasmid with a +1 frameshift in the polylinker (BamHI+1). Blue colonies on

selective plates appearing within 36 hours of incubation at 30º C contained pUT18 plasmids encoding potential genes for polypeptides that interact with *E. coli* YtfE. To isolate the pUT18 plasmids, the colonies were selected on LB-agar plates supplemented with ampicillin, and the plasmids isolated were reintroduced into competent DHM1 cells containing pKT25-YtfE, empty pKT25 vector or pKT25-TorD to confirm the interactions.

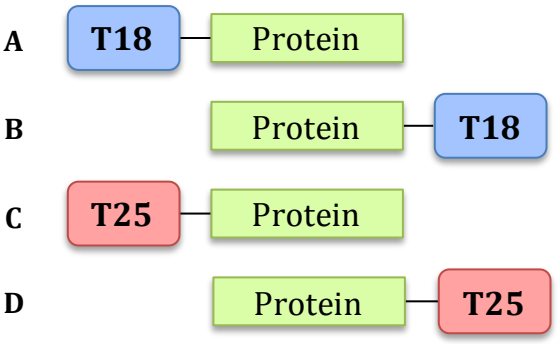


Figure VI.2 – Schematic representation of hybrid proteins obtained after using pUT18, pUT18C, pKT25 and pKNT25 plasmids for cloning. Protein – YtfE, IscS, SufS, IscU or Dps. T25 – fragment T25 from the adenylate cyclase catalytic domain from *B. pertussis*. T18 – fragment T18 from the adenylate cyclase catalytic domain from *B. pertussis*. **A** – cloning using pUT18C plasmid; **B** – cloning using plasmid pUT18; **C** – cloning using plasmid pKT25; **D** – cloning using pKNT25 plasmid.

DHM1 cells co-transformed with the plasmid containing the gene of interest (*ytfE*/*iscS*/*sufS*/*iscU*/*dps*) and the complementary empty plasmid were used as negative controls. DHM1 cells transformed with the plasmid harbouring the gene of interest and the complementary vector containing *torD* were used to test for "false positives". TorD is a protein that binds non-specifically with a wide variety of polypeptides, hence with this experience we could analyse the non-specificity of the interactions (*10*).

The efficiency of the interactions was evaluated by quantifying the β-galactosidase activities in liquid cultures.

β-galactosidase assays

For β-galactosidase assays, 3-4 representative colonies of each transformation plate were inoculated, in duplicate, in LB broth supplemented with the appropriate antibiotics. After an overnight growth at 37º C, the culture was re-inoculated 1/100 into fresh LB supplemented with ampicillin, kanamycin and IPTG. After 16 hours growth, at 30º C, 1 mL of cultures displaying an OD_{600}~0.5 were collected by centrifugation (5 min at 5000 rpm). The pellets were resuspended in 100 µl BugBuster HT 1x (Novagen) for cellular lysis and incubated at 37º C for 30 min. Cellular debris were removed by centrifugation (10 min at 12000 rpm) and 20 µl of each suspension was used in duplicate for the enzymatic reaction in a microtitre plate reader. The β-galactosidase assays were initiated upon addition of the following reaction mixture: 0.27% β-mercaptoethanol (v/v), 0.9 mg/mL ONPG (o-nitrophenyl-β-D-galactopyranoside) in Buffer A (60 mM $Na_2HPO_4.7H_2O$, 40 mM $NaH_2PO_4.H_2O$, 1 mM $MgSO_4.7H_2O$ and 10 mM KCl). The absorbance was measured at 420 nm, each 2 min and the reaction was held for 1.5 h at 28º C. The β-galactosidase specific activity (*11*) is defined in units per milligram of protein. For conversion of microplate reader Abs_{420} values into standard spectrophotometer values, a correction factor of 2.2 was determined using serial dilutions of an O-nitrophenyl (ONP) solution.

The hybrid plasmids extracted from positive clones of the libraries were sequenced using the $T18_{Fw}$ primer, and genes identified by BLAST search using the *E. coli* K12 MG1655 genome database.

VI.3 – Results

E. coli YtfE interacts with IscS and SufS

The formation of YtfE-promoted Fe-S centre in the scaffold protein IscU and in the presence of the cysteine desulphurase IscS led us to analyse via BACTH assays possible interactions of YtfE with IscU, IscS and SufS, a cysteine desulphurase expressed by *E. coli* under oxidative stress conditions ((*3*) and chapter V).

While no interaction was observed between YtfE and IscU, the formation of a complex between YtfE and IscS was inferred by the 4-6 times higher β-galactosidase activity in comparison to that of the control. The interaction seems to be independent of YtfE configuration as judged by the similar values obtained when YtfE was expressed either as N- or C-terminal part of the pUT18 fusion protein (Figure VI.3).

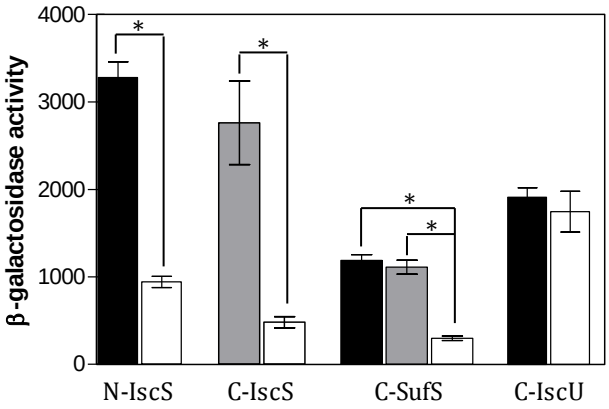


Figure VI.3 – Interaction of *E. coli* YtfE with IscS, IscU and SufS. The interaction of YtfE, cloned in the C-terminal (black bars) or in the N-terminal (grey bars) of Cya domain, was evaluated in cells co-transformed with the complementary plasmids containing *iscS*, *iscU* or *sufS* genes fused to a second Cya domain. Empty vectors co-transformed with vectors containing *iscS*, *sufS* or *IscU* genes (white bars) served as negative controls. Values are mean ± standard error of two independent cultures analyzed in duplicate. *p<0.05 (One-way ANOVA Bonferrani multiple comparison test).

The results also revealed that YtfE interacts with SufS but the formation of the complex was only observed when the N-terminal of SufS

fused to the T25 fragment was complemented with YtfE fused to the T18 fragment in either position (Figure VI.3).

Hence, YtfE interacts *in vivo* with all known sulphur-supply proteins of the iron-sulphur biogenesis systems of *E. coli*.

Identification of protein interactions with YtfE in *E. coli* proteome

To identify possible interactions of YtfE with proteins from *E. coli*, DHM1 cells containing pKT25-YtfE were co-transformed with two libraries BamHI and BamHI+1 (Figure VI.4). The size of each library is approximately 1.7 x 10^3 clones and the plates for the co-transformants of each library resulted in over 3 x 10^3 clones with ~1 blue colony out of a total of 100 colonies. In order to confirm the positive interactions and to exclude "false positives", the plasmids isolated from blue colonies were co-transformed in DHM1 cells harbouring pKT25, pKT25-YtfE or pKT25-TorD and the β-galactosidase activities were measured (Figure VI.4 and VI.5). In this second round, only nine plasmids were selected to be sequenced as they fulfilled two conditions. First, the negative control in which DHM1 cells:pKT25 were co-transformed with the selected plasmids gave β-galactosidase activity lower than the cells containing pKT25-YtfE. Second, the cells containing pKT25-TorD, the control for "false positives", had β-galactosidase activities lower when compared to the cells harbouring pKT25-YtfE. The selected plasmids satisfying these requirements were nominated from A to I and their β-galactosidase activities are represented in Figure VI.5.

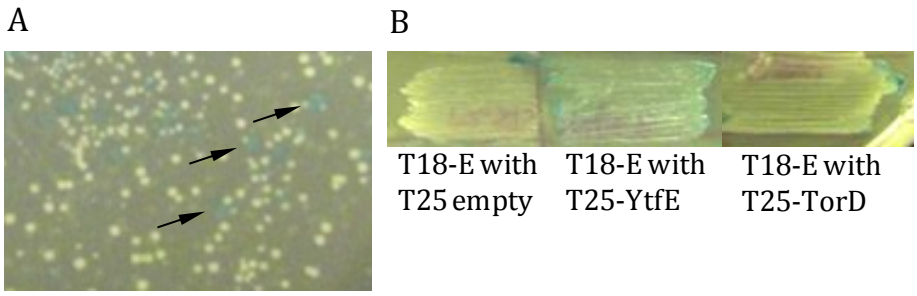


Figure VI.4 – Representative plates obtained during the BACTH assays for *E. coli* YtfE interaction with libraries BamHI and BamHI+1. In **A,** a selective plate obtained for DHM1 cells containing pKT25-YtfE co-transformed with BamHI+1 library is exemplified. Arrows indicate blue colonies (positive interaction). In **B,** the colonies of the interaction of polypeptides from plasmid E (BamHI+1 library) with pKT25 (negative control), pKT25-YtfE or pKT25-TorD (false positive control) are exhibited . All transformations are performed in LB-agar selective plates with IPTG/X-Gal as described in Materials and Methods and are represented for growth after 36 hours at 30º C.

The plasmids were sequenced using the $T18_{Fw}$ primer (Table VI.2) and the results analysed using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for *E. coli* K12 MG1655 genome (Figure VI.6). Plasmid A (BamHI library) and F (BamHI+1 library) lack fragments of *E. coli* genome. The sequencing of plasmids B to E from BamHI library revealed, upstream of the T18 domain, the same fragment of *E. coli* genome, namely *rhtA*, a gene encoding a inner membrane transporter involved in resistance to homoserine and threonine (Figure VI.6, yellow arrow). The chromosomal fragments contained in the vectors have a maximum of 2 kbp, i.e. it also contains part of the gene downstream of *rhtA* that encodes Dps (*D*NA binding *p*rotein from *s*tarved cells) (Figure VI.6).

