Desulfovibrio vulgaris defenses against oxidative and nitrosative stresses

Mafalda Cristina de Oliveira Figueiredo

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

Supervisor: Dr. Lígia M. Saraiva

Co-supervisor: Prof. Miguel Teixeira

Oeiras, September 2013

INSTITUTO
DE TECNOLOGIA
QUÍMICA E BIOLÓGICA
/UNL

Knowledge Creation

From left to right: Carlos Romão (president of the jury), Fernando Antunes (3rd opponent), Ana Melo (4th opponent), Lígia Saraiva (supervisor), Carlos Salgueiro (2nd opponent), Mafalda Figueiredo, Alain Dolla (1st opponent) and Miguel Teixeira (co-supervisor).

17th September 2013

Second edition, October 2013

Molecular Genetics of Microbial Resistance Laboratory

Instituto de Tecnologia Química e Biológica

Universidade Nova de Lisboa

2780-157

Portugal

"Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less."

Marie Curie

Acknowledgments

The present work would not have been possible without the help, the support and the friendship of several people whom I would like to formally express my sincere gratitude:

Dr. Lígia M. Saraiva

Firstly I would like to express my gratitude to my supervisor **Dr. Lígia M. Saraiva**, without her ideas and persistence I would not have come this far. I thank her for the constant support and encouragement when things did not go so well, for the trust she placed in me and in my work and for always being there when I needed over these five years. I have to thank Dr. Lígia for the good advices and for the careful revision of this thesis. Thanks for everything!!!

Prof. Miguel Teixeira

To my co-supervisor **Prof. Miguel Teixeira** I would like to thank for the very interesting and helpful discussions and for all the help given whenever I needed.

Scientific Thesis Commission

To **Prof. Cláudio M. Soares** and **Dr. Cecília M. Arraiano** for being part of my scientific thesis commission and for their suggestions and helpful advices.

Prof. David Turner

To **Prof. David Turner**, from Hemoproteins laboratory at ITQB for reviewing the abstract of this thesis accordingly to the ITQB inter rules.

Prof. Judy D. Wall

To **Prof. Judy D. Wall** for providing the wild type and mutant strains of *D. vulgaris* used in this work and for the collaboration on the work present in Part II - Chapter 2.

João Carita

To **João Carita** for the collaboration on the work presented in Part II - Chapter 1, especially for performing the *Desulfovibrio* growths in the fermenter.

Prof. Simon Andrews

I have also to thank **Prof. Simon Andrews** for the excellent reception in his laboratory and also to the group members of the Bacterial Iron Homeostasis lab in particular to **Helen Goodluck** my companion during the month I was in the University of Reading.

Molecular Genetics of Microbial Resistance Group

I would like to thank to all past and present members of **Molecular Genetics of Microbial Resistance** group for making the lab such an enjoyable and pleasant place to work.

Very special thanks to **Susana** that is my "third supervisor" who taught me everything in the lab, who always helped me and encouraged me, thank you also for being a great friend and confidant.

To **Lígia** by her enthusiasm when discussing ideas, for their laughter that keeps the lab in a good mood and to be a good friend.

To **Marta** I would like to thank for always being available for my questions and issues and to always have a solution to solve the problems.

To **Margarida** for being a good friend who is always ready to help.

To **Sara** that is always a funny presence in the lab, for the conversations at lunch and for being a good colleague.

To **Filipa** for being a great friend and companion, for all conversations until late hours in the lab when I was doing the experiments with macrophages, and for always encouraging me to be better.

To **Joana** by her friendship and companionship, for supporting me whenever it was necessary even though "the other side of the door" and often just talking by signs.

To **Vera** I would like to thank for their help in the early months in the lab, and to always be present whenever I needed.

To **Cláudia** for spend part of their time helping me when I started to give my first steps in the lab, and for over the years to be a friend always ready to help.

To **André,** for most of the time the only male presence in the lab, for always remember us that “The Summer is coming!”.

To the trainees who have passed by the lab, **Fábio** for all the work that we have done together with *Desulfovibrio*, to **Catarina**, **Luís**, **Cláudia** and **Marco**, for all lunch talks and for contributing to the good environment.

Thank you all for your friendship and for the good moments shared over the last years.

Everyone in the 3rd floor of ITQB

I would like to thank also to everyone in the **3rd floor** of ITQB, past and present people, for contributing to the helpful and friendly environment.

Aos meus amigos

Gostaria de agradecer também aos meus amigos de sempre e para sempre, o nosso “núcleo duro” vocês sabem quem são! Obrigado a todos pelo apoio e amizade ao longo destes anos. Quero agradecer especialmente ao **João Daniel** e **Viviane** que mais que amigos neste momento se tornaram família e que sempre estiveram lá quando foi preciso. Ao **Daniel Coelho** quero agradecer por sempre ter demostrado interesse no meu trabalho, por ter feito um esforço para ler os meus artigos e tentar perceber o que faço no laboratório.

À minha Família

À minha família quero agradecer em primeiro lugar aos **meus pais** pelo vosso amor e apoio incondicional, por terem acreditado em mim e me darem força para ir sempre mais longe, é a vocês que devo aquilo que sou hoje.

À minha irmã **Daniela**, quero agradecer pela amizade, por estar sempre disponível para me ajudar e pelo apoio incondicional.

Aos meus **avós**, agradeço por me acompanharem e ajudarem ao longo de toda a minha vida e fico muito feliz de ver o vosso orgulho de terem uma netinha Doutora.

Aos meus tios, em especial à minha **tia Mafalda**, que ao longo destes 29 anos tem sido uma segunda mãe que me tem ajudado muito, sem ti teria sido muito mais difícil chegar onde estou hoje.

À minha prima **Mara** quero agradecer por todo o apoio, companheirismo e amizade.

Francisco

Ao meu marido **Francisco**, quero agradecer por estar sempre ao meu lado, por toda a paciência que tem comigo mesmo quando eu não tenho a mesma com ele, por estar sempre disponível para me ouvir a falar de tudo o que se passou no meu dia, por ser o meu melhor amigo, e por me amar incondicionalmente e me fazer tão feliz.

Fundação para a Ciência e Tecnologia

Fundação para a Ciência e Tecnologia (FCT) for the financial support crucial for the accomplishment of this work and by awarding a PhD grant (SFRH/BD/44140/2008).

This thesis is dedicated to my parents

Thesis Outline

The work presented in this thesis was performed at the Molecular Genetics of Microbial Resistance Laboratory from the Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa under the supervision of Dr. Lígia M. Saraiva and co-supervision of Prof. Miguel Teixeira.

The main objective of this thesis was to elucidate the mechanisms of resistance in *Desulfovibrio vulgaris* Hildenborough against oxidative and nitrosative stresses.

This dissertation is divided in three main parts. Part I consists in three introductory chapters: Chapter 1 describes the sulfate reducing bacteria group with particular focus on *Desulfovibrio* genus; Chapter 2 addresses the iron metabolism in *Desulfovibrio*; and Chapter 3 is an overview on the general responses of *Desulfovibrio* to oxygen and nitric oxide stresses. Part II of this thesis contains the experimental results achieved during this work, which is divided in two chapters based on original publications. Part III presents a general discussion and conclusion of all the work performed.

Thesis Publications

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

Figueiredo, MC, Lobo, SA, Carita, JN, Nobre, LS & Saraiva, LM (2012). Bacterioferritin protects the anaerobe *Desulfovibrio vulgaris* Hildenborough against oxygen. *Anaerobe*, 18:454-458.

Figueiredo, MC, Lobo, SA, Sousa, SH, Pereira, FP, Wall, JD, Nobre, LS & Saraiva, LM (2013). Hybrid Cluster Proteins and Flavodiiron Proteins Afford Protection to *Desulfovibrio vulgaris* upon Macrophage Infection. *J Bacteriol*, 195:2684-2690.

Abstract

The work presented in this dissertation aimed to unravel the defense mechanisms of the anaerobic sulfate reducing bacterium *Desulfovibrio* (*D.*) *vulgaris* Hildenborough against oxidative and nitrosative stresses.

Desulfovibrio spp. are usually found in anaerobic niches in soil, marine and fresh waters and sediments, but also in zones periodically exposed to oxygen. Ecologically, *Desulfovibrio* spp. play an important role in bioremediation of heavy metals due to their capacity to precipitate toxic metal ions from environmental contaminants. However, because of their ability to cause metal corrosion these bacteria have a strong negative economic impact, constituting a major problem in oil and gas industries. These microorganisms are also part of the normal microbial flora present in the gut of animals and humans. Although recent reports revealed that they can act as opportunistic pathogens, the function in the intestinal ecosystem remains essentially unknown.

In spite of *Desulfovibrio* spp. being anaerobic microorganisms, their presence in oxic-anoxic interfaces shows that pathways for defense against toxic reactive oxygen species (ROS) must be active. Hence, in the first part of this work, transcriptomic and physiological approaches were used to evaluate the effect of oxygen on the expression of genes putatively involved in *D. vulgaris* oxygen resistance. The results showed that the gene for the iron storage protein bacterioferritin (*bfr*) exhibited high transcriptional induction upon exposure of cells to high levels of oxygen. To clarify the contribution of bacterioferritin to oxygen resistance, a *bfr* mutant strain was analyzed. The data revealed that the absence of *bfr* significantly decreases the survival of *D. vulgaris* in oxygenated environments. Furthermore, the *bfr* mutant strain exhibited a higher content of

ROS in relation to the wild type indicating that bacterioferritin contributes to avoiding ROS formation.

To assess the regulation of *D. vulgaris* bacterioferritin, we examined the transcription profile of the *bfr* gene in cells of two independent mutant strains inactivated in two general regulators, namely the ferric uptake regulator (Fur) and the peroxide regulator (PerR). While *bfr* gene transcription does not suffer alteration in the Δ*fur* mutant strain, it is repressed in the Δ*perR* mutant, showing that the expression of *D. vulgaris bfr* is under the control of PerR. Analysis of the *bfr* putative promoter region allowed identification of a potential PerR binding site located upstream of the start codon of *bfr*, with the sequence TAAACGAATCTTTACACAC. Altogether, it was demonstrated that the iron storage bacterioferritin provides protection for *D. vulgaris* against oxygen stress.

In recent years, *Desulfovibrio* spp. have been proposed to be associated with gastrointestinal diseases such as inflammatory bowel disease, Crohn's disease and ulcerative colitis. Due to its potential pathogenicity it is crucial to understand the mechanisms whereby these microorganisms overcome the innate immune system of the host. Cells of the innate immune system produce reactive oxygen species and reactive nitrogen species (RNS) to fight pathogens and, in order to detoxify these compounds, bacteria activate several protective systems. Therefore, in the second part of this work, the response of *D. vulgaris* to nitric oxide was studied. *D. vulgaris* was exposed to a nitric oxide donor and the transcription of genes coding for proteins potentially involved in NO protection, such as rubredoxin:oxygen oxidoreductase proteins (*roo1* and *roo2*) and hybrid cluster proteins (*hcp1* and *hcp2*) was analyzed. Under nitrosative stress conditions, the expression of *hcp2* gene is highly induced while those of the other genes analyzed did not vary significantly. Next, we tested the ability of *D. vulgaris* strains with the two *roo* and *hcp* genes deleted to grow in the presence of a nitric oxide donor. The *D. vulgaris hcp2* mutant was found to be the most susceptible strain to nitrosative stress.

Moreover, the contribution of the Hcp and Roo proteins to the NO consumption of *D. vulgaris* was evaluated by means of a NO-electrode in cell lysates of wild type and mutant strains grown anaerobically and treated with a NO donor. Untreated cells of *roo* and *hcp* mutants showed no major differences in the NO consumption rates; however, upon nitrosative stress exposure *roo1* and *roo2* mutant strains exhibited lower NO consumption in comparison with the wild type strain. A strain of *D. vulgaris* with *roo1* deleted had the lowest NO consumption ability, revealing that, among the four proteins, Roo1 is the major contributor to the NO reduction capability of the *D. vulgaris* cells.

In vivo studies performed with animal cells suggest that *D. vulgaris* is not capable of intracellular replication in macrophages but survives in the extracellular environment when co-cultured with macrophages. Importantly, the bacterium proved to be able to activate one of the key weapons of the innate immune system, namely the inducible nitric oxide synthase (iNOS) present in macrophages that releases NO to fight pathogens. Indeed, exposure of *D. vulgaris* wild type to macrophages significantly decreases the survival of the microorganism, which is attenuated upon inhibition of iNOS.

To evaluate the role of Roo and Hcp in *Desulfovibrio* during infection of macrophages, the viability of the mutant strains upon macrophage incubation was analyzed. All mutant strains exhibited viability reduced by approximately 90%, while that of the parental strain decreased by only 30%. This clearly shows that Hcp and Roo proteins protect *D. vulgaris* during exposure to mammalian macrophages.

In conclusion, this thesis contributed to better understanding the defense mechanisms of *D. vulgaris* against oxygen and nitric oxide. In particular, the relation between intracellular iron storage and oxidative stress response was revealed through the role of *D. vulgaris* bacterioferritin in survival upon oxygen exposure. Furthermore, *D. vulgaris* was shown to activate the innate immune

system, with Roo and Hcp proteins contributing to the survival of the bacterium *D. vulgaris* when facing damaging stresses imposed by the host.

Resumo

O trabalho apresentado nesta dissertação tem como objetivo elucidar os mecanismos de defesa da bactéria anaeróbica redutora de sulfato *Desulfovibrio* (*D.*) *vulgaris* contra os stresses oxidativo e nitrosativo.

Os membros do género *Desulfovibrio* são normalmente encontrados em nichos anaeróbios do solo, nomeadamente ambientes marinhos e sedimentos, mas também em zonas periodicamente expostas ao oxigénio. Ecologicamente, as bactérias do género *Desulfovibrio* têm um papel importante, principalmente na biorremediação de metais pesados visto terem a capacidade de separarem por precipitação metais tóxicos de contaminantes ambientais. Contudo, devido também à sua capacidade de causar corrosão de metais, estas bactérias têm um forte impacto económico negativo, constituindo um sério problema para as indústrias de petróleo e gás. Estes microrganismos fazem também parte da flora microbiana normalmente presente no intestino de animais e humanos. Embora estudos recentes tenham revelado que estas bactérias podem atuar como agentes patogénicos oportunistas, a sua função no ecossistema intestinal permanece essencialmente desconhecida.