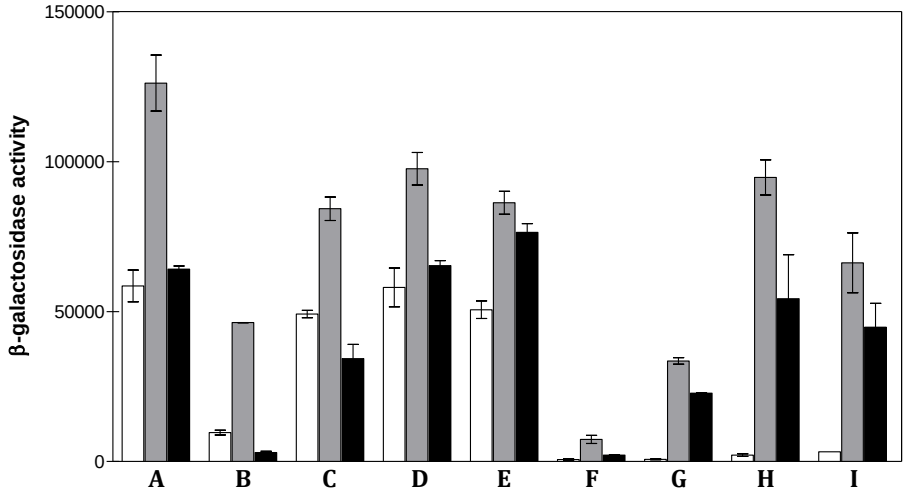


Figure VI.5 – BACTH analysis of interactions with *E. coli* YtfE. The efficiency of functional complementation between the indicated hybrid polypeptides was quantified by β-galactosidase activities in *E. coli* DHM1 cell lysates harbouring the complementing plasmids, as described in Materials and Methods. A to I designates the plasmids extracted from libraries BamHI (A to E) and BamHI+1 (F To I) that were co-transformed with T25 empty plasmid (white bars), T25 fragment associated with YtfE (grey bars) or T25 domain linked to TorD (black bars). Each bar represents the mean value ± standard error from results of at least three independent cultures.

Dps is an iron storage protein formed by 12 subunits capable of holding ~500 iron atoms (*12, 13*). In *E. coli*, Dps has a protective role against oxidative stress as its ferroxidase centre removes Fe^{2+} and H_2O_2 from solution preventing the occurrence of the Fenton reaction (*12, 13*).

The sequencing results of plasmids G to I from BamHI+1 library exhibited, upstream of the T18 fragment, the full sequence of *efp* and *ecnA* and part of *ecnB* (Figure VI.6, yellow arrow). All sequenced fragments are in-frame with the ORF encoding the T18 fragment.

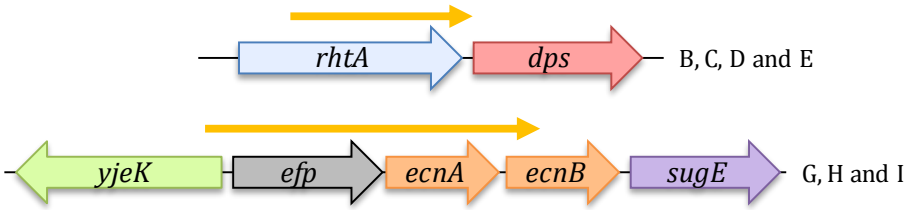


Figure VI.6 – Schematic representation of the results obtained after sequencing the selected plasmids extracted from BamHI and BamHI+1 libraries. The results were acquired by blasting the sequencing results using BLAST against *E. coli* MG1655 genome. Yellow arrow represents the part of the plasmid sequenced which matches the *E. coli* genome. Plasmids B, C, D and E, extracted from BamHI library, and plasmids G, H and I, from BamHI+1 library, contained the same fragment of *E. coli* genome. Genes: *rhtA* – *r*esistance to *h*omoserine and *t*hreonine; *dps* – *D*NA-binding *p*rotein from *s*tarved cells ; *yjeK* – predicted lysine 2,3-aminomutase; *efp* – *e*longation *f*actor-*p*eptidyltransferase; *ecnA* – *e*nterici*d*in *A* ; *ecnB* – *e*nterici*d*in *B* ; sugE – *su*pressor of *g*ro*E*.

The *efp* gene encodes the elongation factor EF-P, a translation factor that facilitates *in vitro* the formation of the first peptide bond (Figure VI.6) (*14, 15*). The *ecnA* gene belongs to the gene cluster *ecnAB* that express two small cell-membrane associated lipoproteins (Figure VI.6). EcnAB form a toxin-antitoxin module which regulates a programmed bacterial cell death under high osmolarity conditions. The entericidin A (EcnA) functions as an antidote to the bacteriolytic entericidin B (EcnB) (*16*).

At first glance, Dps is a protein that may be involved with YtfE in the repair of Fe-S clusters due to its role in iron storage and cellular protection under oxidative stress conditions (*12*). Hence, the direct interaction of YtfE with Dps was tested.

E. coli YtfE interacts with the iron storage protein Dps

The formation of a complex between the two proteins was inferred by the 4-5 times higher β-galactosidase activities in comparison to that of the control (Figure VI.7). The interaction seems to be independent of the YtfE interacting domain as judged by the similar values obtained when YtfE was expressed either as N- or C-terminal part of the pUT18 fusion protein. However, the interaction is dependent on the Dps domain due to the higher values achieved when the C-terminal of Dps was fused to the T25 Cya domain (Figure VI.7).

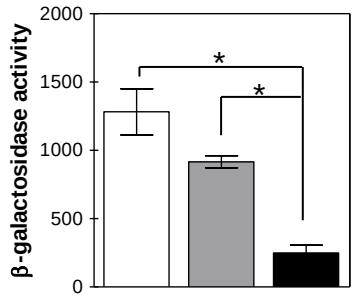


Figure VI.7 – Interaction of *E. coli* YtfE with Dps. The interaction of YtfE cloned in the C-terminal (white bars) or in the N-terminal (grey bars) of Cya Domain was evaluated in cells co-transformed with the complementary plasmid containing *dps* gene fused to the N-terminal of the second Cya domain. Empty vectors co-transformed with vectors containing *dps* or *ytfE* genes served as negative controls. Values are means ± standard error of at least three independent cultures analyzed in duplicate. *p<0.05 (One-way ANOVA Bonferrani multiple comparison test).

In conclusion, YtfE interacts *in vivo* with Dps, confirming the results obtained from the libraries.

VI.4 – Discussion

In this work, *E. coli* was screened *in vivo* for protein interactions with YtfE using a bacterial adenylate cyclase two-hybrid system approach.

The oxidative and nitrosative stresses cause iron-sulphur cluster degradation, therefore creating a demand for their repair to maintain the integrity of the cellular metabolic pathways. According to our data (chapter

V), the recovery of Fe-S clusters via YtfE may occur by transfer of iron to the dismantled cluster. If the damage leads to the complete destruction of the cluster, we showed that YtfE has the ability to interact with IscS/SufS, acting together to provide both the iron and the sulphur required for the reassembling process, through an as yet unknown mechanism. Moreover, the positive interaction with IscS suggests that YtfE may also have a role in the assembly of Fe-S clusters. It is noteworthy that, although deletion of *ytfE* is associated with a clear phenotype under oxidative and nitrosative stress conditions, we observed that non-stressed cells also have lower aconitase and fumarase activity in the absence of *ytfE* (*4*).

The second part of this work involved the identification of novel *E. coli* YtfE interactants by screening the *E. coli* proteome. The results revealed a new interactant, the ferritin-like protein, Dps. Further studies are required to understand how Dps is involved with YtfE in the repair of Fe-S clusters under stress conditions.

Although the bacterial two-hybrid system is an *in vivo* method to identify protein-protein interactions, other *in vitro* and *in vivo* techniques are required to characterize the interaction of YtfE with IscS, SufS and Dps, such as co-immunoprecipitation, Far-Western blotting and surface plasmon resonance (Biacore). In summary, by providing evidence for interaction with IscS, SufS and Dps, the present work helped to identify other players that could be involved with YtfE in Fe-S cluster assembly/repair.

VI.5 – References

(1) Hromatka, B. S., Noble, S. M., and Johnson, A. D. (2005) Transcriptional response of Candida albicans to nitric oxide and the role of the YHB1 gene in nitrosative stress and virulence. *Mol Biol Cell 16*, 4814-26.

(2) Lombard, M., Touati, D., Fontecave, M., and Niviere, V. (2000) Superoxide reductase as a unique defense system against superoxide stress in the microaerophile Treponema pallidum. *J Biol Chem 275*, 27021-6.

(3) Py, B., and Barras, F. (2010) Building Fe-S proteins: bacterial strategies. *Nat Rev Microbiol 8*, 436-46.

(4) Justino, M. C., Almeida, C. C., Goncalves, V. L., Teixeira, M., and Saraiva, L. M. (2006) Escherichia coli YtfE is a di-iron protein with an important function in assembly of iron-sulphur clusters. *FEMS Microbiol Lett 257*, 278-84.

(5) Justino, M. C., Almeida, C. C., Teixeira, M., and Saraiva, L. M. (2007) Escherichia coli di-iron YtfE protein is necessary for the repair of stress-damaged iron-sulfur clusters. *J Biol Chem 282*, 10352-9.

(6) Justino, M. C., Vicente, J. B., Teixeira, M., and Saraiva, L. M. (2005) New genes implicated in the protection of anaerobically grown Escherichia coli against nitric oxide. *J Biol Chem 280*, 2636-43.

(7) Karimova, G., Pidoux, J., Ullmann, A., and Ladant, D. (1998) A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proc Natl Acad Sci U S A 95*, 5752-6.

(8) Ladant, D., and Ullmann, A. (1999) Bordatella pertussis adenylate cyclase: a toxin with multiple talents. *Trends Microbiol 7*, 172-6.

(9) Ullmann, A., and Danchin, A. (1983) *Role of cyclic AMP in Bacteria*, New York.

(10) Pommier, J., Mejean, V., Giordano, G., and Iobbi-Nivol, C. (1998) TorD, a cytoplasmic chaperone that interacts with the unfolded trimethylamine N-oxide reductase enzyme (TorA) in Escherichia coli. *J Biol Chem 273*, 16615-20.

(11) Thibodeau, S. A., Fang, R., and Joung, J. K. (2004) High-throughput beta-galactosidase assay for bacterial cell-based reporter systems. *Biotechniques 36*, 410-5.

(12) Calhoun, L. N., and Kwon, Y. M. (2010) Structure, function and regulation of the DNA-binding protein Dps and its role in acid and oxidative stress resistance in Escherichia coli: a review. *J Appl Microbiol 110*, 375-86.

(13) Chiancone, E., and Ceci, P. (2010) The multifaceted capacity of Dps proteins to combat bacterial stress conditions: Detoxification of iron and hydrogen peroxide and DNA binding. *Biochim Biophys Acta 1800*, 798-805.

(14) Blaha, G., Stanley, R. E., and Steitz, T. A. (2009) Formation of the first peptide bond: the structure of EF-P bound to the 70S ribosome. *Science 325*, 966-70.

(15) Ganoza, M. C., and Aoki, H. (2000) Peptide bond synthesis: function of the efp gene product. *Biol Chem 381*, 553-9.

(16) Bishop, R. E., Leskiw, B. K., Hodges, R. S., Kay, C. M., and Weiner, J. H. (1998) The entericidin locus of Escherichia coli and its implications for programmed bacterial cell death. *J Mol Biol 280*, 583-96.

VI.6 – Acknowledgments

The author is grateful to André Fernandes for the participation in this work.

The author is also grateful to Prof. Simon C. Andrews for the BACTH system plasmids: pUT18, pUT18C, pKT25, pKNT25, for providing the libraries BamHI and BamHI+1 plasmids, and for his support in this work.

Discussion

Chapter VII

Discussion and general conclusions

VII Discussion and general conclusions

Chapter VII

Discussion and general conclusions

VII.1 – Discussion

VII.1.1 – The role of *Escherichia coli* flavorubredoxin

One of the main purposes of this dissertation was to determine the role of flavorubredoxin in the protection of bacteria upon nitrosative and oxidative stress. Bacteria are exposed to nitric oxide generated from mainly two sources: from nitrite and nitrate reduction (denitrification pathway), or as products of the inducible nitric oxide synthase, one of the mammalian host defence mechanisms (*1-6*).

To survive the deleterious effects of nitric oxide, microbes are able to detect and detoxify NO and to repair the damage provoked in the cellular targets (*5-8*). One important enzyme involved in nitric oxide detoxification is *Escherichia coli* flavorubredoxin (*9, 10*). The results obtained in this thesis show that *norV* transcription is hindered in the presence of oxidative and nitrosative stress. Moreover, it was demonstrated that the lack of *norV* transcription is related to its regulator, NorR (*11-14*). Upon oxidation of the NorR mono-iron centre, nitric oxide no longer binds, compromising the activation of *norV* (chapter III).

Consistent with the data, the mononuclear iron centres were shown to be damaged by oxidative stress and, in the presence of traces of oxygen, NorR loses its iron atoms (*15, 16*). Furthermore, purification of the recombinant protein under aerobic condition yields a NorR protein devoid

of iron (*17*). However, the nitrosylated mono-iron centre of *Ralstonia* NorR is stable under aerobic conditions (*16*). When the levels of oxidative stress are low, *E. coli* can reduce the oxidized mono-iron centre through an iron reductase that would lead to NO binding and consequently activation of *norV* transcription. In order to comprehend the instability of the mono-iron centre, the structure of NorR must be determined to unveil the iron centre position and exposure.

Besides flavorubredoxin, two other major nitric oxide detoxifying systems are present in *E. coli*: the periplasmic formate dependent cytochrome c nitrite reductase, NrfA, and the flavohaemoglobin, Hmp (Figure VII.1)(*18*). The combined activity of the three enzymes allows *E. coli* to be flexible in its metabolism and hence helps its survival in a range of different environments. For example, upon infection, bacteria are exposed to high concentrations of nitric oxide generated by macrophages in low dioxygen environments (~1% of oxygen within infected tissues). Under these conditions, the three enzymes are catalytically efficient and FlRd and NrfA act as NO reductases whether Hmp acts as NO denitrosylase or reductase (*9, 18-21*).

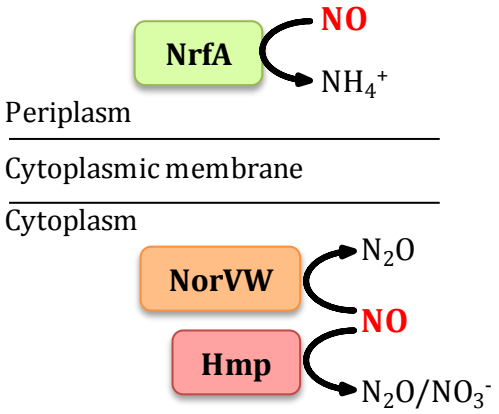


Figure VII.1 – The cellular location of proteins involved in the detoxification of nitric oxide in *Escherichia coli*. NrfA – Periplasmic nitrite reductase; NorVW – flavorubredoxin, NorV, and its reductase NorW; Hmp – flavohaemoglobin; NO – nitric oxide; NH_4^+ - ammonium; N_2O – nitrous oxide; NO_3^- - nitrate. Adapted from [18].

A study revealed that deletion of *nrfA* slightly attenuated the virulence of *Salmonella enterica* in mice (*22*). It has been proposed that NrfA in the periplasm provides the first line of defence against external NO generated by the host. Thus, the lack of NrfA is not critical for virulence, as Flrd and Hmp are present in the cytoplasm to detoxify the NO that escapes NrfA control (*19*). In *E. coli*, Hmp was shown to have a role in bacterial viability and NO detoxification in macrophages (*23*). Moreover, Hmp was shown to be important for pathogenicity in *Erwinia chrysanthemi* and *Salmonella enterica* (*24, 25*). Our data reveal that Hmp is able to protect bacteria at all stages of macrophage infection and FlRd protects bacteria against the attack of macrophages depending on the stage of infection. The requirement of flavorubredoxin and flavohaemoglobin to scavenge nitric oxide under different phases of infection is schematized in Figure VII.2.

To understand how bacteria respond upon macrophage infection, microarrays studies have been performed using different pathogens. In the earlier phase of infection (oxidative burst), microbes activate systems that detoxify ROS such as superoxide dismutase B and C, catalase, peroxidases (AhpC and Tpx) and SoxRS (the superoxide regulator and sensor that regulates genes involved in oxidative and nitrosative stress protection) (*26-30*). Upon nitric oxide burst, the microorganisms activate the inducible NO detoxifying systems flavohaemoglobin and flavorubredoxin (*27, 31, 32*). However, according to our results, Hmp is able to protect bacteria in all stages of macrophage infection. The regulation of *hmp* is complex since the response to nitric oxide inducers involves at least three repressors FNR, NsrR and the methionine repressor MetR (*33, 34*) (Table VII.1). Microarray experiments performed with several microorganisms revealed that *hmp* is one of the few genes whose expression is always induced by NO, under aerobic and anaerobic conditions, independently of the agent used to

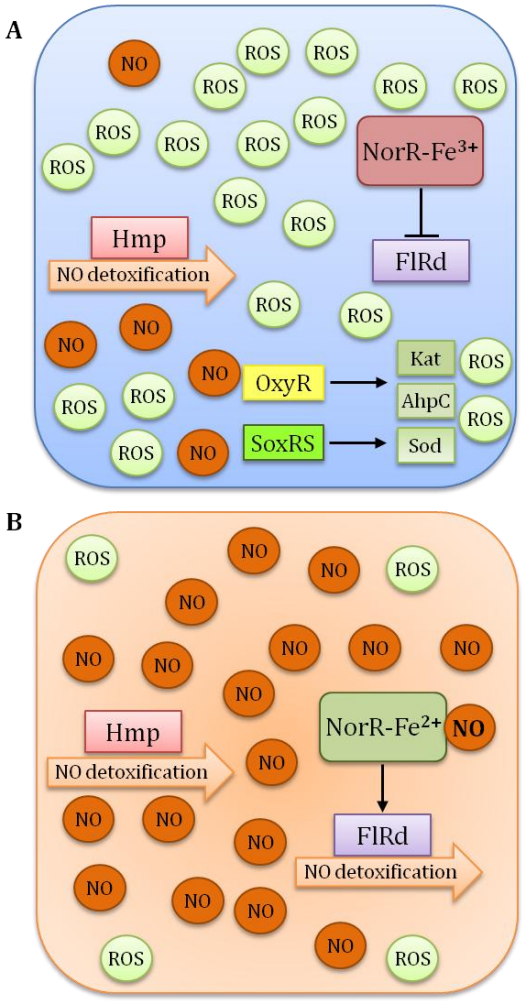


Figure VII.2 – Schematic representation of nitric oxide detoxification during macrophage infection. (A) In the early phase of macrophage infection, upon oxidative burst, Hmp detoxifies NO and NorR iron centre is oxidized. Consequently, FlRd is not any longer expressed. The nitric oxide present activates transcription factors, like OxyR and SoxRS, whose regulons encode ROS detoxificants, such as KatG, AhpC and SodA. **(B)** Upon NO burst, the latter phase of macrophage infection, the mono-iron centre of NorR is nitrosylated and FlRd is expressed, as well as Hmp, which are required for NO detoxification. ROS – reactive oxygen species; NO – nitric oxide; NorR – Nitric oxide reductase Regulator; NorR-Fe^{3+} - NorR with mono-iron centre oxidized; FlRd – flavorubredoxin; Hmp – flavohaemoglobin; OxyR – Oxidative stress Regulator; SoxRS – Superoxide Response and Sensor; KatG – Catalase; AhpC – alkyl hydroperoxide reductase; SodA – Superoxide dismutase A; NorR-Fe^{2+}-NO – nitrosylated NorR mono-iron centre.

generate nitrosative stress (*8, 13, 35-39*). Besides NO induction, *hmp* is also up-regulated upon iron limitation (*23, 24, 40*). Interestingly, *hmp* induction occurs only when NO is present in the cell environment, as constitutive *hmp* transcription in *E. coli* in the absence of NO generates oxidative stress by virtue of oxygen reduction to superoxide anion by Hmp (*41, 42*).

E. coli FNR contains a Fe-S cluster and in the presence of nitrosative stress the centre is nitrosylated resulting in FNR derepression of its regulon. Both *hmp* and *norV* were shown to be regulated by FNR (*43-45*).

However, studies suggest that the repression of *norV* by FNR occurs due to an indirect effect (*43, 45*). Therefore, the NO-regulation of *norV* is mainly performed by NorR, and FNR does not possess a relevant role (Table VII.1).

Homologues of the NorR regulatory protein are found in some gamma- and beta-proteobacteria and their regulon contains various enzymes involved in nitric oxide detoxification (*46*). The NorR-encoding gene is located upstream and is divergently transcribed from the *norVW* operon in enterobacteria, like *E. coli*, *Salmonella enterica*, *Hahella chejuensis*, *Shigella flexneri*, *Vibrio fischeri*, among others. In addition, *norR* is upstream of the flavohaemoglobin encoding gene (*hmp* or *fhp*) in *Pseudomonas spp. Polaromonas sp.*, *Azotobacter vinelandii*, *Burkholderia fungorum* and *Vibrio cholerae* (*46*). Work with *V. cholerae* showed that NorR is the regulator of *hmpA* in this organism (*47*). In *Pseudomonas aeruginosa*, *fhp* (*f*lavo*h*aemoglobin *p*rotein) is regulated by FhpR (a NorR type σ^{54}-dependent activator) in the presence of nitric oxide inducers (*48*). Further studies are required to understand if the NorR sensitivity to oxidative stress also occurs in organisms where NorR regulates other genes besides *norV*.