Apesar de as espécies de *Desulfovibrio* serem microrganismos anaeróbios, a sua presença em habitats expostos ao oxigénio demonstra que contêm mecanismos de defesa contra as espécies reativas de oxigénio (ERO) tóxicas. Assim, na primeira parte deste trabalho realizaram-se estudos transcriptómicos e fisiológicos para avaliar o efeito do oxigénio na expressão de genes hipoteticamente envolvidos na resistência ao oxigénio em *D. vulgaris*. Os resultados mostraram que quando as células de *D. vulgaris* foram expostas a altas concentrações de oxigénio o gene que codifica para a proteína armazenadora de ferro bacterioferritina (*bfr*) exibiu uma elevada indução da transcrição. Para

clarificar a contribuição da bacterioferritina para a resistência ao oxigénio, uma estirpe mutada no gene *bfr* foi analisada. Os dados revelaram que a ausência do gene *bfr* diminuiu significativamente a sobrevivência de *D. vulgaris* em ambientes oxigenados. Além disso, a estirpe mutada no gene *bfr* apresentou um teor mais elevado de ERO em relação à estirpe selvagem indicando que a bacterioferritina contribui para evitar a acumulação de ERO.

Para avaliar a regulação da bacterioferritina de *D. vulgaris*, examinou-se o perfil de transcrição do gene *bfr* em células de duas estirpes de *D. vulgaris* mutadas independentemente em dois reguladores gerais, nomeadamente no regulador de absorção de ferro (Fur) e no regulador de peróxido (PerR). Enquanto a transcrição do gene *bfr* não sofre alteração na estirpe mutante Δ*fur*, a sua expressão é repressa no mutante Δ*perR*, demonstrando que a expressão da *bfr* de *D. vulgaris* está sob o controlo do regulador PerR. Adicionalmente, a análise da região promotora putativa do gene *bfr* permitiu a identificação de um potencial local de ligação localizado a montante do codão de iniciação da *bfr*, com a sequência TAAACGAATCTTTACACAC. Com estes estudos demonstramos que a bacterioferritina protege *D. vulgaris* contra o stress oxidativo.

Nos últimos anos, algumas estirpes de *Desulfovibrio* têm sido propostas como estando associadas a doenças gastrointestinais tais como doença inflamatória do intestino, doença de Crohn e colite ulcerosa. Devido à sua potencial patogenicidade é crucial compreender os mecanismos usados por estes microrganismos para superar o sistema imune inato do hospedeiro. As células do sistema imune inato produzem ERO e espécies reativas de nitrogénio (ERN) para combater os patogénicos. Por forma a fazer a destoxificação destes compostos, as bactérias contra-atacam ativando vários sistemas de proteção. Assim na segunda parte deste trabalho, foi estudada a resposta de *D. vulgaris* ao óxido nítrico (NO). Para isso, *D. vulgaris* foi exposto a um dador de óxido nítrico e a transcrição de genes que codificam para proteínas potencialmente envolvidas na proteção contra

o NO, tais como as proteínas rubredoxina:oxigénio oxidoreductase (Roo1 e Roo2) e "hybrid cluster protein" (Hcp1 e Hcp2), foi analisada. Sob condições de stress nitrosativo, a expressão do gene *hcp2* é altamente induzida enquanto a expressão dos outros genes analisados não variou significativamente. Em seguida, foi testada a capacidade de estirpes de *D. vulgaris* mutadas nos genes *roo* e *hcp* para crescer na presença de um dador de óxido nítrico. Verificou-se que a estirpe de *D. vulgaris* mutada no gene *hcp2* é a mais suscetível ao stress nitrosativo. A contribuição das proteínas Roo e Hcp para o consumo de NO em *D. vulgaris* foi também avaliada, por meio de um eléctrodo de NO, em lisados de células da estirpe selvagem e das estirpes mutantes quando crescidas anaerobicamente e tratadas com um doador de NO. As células não tratadas dos mutantes *roo* e *hcp* não apresentaram grandes diferenças nas taxas de consumo de NO. No entanto, após a exposição ao stress nitrosativo as estirpes mutantes *roo1* e *roo2* apresentaram menor consumo de NO em comparação com a estirpe selvagem. A estirpe de *D. vulgaris* deletada no gene *roo1* exibiu a menor capacidade de consumo de NO revelando que, de entre as quatro proteínas, a Roo1 é a principal proteína responsável pela capacidade de redução de NO nas células de *D. vulgaris*.

Estudos *in vivo* realizados com células animais sugerem que *D. vulgaris* não é capaz de replicação intracelular nos macrófagos, mas sobrevive no ambiente extracelular quando co-cultivado com os macrófagos. Para além disso, estas bactérias mostraram ser capazes de ativar uma das principais armas do sistema imune inato, nomeadamente a proteína produtora de óxido nítrico (iNOS) presente nos macrófagos, que liberta NO para combater organismos patogénicos. De facto, a exposição da estirpe selvagem *D. vulgaris* aos macrófagos reduziu significativamente a sobrevivência deste microrganismo, sendo atenuada com a inibição da iNOS.

Para avaliar o papel de Roo e Hcp em *Desulfovibrio* durante a infeção dos macrófagos, a viabilidade das estirpes mutantes mediante a incubação com

macrófagos foi analisada. Todas as estirpes mutantes exibiram a viabilidade reduzida em aproximadamente 90%, enquanto a estirpe selvagem diminuiu apenas 30%, mostrando claramente que as proteínas Roo e Hcp protegem *D. vulgaris* durante a exposição a macrófagos de mamíferos.

Em conclusão, este trabalho contribuiu para uma melhor compreensão dos mecanismos de defesa de *D. vulgaris* contra o stress oxidativo e nitrosativo. Em particular, a relação entre a armazenagem de ferro intracelular e resposta ao stress oxidativo foi revelada através do papel da bacterioferritina na sobrevivência de *D. vulgaris* quando exposto ao oxigénio. Além disso, *D. vulgaris* mostrou ativar o sistema imunitário inato, e que as proteínas Roo e Hcp contribuem para a sobrevivência da bactéria *D. vulgaris* quando este enfrenta stresses prejudiciais impostos pelo hospedeiro.

Abbreviations list

ALA	δ-Aminoleaevulinic acid
AMP	Adenosine-5´-phosphate
APS	Adenosine-5´-phophosulfate
ATCC	American type culture collection
ATP	Adenosine triphosphate
Bp	Base pair
cDNA	Complementary DNA
CFU	Colony Forming Units
CO	Carbon monoxide
CO_2	Carbon dioxide
CoA	Coenzyme
DCFH-DA	2´,7´-dichlorofluorescein diacetate
DMEM	Dulbecco´s Modified Eagle medium
DNA	Deoxyribonucleic acid
e^-	Electron
eNOS	Endothelial nitric oxide synthase
FDP	Flavodiiron protein
Fe	Iron
Fe-S	Iron-sulfur cluster
FI	Fluorescence intensity
FMN	Flavin mononucleotide
GSNO	S-Nitrosoglutathione
H^+	Proton
H_2	Hydrogen
H_2O	Water
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulfide
HMB	Hydroxymethylbilane
HS^-	Sulfide
HSO_3	Bisulfite
INF-γ	Interferon-γ
iNOS	Inducible nitric oxide synthase
Km	Kanamycin
L-NMMA	N^G-monomethyl-L-arginine acetate salt

LPS	Lipopolysaccharides
M	Molar
Mb	Mega base pair
MOI	Multiplicity of infection
mRNA	messenger RNA
N_2O_3	Nitrogen trioxide
NADH	β-Nicotinamide adenine dinucleotide, reduced form
NADPH	β-Nicotinamide adenine dinucleotide phosphate, reduced form
ND	Not determined
NH_3	Ammonia
NH_4^+	Ammonium ion
nm	Nanometer
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
$^{\bullet}NO_2$	Nitrogen dioxide
NO_2	Nitrite
NO_3^-	Nitrate
NOS	Nitric oxide synthase
O_2	Dyoxygen
$O_2^{\bullet-}$	Superoxide anion
OD	Optical density
$^{\bullet}OH$	Hydroxyl radical
OH^-	Hydroxide ion
$OHOO^-$	Peroxynitrite
PBG	Porphobilinogen
PBS	Phosphate buffer
PCR	Polymerase chain reaction
Phox	NADPH oxidase
Pi	Inorganic phosphate
PPi	Pyrophosphate
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase-polimerase chain reaction
S	Supplemental
S^{2-}	Sulfide
$S_2O_3^{2-}$	Thiosulfate

$S_3O_6^{2-}$	Trithionate
SH	Siroheme
SO_4^{2-}	Sulfate
spp.	Species
SRB	Sulfate reducing bacteria
SRO	Sulfate reducing organisms
TSAS	Tryptic Soy Agar medium Supplemented
wt	Wild type
Δ	Deletion

Latin abbreviations

e.g.	exempli gratia, for example
et al.	et alia, and other people

Strains

B. brevis	*Bacillus brevis*
B. melitensis	*Brucella melitensis*
B. subtilis	*Bacillus subtilis*
C.	*Campylobacter*
D.	*Desulfovibrio*
E.	*Escherichia*
H.	*Helicobacter*
N.	*Neisseria*
P.	*Pseudomonas*
S. aureus	*Staphylococcus aureus*
S. enterica	*Salmonella enterica* serovar Typhimurium

Amino acids

A	**Ala**	Alanine	**M**	**Met**	Methionine
C	**Cys**	Cysteine	**N**	**Asn**	Asparagine
D	**Asp**	Aspartic acid	**P**	**Pro**	Proline
E	**Glu**	Glutamic acid	**Q**	**Gln**	Glutamine
F	**Phe**	Phenylalanine	**R**	**Arg**	Arginine
G	**Gly**	Glycine	**S**	**Ser**	Serine
H	**His**	Histidine	**T**	**Thr**	Threonine
I	**Ile**	Isoleucine	**V**	**Val**	Valine
K	**Lys**	Lysine	**W**	**Trp**	Tryptophan
L	**Leu**	Leucine	**Y**	**Tyr**	Tyrosine

Table of Contents

Part 1 – Introduction

Chapter 1

Sulfate Reducing Bacteria

Chapter 2

Desulfovibrio and iron homeostasis

Chapter 3

Desulfovibrio responses to oxidative and nitrosative stresses

Part II – Results

Chapter 1

Bacterioferritin protects the anaerobe *Desulfovibrio vulgaris* Hildenborough against oxygen

Chapter 2

Hybrid cluster proteins and flavodiiron proteins afford protection to *Desulfovibrio vulgaris* upon macrophage infection

Part III – General discussion

Chapter 1

General discussion

Introduction

Part 1

Chapter 1

Sulfate Reducing Bacteria

Chapter 1

Sulfate Reducing Bacteria

1.1 - General overview of Sulfate Reducing Bacteria

Sulfate reducing bacteria (SRB) are anaerobic prokaryotic microorganisms capable of dissimilatory sulfate reduction. This reduction is coupled with the oxidation of a wide range of electron donors, such as organic acids (*e.g.* formate, fumarate, or lactate), alcohols, fatty acids and molecular hydrogen. Although sulfate is the primary electron acceptor, SRB are metabolic versatile and therefore can use other terminal electron acceptors such as elemental sulfur, fumarate, nitrate, nitrite and transition metal ions (Widdel & Bak, 1992; Thauer *et al.*, 2007; Gilmour *et al.*, 2011).

This group of microorganisms is widespread in a multitude of environments such as soil, sediments, marine and fresh waters as well as in the gut of animals and humans (Beerens & Romond, 1977; Postgate, 1979; Thauer *et al.*, 2007). These bacteria are major contributors to the global carbon and sulfur cycles, participating in the recycling of sulfur compounds by using simple organics as electron donors (Zhou *et al.*, 2011). SRB are involved in biodegradation of environmental contaminants, having the capacity to reduce and precipitate toxic heavy metals such as uranium(VI), copper(II), chromium(VI) and manganese(II) (Hockin & Gadd, 2007; Muyzer & Stams, 2008). Nevertheless, SRB constitute a problem in oil and gas industries due to their metal corrosion ability, which causes serious deterioration of pipelines and industrial equipment (Hamilton, 1985; Beech & Sunner, 2007).

The first sulfate reducing organism, the *Spirillum desulfuricans* (later named *Desulfovibrio desulfuricans*) (Figure. 1.1), was isolated in 1985 by W. M. Beijerinck a Dutch microbiologist (Beijerinck, 1895). While he was studying the annual phenomenon “eine wahren Scherecken” (a true horror), which consists

in the contamination of sewage caused by large amounts of hydrogen sulfide produced by microorganisms, he detected isolated colonies with a surrounding black iron sulfide precipitate. Beijerinck observed that *Spirillum desulfuricans* grew only in the presence of aerobic bacteria, by consuming oxygen of the medium, which lead to the classification of this microorganism as strict anaerobe.

Figure 1.1 - Scanning electron microscopy image of *D. desulfuricans*. Adapted from (Gilmour *et al.*, 2011).

The taxonomic classification of SRB has been changing a long time, and in the 1960s these microorganisms were classified according to cell morphology and ability to form spores. Despite SRB being a diverse group in relation to cell morphology (*e.g.* cocci, oval, rods, curved (vibrio), cell aggregates and filaments), these bacteria were first divided only in two genera: the nonspore-forming vibrio-shaped isolates described as *Desulfovibrio* species, and the rod-shaped spore-forming strains that were included in the new *Desulfotomaculum* genus (Campbell & Postgate, 1965; Postgate & Campbell, 1966). Nowadays, phylogenic classification of SRB is based on different taxonomic markers such as nutrition, morphology, chemical and biochemical composition (presence of desulfoviridin, lipid fatty acids and menaquinones), the GC content of the DNA and the 16S rRNA sequence analysis (Widdel & Hansen, 1992). The 16S rRNA

comparative analysis of the sulfate reducing organisms (SRO) revealed that this group is formed by seven phylogenetic lineages, five within Bacteria (Deltaproteobacteria, Clostridia, Nitrospirae, Thermodesulfobacteria and Thermodesulfobiaceae) and two within Archaea (Euryarchaeota and Crenarchaeota) (Muyzer & Stams, 2008). The group that includes only bacteria is commonly named sulfate reducing bacteria (SRB). In spite of the 16S rRNA analysis being the most utilized technique in phylogenetic profiles of SRB, studies on lateral gene transfer events among the different species of essential genes have also been done. Enzymes of the sulfate respiration pathway like dissimilatory sulfide reductase (DsrAB) and dissimilatory adenosine-5'-phosphosulfate (APS) reductase (AprBA) have been used as molecular markers providing a more precise evolutionary classification (Wagner *et al.*, 2005; Meyer & Kuever, 2007).

1.2 - Environmental impact of SRB

The importance of SRB in the environment goes further beyond their role in the biogeochemical cycling of carbon and sulfur, mainly because the end product of sulfate reduction, hydrogen sulfide, can be harmful or beneficial.

The economical interest in SRB is related with their involvement in biocorrosion of ferrous metal installations in the gas and petroleum industries. This problem results in major financial losses that in United States reaches 100 M $ per year (Beech & Sunner, 2007). Steel and iron surfaces act as substratum for microbial communities to form biofilms, which create an anaerobic atmosphere favoring the production of hydrogen sulfide by SRB. Hydrogen sulfide leads to microbial reservoir souring, a process that decreases the quality of oil and gas and causes corrosion (Muyzer & Stams, 2008). Souring can be prevented or reduced by continuous injection of low nitrate concentrations. Nitrate injection stimulates nitrate-reducing, sulfide-oxidizing bacteria (NR-SOB)

and heterotrophic nitrate-reducing bacteria (hNRB), which remove sulfide by the production of nitrite and other reactive nitrogen species. Nitrite reduces the SRB activity and the corrosion rates, affecting especially the dissimilatory sulfite reductase, an enzyme that produces sulfide. With the use of this methodology, biocorrosion is significantly reduced when compared with the traditional biocide treatment (Bodtker *et al.*, 2008; Voordouw, 2008).

Sulfate reduction can be applied beneficially to biotechnology, for instance in the removal of heavy metals. The ability of SRB to remediate heavy metals is dependent on the metal concentration in solution since the effect of these metals are toxic/inhibitory at higher concentrations and stimulatory at lower concentrations (Cabrera *et al.*, 2006). Metal sulfates like cadmium, cobalt, copper, iron, nickel, uranium and zinc are highly soluble but the corresponding metal sulfides have low solubility. Hence, the sulfide produced during microbial sulfate reduction promotes metal precipitation allowing the recovery and reuse of the metals. The production of the insoluble mineral has been used as a mechanism for metal sequestration in metal-processing wastes and acid mine-drainage waters (Hockin & Gadd, 2007; Muyzer & Stams, 2008). This bioremediation process is performed in bioreactors that were designed for optimum hydrogen sulfide (H_2S) production (Malik, 2004). Besides the chemical indirect reduction of metals and metalloids due to the production of H_2S, these sulfate reducers can also reduce metals indirectly by an enzymatic way. Metalloenzymes, like hydrogenases and cytochromes, are involved in this reduction process. For example, in *D. vulgaris*, the tetraheme cytochrome c_3 participates in reduction of U(VI) to U(V) (Cabrera *et al.*, 2006) and both cytochrome c_3 and Fe-hydrogenase reduce Cr(VI) to Cr(III) (Michel *et al.*, 2003).

1.3 - *Desulfovibrio* genus

The most extensively studied genus among SRB is *Desulfovibrio* that belongs to the class of δ-proteobacteria and is usually found in aquatic habitats, soil and sediments, oil and natural gas wells, sewages and in the digestive tract of animals and humans (Muyzer & Stams, 2008; Zhou *et al.*, 2011). Cells of *Desulfovibrio* have a Gram-negative staining, are curved (vibrio) or rod-shaped (average width of 0.5 – 1.3 x 0.8 - 5 µm), often motile with a single polar flagellum (Figure 1.2). The DNA GC content is between 49 - 65% and the optimal growth temperature is from 30 to 38 ºC (Widdel & Bak, 1992).

Figure 1.2 – Scanning electron microscopy image of *D. desulfuricans*. Adapted from (Gilmour *et al.*, 2011).

Desulfovibrio species have been studied from the metabolic and biochemical point of view due to their widespread environmental distribution and the molecular methods for engineering this genus that have becoming accessible, such as chromosomal modifications and deletions, plasmid insertion and replication (van den Berg *et al.*, 1989; Keller *et al.*, 2011). Advances in genome sequencing during the last ten years also contributed for a better understanding of these microorganisms behavior. Presently, there are 12 strains of *Desulfovibrio* with its genome fully sequenced (Zhou *et al.*, 2011). *Desulfovibrio vulgaris* Hildenborough (ahead annotated *D. vulgaris*), first isolated from clay soil near Hildenborough, UK, in 1964 (Postgate, 1979), was the first SRO with the genome completed sequenced (Heidelberg *et al.*, 2004). For this reason, *D. vulgaris* Hildenborough was been used as a model organism

of sulfate reducing bacteria in a larger number of physiological and biochemical studies.

The work presented in this thesis was performed in *D. vulgaris* with the aim of understanding the behavior of sulfate reducers when exposed to oxidative and nitrosative stress conditions.

1.3.1 - Metabolism

Energy transduction

Energy transduction models

Desulfovibrio spp. are able to oxidize several substrates, such as, lactate, pyruvate, ethanol, molecular hydrogen, formate, malate, sugars, amino acids, aromatic hydrocarbons or alkanes (Rabus *et al.*, 2006). The first mechanism proposed for energy conservation in *Desulfovibrio* spp. growing in lactate and sulfate is the so-called "hydrogen cycling" (Odom & Jr., 1981). Molecular hydrogen (H_2) besides serving as sole electron donor with carbon dioxide (CO_2) and acetate as carbon sources (chemolithoheterotrophy) or only carbon dioxide (autotrophy), is also proposed to play a central role in the generation of a chemiosmotic gradient required for the reduction of sulfate (Odom & Jr., 1981; Liamleam & Annachhatre, 2007; Kanekar *et al.*, 2012). In this model, the electrons produced during the oxidation of lactate to pyruvate and of pyruvate to acetyl-CoA are used by cytoplasmic hydrogenases to form H_2, which diffuses across the cytoplasmic membrane and is then oxidized by the periplasmic hydrogenases and used to reduce sulfate (Odom & Jr., 1981). The gradient generated in this process by proton translocation is coupled with energy generation (Badziong & Thauer, 1980). *D. vulgaris* contains at least six hydrogenases: four of them are periplasmic and therefore probably involved in hydrogen oxidation, namely [Fe] hydrogenase, [NiFeSe] hydrogenase, and two

[NiFe] hydrogenases (Fauque *et al.*, 1988; Heidelberg *et al.*, 2004; Caffrey *et al.*, 2007). The other two hydrogenases are included in cytoplasmic-facing membrane-bound associated complexes EchABCDEF and CooMKLXUH, and proposed to be involved in cytoplasmic proton reduction (Pereira *et al.*, 2007). The principal drawback of this proposal is that the genes coding for these hydrogenases are not widespread in SRO (Pereira *et al.*, 2011), and the production of hydrogen from lactate oxidation is an energetically unfavorable process that probably occurs only when the partial pressure of hydrogen is maintained at low levels (Traore *et al.*, 1981; Odom & Peck, 1984). Hence, this mechanism is probably only one of the several possible pathways for energy conservation in *Desulfovibrio*.

Besides hydrogen cycling, other alternative mechanisms for energy conservation have been considered, such as formate and carbon monoxide (CO) cycling (Voordouw, 2002). The oxidation of formate to CO_2 and H^+ is catalyzed by formate dehydrogenases (Fdh). In *D. vulgaris* there are three Fdh, being two of them periplasmic and the other a membrane-associated facing the periplasm (Sebban *et al.*, 1995; Heidelberg *et al.*, 2004; ElAntak *et al.*, 2005). As well as hydrogen cycling, the formate cycling model proposes that formate is formed in the cytoplasm from lactate oxidation and then diffuses to the periplasm where is oxidized by formate dehydrogenases. The generated protons are used in adenosine-5'-triphosphate (ATP) synthesis and the electrons are donated to the periplasmic *c*-type cytochromes and membrane-bound complexes (Voordouw, 2002). The mechanism of CO cycling consists in cytoplasmic formation of CO from pyruvate generated in lactate oxidation, which is subsequent transformed in CO_2 and H_2 by a cytoplasmic CO dehydrogenase and a CO-dependent hydrogenase. The hydrogen produced is then oxidized by periplasmic hydrogenases to reduce sulfate (Voordouw, 2002). However, a recent study revealed that *D. vulgaris* had a very limited growth on CO, thus suggesting that

CO cycling does not confer significant energetic advances, and probably, it does not serve as growth substrate (Rajeev *et al.*, 2012).

Electron carriers and transmembrane electron transport

In terms of energy conservation, the components involved in the respiratory chain of SRB are not yet fully understood. Contrary to other anaerobic respiratory processes, in SRB the terminal reductases (APS reductase and DsrAB) are not directly involved in membrane charge translocation as they occur in the cytoplasm (Matias *et al.*, 2005). Nevertheless, several membrane complexes had been proposed to contribute to the electron transfer during sulfate respiration (Pereira *et al.*, 2006; Pereira, 2008). Among sulfate reducers two conserved membrane complexes have been identified: QmoABC (quinine-interacting membrane-bound oxidoreductase complex), which was first isolated from *D. desulfuricans* ATCC27774 (Pires *et al.*, 2003), and the DsrMKJOP complex, first purified from *Archaeoglobus fulgidus* and named Hme complex for "Hdr-like menaquinol-oxidizing enzyme" (Mander *et al.*, 2002). Subsequently, in 2006, this complex was also isolated from *D. desulfuricans* ATCC27774 (Pires *et al.*, 2006). QmoABC is proposed to transfer electrons from menaquinone pool to APS reductase (Pires *et al.*, 2003), and DsrMKJOP complex, may be involved in electron transfer to the sulfite reductase (Pires *et al.*, 2006).

Desulfovibrio spp. also contain a high number of periplasmic or membrane-bound *c*-type cytochromes that mediate the electron transfer. The *c*-type cytochromes include the monoheme cytochrome c_{553} and several multiheme cytochromes, such as the tetraheme cytochrome c_3 type I (TpIc_3) and type II (TpIIc_3). Interestingly, the first multiheme cytochrome to be successfully expressed in *E. coli* was the *D. desulfuricans* ATCC 27774 tetraheme cytochrome c_3 (da Costa *et al.*, 2000). In *Desulfovibrio* spp., the periplasmic cytochromes *c*

acts as electron acceptors for periplasmic hydrogenases and formate dehydrogenases. The periplasmic cytochromes c_{553} and c_3 are proposed to transfer electrons to the membrane-bound associated complexes, which contain a soluble cytochrome subunit of the c_3 type family as electron acceptor. The first transmembrane complex recognized in *Desulfovibrio* was the high molecular mass cytochrome *c* (Hmc) that contains 16 *c*-type hemes (Matias *et al.*, 2005; Pereira, 2008). In *D. desulfuricans* the Hmc complex is not present, which instead contains a nine-heme cytochrome complex (9Hc) (Saraiva *et al.*, 2001). Interestingly, the presence of both complexes in the same bacterium was not yet observed (Pereira *et al.*, 2011). The third example is the transmembrane complex Tmc that was first isolated from *D. vulgaris* in 2006, this complex includes a tetraheme cytochrome c_3 type subunit (TmcA or Type II cytochrome c_3), an integral membrane cytochrome *b* protein, and two cytoplasmic proteins (Pereira *et al.*, 2006). The expression of *tmc* genes is higher in cells growing in hydrogen/sulfate when compared with lactate/sulfate, suggesting a role of Tmc complex in hydrogen oxidation (Pereira, 2008).

Given the metabolic diversity of SRB, it is likely that, according to the electron donor used, several respiratory chains exist making difficult the development of a unique model of electron transport. The exception is the last set of reactions involved in sulfate activation and reduction which are common to all pathways (Rabus *et al.*, 2006).

Sulfate reduction

Desulfovibrio spp. obtain energy by performing dissimilatory sulfate reduction, using sulfate as electron acceptor to produce hydrogen sulfide. Hydrogen sulfide has a typical smell and in the presence of iron minerals forms black precipitates of ferrous sulfide, which makes easy the recognition of these microorganisms (Rabus *et al.*, 2006). The reduction of sulfate to sulfide is an

eight electron process that occurs in the cytoplasm (Figure 1.3). After sulfate has been transported into the cell, it needs to be activated since this molecule is thermodynamically stable and by itself is not a suitable electron acceptor. Sulfate is first activated with ATP to yield adenosine phosphosulfate (APS), a reaction catalyzed by the ATP sulfurylase. The next steps of the pathway are the reduction of APS to bisulfite and adenosine-5´-phosphate (AMP) catalyzed by APS reductase, and the reduction of bisulfite to sulfide by the enzyme sulfite reductase, which in *Desulfovibrio* is known as desulfoviridin. Two different pathways for the reduction of bisulfite have been proposed: i) the sequential reduction in three two-electron reduction steps, with the formation of the intermediates trithionate and thiosulfate by trithionate and thiosulfate reductases; ii) a direct six-electron reduction without the formation of any intermediates (Figure 1.3) (Hansen, 1994; Rabus *et al.*, 2006; Thauer *et al.*, 2007).