Table VII.1 - Transcriptional machinery involved in NO-regulated Hmp and FlRd expression. Adapted from (*57*).

Organism	Signal/ Stimulus	Transcription factor/Component	Activator or Repressor	Molecular mechanism	Refs
Hmp					
Escherichia coli	Exogenous NO, murine macrophages, NO_2^- respiration	NsrR	Repressor	NO ligation to Fe–S cluster	(*46, 49, 50*)
E. coli	Exogenous NO	MetR	Activator	Unknown	(*51*)
E. coli	Anaerobiosis, nitrate respiration	FNR	Repressor	O_2 and/or NO ligation to Fe–S cluster	(*40, 52*)
Salmonella enterica	Exogenous NO, stimulated macrophages	NsrR	Repressor	NO ligation to Fe–S cluster	(*42*)
Pseudomonas aeruginosa	Exogenous NO, denitrification	FhpR	Activator	Activation via a conserved sequence in flavoHb	(*48, 53*)
Bacillus subtilus	Exogenous NO	ResDE (two-component kinase)	Activator	Unknown	(*54, 55*)
FlRd					
E. coli	Exogenous NO	NorR	Activator	NO ligation to Fe-S cluster	(*12, 56*)
S. enterica	Murine macrophages	NorR	Activator	NO ligation to Fe-S cluster	(*27*)

The *norV* gene is not located downstream of *norR* in microorganisms like *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Clostridium acetobutylicum*, *Photobacterium profundum* and *Rhodobacter capsulatus*. For example, in *Ph. profundum*, upstream of the *norVW* operon are two regulatory binding sites for NsrR (*46*), the regulator of NO metabolism in gamma- and beta-proteobacteria (*46*). In *C. acetobutylicum*, the *norV* gene is downstream and divergently transcribed from *soxR* encoding a Fe-S containing regulatory protein involved in oxidative stress protection (*33*).

The detoxification of nitric oxide is clearly implicated in the resistance of bacteria against the mammalian immune system. Nonetheless, some microorganisms possess apparently only one detoxifying system, like *Porphyromonas gingivalis* and *Fusobacterium nucleatum*, two human pathogens, which apparently contain only flavorubredoxin in their

genomes. Interestingly, the *norV* gene is not divergently transcribed from *norR* in these organisms, which could indicate that NorR is not the regulator of flavorubredoxin gene.

The results obtained in chapter III reveal the importance of flavorubredoxin for bacteria survival inside macrophages. Future studies are required to further understand the advantage of NorR sensitivity to ROS and if this inactivation mechanism also occurs for other mono-iron containing regulators/enzymes.

VII.1.2 – The function of the Ric family of proteins

Previous work in our laboratory with *E. coli* YtfE revealed a role for this protein in the repair of oxidatively and nitrosatively damaged Fe-S clusters (*8, 58, 59*). A homologue of *E. coli* YtfE was recognised in *Staphylococcus aureus*, the previously annotated ScdA protein, and our data showed that this enzyme is also involved in the repair of Fe-S centres. Although *S. aureus* is highly resistant to nitrosative stress (*35*), this bacterium only contains apparently a flavohaemoglobin system to detoxify NO (*35, 60, 61*). Moreover, it contains a nitroreductase that protects against GSNO (S-Nitrosoglutathione) (*62*). The discovery of a novel mechanism used by *S. aureus* to resist nitrosative stress is of high relevance to the study of this pathogen. The homologues of *E. coli* YtfE and *S. aureus* ScdA are widespread in bacteria, fungi and protozoa and we suggested that this family of proteins be named Ric for Repair of Iron Centres.

In *Haemophilus influenzae*, the *ric* mutant strain is less resistant to NO donors and this gene is critical for survival in activated NO-producing macrophages (*63*). Before the reports on *E. coli ytfE*, Vollack and Zumft

published that *Pseudomonas stutzeri* DnrN, a Ric homologue, controlled the transcription of the *nirS* operon (coding for cytochrome *cd1* nitrite reductase), as *P. stutzeri dnrN* mutant had a higher induction of the *nirS* operon after challenge with nitric oxide (*64*). At the time, no explanation for this effect was put forward; however, the behaviour is similar to what occurs with *norB* gene in *N. gonorrhoeae* (Chapter IV). In the gonococcus, *dnrN* deletion affects the regulation of *norB* as its regulator, NsrR, suffers more pronounced nitrosylation, leading to its inactivation and consequently derepression of *norB*. Hence, in the wild type strain, the recovery of the NsrR cluster allows reactivation of its repressor function, while in the mutant strain high levels of *norB* were still observed due to the failure to repair the nitrosylated NsrR centre (Chapter IV).

R. eutropha NorA shares a high degree of identity with *E. coli* Ric (49%) and also contains a di-iron centre (*65*). A study showed that this protein binds nitric oxide, a general characteristic of the di-iron proteins, and its high cytoplasmic concentration (~20 μM) led the authors to propose that NorA is a NO scavenger (*65*). Contrary to what occurs in *E. coli*, the growth of *R. eutropha norA* mutant in media supplemented with nitrate was not impaired significantly (*65, 66*), showing that the activity of nitrate reductase (Nar), an Fe-S containing enzyme, does not depend on NorA. Although the two proteins are highly similar in their amino acid sequence, more studies have to be performed to understand if *R. eutropha* NorA and *E. coli* YtfE serve similar physiological functions.

As previously reported, *E. coli*, *S. aureus* and *N. gonorrhoeae ric* are induced upon exposure to nitric oxide. Moreover, in *S. enterica* and *Yersinia pestis*, the *ric* genes were significantly up-regulated during host-pathogen interaction (*27, 67, 68*).

A bioinformatic study firstly raised the hypothesis that the nitric oxide-sensitive transcriptional regulator NsrR controlled the expression of *E. coli ytfE*, based on the presence of NsrR binding motifs in the *ric* promoter region (*46*). This prediction was confirmed as *E. coli* NsrR was able to bind to the *ytfE* promoter region and in the absence of NsrR, the *ytfE* expression was up-regulated (*69, 70*). In other enterobacteria, *N. gonorrhoeae* and *N. meningitidis*, the expression of the *ric* genes is also regulated by NsrR (*50, 71, 72*). In *P. stutzeri*, the induction of the *ric* gene (whose role is similar to *E. coli ric*) in response to NO depends on the DnrD regulator (*64*).

The *E. coli ric* gene is up-regulated when Fur is absent (*58*); nevertheless, no binding of Fur to the *ric* promoter could be observed. Hence, the derepression of *ric* in *E. coli fur* mutant strain might be the product of indirect effects, possibly at the level of the regulation of NsrR. Also in an *E. coli fnr* mutant strain the *ric* gene is derepressed (*58*) and FNR binds to the *ric* promoter (*45*). However, no canonical FNR binding sites were found in the *E. coli ric* regulatory region and FNR binds to a site with poorer match to FNR consensus sequence (*45*).

A comprehensive analysis of all genomes available shows a high variability of the *ric* gene organization. Figure VII.3 displays the genomes in which *ric* is located near genes encoding proteins related to oxidative and nitrosative stress resistance. For example, in *Neisseria* sp., a thioredoxin gene is found upstream and divergently transcribed from *ric*, while in fungi a *kat* gene (encoding the hydrogen peroxide-detoxifying enzyme catalase) precedes *ric*. In *R. eutropha*, *Acidobacteria bacterium* and *Hahella chejuensis*, the gene *norB*, encoding a NO reductase, is located downstream of *ric*. A different organization is found in the *Bacillus* genus, where the genes following the *ric* gene encode a nitrite reductase. In *Acidiphilium cryptum*,

the *ric* gene is located downstream of a gene encoding a truncated globin, a protein that might be involved in nitric oxide detoxification (*73*), and upstream of a putative [4Fe-4S] cluster-containing ferredoxin. In *Pelobacter propionicus*, *ric* is included in a gene cluster that contains *hcp* (encoding the hybrid cluster protein, that in *E. coli* affords protection against oxidative stress (*74*)), and *fdp*, which codes for a putative nitric oxide reductase of the flavodiiron protein family (*75, 76*). In *S. aureus*, the *lytSR* cluster that encodes for proteins involved in the protection of bacteria from host attack and antibiotic resistance is located upstream of the *ric* gene (*77*). Previously, *scdA* (the *ric* homologue in *S. aureus*) was annotated as having a role in cell morphogenesis, due to the observation that a mutation in *scdA* caused a morphological defect (*78*). However, this phenotype probably resulted from a polar effect on the downstream *lytSR* genes as our results revealed that a *S. aureus scdA* mutant strain showed no morphological defects (chapter IV), while *lytS* mutants were shown to possess a morphological deficiency (*79*).

In the genomes of two *Ralstonia* species, of *Herminiimonas arsenicoxydans*, *Hahella chejuensis*, *Anaeromyxobacter dehalogenans* and *Idiomarina ihoihensis*, *ric* is downstream and divergently transcribed from the NO-sensor NorR, which suggests that NorR is a regulator of *ric* genes. In fact, the *ric* gene (*norA*) in *Ralstonia eutropha* was demonstrated to be regulated by NorR (*66*). Among all presently available genomes, including all enterobacteria, *ric* is never found in the close vicinity of the NsrR regulator.