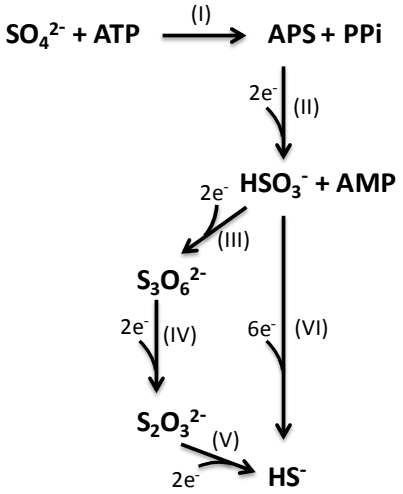


Figure 1.3 – Pathway of dissimilatory sulfate reduction.
SO_4^{2-} - sulfate; ATP - adenosine-5'-triphosphate; APS - adenosine-5´-phosphosulfate; PPi - pyrophosphate; HSO_3 - bisulfite; AMP - adenosine-5´-phosphate; $S_3O_6^{2-}$ - trithionate; $S_2O_3^{2-}$ - thiosulfate; HS^- - sulfide; e^- - electron. Reaction catalyzed by (I) - ATP sulfurylase; (II) - APS reductase; (III) - sulfite reductase; (IV) - trithionate reductase; (V) - thiosulfate reductase and (VI) - sulfite reductase (desulfoviridin). Adapted from (Hansen, 1994).

Nitrate and nitrite reduction

Besides sulfate, *Desulfovibrio* spp. use several other electron acceptors, such as sulfite, thiosulfate, sulfur, nitrate and nitrite, elemental Fe^{3+}, CO_2 and

fumarate. The dissimilatory reduction of nitrate and nitrite (also called ammonification) can function as the sole energy-conserving process in some SRB such as *D. desulfuricans* ATCC 27774 and *D. gigas* (Moura *et al.*, 1997). This reaction occurs in a two step process (Figure 1.4), being the first the conversion of nitrate to nitrite, via two-electron reduction, catalyzed by the enzyme nitrate reductase. The crystal structure of the periplasmic nitrate reductase (Nap) from *D. desulfuricans* ATCC 27774 was the first to be solved for a dissimilatory nitrate reductase. The protein contains one molybdenum cofactor in the active site and a $[4Fe\text{-}4S]^{+2/+1}$ cluster (Dias *et al.*, 1999). Interestingly, nitrate reductase genes are absent from *D. vulgaris* genome, and contrary to *D. desulfuricans* ATCC 27774, this strain is not able to grow on nitrate being a non-ammonifying microorganism (Pereira *et al.*, 2000; Marietou *et al.*, 2005). The second step consists in the six-electron reduction of nitrite to ammonia and is performed by nitrite reductase enzymes (NiR). Cytochrome *c* nitrite reductase (ccNiR) from *D. desulfuricans* ATCC 27774 is a membrane-bound protein composed by two subunits, the periplasmic pentaheme catalytic subunit NrfA and the transmembrane tetraheme subunit NrfH (Almeida *et al.*, 2003). Although *D. vulgaris* Hildenborough is not capable of growing on nitrate, it contains a membrane protein complex identified as a cytochrome *c* nitrite reductase (NiR) formed by two cytochrome *c* subunits with nitrite and sulfite reductase activities, which reduce nitrite to ammonium in a reaction that does not sustain growth (Pereira *et al.*, 2000).

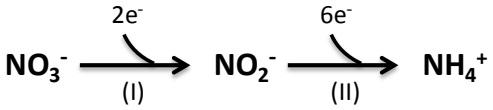


Figure 1.4 – Pathway of dissimilatory nitrate reduction.
NO_3^- - nitrate; NO_2^- - nitrite; NH_4^+ - ammonium ion. Reaction catalyzed by (I) nitrate reductase (Nap) and (II) cytochrome *c* nitrite reductase (NiR). Adapted from (Moura *et al.*, 2007).

Heme biosynthesis

The genus *Desulfovibrio* contains several single and multi-heme proteins. The most common heme containing proteins are the *c*-type cytochromes containing 1 (cytochrome c_{553}), 4 (cytochrome c_3), 9 (nine-heme cytochrome *c*), and 16 (dodecaheme cytochrome *c*) heme groups (Pereira & Xavier, 2005). In addition to heme *c*, other heme forms have been isolated from *Desulfovibrio* proteins, such as heme *b*, which is present in the membrane subunits of several respiratory complexes and enzymes, and heme *d* in the quinol oxygen reductases of the *bd* family (Lemos *et al.*, 2001). Recently, it was shown the presence of an *o*-type heme associated with the protein heme-copper oxygen reductase (Lamrabet *et al.*, 2011). This genus also contains unique heme derivatives such as Fe-coproporphyrin III isolated from *D. desulfuricans* ATCC 27774 bacterioferritin, (Romão *et al.*, 2000a), and iron-uroporphyrin I present in *D. gigas* rubredoxin:oxygen oxidoreductase (Romão *et al.*, 2000a). Siroheme also occurs in *Desulfovibrio* as a cofactor of the assimilatory and dissimilatory sulfite and nitrite reductases (Oliveira *et al.*, 2008).

Desulfovibrio genomes lack the genes encoding enzymes needed to produce heme from uroporphyrinogen III (UROGEN III) through the classical pathway. Instead, in *Desulfovibrio* spp. the heme synthesis occurs via an alternative pathway that involves the transformation of precorrin-2 (PC-2) into heme (Figure 1.5) (Lobo *et al.*, 2009; Bali *et al.*, 2011). Although, the first suggestions for an alternative heme biosynthetic pathway in *Desulfovibrio* were made in the 1990s, only recently the existence of an alternative heme branch was shown (Bali *et al.*, 2011). The confirmation of this alternative pathway arose when *D. vulgaris* cells lysates incubated with sirohydrochlorin (SHC) and siroheme (SH) allowed the identification of the different intermediates of this novel pathway and also the final product heme *b* (Bali *et al.*, 2011).

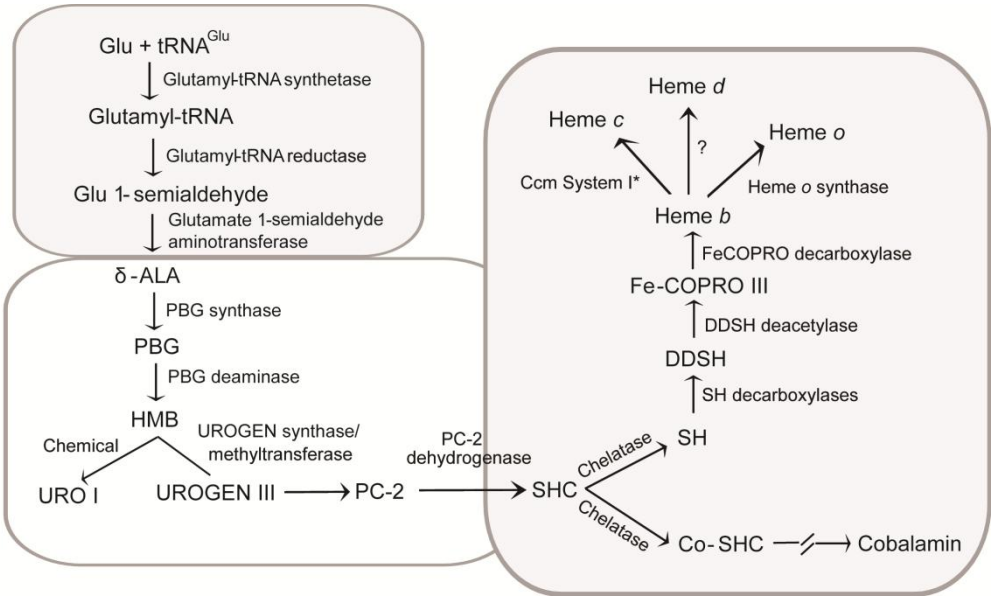


Figure 1.5 - Heme biosynthesis in *Desulfovibrio*.
δ-Aminolevulinic acid (δ-ALA), porphobilinogen (PBG), hydroxymethylbilane (HMB), uroporphyrinogen III (UROGEN III), uroporphyrin I (URO I), precorrin-2 (PC-2), sirohydrochlorin (SHC), cobaltsirohydrochlorin (Co-SHC), siroheme (SH), 12,18-didecarboxysiroheme (DDSH), and Fe-coproporphyrin III (Fe-COPRO III). Adapted from (Lobo *et al.*, 2012).

1.3.2 - *Desulfovibrio* in humans

Desulfovibrio spp. are normal inhabitants of the microbial flora of the human intestine together with other SRB. As previously mentioned, these bacteria are able to use as electron donors different products of the anaerobic fermentation such as lactate, acetate, pyruvate and ethanol (Thauer *et al.*, 2007). The electron acceptor sulfate is also available in the human lumen, and is obtained from drinking water, vegetables and food additives. Other potential sources of sulfate utilized by intestinal bacteria are host glycoproteins (*e.g*. mucins secreted by the gut epithelium cells) and acidic mucopolysaccharids (*e.g*. chondroitin sulfate present in epithelial cells lining the gut) (Macfarlane *et al.*, 2007). The predominant genus of SRB present in the human intestinal mucosa is *Desulfovibrio* (Gibson *et al.*, 1993). Early reports have shown the isolation of SRB from human feces from healthy individuals (Moore *et al.*, 1976). Since then

several isolates confirmed the presence of these bacteria in human feces (Beerens & Romond, 1977; Gibson *et al.*, 1988; Gibson *et al.*, 1993; Loubinoux *et al.*, 2002b; Jia *et al.*, 2012). Moreover, *Desulfovibrio* seem to be not only present in human feces but also in the gut. Measurements over 12 months revealed that mucosal *Desulfovibrio* numbers may vary significantly along time (Fite *et al.*, 2004). In addition, fecal *Desulfovibrios* are also present in relatively high levels in children younger than six months reveling that SRB are acquired very early in life (Hopkins *et al.*, 2005).

The implication of *Desulfovibrio* spp. in inflammatory bowel diseases has been suggested due to their metabolic end product hydrogen sulfide that at high concentrations promotes the damage of DNA and inhibits the butyrate oxidation pathway, which is an important energetic process for colonic epithelial cells (Pitcher & Cummings, 1996). Furthermore, patients presenting inflammatory bowel diseases showed significant high prevalence of *Desulfovibrio* strains (55%) in their feces when compared with healthy individuals (12%) (Loubinoux *et al.*, 2002b). In spite of these results, a recent study does not show a clear correlation between *Desulfovibrio* numbers and inflammatory bowel diseases (Jia *et al.*, 2012). This might be related with the differences in the methodologies used and the individual diversity in microbial flora within groups.

Over the last years, the presence of *Desulfovibrio* associated with different diseases, such as bacteremia, brain and intra-abdominal abscess, appendicitis and periodontitis have been reported (Baron *et al.*, 1992; Lozniewski *et al.*, 1999; Langendijk *et al.*, 2000; Verstreken *et al.*, 2012). Most recently, *Desulfovibrio* spp. were reported to have higher incidence in the bowel flora of autistic individuals when compared with a healthy group. However, these differences may be a result of the use of an antimicrobial drug treatment that modifies the intestinal bacterial flora and these augment may be casuistic

(Finegold, 2011b; Finegold, 2011a; Finegold *et al.*, 2012). In spite of the proposals that *Desulfovibrio* spp. may be involved in disorders of the human gut tract, their role in the intestinal ecosystem remain unclear, as these microorganisms may be carried asymptomatic in the gastrointestinal tract or act as opportunistic pathogens. So far, five species of *Desulfovibrio* were isolated from human samples: *D. piger* (peritoneal fluid, abdominal collection and feces), *D. desulfuricans* (blood, peritoneal fluid and abdominal collection), *D. fairfieldensis* (blood, peritoneal fluid, abdominal collection, pelvic collection, colorectal collection, liver abscess, urine, and periodontal pockets), *D. vulgaris* (peritoneal fluid and abdominal collection) and, more recently, *D. intestinalis* from the vaginal flora (Johnson & Finegold, 1987; Tee *et al.*, 1996; La Scola & Raoult, 1999; Loubinoux *et al.*, 2000; Loubinoux *et al.*, 2002a; Loubinoux *et al.*, 2003; Goldstein *et al.*, 2003; Pimentel & Chan, 2007; Urata *et al.*, 2008; Ichiishi *et al.*, 2010; Verstreken *et al.*, 2012; Jia *et al.*, 2012). Due to the slow growth of these microorganisms, they are difficult to identify and frequently overlooked in mixed cultures. Hence, it is possibly that their incidence in human remains underestimated (Goldstein *et al.*, 2003; Verstreken *et al.*, 2012).

Susceptibilities studies with commonly used antimicrobial agents have been done with clinical isolates from *Desulfovibrio* isolated from humans (Table 1.1) (Lozniewski *et al.*, 2001; Warren *et al.*, 2005; Nakao *et al.*, 2009). In general, the results demonstrated that the *Desulfovibrio* isolates are susceptible to metronidazole, chloramphenicol, clindamycin and imipenem. In these studies *Desulfovibrio* spp. showed to be resistant to various antimicrobial agents commonly used to treat mixed infections such as β-lactams (penicillin derivates) and also β-lactams combined with β-lactamase inhibitors. Despite this resistance to β-lactam antibiotics, only some of the analyzed isolates exhibit nitrocefin disk test positive indicative of the presence of β-lactamase enzymes. Thus, the mechanism underlying the trend of resistance to β-lactams needs to

be further investigated (Lozniewski *et al.*, 2001; Warren *et al.*, 2005; Nakao *et al.*, 2009). So far, among all *Desulfovibrio* spp. *D. fairfieldensis* seems to be the species with higher pathogenic potential, possessing the higher antimicrobial resistance to the antibiotics tested. These differences in susceptibility pattern to antibiotics among *Desulfovibrio* species reveal that is important to identify the clinical isolates at the species level in order to perform the correct antibiotic administration (Loubinoux *et al.*, 2000; Warren *et al.*, 2005; Nakao *et al.*, 2009).

Table 1.1 – Studies of antimicrobial susceptibilities of *Desulfovibrio* spp.

	(Lozniewski *et al.*, 2001)[1]	(Warren *et al.*, 2005)[2]	(Nakao *et al.*, 2009)[3]
Susceptible	Metronidazole Chloramphenicol Clindamycin Imipenem Amoxicillin-clavulanate Ticarcillin-clavulanate	Metronidazole Chloramphenicol Clindamycin Imipenem	Metronidazole Chloramphenicol Clindamycin Sulbactam-ampicilin Meropenem
Resistant	Penicillin G Piperacillin Piperacilin-tazobactam Cefoxitin Cefotetan	Penincillin Piperacillin-tazobactam Cefoxitin Ceftriaxone Ertapenem Ticarcillin-clavulanic acid	Piperacillin Piperacillin-tazobactam Cefoxitin Cefotaxime

[1] 16 strains of *Desulfovibrio* spp. isolated from patients hospitalized.
[2] Human clinical isolates: *D. fairfieldensis* (n=10), *D. piger* (n = 2), *D. desulfuricans* (n = 3), *D. vulgaris* (n = 3).
[3] 23 *Desulfovibrio* isolates from humans: *D. fairfieldensis* (n = 8), *D. desulfuricans* Essex 6 (n = 7), *D. desulfuricans* MB (n = 6), *D. piger* (n = 2).

In spite of its potential pathogenicity, the ability of *Desulfovibrio* spp. to survive when in contact with professional cells of the innate immune system such as macrophages was not yet addressed. The work presented in this thesis in Part II - Chapter 2 shows, for the first time, the behavior of *D. vulgaris* upon incubation with murine macrophages.

1.4 – References

Almeida, MG, Macieira, S, Goncalves, LL, Huber, R, Cunha, CA, Romão, MJ, Costa, C, Lampreia, J, Moura, JJ & Moura, I (2003). The isolation and characterization of cytochrome *c* nitrite reductase subunits (NrfA and NrfH) from *Desulfovibrio desulfuricans* ATCC 27774. Re-evaluation of the spectroscopic data and redox properties. *Eur J Biochem*, **270**:3904-3915.

Badziong, W & Thauer, RK (1980). Vectorial electron transport in *Desulfovibrio vulgaris* (Marburg), growing on hydrogen plus sulfate as sole energy source. *Arch Microbiol* **125**:167-174.

Bali, S, Lawrence, AD, Lobo, SA, Saraiva, LM, Golding, BT, Palmer, DJ, Howard, MJ, Ferguson, SJ & Warren, MJ (2011). Molecular hijacking of siroheme for the synthesis of heme and d_1 heme. *Proc Natl Acad Sci U S A*, **108**:18260-18265.

Baron, EJ, Bennion, R, Thompson, J, Strong, C, Summanen, P, McTeague, M & Finegold, SM (1992). A microbiological comparison between acute and complicated appendicitis. *Clin Infect Dis*, **14**:227-231.

Beech, IB & Sunner, JA (2007). Sulphate-reducing bacteria and their role in corrosion of ferrous materials In *Sulphate-reducing Bacteria: Environmental and Engineered Systems*, pp. 459-482. Edited by L. L. Barton & W. A. Hamilton. New York: Cambridge University Press.

Beerens, H & Romond, C (1977). Sulfate-reducing anaerobic bacteria in human feces. *Am J Clin Nutr*, **30**:1770-1776.

Beijerinck, WM (1895). Ueber *Spirillum desulfuricans* als ursache von sulfatreduction. *Zentralbl Bakteriol Parasitenkd*, **1**: 1-9, 49-59, 104-114.

Bodtker, G, Thorstenson, T, Lillebo, BL, Thorbjornsen, BE, Ulvoen, RH, Sunde, E & Torsvik, T (2008). The effect of long-term nitrate treatment on SRB activity, corrosion rate and bacterial community composition in offshore water injection systems. *J Ind Microbiol Biotechnol*, **35**:1625-1636.

Cabrera, G, Perez, R, Gomez, JM, Abalos, A & Cantero, D (2006). Toxic effects of dissolved heavy metals on *Desulfovibrio vulgaris* and *Desulfovibrio* sp. strains. *J Hazard Mater*, **135**:40-46.

Caffrey, SM, Park, HS, Voordouw, JK, He, Z, Zhou, J & Voordouw, G (2007). Function of periplasmic hydrogenases in the sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough. *J Bacteriol*, **189**:6159-6167.

Campbell, LL & Postgate, JR (1965). Classification of the spore-forming sulfate-reducing bacteria. *Bacteriol Rev*, **29**:359-363.

da Costa, PN, Conte, C & Saraiva, LM (2000). Expression of a *Desulfovibrio* tetraheme cytochrome *c* in *Escherichia coli*. *Biochem Biophys Res Commun*, **268**:688-691.

Dias, JM, Than, ME, Humm, A, Huber, R, Bourenkov, GP, Bartunik, HD, Bursakov, S, Calvete, J, Caldeira, J, Carneiro, C, Moura, JJ, Moura, I & Romão, MJ (1999). Crystal structure of the first dissimilatory nitrate reductase at 1.9 Å solved by MAD methods. *Structure*, **7**:65-79.

ElAntak, L, Dolla, A, Durand, MC, Bianco, P & Guerlesquin, F (2005). Role of the tetrahemic subunit in *Desulfovibrio vulgaris* Hildenborough formate dehydrogenase. *Biochemistry*, **44**:14828-14834.

Fauque, G, Peck, HD, Jr., Moura, JJ, Huynh, BH, Berlier, Y, DerVartanian, DV, Teixeira, M, Przybyla, AE, Lespinat, PA, Moura, I & et al. (1988). The three classes of hydrogenases from sulfate-reducing bacteria of the genus *Desulfovibrio*. *FEMS Microbiol Rev*, **4**:299-344.

Finegold, SM (2011a). *Desulfovibrio* species are potentially important in regressive autism. *Med Hypotheses*, **77**:270-274.

Finegold, SM (2011b). State of the art; microbiology in health and disease. Intestinal bacterial flora in autism. *Anaerobe*, **17**:367-368.

Finegold, SM, Downes, J & Summanen, PH (2012). Microbiology of regressive autism. *Anaerobe*, **18**:260-262.

Fite, A, Macfarlane, GT, Cummings, JH, Hopkins, MJ, Kong, SC, Furrie, E & Macfarlane, S (2004). Identification and quantitation of mucosal and faecal desulfovibrios using real time polymerase chain reaction. *Gut*, **53**:523-529.

Gibson, GR, Macfarlane, GT & Cummings, JH (1988). Occurrence of sulphate-reducing bacteria in human faeces and the relationship of dissimilatory sulphate reduction to methanogenesis in the large gut. *J Appl Bacteriol*, **65**:103-111.

Gibson, GR, Macfarlane, S & Macfarlane, GT (1993). Metabolic interactions involving sulphate-reducing and methanogenic bacteria in the human large intestine. *FEMS Microbiol Ecol*, **12**:117-125.

Gilmour, CC, Elias, DA, Kucken, AM, Brown, SD, Palumbo, AV, Schadt, CW & Wall, JD (2011). Sulfate-reducing bacterium *Desulfovibrio desulfuricans* ND132 as a model for understanding bacterial mercury methylation. *Appl Environ Microbiol*, **77**:3938-3951.

Goldstein, EJ, Citron, DM, Peraino, VA & Cross, SA (2003). *Desulfovibrio desulfuricans* bacteremia and review of human *Desulfovibrio* infections. *J Clin Microbiol*, **41**:2752-2754.

Hamilton, WA (1985). Sulphate-reducing bacteria and anaerobic corrosion. *Annu Rev Microbiol*, **39**:195-217.

Hansen, TA (1994). Metabolism of sulfate-reducing prokaryotes. *Antonie Van Leeuwenhoek*, **66**:165-185.

Heidelberg, JF, Seshadri, R, Haveman, SA, Hemme, CL, Paulsen, IT, Kolonay, JF, Eisen, JA, Ward, N, Methe, B, Brinkac, LM, Daugherty, SC, Deboy, RT, Dodson, RJ, Durkin, AS, Madupu, R, Nelson, WC, Sullivan, SA, Fouts, D, Haft, DH, Selengut, J, Peterson, JD, Davidsen, TM, Zafar, N, Zhou, L, Radune, D, Dimitrov, G, Hance, M, Tran, K, Khouri, H, Gill, J, Utterback, TR, Feldblyum, TV, Wall, JD, Voordouw, G & Fraser, CM (2004). The genome sequence of the anaerobic, sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough. *Nat Biotechnol*, **22**:554-559.

Hockin, SL & Gadd, GM (2007). Bioremediation of metals and metalloids by precipitation and cellular binding. In *Sulphate-reducing Bacteria: Environmental and Engineered Systems*, pp. 405-434. Edited by L. L. Barton & W. A. Hamilton. New York: Cambridge University Press.

Hopkins, MJ, Macfarlane, GT, Furrie, E, Fite, A & Macfarlane, S (2005). Characterisation of intestinal bacteria in infant stools using real-time PCR and northern hybridisation analyses. *FEMS Microbiol Ecol*, **54**:77-85.

Ichiishi, S, Tanaka, K, Nakao, K, Izumi, K, Mikamo, H & Watanabe, K (2010). First isolation of *Desulfovibrio* from the human vaginal flora. *Anaerobe*, **16**:229-233.

Jia, W, Whitehead, RN, Griffiths, L, Dawson, C, Bai, H, Waring, RH, Ramsden, DB, Hunter, JO, Cauchi, M, Bessant, C, Fowler, DP, Walton, C, Turner, C & Cole, JA (2012). Diversity and distribution of sulphate-reducing bacteria in human faeces from healthy subjects and patients with inflammatory bowel disease. *FEMS Immunol Med Microbiol*, **65**:55-68.

Johnson, CC & Finegold, SM (1987). Uncommonly encountered, motile, anaerobic gram-negative bacilli associated with infection. *Rev Infect Dis*, **9**:1150-1162.

Kanekar, PP, Kanekar, SP, Kelkar, AS & Dhakephalkar, PK (2012). Halophiles - Taxonomy. Diversity, Physiology and Applications. In *Microorganisms in Environmental Management: Microbes and Environment*, pp. 1-34. Edited by T. Satyanarayana, B. N. Johri & A. Prakash. New York: Springer.

Keller, KL, Wall, JD & Chhabra, S (2011). Methods for engineering sulfate reducing bacteria of the genus *Desulfovibrio*. *Methods Enzymol*, **497**:503-517.

La Scola, B & Raoult, D (1999). Third human isolate of a *Desulfovibrio* sp. identical to the provisionally named *Desulfovibrio fairfieldensis*. *J Clin Microbiol*, **37**:3076-3077.

Lamrabet, O, Pieulle, L, Aubert, C, Mouhamar, F, Stocker, P, Dolla, A & Brasseur, G (2011). Oxygen reduction in the strict anaerobe *Desulfovibrio vulgaris* Hildenborough: characterization of two membrane-bound oxygen reductases. *Microbiology*, **157**:2720-2732.

Langendijk, PS, Hanssen, JT & Van der Hoeven, JS (2000). Sulfate-reducing bacteria in association with human periodontitis. *J Clin Periodontol*, **27**:943-950.

Lemos, RS, Gomes, CM, Santana, M, LeGall, J, Xavier, AV & Teixeira, M (2001). The 'strict' anaerobe *Desulfovibrio gigas* contains a membrane-bound oxygen-reducing respiratory chain. *FEBS Lett*, **496**:40-43.

Liamleam, W & Annachhatre, AP (2007). Electron donors for biological sulfate reduction. *Biotechnol Adv*, **25**:452-463.

Lobo, SA, Brindley, A, Warren, MJ & Saraiva, LM (2009). Functional characterization of the early steps of tetrapyrrole biosynthesis and modification in *Desulfovibrio vulgaris* Hildenborough. *Biochem J*, **420**:317-325.

Lobo, SAL, Warren, MJ & Saraiva, LM (2012). Sulfate-Reducing Bacteria Reveal a New Branch of Tetrapyrrole Metabolism. In *Advances in Microbial Physiology*, pp. 267-295. Edited by R. K. Poole. Amsterdam, The Netherlands.

Loubinoux, J, Mory, F, Pereira, IA & Le Faou, AE (2000). Bacteremia caused by a strain of *Desulfovibrio* related to the provisionally named *Desulfovibrio fairfieldensis*. *J Clin Microbiol*, **38**:931-934.

Loubinoux, J, Bisson-Boutelliez, C, Miller, N & Le Faou, AE (2002a). Isolation of the provisionally named *Desulfovibrio fairfieldensis* from human periodontal pockets. *Oral Microbiol Immunol*, **17**:321-323.

Loubinoux, J, Bronowicki, JP, Pereira, IA, Mougenel, JL & Faou, AE (2002b). Sulfate-reducing bacteria in human feces and their association with inflammatory bowel diseases. *FEMS Microbiol Ecol*, **40**:107-112.

Loubinoux, J, Jaulhac, B, Piemont, Y, Monteil, H & Le Faou, AE (2003). Isolation of sulfate-reducing bacteria from human thoracoabdominal pus. *J Clin Microbiol*, **41**:1304-1306.

Lozniewski, A, Maurer, P, Schuhmacher, H, Carlier, JP & Mory, F (1999). First isolation of *Desulfovibrio* species as part of a polymicrobial infection from a brain abscess. *Eur J Clin Microbiol Infect Dis*, **18**:602-603.

Lozniewski, A, Labia, R, Haristoy, X & Mory, F (2001). Antimicrobial susceptibilities of clinical *Desulfovibrio* isolates. *Antimicrob Agents Chemother*, **45**:2933-2935.

Macfarlane, GT, Cummings, JH & Macfarlane, S (2007). Sulphate-reducing bacteria and the human large intestine. In *Sulphate-reducing Bacteria: Environmental and Engineered Systems*, pp. 503-521. Edited by L. L. Barton & W. A. Hamilton. New York: Cambridge University Press.

Malik, A (2004). Metal bioremediation through growing cells. *Environ Int*, **30**:261-278.

Mander, GJ, Duin, EC, Linder, D, Stetter, KO & Hedderich, R (2002). Purification and characterization of a membrane-bound enzyme complex from the sulfate-reducing archaeon *Archaeoglobus fulgidus* related to heterodisulfide reductase from methanogenic archaea. *Eur J Biochem*, **269**:1895-1904.