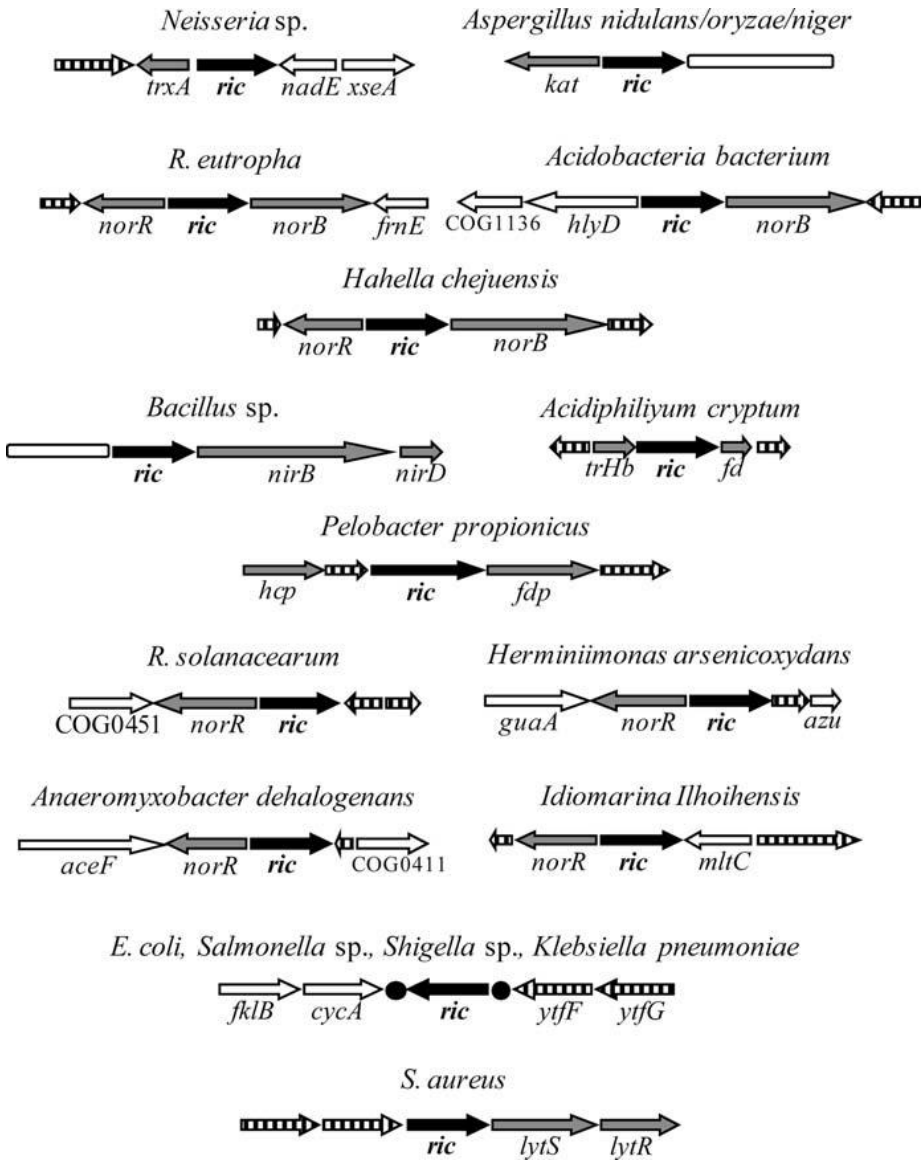


Figure VII.3 – Genomic organization of the *ric* genes. Arrows depict genes, white blocks indicate regions not conserved, and black dots indicate regions of gene insertion. Black arrows indicate the *ric* genes, stripped arrows represent hypothetical genes, and grey arrows highlight the following genes: *fd*, for a putative [4Fe-4S] ferredoxin; *fdp*, for a putative flavodiiron NO reductase; *hcp*, for a hybrid cluster protein; *kat*, catalase; *lytR* and *lytS*, autolysis regulatory system; *nirB* and *nirD*, nitrite reductase; *norB*, NO reductase; *norR*, for the NO sensor/regulator; *trHb*, putative truncated globin; and *trxA*, thioredoxin I.

Previous studies (*8, 58, 59*) and the work presented in chapter IV showed the importance of Rics in the repair of Fe-S clusters. As described in chapter II, upon oxidative and nitrosative stress, [4Fe-4S] clusters are damaged differently (Figure VII.4). In order to obtain a repaired Fe-S centre, it is not necessary to assemble *de novo* a completely new centre. In fact, an *E. coli* strain mutated in *ytfE* in the presence of hydrogen peroxide contains oxidatively damaged Fe-S clusters (*59*), and the Isc and Suf Fe-S clusters assembly systems were not able to complement the mutated strain (Chapter IV). In conclusion, the role of YtfE in *E. coli* is different from that of the Isc and Suf assembly systems.

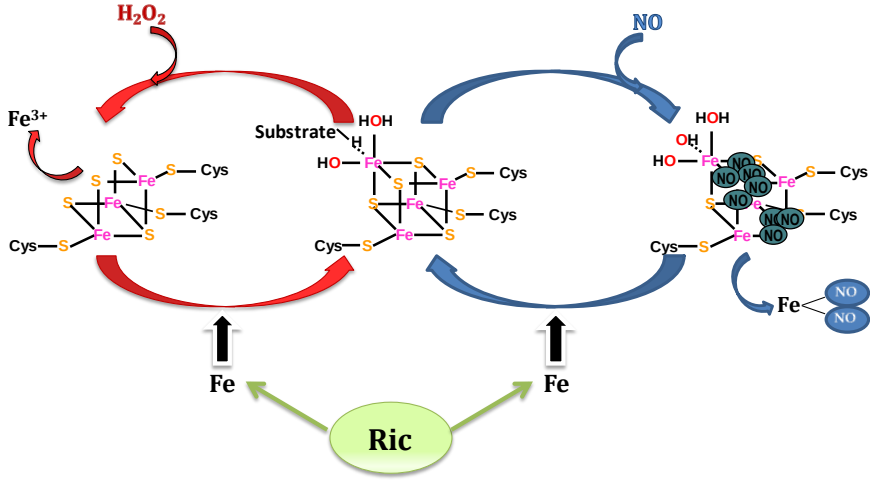


Figure VII.4 – Schematic representation of the oxidative and nitrosative damage to [4Fe-4S] cluster of dehydratase enzymes and the model mechanism for the repair of Fe-S clusters with the proposal for the intervention of the Ric proteins. The depicted effects for the nitrosative damage upon the [4Fe-4S] cluster are only an illustrative representation of DNIC formation. Fe – iron; Fe^{3+} - ferric iron; NO – nitric oxide; H_2O_2 – hydrogen peroxide; Cys – Cysteine residues; S – sulphur atom; H – hydrogen bond; HOH – water molecule; and OH – hydroxide molecule.

Although the repair of oxidatively and nitrosatively damaged Fe-S centres most probably occurs through different mechanisms, the iron

reinsertion is always required; therefore, we previously proposed the involvement of Ric proteins in the enzymatic process needed to recruit and integrate iron (*59*) (Figure VII.4).

The assembly of Fe-S clusters *in vivo* requires specific machineries as iron and sulphur are toxic to the cells in the concentrations necessary for the formation of this prosthetic group (*80*). The source of the sulphur atom in Fe-S clusters is L-cysteine, as a result of the action of cysteine desulphurases (IscS) (Figure VII.5). These enzymes degrade L-cysteine into L-alanine, sequestering the released sulphur anion (S^{2-}) on a specific cysteine residue, making sulphur atoms available without releasing it in solution (*81*). However, the iron source used to build Fe-S clusters is still a matter of debate. Current models propose that a metallochaperone acquires iron and directly donates the metal for the assembly pathway by interacting with Fe-S clusters assembly proteins. This pathway would protect iron from chelation by other cellular components and limit the reaction of iron with oxygen and reactive oxygen species (*81*).

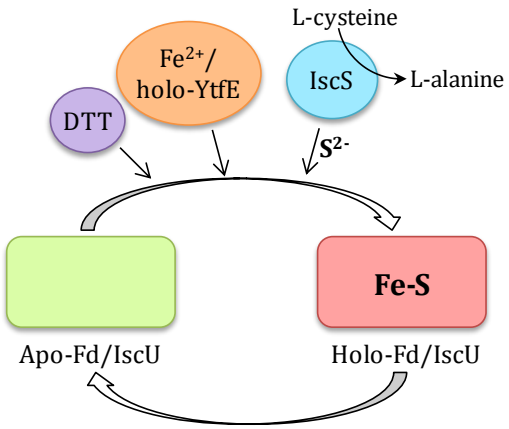


Figure VII.5 – Biosynthesis of Fe-S clusters performed in chapter V. Apo-Fd – Apo-ferredoxin; Holo-Fd – holo-ferredoxin; IscU – scaffold protein of Isc system; Fe-S – iron-sulphur cluster; IscS – cysteine desulphurase of Isc system; S^{2-} - sulphide; Fe^{2+} - inorganic iron; holo-YtfE – di-iron containing YtfE protein; DTT – reducing agent dithiothreitol.

Some proteins were proposed to be the iron donors for the assembly of Fe-S clusters in bacteria. CyaY, the frataxin homologue in

bacteria, was shown *in vitro* to bind iron and to be required for Fe-S clusters assembly (*82*). However, this enzyme has a weak iron binding constant ($2.6x10^5$ M^{-1}) and when *E. coli* cells are supplemented with exogenous iron, CyaY is not able to bind iron (*83-85*). Moreover, deletion of *cyaY* in *E. coli* does not cause a phenotype associated with Fe-S clusters assembly deficiency and recently it was proposed that CyaY acts instead as an inhibitor of Fe-S clusters assembly (*86, 87*). Other predicted iron donor proteins are IscA and SufA, which belong to the Isc and Suf pathways, respectively, and are able to bind iron, possibly donating the iron for the assembly of Fe-S clusters *in vitro*. Nonetheless, *in vivo* phenotypic analyses failed to provide any evidence favouring a role of either proteins in Fe-S cluster assembly (*82, 88*). Moreover, IscA and SufA are not able to interact with cysteine desulphurases or scaffold proteins (*81, 89*). IscA binds iron with a very high affinity constant ($3x10^{19}$ M^{-1} (*90-92*)) and the maximal iron binding is 0.45 Fe per monomer of IscA (*90*).

The work presented in this dissertation showed that in the presence of IscS, L-cysteine, the reducing agent DTT and an excess of inorganic iron, Fe-S clusters were assembled in apo-ferredoxin (chapter V). Replacement of the inorganic iron by holo-YtfE, promotes the assembly of Fe-S clusters in the apo-ferredoxin and also in the scaffold protein IscU (Figure VII.5) (chapter V).

Although preliminary data, our results and the previous data obtained in our laboratory (chapter V and (*58, 59*)) suggest that YtfE may function as the iron donor for the repair of Fe-S clusters as: 1)YtfE is an iron binding protein with two iron atoms per monomer; and 2) the di-iron YtfE form is able to promote the assembly of iron-sulphur centres in IscU and apo-ferredoxin.

To identify whether other proteins were required for YtfE repair function, we analyzed the possible interaction of *E. coli* YtfE with proteins of Fe-S cluster assembly systems and explored the *E. coli* proteome to find YtfE interactants. The experiments were performed using the bacterial adenylate cyclase two hybrid (BACTH) system.

Our data revealed that *E. coli* YtfE was able to interact with IscS but not with IscU. Consistent with these results YtfE was able to promote the formation of Fe-S clusters either in the presence of IscS and L-cysteine or using an inorganic source of sulphur (Na_2S) in the scaffold protein IscU. However, the Fe-S clusters assembly was more efficient in the presence of IscS (100% of $[2Fe\text{-}2S]^{2+/1+}$ cluster formed per IscU dimer versus 50% when using Na_2S). In addition, spectroscopic studies by Ding et al. revealed that *E. coli* IscS along with L-cysteine were required to repair the nitrosatively damaged $[4Fe\text{-}4S]^{2+/1+}$ and $[2Fe\text{-}2S]^{2+/1+}$ centres of endonuclease III and ferredoxin, respectively (*93, 94*).

The results also showed that YtfE is able to interact with SufS, the cysteine desulphurase of the operative mechanism under oxidative and iron limited conditions (*82*). In conclusion, YtfE interacts *in vivo* with all known cysteine desulphurases of the iron-sulphur biogenesis system in *E. coli*. When the oxidative and nitrosative stress persist, the Fe-S clusters can be completely degraded. At this point, YtfE can interact with IscS/SufS to provide both the iron and the sulphur required for the reassembling process. Moreover, the interaction of YtfE and IscS suggests a role for YtfE in the assembly of Fe-S clusters under non-stress conditions.

In this dissertation, using the BACTH system, our data revealed that the ferritin-like protein Dps (*D*NA-binding *p*rotein from *s*tarved cells) interacts with YtfE. The first identified role of Dps in *E. coli* was in protection of the cell against oxidative stress (*95, 96*). The Dps crystal

structure revealed similarity to the iron storage protein ferritin and that it is able to accommodate ~500 iron atoms (*97, 98*). The ferroxidase activity of Dps uses hydrogen peroxide, removing Fe(II) and H_2O_2 from solution, which results in a strong inhibition of the Fenton reaction that leads to the protection of DNA, RNA, proteins and lipids (*98-100*).

It is interesting that *E. coli* YtfE is able to interact with Dps as both proteins are involved in the protection of bacteria upon oxidative stress which is directly related to the iron metabolism (*99, 101*). Under oxidative stress conditions, Dps could store free iron that is released from several sources like damaged iron-sulphur clusters (*102*). Once the iron is "safely" stored in Dps, YtfE could interact with the iron storage protein to provide the iron required for the repair and reassembly of Fe-S clusters. This process could involve the donation of iron from Dps to YtfE. To rescue the iron from ferritin-like proteins (e.g. Dps), other enzymes are needed. When the iron is required for cell metabolism, as for Fe-S clusters assembly, the metal is mobilized upon reduction of the oxy-hydroxide core of the ferritin-like proteins, using iron reductase systems (*103, 104*). Hence, if Dps and YtfE acted together to provide the iron required for the reassembly/repair of Fe-S clusters, an iron reductase system should be present during this process to recover the iron that is stored in Dps making it available for YtfE to deliver it, for example, to the scaffold protein IscU.

A similar model was proposed in a study by Ding et al. which showed that the iron stored in the *E. coli* iron storage protein ferritin A could be retrieved by the proposed iron donor IscA to re-assemble Fe-S clusters in the scaffold protein IscU (*102*).

Future work needs to be developed to understand the underlying mechanisms of *E. coli* YtfE in the assembly/repair of Fe-S clusters and to elucidate the relevance of *E. coli* YtfE interaction with IscS, SufS and Dps.

VII.2 – General conclusions

This dissertation contributed to further understand the mechanisms used by bacteria to resist the deleterious effects of nitrosative and oxidative stress, by studying the role of two di-iron proteins: flavorubredoxin and Ric (in *E. coli* and *S. aureus*). Overall, the results obtained showed that:

- When cells are exposed to oxidative and nitrosative stress, the transcription and expression of flavorubredoxin is hindered. The lower FlRd expression is related to the oxidation of NorR mono-iron centre that loses the ability to bind nitric oxide. Upon macrophage infection, *E. coli* FlRd protects bacteria in a later phase of infection when the oxidative burst is not present.
- A homologue of *E. coli* Ric was found in *S. aureus*. The deletion of this gene resulted in a staphylococcal strain more sensitive to hydrogen peroxide and unable to repair Fe-S clusters. Homologues of the two diiron proteins were found distributed in several pathogenic microorganisms and the family of proteins was named *Ric* for *R*epair of *i*ron *c*entres.
- The *E. coli* Ric protein was able to promote the assembly of Fe-S clusters in the scaffold protein IscU and in the apoform of ferredoxin.
- Finally, *E. coli* Ric was found to interact *in vivo* with the cysteine desulphurases IscS and SufS, and with Dps, a ferritin-like protein.

VII.3 – References

(1) Watmough, N. J., Butland, G., Cheesman, M. R., Moir, J. W., Richardson, D. J., and Spiro, S. (1999) Nitric oxide in bacteria: synthesis and consumption. *Biochim Biophys Acta 1411*, 456-74.

(2) Zumft, W. G. (1997) Cell biology and molecular basis of denitrification. *Microbiol Mol Biol Rev 61*, 533-616.

(3) Ji, X. B., and Hollocher, T. C. (1988) Reduction of nitrite to nitric oxide by enteric bacteria. *Biochem Biophys Res Commun 157*, 106-8.

(4) Corker, H., and Poole, R. K. (2003) Nitric oxide formation by Escherichia coli. Dependence on nitrite reductase, the NO-sensing regulator Fnr, and flavohemoglobin Hmp. *J Biol Chem 278*, 31584-92.

(5) Nathan, C., and Shiloh, M. U. (2000) Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc Natl Acad Sci U S A 97*, 8841-8.

(6) Shiloh, M. U., and Nathan, C. F. (2000) Reactive nitrogen intermediates and the pathogenesis of Salmonella and mycobacteria. *Curr Opin Microbiol 3*, 35-42.

(7) Fang, F. C. (2004) Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nat Rev Microbiol 2*, 820-32.

(8) Justino, M. C., Vicente, J. B., Teixeira, M., and Saraiva, L. M. (2005) New genes implicated in the protection of anaerobically grown Escherichia coli against nitric oxide. *J Biol Chem 280*, 2636-43.

(9) Gomes, C. M., Giuffre, A., Forte, E., Vicente, J. B., Saraiva, L. M., Brunori, M., and Teixeira, M. (2002) A novel type of nitric-oxide reductase. Escherichia coli flavorubredoxin. *J Biol Chem 277*, 25273-6.

(10) Gardner, A. M., Helmick, R. A., and Gardner, P. R. (2002) Flavorubredoxin, an inducible catalyst for nitric oxide reduction and detoxification in Escherichia coli. *J Biol Chem 277*, 8172-7.

(11) Mukhopadhyay, P., Zheng, M., Bedzyk, L. A., LaRossa, R. A., and Storz, G. (2004) Prominent roles of the NorR and Fur regulators in the Escherichia coli transcriptional response to reactive nitrogen species. *Proc Natl Acad Sci U S A 101*, 745-50.

(12) Hutchings, M. I., Mandhana, N., and Spiro, S. (2002) The NorR protein of Escherichia coli activates expression of the flavorubredoxin gene norV in response to reactive nitrogen species. *J Bacteriol 184*, 4640-3.

(13) Pullan, S. T., Gidley, M. D., Jones, R. A., Barrett, J., Stevanin, T. M., Read, R. C., Green, J., and Poole, R. K. (2007) Nitric oxide in chemostat-cultured Escherichia coli is sensed by Fnr and other global regulators: unaltered methionine biosynthesis indicates lack of S nitrosation. *J Bacteriol 189*, 1845-55.

(14) Justino, M. C., Goncalves, V. M., and Saraiva, L. M. (2005) Binding of NorR to three DNA sites is essential for promoter activation of the flavorubredoxin gene, the nitric oxide reductase of Escherichia coli. *Biochem Biophys Res Commun 328*, 540-4.

(15) Anjem, A., and Imlay, J. A. (2012) Mononuclear iron enzymes are primary targets of hydrogen peroxide stress. *J Biol Chem 287*, 15544-56.
(16) Klink, A., Elsner, B., Strube, K., and Cramm, R. (2007) Characterization of the signaling domain of the NO-responsive regulator NorR from Ralstonia eutropha H16 by site-directed mutagenesis. *J Bacteriol 189*, 2743-9.
(17) D'Autreaux, B., Tucker, N. P., Dixon, R., and Spiro, S. (2005) A non-haem iron centre in the transcription factor NorR senses nitric oxide. *Nature 437*, 769-72.
(18) Vine, C. E., and Cole, J. A. (2011) Unresolved sources, sinks, and pathways for the recovery of enteric bacteria from nitrosative stress. *FEMS Microbiol Lett 325*, 99-107.
(19) van Wonderen, J. H., Burlat, B., Richardson, D. J., Cheesman, M. R., and Butt, J. N. (2008) The nitric oxide reductase activity of cytochrome c nitrite reductase from Escherichia coli. *J Biol Chem 283*, 9587-94.
(20) Bowman, L. A., McLean, S., Poole, R. K., and Fukuto, J. M. (2011) The diversity of microbial responses to nitric oxide and agents of nitrosative stress close cousins but not identical twins. *Adv Microb Physiol 59*, 135-219.
(21) Hausladen, A., Gow, A., and Stamler, J. S. (2001) Flavohemoglobin denitrosylase catalyzes the reaction of a nitroxyl equivalent with molecular oxygen. *Proc Natl Acad Sci U S A 98*, 10108-12.
(22) Bang, I. S., Liu, L., Vazquez-Torres, A., Crouch, M. L., Stamler, J. S., and Fang, F. C. (2006) Maintenance of nitric oxide and redox homeostasis by the salmonella flavohemoglobin hmp. *J Biol Chem 281*, 28039-47.
(23) Stevanin, T. M., Read, R. C., and Poole, R. K. (2007) The hmp gene encoding the NO-inducible flavohaemoglobin in Escherichia coli confers a protective advantage in resisting killing within macrophages, but not in vitro: links with swarming motility. *Gene 398*, 62-8.
(24) Stevanin, T. M., Poole, R. K., Demoncheaux, E. A., and Read, R. C. (2002) Flavohemoglobin Hmp protects Salmonella enterica serovar typhimurium from nitric oxide-related killing by human macrophages. *Infect Immun 70*, 4399-405.
(25) Favey, S., Labesse, G., Vouille, V., and Boccara, M. (1995) Flavohaemoglobin HmpX: a new pathogenicity determinant in Erwinia chrysanthemi strain 3937. *Microbiology 141 (Pt 4)*, 863-71.
(26) Faucher, S. P., Porwollik, S., Dozois, C. M., McClelland, M., and Daigle, F. (2006) Transcriptome of Salmonella enterica serovar Typhi within macrophages revealed through the selective capture of transcribed sequences. *Proc Natl Acad Sci U S A 103*, 1906-11.
(27) Eriksson, S., Lucchini, S., Thompson, A., Rhen, M., and Hinton, J. C. (2003) Unravelling the biology of macrophage infection by gene expression profiling of intracellular Salmonella enterica. *Mol Microbiol 47*, 103-18.
(28) Poirier, K., Faucher, S. P., Beland, M., Brousseau, R., Gannon, V., Martin, C., Harel, J., and Daigle, F. (2008) Escherichia coli O157:H7 survives within

human macrophages: global gene expression profile and involvement of the Shiga toxins. *Infect Immun 76*, 4814-22.