Marietou, A, Richardson, D, Cole, J & Mohan, S (2005). Nitrate reduction by *Desulfovibrio desulfuricans*: a periplasmic nitrate reductase system that lacks NapB, but includes a unique tetraheme *c*-type cytochrome, NapM. *FEMS Microbiol Lett*, **248**:217-225.

Matias, PM, Pereira, IA, Soares, CM & Carrondo, MA (2005). Sulphate respiration from hydrogen in *Desulfovibrio* bacteria: a structural biology overview. *Prog Biophys Mol Biol*, **89**:292-329.

Meyer, B & Kuever, J (2007). Phylogeny of the alpha and beta subunits of the dissimilatory adenosine-5'-phosphosulfate (APS) reductase from sulfate-reducing prokaryotes-origin and evolution of the dissimilatory sulfate-reduction pathway. *Microbiology*, **153**:2026-2044.

Michel, C, Giudici-Orticoni, M-T, Baymann, F & Bruschi, M (2003). Bioremediation of Chromate by Sulfate-Reducing Bacteria, Cytochromes c_3 and hydrogenases. *Water, Air, & Soil Pollution: Focus*, **3**:161-169.

Moore, WEC, Johnson, JL & Holdeman, LV (1976). Emendation of *Bacteroidaceae* and *Butyrivibrio* and Descriptions of *Desulfomonas* gen. nov. and Ten New Species in the Genera *Desulfomonas*,

Butyrivibrio, *Eubacterium*, *Clostridium*, and *Ruminococcus*. *International Journal of Systematic and Evolutionary Microbiology*, **26**:238-252

Moura, I, Bursakov, S, Costa, C & Moura, JJ (1997). Nitrate and nitrite utilization in sulfate-reducing bacteria. *Anaerobe*, **3**:279-290.

Moura, JJG, Gonzalez, P, Moura, I & Fauque, G (2007). Dissimilatory nitrate and nitrite ammonification by sulphate-reducing bacteria. In *Sulphate-reducing Bacteria: Environmental and Engineered Systems*, pp. 241-264. Edited by L. L. Barton & W. A. Hamilton. New York: Cambridge University Press.

Muyzer, G & Stams, AJ (2008). The ecology and biotechnology of sulphate-reducing bacteria. *Nat Rev Microbiol*, **6**:441-454.

Nakao, K, Tanaka, K, Ichiishi, S, Mikamo, H, Shibata, T & Watanabe, K (2009). Susceptibilities of 23 *Desulfovibrio* isolates from humans. *Antimicrob Agents Chemother*, **53**:5308-5311.

Odom, JM & Jr., HDP (1981). Hydrogen cycling as a general mechanism for energy coupling in the sulfate-reducing bacteria, *Desulfovibrio* sp. **194**:5783-5793.

Odom, JM & Peck, HD, Jr. (1984). Hydrogenase, electron-transfer proteins, and energy coupling in the sulfate-reducing bacteria *Desulfovibrio*. *Annu Rev Microbiol*, **38**:551-592.

Oliveira, TF, Vonrhein, C, Matias, PM, Venceslau, SS, Pereira, IA & Archer, M (2008). The crystal structure of *Desulfovibrio vulgaris* dissimilatory sulfite reductase bound to DsrC provides novel insights into the mechanism of sulfate respiration. *J Biol Chem*, **283**:34141-34149.

Pereira, IA, LeGall, J, Xavier, AV & Teixeira, M (2000). Characterization of a heme *c* nitrite reductase from a non-ammonifying microorganism, *Desulfovibrio vulgaris* Hildenborough. *Biochim Biophys Acta*, **1481**:119-130.

Pereira, IA, Ramos, AR, Grein, F, Marques, MC, da Silva, SM & Venceslau, SS (2011). A comparative genomic analysis of energy metabolism in sulfate reducing bacteria and archaea. *Front Microbiol*, **2**:69.

Pereira, IAC & Xavier, AV (2005). Multi-Heme Cytochromes and Enzymes. In *Encyclopedia of Inorganic Chemistry*, pp. 33-72. Edited by R. B. King. New York: John Wiley & Sons.

Pereira, IAC, Haveman, SA & Voordouw, G (2007). Biochemical, genetic and genomic characterization of anaerobic electron transport pathways in sulphate-reducing *Delta proteobacteria*. In *Sulphate-reducing Bacteria: Environmental and Engineered Systems*, pp. 215-240. Edited by L. L. Barton & W. A. Hamilton. New York: Cambridge University Press.

Pereira, IAC (2008). Respiratory Membrane complexes of *Desulfovibrio*. In *Microbial Sulfur Metabolism*, pp. 24-35. Edited by C. Dahl & C. G. Friedrich. New York: Springer.

Pereira, PM, Teixeira, M, Xavier, AV, Louro, RO & Pereira, IA (2006). The Tmc complex from *Desulfovibrio vulgaris* Hildenborough is involved in transmembrane electron transfer from periplasmic hydrogen oxidation. *Biochemistry*, **45**:10359-10367.

Pimentel, JD & Chan, RC (2007). *Desulfovibrio fairfieldensis* bacteremia associated with choledocholithiasis and endoscopic retrograde cholangiopancreatography. *J Clin Microbiol*, **45**:2747-2750.

Pires, RH, Lourenco, AI, Morais, F, Teixeira, M, Xavier, AV, Saraiva, LM & Pereira, IA (2003). A novel membrane-bound respiratory complex from *Desulfovibrio desulfuricans* ATCC 27774. *Biochim Biophys Acta*, **1605**:67-82.

Pires, RH, Venceslau, SS, Morais, F, Teixeira, M, Xavier, AV & Pereira, IA (2006). Characterization of the *Desulfovibrio desulfuricans* ATCC 27774 DsrMKJOP complex-a membrane-bound redox complex involved in the sulfate respiratory pathway. *Biochemistry*, **45**:249-262.

Pitcher, MC & Cummings, JH (1996). Hydrogen sulphide: a bacterial toxin in ulcerative colitis? *Gut*, **39**:1-4.

Postgate, JR & Campbell, LL (1966). Classification of *Desulfovibrio* species, the nonsporulating sulfate-reducing bacteria. *Bacteriol Rev*, **30**:732-738.

Postgate, JR (1979). *The Sulphate-Reducing Bacteria*. New York: Cambridge University Press.

Rabus, R, Hansen, TA & Widdel, F (2006). Dissimilatory Sulfate- and Sulfur-Reducing Prokaryotes. In *The Prokaryotes*, pp. 659-768. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer & E. Stackebrandt. New York: Springer.

Rajeev, L, Hillesland, KL, Zane, GM, Zhou, A, Joachimiak, MP, He, Z, Zhou, J, Arkin, AP, Wall, JD & Stahl, DA (2012). Deletion of the *Desulfovibrio vulgaris* carbon monoxide sensor invokes global changes in transcription. *J Bacteriol*, **194**:5783-5793.

Romão, CV, Louro, R, Timkovich, R, Lubben, M, Liu, MY, LeGall, J, Xavier, AV & Teixeira, M (2000). Iron-coproporphyrin III is a natural cofactor in bacterioferritin from the anaerobic bacterium *Desulfovibrio desulfuricans*. *FEBS Lett*, **480**:213-216.

Saraiva, LM, da Costa, PN, Conte, C, Xavier, AV & LeGall, J (2001). In the facultative sulphate/nitrate reducer *Desulfovibrio desulfuricans* ATCC 27774, the nine-haem cytochrome *c* is part of a membrane-bound redox complex mainly expressed in sulphate-grown cells. *Biochim Biophys Acta*, **1520**:63-70.

Sebban, C, Blanchard, L, Bruschi, M & Guerlesquin, F (1995). Purification and characterization of the formate dehydrogenase from *Desulfovibrio vulgaris* Hildenborough. *FEMS Microbiol Lett*, **133**:143-149.

Tee, W, Dyall-Smith, M, Woods, W & Eisen, D (1996). Probable new species of *Desulfovibrio* isolated from a pyogenic liver abscess. *J Clin Microbiol*, **34**:1760-1764.

Thauer, RK, Stackebrandt, E & Hamilton, AW (2007). Energy metabolism and phylogenetic diversity of sulphate-reducing bacteria. In *Sulphate-reducing Bacteria: Environmental and Engineered Systems*, pp. 1-38. Edited by L. L. Barton & W. A. Hamilton. New York: Cambridge University Press.

Traore, AS, Hatchikian, CE, Belaich, JP & Le Gall, J (1981). Microcalorimetric studies of the growth of sulfate-reducing bacteria: energetics of *Desulfovibrio vulgaris* growth. *J Bacteriol*, **145**:191-199.

Urata, T, Kikuchi, M, Hino, T, Yoda, Y, Tamai, K, Kodaira, Y & Hitomi, S (2008). Bacteremia caused by *Desulfovibrio fairfieldensis*. *J Infect Chemother*, **14**:368-370.

van den Berg, WAM, Stokkermans, JPWG & van Dongen, WMAM (1989). Development of a plasmid transfer system for the anaerobic sulphate reducer, *Desulfovibrio vulgaris*. *Journal of Biotechnology*, **12**:173-184.

Verstreken, I, Laleman, W, Wauters, G & Verhaegen, J (2012). *Desulfovibrio desulfuricans* bacteremia in an immunocompromised host with a liver graft and ulcerative colitis. *J Clin Microbiol*, **50**:199-201.

Voordouw, G (2002). Carbon monoxide cycling by *Desulfovibrio vulgaris* Hildenborough. *J Bacteriol*, **184**:5903-5911.

Voordouw, G (2008). Impact of Nitrate on the Sulfur Cycle in Oil Fields. In *Microbial Sulfur Metabolism*, pp. 296-302 Edited by C. Dahl & C. G. Friedrich. New York: Springer.

Wagner, M, Loy, A, Klein, M, Lee, N, Ramsing, NB, Stahl, DA & Friedrich, MW (2005). Functional marker genes for identification of sulfate-reducing prokaryotes. *Methods in Enzymology*, **397**:469-489.

Warren, YA, Citron, DM, Merriam, CV & Goldstein, EJ (2005). Biochemical differentiation and comparison of *Desulfovibrio* species and other phenotypically similar genera. *J Clin Microbiol*, **43**:4041-4045.

Widdel, F & Bak, F (1992). Gram-negative Mesophilic Sulfate-Reducing Bacteria In *The Prokaryotes: A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Application*, pp. 3353-3378. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer Verlag.

Widdel, F & Hansen, TA (1992). The Dissimilatory Sulfate- and Sulfur-Reducing Bacteria. In *The Prokaryotes*, pp. 583-624. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: ???

Zhou, J, He, Q, Hemme, CL, Mukhopadhyay, A, Hillesland, K, Zhou, A, He, Z, Van Nostrand, JD, Hazen, TC, Stahl, DA, Wall, JD & Arkin, AP (2011). How sulphate-reducing microorganisms cope with stress: lessons from systems biology. *Nat Rev Microbiol*, **9**:452-466.

Part 1

Chapter 2

Desulfovibrio and iron homeostasis

Chapter 2

Desulfovibrio and iron homeostasis

2.1 - Iron is an essential element

Iron is the 26th element of the periodic table being the fourth most represented element in the earth's crust with its abundance only overtaken by aluminum, silicon, and oxygen (Neilands, 1981). In biological systems, iron is commonly found in two redox states: the reduced ferrous form (Fe^{2+}) and the oxidized ferric form (Fe^{3+}). At physiological pH, Fe^{2+} is soluble while Fe^{3+} is highly insoluble, precipitating as ferric hydroxide. Hence, under aerobic conditions the bioavailability of iron is low and its toxicity arises from its reaction with oxygen that yields deleterious reactive oxygen species (ROS) (Prousek, 2007). In the presence of superoxide ($O_2^{\bullet -}$), Fe^{3+} is reduced (Eq. A), that in the presence of hydrogen peroxide (H_2O_2) produces the highly damaging hydroxyl radical ($HO^{\bullet}$), via the so-called Fenton reaction (Eq. B). The combination of these two reactions, is the so-called Haber–Weiss reaction (Eq. C) (Andrews *et al.*, 2003).

$$O_2^{\bullet -} + Fe^{3+} \longrightarrow Fe^{2+} + O_2 \qquad \textbf{(Eq. A)}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + HO^{\bullet} \qquad \textbf{(Eq. B)}$$

$$O_2^{\bullet -} + H_2O_2 \xrightarrow[\text{Fe catalysis}]{} O_2 + HO^{\bullet} + OH^- \qquad \textbf{(Eq. C)}$$

Scheme 2.1 - Iron reactivity.
Equation A) Iron reduction, **Equation B)** Fenton reaction, **Equation C)** Haber–Weiss reaction. Adapted from (Andrews *et al.*, 2003).

Iron is an essential element for almost living organisms. It is a versatile prosthetic component that is found incorporated into proteins as mono and diiron centers, iron-sulfur clusters and heme groups that act as biocatalysts or electron

carriers. Hence, iron is involved in a variety of biological processes such as respiration, nitrogen fixation, oxygen transport, gene regulation, DNA biosynthesis and photosynthetic reactions (Crichton & Ward, 1992; Andrews *et al.*, 2003). Albeit the vital role of iron, its toxicity and low solubility, requires a tight control which in bacteria involves systems for uptake, transport, storage and regulation.

2.2 - Proteins involved in iron metabolism

2.2.1 - Iron acquisition systems

In aerobic environments, bacteria acquire iron by secreting high-affinity extracellular ferric chelators, named siderophores. Siderophores are iron carriers of low molecular mass that are synthesize under low iron conditions (Andrews *et al.*, 2003; Rodionov *et al.*, 2004). In *Desulfovibrio* available genomes, genes for siderophore synthesis have not been identified and the production of siderophores has not been reported. However, the presence of two copies in *D. vulgaris* of the *fepC* gene (DVU0103 and DVU0648), which is predicted to encode an ABC transporter for the siderophore enterobactin, suggests that *Desulfovibrio* spp. may acquire Fe^{3+} through siderophores produced by other bacteria (Rodionov *et al.*, 2004; Bender *et al.*, 2007).