(29) Echenique-Rivera, H., Muzzi, A., Del Tordello, E., Seib, K. L., Francois, P., Rappuoli, R., Pizza, M., and Serruto, D. (2011) Transcriptome analysis of Neisseria meningitidis in human whole blood and mutagenesis studies identify virulence factors involved in blood survival. *PLoS Pathog 7*, e1002027.

(30) Staudinger, B. J., Oberdoerster, M. A., Lewis, P. J., and Rosen, H. (2002) mRNA expression profiles for Escherichia coli ingested by normal and phagocyte oxidase-deficient human neutrophils. *J Clin Invest 110*, 1151-63.

(31) Schnappinger, D., Ehrt, S., Voskuil, M. I., Liu, Y., Mangan, J. A., Monahan, I. M., Dolganov, G., Efron, B., Butcher, P. D., Nathan, C., and Schoolnik, G. K. (2003) Transcriptional Adaptation of Mycobacterium tuberculosis within Macrophages: Insights into the Phagosomal Environment. *J Exp Med 198*, 693-704.

(32) Snyder, J. A., Haugen, B. J., Buckles, E. L., Lockatell, C. V., Johnson, D. E., Donnenberg, M. S., Welch, R. A., and Mobley, H. L. (2004) Transcriptome of uropathogenic Escherichia coli during urinary tract infection. *Infect Immun 72*, 6373-81.

(33) Spiro, S. (2007) Regulators of bacterial responses to nitric oxide. *FEMS Microbiol Rev 31*, 193-211.

(34) Poole, R. K. (2005) Nitric oxide and nitrosative stress tolerance in bacteria. *Biochem Soc Trans 33*, 176-80.

(35) Richardson, A. R., Dunman, P. M., and Fang, F. C. (2006) The nitrosative stress response of Staphylococcus aureus is required for resistance to innate immunity. *Mol Microbiol 61*, 927-39.

(36) Moore, C. M., Nakano, M. M., Wang, T., Ye, R. W., and Helmann, J. D. (2004) Response of Bacillus subtilis to nitric oxide and the nitrosating agent sodium nitroprusside. *J Bacteriol 186*, 4655-64.

(37) Flatley, J., Barrett, J., Pullan, S. T., Hughes, M. N., Green, J., and Poole, R. K. (2005) Transcriptional responses of Escherichia coli to S-nitrosoglutathione under defined chemostat conditions reveal major changes in methionine biosynthesis. *J Biol Chem 280*, 10065-72.

(38) Firoved, A. M., Wood, S. R., Ornatowski, W., Deretic, V., and Timmins, G. S. (2004) Microarray analysis and functional characterization of the nitrosative stress response in nonmucoid and mucoid Pseudomonas aeruginosa. *J Bacteriol 186*, 4046-50.

(39) Hromatka, B. S., Noble, S. M., and Johnson, A. D. (2005) Transcriptional response of Candida albicans to nitric oxide and the role of the YHB1 gene in nitrosative stress and virulence. *Mol Biol Cell 16*, 4814-26.

(40) Poole, R. K., Anjum, M. F., Membrillo-Hernandez, J., Kim, S. O., Hughes, M. N., and Stewart, V. (1996) Nitric oxide, nitrite, and Fnr regulation of hmp (flavohemoglobin) gene expression in Escherichia coli K-12. *J Bacteriol 178*, 5487-92.

(41) Poole, R. K., Rogers, N. J., D'Mello R, A., Hughes, M. N., and Orii, Y. (1997) Escherichia coli flavohaemoglobin (Hmp) reduces cytochrome c and Fe(III)-hydroxamate K by electron transfer from NADH via FAD: sensitivity of oxidoreductase activity to haem-bound dioxygen. *Microbiology 143 (Pt 5)*, 1557-65.

(42) Wu, G., Corker, H., Orii, Y., and Poole, R. K. (2004) Escherichia coli Hmp, an "oxygen-binding flavohaemoprotein", produces superoxide anion and self-destructs. *Arch Microbiol 182*, 193-203.

(43) Overton, T. W., Griffiths, L., Patel, M. D., Hobman, J. L., Penn, C. W., Cole, J. A., and Constantinidou, C. (2006) Microarray analysis of gene regulation by oxygen, nitrate, nitrite, FNR, NarL and NarP during anaerobic growth of Escherichia coli: new insights into microbial physiology. *Biochem Soc Trans 34*, 104-7.

(44) da Costa, P. N., Teixeira, M., and Saraiva, L. M. (2003) Regulation of the flavorubredoxin nitric oxide reductase gene in Escherichia coli: nitrate repression, nitrite induction, and possible post-transcription control. *FEMS Microbiol Lett 218*, 385-93.

(45) Constantinidou, C., Hobman, J. L., Griffiths, L., Patel, M. D., Penn, C. W., Cole, J. A., and Overton, T. W. (2006) 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*, 4802-15.

(46) Rodionov, D. A., Dubchak, I. L., Arkin, A. P., Alm, E. J., and Gelfand, M. S. (2005) Dissimilatory metabolism of nitrogen oxides in bacteria: comparative reconstruction of transcriptional networks. *PLoS Comput Biol 1*, e55.

(47) Stern, A. M., Hay, A. J., Liu, Z., Desland, F. A., Zhang, J., Zhong, Z., and Zhu, J. (2012) The NorR regulon is critical for Vibrio cholerae resistance to nitric oxide and sustained colonization of the intestines. *MBio 3*, e00013-12.

(48) Arai, H., Hayashi, M., Kuroi, A., Ishii, M., and Igarashi, Y. (2005) Transcriptional regulation of the flavohemoglobin gene for aerobic nitric oxide detoxification by the second nitric oxide-responsive regulator of Pseudomonas aeruginosa. *J Bacteriol 187*, 3960-8.

(49) Gilberthorpe, N. J., and Poole, R. K. (2008) Nitric oxide homeostasis in Salmonella typhimurium: roles of respiratory nitrate reductase and flavohemoglobin. *J Biol Chem 283*, 11146-54.

(50) Gilberthorpe, N. J., Lee, M. E., Stevanin, T. M., Read, R. C., and Poole, R. K. (2007) NsrR: a key regulator circumventing Salmonella enterica serovar Typhimurium oxidative and nitrosative stress in vitro and in IFN-gamma-stimulated J774.2 macrophages. *Microbiology 153*, 1756-71.

(51) Membrillo-Hernandez, J., Coopamah, M. D., Channa, A., Hughes, M. N., and Poole, R. K. (1998) A novel mechanism for upregulation of the Escherichia coli K-12 hmp (flavohaemoglobin) gene by the 'NO releaser', S-nitrosoglutathione: nitrosation of homocysteine and modulation of MetR binding to the glyA-hmp intergenic region. *Mol Microbiol 29*, 1101-12.

(52) Cruz-Ramos, H., Crack, J., Wu, G., Hughes, M. N., Scott, C., Thomson, A. J., Green, J., and Poole, R. K. (2002) NO sensing by FNR: regulation of the Escherichia coli NO-detoxifying flavohaemoglobin, Hmp. *Embo J 21*, 3235-44.

(53) Koskenkorva, T., Aro-Karkkainen, N., Bachmann, D., Arai, H., Frey, A. D., and Kallio, P. T. (2008) Transcriptional activity of Pseudomonas aeruginosa fhp promoter is dependent on two regulators in addition to FhpR. *Arch Microbiol 189*, 385-96.

(54) Kommineni, S., Yukl, E., Hayashi, T., Delepine, J., Geng, H., Moenne-Loccoz, P., and Nakano, M. M. (2010) Nitric oxide-sensitive and -insensitive interaction of Bacillus subtilis NsrR with a ResDE-controlled promoter. *Mol Microbiol 78*, 1280-93.

(55) Nakano, M. M., Geng, H., Nakano, S., and Kobayashi, K. (2006) The nitric oxide-responsive regulator NsrR controls ResDE-dependent gene expression. *J Bacteriol 188*, 5878-87.

(56) Gardner, A. M., Gessner, C. R., and Gardner, P. R. (2003) Regulation of the nitric oxide reduction operon (norRVW) in Escherichia coli. Role of NorR and sigma54 in the nitric oxide stress response. *J Biol Chem 278*, 10081-6.

(57) Forrester, M. T., and Foster, M. W. (2012) Protection from nitrosative stress: A central role for microbial flavohemoglobin. *Free Radic Biol Med 52*, 1620-33.

(58) Justino, M. C., Almeida, C. C., Goncalves, V. L., Teixeira, M., and Saraiva, L. M. (2006) Escherichia coli YtfE is a di-iron protein with an important function in assembly of iron-sulphur clusters. *FEMS Microbiol Lett 257*, 278-84.

(59) Justino, M. C., Almeida, C. C., Teixeira, M., and Saraiva, L. M. (2007) Escherichia coli di-iron YtfE protein is necessary for the repair of stress-damaged iron-sulfur clusters. *J Biol Chem 282*, 10352-9.

(60) Goncalves, V. L., Nobre, L. S., Vicente, J. B., Teixeira, M., and Saraiva, L. M. (2006) Flavohemoglobin requires microaerophilic conditions for nitrosative protection of Staphylococcus aureus. *FEBS Lett 580*, 1817-21.

(61) Nobre, L. S., Goncalves, V. L., and Saraiva, L. M. (2008) Flavohemoglobin of Staphylococcus aureus. *Methods Enzymol 436*, 203-16.

(62) Tavares, A. F., Nobre, L. S., Melo, A. M., and Saraiva, L. M. (2009) A novel nitroreductase of Staphylococcus aureus with S-nitrosoglutathione reductase activity. *J Bacteriol 191*, 3403-6.