Under anaerobic conditions, ferrous iron ion is the predominant form of iron that can be transported by the ferrous iron transport system (Feo). Anaerobic δ-proteobacteria contain multiple copies of the genes coding for bacterial ferrous iron transporter system. In *D. vulgaris*, the *feo* locus contains the *feo*AB operon (DVU2572-71) and a closely associated second copy of the *feo*A gene (DVU2574) (Rodionov *et al.*, 2004; Bender *et al.*, 2007). In fact, the derepression of the *feo*AB operon in a strain that lacks the gene for the ferric uptake regulator (Fur) suggests that the FeoAB system is the primary iron uptake mechanism in *D. vulgaris* (Bender *et al.*, 2007).

2.2.2 - Iron storage proteins

The iron storage proteins play a key role in the biological systems, as they have the ability of oxidize the ferrous ions and to accumulate large amounts of iron in a ferric oxide mineral form for future intracellular needs. There are three types of iron storage proteins, which may exist in multiple copies or simultaneously in a single organism. These are: ferritins (Ftn), which are present in bacteria and eukaryotes; bacterioferritins (Bfr), found in eubacteria and in the eukaryotic organism *Absidia spinosa* (Carrano *et al.*, 1996), and the DNA protecting proteins under starved conditions (Dps) so far only present in prokaryotic microorganisms (Andrews *et al.*, 2003). These three types of iron storage proteins have structural and functional similarities, but at the same time possess distinctive characteristics that confer different physiological purposes beyond iron accumulation.

The ferritin superfamily

Ferritins are present in almost all organisms that utilize iron. Ferritin was first isolated in mammals, in 1894, by Naunyn Schmiedeberg, a german pharmacologist, who called it “ferratin”. The protein was purified from horse spleen by Laufberger in 1937, which named it ferritin and suggested an iron storage function (Andrews, 1998). Iron storage proteins were later found to be also present in plants and fungi, in which were initially termed “phytoferritins” (Hyde *et al.*, 1963; David & Easterbrook, 1971).

The first report of a bacterial ferritin-like protein was in 1979, when Stiefel and Watt isolated the bacterioferritin from the nitrogen-fixing bacteria *Azotobacter vinelandii* (Stiefel & Watt, 1979). In fact, this protein which contains a non-heme iron center was first isolated by Bulen and co-workers but described as a cytochrome b_1 (Bulen *et al.*, 1973). Also, the *Escherichia* (*E.*) *coli* Bfr was firstly

isolated and studied as a cytochrome b_1 but was only defined as a bacterioferritin in 1988 (Keilin, 1934; Smith *et al.*, 1988).

Although bacterioferritins and ferritins share common characteristics they are distantly related in evolution, due to the low amino acid sequence similarity. For example, *E. coli* bacterioferritin and ferritins (*ftnA* and *ftnB*) have low amino acid sequence identity (12-21%). The limited identity between ferritins and bacterioferritins indicates that these proteins form distinct families within a superfamily. Ferritin-like proteins are proposed to have evolved from a rubrerythrin-like ancestor that is a simpler protein which is involved in defense against toxic oxygen species (Andrews *et al.*, 1991; Andrews, 1998; Andrews, 2010).

Ferritins and bacterioferritins are composed by 24 identical subunits arranged into a spherical protein shell surrounding a central cavity, that stores up to 4500 Fe^{3+} (Figure 2.1A). The subunits are associate as dimers, each one with a molecular mass of 20 kDa. These proteins have a four-helix bundle structure, with four main helices A-D and a short fifth helix (helix E) (Figure 2.1B) (Harrison & Arosio, 1996; Andrews *et al.*, 2003; Carrondo, 2003). The oxidation of ferrous ions to the ferric form occurs in the diiron ferroxidase center located in the middle of the four-helical bundle of each subunit, which is generally coordinated by six highly conserved residues (Figure 2.1B-C) (Andrews, 1998). Interestingly**,** ferritins from *Pyrococcus furiosus*, *Archaeoglobus fulgidus*, *E. coli* and *D. vulgaris* present a third mononuclear iron binding site, which is proposed to be involved in transporting electrons to the stable diiron ferroxidase center (Stillman *et al.*, 2001; Johnson *et al.*, 2005; Tatur *et al.*, 2007; Honarmand Ebrahimi *et al.*, 2009; Pereira *et al.*, 2012).

As previously mentioned, in the bacterial ferritin superfamily the iron storage process requires a ferroxidation step that takes place in the diiron center. The broadly accepted ferroxidation model consists in three separated kinetic phases: the rapid binding of Fe^{2+} to the binuclear center; a second slower step consisting in

the oxidation of the bound ferrous ion pair by O_2 or H_2O_2 to yield an μ-oxo-bridged di-ferric intermediate; and a much slower final phase that involves the migration of Fe^{3+} to the central cavity to form the mineral core (Crow *et al.*, 2009). After the initiation of the build of the core, the oxidation of Fe^{2+} occurs directly in an autocatalytic reaction that takes place at the surface of the growing mineral core (Brun *et al.*, 1997). Recent studies in *D. vulgaris* and *E. coli*, showed that Bfr uses H_2O_2 as a co-substrate to oxidize Fe^{2+} ions at the ferroxidase center, therefore avoiding the production of toxic hydroxyl radicals derived from the oxygen chemistry of the Fenton reaction (Bou-Abdallah *et al.*, 2002; Timóteo *et al.*, 2012).

The major difference between Ftn and Bfr is the presence in bacterioferritins of 12 non-covalently bound heme groups located in the interface of two subunits (Figure 2.1B) (Frolow *et al.*, 1994). The heme is, in general, in the form of protoporphyrin IX (*b*-type). Nevertheless, the Bfr of *D. desulfuricans* contains a distinct form of heme, the iron-coproporphyrin III (Romão *et al.*, 2000a). It is now known that the heme groups present in Bfr play an important role in the release of iron from the mineral core. Heme catalyses the transfer of electrons from the outside of the protein to the internal cavity which are used to reduce Fe^{3+} to Fe^{2+}. The low affinity of Fe^{2+} to the central cavity results in a movement via protein channels to the external protein surface, where it is again available for chelation (Yasmin *et al.*, 2011). Another function attributed to the heme present in *E. coli* Bfr is to accelerate the iron core formation by an electron transfer mechanism. This hypothesis was formulated upon observation that the heme bound to Bfr decreases the rate of iron oxidation at the dinuclear iron ferroxidase sites but increases the rate of iron core formation (Wong *et al.*, 2012).

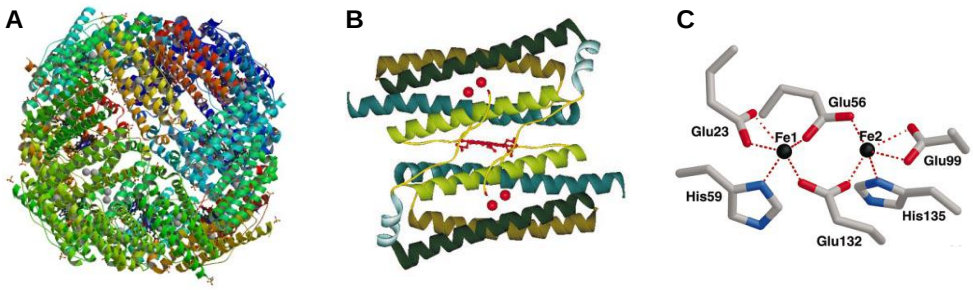


Figure 2.1 - Structural characteristics of *Desulfovibrio desulfuricans* Bfr.
A) Crystallographic structure of *D. desulfuricans* Bfr, illustrating the overall quaternary structure with the 12 dimers shown in different colors; **B)** Bfr homodimers formed by two subunits, the Fe-coproporphyrin III heme is represented between the two subunits and the red spheres are the diiron ferroxidase centers of each monomer; **C)** Structure of the diiron center "as isolated" with ligands. Adapted from (Frazão *et al.*, 2000; Macedo *et al.*, 2003; Carrondo, 2003).

In spite of the primary function of ferritins as iron storage proteins, a secondary role is associated with these proteins, namely in protection against oxidative stress as the mechanism of iron storage prevents the formation of ROS by the Fenton reaction (Harrison & Arosio, 1996; Andrews *et al.*, 2003; Carrondo, 2003).

The study of *E. coli, Porphyromonas gingivalis* and *Campylobacter* (*C.*) *jejuni* FtnA suggested that its main function is the accumulation of iron for subsequence use under iron depletion conditions (Wai *et al.*, 1996; Abdul-Tehrani *et al.*, 1999; Ratnayake *et al.*, 2000). Moreover, *C. jejuni* FtnA also revealed a role in iron-mediated oxidative stress showing a ferritin-deficient mutant higher sensitivity to H_2O_2 and the superoxide generating agent paraquat than the wild type strain (Wai *et al.*, 1996).

Bfr proteins from *Neisseria* (*N.*) *gonorrhoeae* and *Pseudomonas* (*P.*) *aeruginousa* have been implicated in redox stress resistance (Chen & Morse, 1999; Ma *et al.*, 1999). *N. gonorrhoeae bfr*-deficient mutant proved to be more susceptible to H_2O_2 and paraquat than the parental strain (Chen & Morse, 1999). In the case of *P. aeruginousa*, a mutant lacking *bfrA* has half of the catalase activity and is more susceptible to peroxides. The authors hypothesized that this lack of

activity is due to the indirect role of Bfr in hydrogen peroxide resistance (Ma *et al.*, 1999).

So far, no stress-associated phenotype was identified for *E. coli* and *Brucella* (*B.*) *melitensis bfr* mutants (Denoel *et al.*, 1997; Abdul-Tehrani *et al.*, 1999). Furthermore, deletion of *bfr* in *B. melitensis* does not affect the colonization capacity of the strain in human macrophages showing that Bfr is not essential for the intracellular survival of *B. melitensis* (Denoel *et al.*, 1997).

Detoxification of iron in anaerobic bacteria seems to be unnecessary as these microorganisms live in absence of oxygen. Nevertheless, anaerobic bacteria are periodically exposed to oxygen, and consequently submitted to reactive oxygen species. Hence, these bacteria need oxygen detoxification systems to avoid the production of the deleterious species catalyzed by Fe^{2+}. The ferritin from *Bacteroides fragilis* was the first example of an iron storage protein isolated from an anaerobe (Rocha *et al.*, 1992). Later, the mRNA levels of the gene that encodes this protein proved to be controlled by both iron and oxidative stress (Rocha & Smith, 2004). The *D. vulgaris* genome contains Bfr and Ftn encoding genes. Recent work established that *D. vulgaris* Bfr uses H_2O_2 as the Fe^{2+} oxidant in the ferroxidase reaction, and has the ability to bind DNA. These two combined activities lead the authors to suggest for Bfr a protective detoxification role similar to the one attributed to Dps proteins (Timóteo *et al.*, 2012).

The Dps family

The *E. coli* Dps was the first member of this family of proteins to be isolated. Almiron and co-workers obtained the protein in 1992 from a 3-day old cell culture that aimed mimicking the natural environmental conditions in which bacteria under starved conditions have to compete for nutrients. This protein showed to be capable of binding DNA without apparent sequence specificity and therefore was named DNA protecting protein under starved conditions (Almiron *et al.*, 1992).

Dps proteins are composed of 12 subunits (Figure 2.2A) and have a storage capacity of approximately 500 iron atoms. Each subunit has four helix-bundle structure, similar to the folding features of ferritins but lacking the E helix and containing an additional short helical region in the middle of the BC loop (Figure 2.2C) (Grant *et al.*, 1998; Ilari *et al.*, 2000). Another difference from ferritins is the location of the diiron site, which in Dps is at the interface between the two-fold related subunits (Figure 2.2B) (Ilari *et al.*, 2000). Furthermore, Dps proteins prefer H_2O_2 as the ferrous ions oxidant instead of O_2, which is used by most ferritins (Zhao *et al.*, 2002).

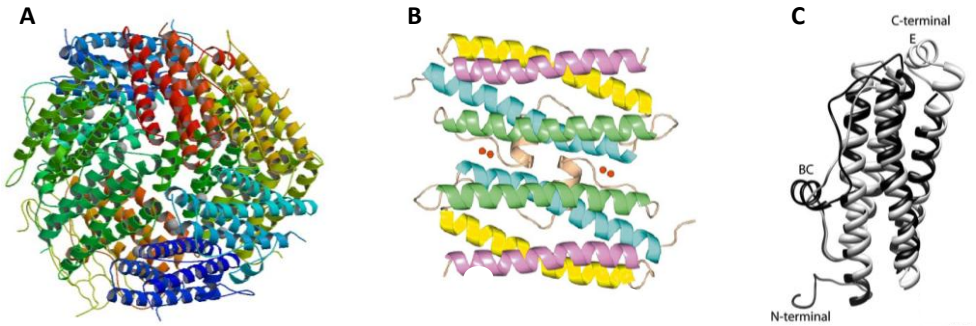


Figure 2.2 - Schematic representation of Dps.
A) Crystal structure of *Bacillus* (*B.*) *brevis* Dps; **B)** *B. brevis* Dps dimer, the iron ions of the two ferroxidase centers are shown as red spheres; **C)** Superimposition of *E. coli* Bfr (grey) and Dps (black) monomers revealing the differences in helix E and BC loop between the two monomers. Adapted from (Ren *et al.*, 2003; Zhang *et al.*, 2011)

Dps are proposed to have several functions such as: (i) preserve the stability of the DNA during the stationary growth phase by forming DNA-protein complexes that protect DNA from ROS induced damages caused; (ii) perform direct ROS detoxification through peroxidase activity, and (iii) rapidly remove free Fe^{2+} (Andrews *et al.*, 2003; Zeth, 2012). In pathogenic bacteria, such as *Porphyromonas gengivalis*, the ability of Dps to protect from ROS damage seems to be vital since the bacterium does not possess catalase. Furthermore, Dps contributes to the survival of these bacteria to the oxidative stress imposed by macrophages

(Ueshima *et al.*, 2003). *Desulfovibrio* spp. apparently do not encode Dps-like proteins; nevertheless, it was proposed that in bacteria that do not express Dps, Bfr may adopt such a function (Timóteo *et al.*, 2012).