(63) Harrington, J. C., Wong, S. M., Rosadini, C. V., Garifulin, O., Boyartchuk, V., and Akerley, B. J. (2009) Resistance of Haemophilus influenzae to reactive nitrogen donors and gamma interferon-stimulated macrophages requires the formate-dependent nitrite reductase regulator-activated ytfE gene. *Infect Immun 77*, 1945-58.

(64) Vollack, K. U., and Zumft, W. G. (2001) Nitric oxide signaling and transcriptional control of denitrification genes in Pseudomonas stutzeri. *J Bacteriol 183*, 2516-26.

(65) Strube, K., de Vries, S., and Cramm, R. (2007) Formation of a dinitrosyl iron complex by NorA, a nitric oxide-binding di-iron protein from Ralstonia eutropha H16. *J Biol Chem 282*, 20292-300.
(66) Pohlmann, A., Cramm, R., Schmelz, K., and Friedrich, B. (2000) A novel NO-responding regulator controls the reduction of nitric oxide in Ralstonia eutropha. *Mol Microbiol 38*, 626-38.
(67) Kim, C. C., Monack, D., and Falkow, S. (2003) Modulation of virulence by two acidified nitrite-responsive loci of Salmonella enterica serovar Typhimurium. *Infect Immun 71*, 3196-205.
(68) Sebbane, F., Lemaitre, N., Sturdevant, D. E., Rebeil, R., Virtaneva, K., Porcella, S. F., and Hinnebusch, B. J. (2006) Adaptive response of Yersinia pestis to extracellular effectors of innate immunity during bubonic plague. *Proc Natl Acad Sci U S A 103*, 11766-71.
(69) Bodenmiller, D. M., and Spiro, S. (2006) The yjeB (nsrR) gene of Escherichia coli encodes a nitric oxide-sensitive transcriptional regulator. *J Bacteriol 188*, 874-81.
(70) Partridge, J. D., Bodenmiller, D. M., Humphrys, M. S., and Spiro, S. (2009) NsrR targets in the Escherichia coli genome: new insights into DNA sequence requirements for binding and a role for NsrR in the regulation of motility. *Mol Microbiol 73*, 680-94.
(71) Overton, T. W., Whitehead, R., Li, Y., Snyder, L. A., Saunders, N. J., Smith, H., and Cole, J. A. (2006) Coordinated regulation of the Neisseria gonorrhoeae-truncated denitrification pathway by the nitric oxide-sensitive repressor, NsrR, and nitrite-insensitive NarQ-NarP. *J Biol Chem 281*, 33115-26.
(72) Heurlier, K., Thomson, M. J., Aziz, N., and Moir, J. W. (2008) The nitric oxide (NO)-sensing repressor NsrR of Neisseria meningitidis has a compact regulon of genes involved in NO synthesis and detoxification. *J Bacteriol 190*, 2488-95.
(73) Angelo, M., Hausladen, A., Singel, D. J., and Stamler, J. S. (2008) Interactions of NO with hemoglobin: from microbes to man. *Methods Enzymol 436*, 131-68.
(74) Almeida, C. C., Romao, C. V., Lindley, P. F., Teixeira, M., and Saraiva, L. M. (2006) The role of the hybrid cluster protein in oxidative stress defense. *J Biol Chem 281*, 32445-50.
(75) Saraiva, L. M., Vicente, J. B., and Teixeira, M. (2004) The role of the flavodiiron proteins in microbial nitric oxide detoxification. *Adv Microb Physiol 49*, 77-129.
(76) Vicente, J. B., Justino, M. C., Goncalves, V. L., Saraiva, L. M., and Teixeira, M. (2008) Biochemical, spectroscopic, and thermodynamic properties of flavodiiron proteins. *Methods Enzymol 437*, 21-45.
(77) Sharma-Kuinkel, B. K., Mann, E. E., Ahn, J. S., Kuechenmeister, L. J., Dunman, P. M., and Bayles, K. W. (2009) The Staphylococcus aureus LytSR two-component regulatory system affects biofilm formation. *J Bacteriol 191*, 4767-75.

(78) Brunskill, E. W., de Jonge, B. L., and Bayles, K. W. (1997) The Staphylococcus aureus scdA gene: a novel locus that affects cell division and morphogenesis. *Microbiology 143 (Pt 9)*, 2877-82.
(79) Brunskill, E. W., and Bayles, K. W. (1996) Identification of LytSR-regulated genes from Staphylococcus aureus. *J Bacteriol 178*, 5810-2.
(80) Bandyopadhyay, S., Chandramouli, K., and Johnson, M. K. (2008) Iron-sulfur cluster biosynthesis. *Biochem Soc Trans 36*, 1112-9.
(81) Py, B., and Barras, F. (2010) Building Fe-S proteins: bacterial strategies. *Nat Rev Microbiol 8*, 436-46.
(82) Ayala-Castro, C., Saini, A., and Outten, F. W. (2008) Fe-S cluster assembly pathways in bacteria. *Microbiol Mol Biol Rev 72*, 110-25, table of contents.
(83) Adinolfi, S., Trifuoggi, M., Politou, A. S., Martin, S., and Pastore, A. (2002) A structural approach to understanding the iron-binding properties of phylogenetically different frataxins. *Hum Mol Genet 11*, 1865-77.
(84) Bou-Abdallah, F., Adinolfi, S., Pastore, A., Laue, T. M., and Dennis Chasteen, N. (2004) Iron binding and oxidation kinetics in frataxin CyaY of Escherichia coli. *J Mol Biol 341*, 605-15.
(85) Wang, W., Huang, H., Tan, G., Si, F., Liu, M., Landry, A. P., Lu, J., and Ding, H. (2010) In vivo evidence for the iron-binding activity of an iron-sulfur cluster assembly protein IscA in Escherichia coli. *Biochem J 432*, 429-36.
(86) Iannuzzi, C., Adinolfi, S., Howes, B. D., Garcia-Serres, R., Clemancey, M., Latour, J. M., Smulevich, G., and Pastore, A. (2011) The role of CyaY in iron sulfur cluster assembly on the E. coli IscU scaffold protein. *PLoS One 6*, e21992.
(87) Li, D. S., Ohshima, K., Jiralerspong, S., Bojanowski, M. W., and Pandolfo, M. (1999) Knock-out of the cyaY gene in Escherichia coli does not affect cellular iron content and sensitivity to oxidants. *FEBS Lett 456*, 13-6.
(88) Lu, J., Yang, J., Tan, G., and Ding, H. (2008) Complementary roles of SufA and IscA in the biogenesis of iron-sulfur clusters in Escherichia coli. *Biochem J 409*, 535-43.
(89) Tokumoto, U., Nomura, S., Minami, Y., Mihara, H., Kato, S., Kurihara, T., Esaki, N., Kanazawa, H., Matsubara, H., and Takahashi, Y. (2002) Network of protein-protein interactions among iron-sulfur cluster assembly proteins in Escherichia coli. *J Biochem 131*, 713-9.
(90) Ding, H., and Clark, R. J. (2004) Characterization of iron binding in IscA, an ancient iron-sulphur cluster assembly protein. *Biochem J 379*, 433-40.
(91) Ding, H., Harrison, K., and Lu, J. (2005) Thioredoxin reductase system mediates iron binding in IscA and iron delivery for the iron-sulfur cluster assembly in IscU. *J Biol Chem 280*, 30432-7.
(92) Ding, H., Yang, J., Coleman, L. C., and Yeung, S. (2007) Distinct iron binding property of two putative iron donors for the iron-sulfur cluster assembly: IscA and the bacterial frataxin ortholog CyaY under physiological and oxidative stress conditions. *J Biol Chem 282*, 7997-8004.

(93) Rogers, P. A., Eide, L., Klungland, A., and Ding, H. (2003) Reversible inactivation of E. coli endonuclease III via modification of its [4Fe-4S] cluster by nitric oxide. *DNA Repair (Amst) 2*, 809-17.

(94) Yang, W., Rogers, P. A., and Ding, H. (2002) Repair of nitric oxide-modified ferredoxin [2Fe-2S] cluster by cysteine desulfurase (IscS). *J Biol Chem 277*, 12868-73.

(95) Choi, S. H., Baumler, D. J., and Kaspar, C. W. (2000) Contribution of dps to acid stress tolerance and oxidative stress tolerance in Escherichia coli O157:H7. *Appl Environ Microbiol 66*, 3911-6.

(96) Martinez, A., and Kolter, R. (1997) Protection of DNA during oxidative stress by the nonspecific DNA-binding protein Dps. *J Bacteriol 179*, 5188-94.

(97) Grant, R. A., Filman, D. J., Finkel, S. E., Kolter, R., and Hogle, J. M. (1998) The crystal structure of Dps, a ferritin homolog that binds and protects DNA. *Nat Struct Biol 5*, 294-303.

(98) Zhao, G., Ceci, P., Ilari, A., Giangiacomo, L., Laue, T. M., Chiancone, E., and Chasteen, N. D. (2002) Iron and hydrogen peroxide detoxification properties of DNA-binding protein from starved cells. A ferritin-like DNA-binding protein of Escherichia coli. *J Biol Chem 277*, 27689-96.

(99) Calhoun, L. N., and Kwon, Y. M. (2010) Structure, function and regulation of the DNA-binding protein Dps and its role in acid and oxidative stress resistance in Escherichia coli: a review. *J Appl Microbiol 110*, 375-86.

(100) Franceschini, S., Ceci, P., Alaleona, F., Chiancone, E., and Ilari, A. (2006) Antioxidant Dps protein from the thermophilic cyanobacterium Thermosynechococcus elongatus. *Febs J 273*, 4913-28.

(101) Justino, M. C., Baptista, J. M., and Saraiva, L. M. (2009) Di-iron proteins of the Ric family are involved in iron-sulfur cluster repair. *Biometals 22*, 99-108.

(102) Bitoun, J. P., Wu, G., and Ding, H. (2008) Escherichia coli FtnA acts as an iron buffer for re-assembly of iron-sulfur clusters in response to hydrogen peroxide stress. *Biometals 21*, 693-703.

(103) Chiancone, E., Ceci, P., Ilari, A., Ribacchi, F., and Stefanini, S. (2004) Iron and proteins for iron storage and detoxification. *Biometals 17*, 197-202.

(104) Schroder, I., Johnson, E., and de Vries, S. (2003) Microbial ferric iron reductases. *FEMS Microbiol Rev 27*, 427-47.