2.2.3 - Regulation of iron homeostasis

As mentioned above, it is crucial to microorganisms to have a tight control of the intracellular iron concentration as the accumulation of free iron leads to the formation of harmful species. In most bacteria, this complex regulation is controlled by the ferric uptake regulator (Fur).

Fur is a highly conserved protein widespread in a large number of Gram-positive and Gram-negative bacteria, which usually acts as a repressor of iron uptake genes, but may also have an activator function (Andrews *et al.*, 2003; Cornelis *et al.*, 2011). This protein is a homodimer binding one structural Zn^{2+} and one regulatory Fe^{2+} per monomer. The repression is usually achieved by a direct mechanism that involves binding to its co-repressor (Fe^{2+}). Thus, under iron-replete conditions the iron-loaded Fur protein blocks the transcription of the target genes (Figure 2.3). In general, the Fur-Fe^{2+} complex binds to a 19-bp palindromic consensus sequence known as the "iron box" or "Fur box" (GATAATGAT(A/T)ATCATTATC), which typically overlaps the promoter region of genes under Fur direct control. In iron-limiting conditions, the apo-Fur is displaced from the Fur box and the RNA polymerase can access the promoter region initiating transcription of the downstream gene(s) (Figure 2.3) (Andrews *et al.*, 2003; Lee & Helmann, 2007; Tong & Guo, 2009). Interestingly, in *Helicobacter* (*H.*) *pylori*, the apo-form of Fur works as a repressor. In this organism, the expression of the *ferritin* gene (*pfr*) is activated by iron in a Fur-dependent manner. The gene is repressed by the iron-free form of Fur that interacts directly with the *pfr* promoter at a different Fur binding site, which is not yet well defined (Delany *et al.*, 2001). To date, the mechanism of Fur apo-regulation was not identified in

other bacteria suggesting that *H. pylori* Fur possesses distinctive features that makes possible this unique type of regulation (Miles *et al.*, 2010).

Fur is also able to induce de expression of iron related genes through direct or indirect mechanisms. In general, the Fur-Fe^{2+} complex functions as an activator by an indirect process mediated by the repression of small regulatory RNA molecules, called RyhB. This molecule acts post-transcriptionally promoting the degradation of the mRNAs of target genes (Figure 2.3) (Masse & Gottesman, 2002; Masse *et al.*, 2005). A recent study revealed that RyhB is not involved in the positive regulation of *E. coli ftnA* by Fur. Instead, Fur-Fe^{2+} acts directly by binding to an "extended" Fur binding site located upstream of *ftnA* start codon. This Fur box binding site is composed by five tandem Fur-box-like sequences that overlap the binding region of a direct repressor of the *ftnA* transcription (histone-like protein; H-NS). Consequently, under iron rich conditions, H-NS displaces from the *ftnA* promoter, allowing the access of the RNA polymerase (Nandal *et al.*, 2010).

It is common to find in the genome of several microorganisms more than one Fur-like regulator. In *E. coli*, a *fur*-like gene, named *zur,* controls zinc levels (Patzer & Hantke, 1998). In *B. subtilis*, three *fur*-like genes have been sequenced (Fur/PerR/Zur): Fur, which is associated with the regulation of siderophore biosynthesis and iron uptake (Bsat *et al.*, 1998); Zur, that regulates two zinc uptake systems (Gaballa & Helmann, 1998), and PerR that controls the genes involved in oxidative-stress response (Bsat *et al.*, 1998). In δ-proteobacteria, the presence of Fur/PerR/Zur family of transcriptional regulators is ubiquitous comprising different functions including regulation of iron homeostasis (Fur), oxidative stress response (PerR), and zinc homeostasis (Zur) (Rodionov *et al.*, 2004; Bender *et al.*, 2007; Zhou *et al.*, 2010; Brioukhanov *et al.*, 2010; Wildschut *et al.*, 2012). The genome of *D. vulgaris* encodes the same three *fur* paralogs, *fur* (DVU0942), *perR* (DVU3095), and *zur* (DVU1340) (Rodionov *et al.*, 2004).

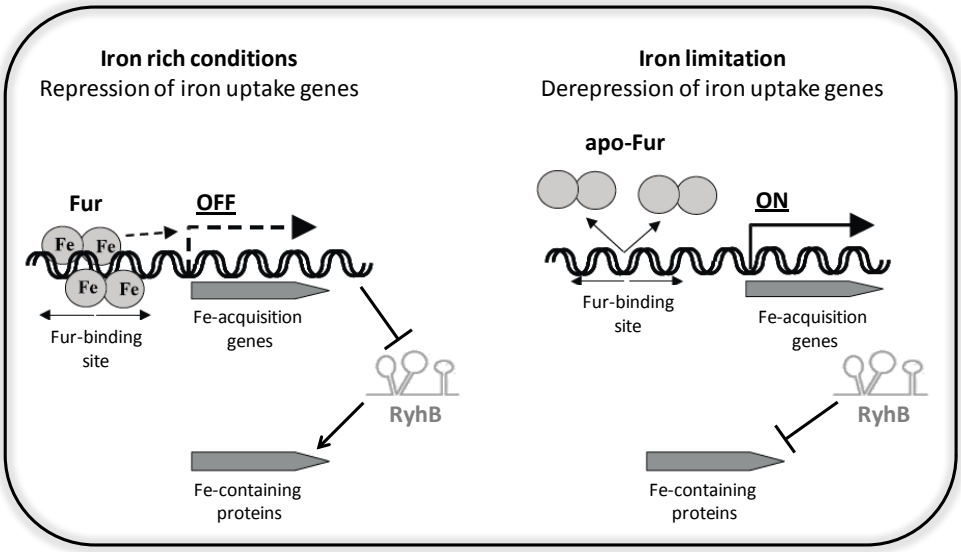


Figure 2.3 - General regulation mechanism of iron related genes by *E. coli* Fur.
Under iron rich conditions: Fur binds Fe^{2+} blocking the transcription of Fe-acquisition genes. Moreover, the Fur-Fe^{2+} complex repress the expression of the small RNA RyhB, resulting in the transcription of Fe-containing proteins, thereby acting indirectly as a positive regulator.
Under iron limitation: Fur loses its co-repressor and the apo-Fur is released from Fur box allowing the transcription of Fe-acquisition genes. Additionally, the apo-Fur is not capable of repressing the transcription of RyhB leading to destabilization of mRNA of Fe-containing proteins. Adapted from (Andrews *et al.*, 2003; Masse *et al.*, 2007)

The physiological and transcriptional profile of a *D. vulgaris* strain deleted in the *fur* gene confirmed that Fur is involved not only in iron metabolism having a global regulatory role. However, the transcription of the *bfr* and *ftn* genes, seemed to be not regulated by Fur (Bender *et al.*, 2007). The involvement of PerR in the regulation of iron storage proteins was reported for ferritin-like Dps homolog (*mrgA*) of *Staphylococcus* (*S.*) *aureus*, *Streptococcus pyogenes* and *Bacillus subtilis* and also for *S. aureus* ferritin, revealing that this regulator responds to the metal balance within the cell (Horsburgh *et al.*, 2001). In *D. vulgaris* Δ*perR* strain the genes for ferrous iron transport protein *feo*A and *feo*B were strongly down-regulated, sustaining the hypothesis that PerR also participates in the control of the intracellular iron levels in the cell (Zhou *et al.*, 2010; Wildschut *et al.*, 2012).

The work presented in this dissertation gives an important contribution to the knowledge of bacterioferritin role in the oxygen response of the anaerobic bacteria *D. vulgaris* and in its regulation by PerR (Part II - Chapter 1).

2.3 - References

Abdul-Tehrani, H, Hudson, AJ, Chang, YS, Timms, AR, Hawkins, C, Williams, JM, Harrison, PM, Guest, JR & Andrews, SC (1999). Ferritin mutants of *Escherichia coli* are iron deficient and growth impaired, and fur mutants are iron deficient. *J Bacteriol*, **181**:1415-1428.

Almiron, M, Link, AJ, Furlong, D & Kolter, R (1992). A novel DNA-binding protein with regulatory and protective roles in starved *Escherichia coli*. *Genes Dev*, **6**:2646-2654.

Andrews, SC, Smith, JM, Yewdall, SJ, Guest, JR & Harrison, PM (1991). Bacterioferritins and ferritins are distantly related in evolution. Conservation of ferroxidase-centre residues. *FEBS Lett*, **293**:164-168.

Andrews, SC (1998). Iron storage in bacteria. *Adv Microb Physiol*, **40**:281-351.

Andrews, SC, Robinson, AK & Rodriguez-Quinones, F (2003). Bacterial iron homeostasis. *FEMS Microbiol Rev*, **27**:215-237.

Andrews, SC (2010). The Ferritin-like superfamily: Evolution of the biological iron storeman from a rubrerythrin-like ancestor. *Biochim Biophys Acta*, **1800**:691-705.

Bender, KS, Yen, HC, Hemme, CL, Yang, Z, He, Z, He, Q, Zhou, J, Huang, KH, Alm, EJ, Hazen, TC, Arkin, AP & Wall, JD (2007). Analysis of a ferric uptake regulator (Fur) mutant of *Desulfovibrio vulgaris* Hildenborough. *Appl Environ Microbiol*, **73**:5389-5400.

Bou-Abdallah, F, Lewin, AC, Le Brun, NE, Moore, GR & Chasteen, ND (2002). Iron detoxification properties of *Escherichia coli* bacterioferritin. Attenuation of oxyradical chemistry. *J Biol Chem*, **277**:37064-37069.

Brioukhanov, AL, Durand, MC, Dolla, A & Aubert, C (2010). Response of *Desulfovibrio vulgaris* Hildenborough to hydrogen peroxide: enzymatic and transcriptional analyses. *FEMS Microbiol Lett*, **310**:175-181.

Brun, NEL, Thomson, AJ & Moore, Gg (1997). Metal Centres of Bacterioferritins or Non-Heam-Iron-Containing Cytochromes b_{557}. In *Metal Sites in Proteins and Models*, pp. 103-138. Edited by H. A. O. Hill, P. J. Sadler & A. J. Thomson. Berlin Heidelberg: Springer Verlag

Bsat, N, Herbig, A, Casillas-Martinez, L, Setlow, P & Helmann, JD (1998). *Bacillus subtilis* contains multiple Fur homologues: identification of the iron uptake (Fur) and peroxide regulon (PerR) repressors. *Mol Microbiol*, **29**:189-198.

Bulen, WA, LeComte, JR & Lough, S (1973). A hemoprotein from azotobacter containing non-heme iron: isolation and crystallization. *Biochem Biophys Res Commun*, **54**:1274-1281.

Carrano, CJ, Bohnke, R & Matzanke, BF (1996). Fungal ferritins: the ferritin from mycelia of *Absidia spinosa* is a bacterioferritin. *FEBS Lett*, **390**:261-264.

Carrondo, MA (2003). Ferritins, iron uptake and storage from the bacterioferritin viewpoint. *Embo J*, **22**:1959-1968.

Chen, CY & Morse, SA (1999). *Neisseria gonorrhoeae* bacterioferritin: structural heterogeneity, involvement in iron storage and protection against oxidative stress. *Microbiology*, **145**:2967-2975.

Cornelis, P, Wei, Q, Andrews, SC & Vinckx, T (2011). Iron homeostasis and management of oxidative stress response in bacteria. *Metallomics*, **3**:540-549.

Crichton, RR & Ward, RJ (1992). Iron metabolism - new perspectives in view. *Biochemistry*, **31**:11255-11264.

Crow, A, Lawson, TL, Lewin, A, Moore, GR & Le Brun, NE (2009). Structural basis for iron mineralization by bacterioferritin. *J Am Chem Soc*, **131**:6808-6813.

David, CN & Easterbrook, K (1971). Ferritin in the fungus *Phycomyces*. *J Cell Biol*, **48**:15-28.

Delany, I, Spohn, G, Rappuoli, R & Scarlato, V (2001). The Fur repressor controls transcription of iron-activated and -repressed genes in *Helicobacter pylori*. *Mol Microbiol*, **42**:1297-1309.

Denoel, PA, Crawford, RM, Zygmunt, MS, Tibor, A, Weynants, VE, Godfroid, F, Hoover, DL & Letesson, JJ (1997). Survival of a bacterioferritin deletion mutant of *Brucella melitensis* 16M in human monocyte-derived macrophages. *Infect Immun*, **65**:4337-4340.

Frazão, C, Silva, G, Gomes, CM, Matias, P, Coelho, R, Sieker, L, Macedo, S, Liu, MY, Oliveira, S, Teixeira, M, Xavier, AV, Rodrigues-Pousada, C, Carrondo, MA & Le Gall, J (2000). Structure of a dioxygen reduction enzyme from *Desulfovibrio gigas*. *Nat Struct Biol*, **7**:1041-1045.

Frolow, F, Kalb, AJ & Yariv, J (1994). Structure of a unique twofold symmetric haem-binding site. *Nat Struct Biol*, **1**:453-460.

Gaballa, A & Helmann, JD (1998). Identification of a zinc-specific metalloregulatory protein, Zur, controlling zinc transport operons in *Bacillus subtilis*. *J Bacteriol*, **180**:5815-5821.

Grant, RA, Filman, DJ, Finkel, SE, Kolter, R & Hogle, JM (1998). The crystal structure of Dps, a ferritin homolog that binds and protects DNA. *Nat Struct Biol*, **5**:294-303.

Harrison, PM & Arosio, P (1996). The ferritins: molecular properties, iron storage function and cellular regulation. *Biochim Biophys Acta*, **1275**:161-203.

Honarmand Ebrahimi, K, Hagedoorn, PL, Jongejan, JA & Hagen, WR (2009). Catalysis of iron core formation in *Pyrococcus furiosus* ferritin. *J Biol Inorg Chem*, **14**:1265-1274.

Horsburgh, MJ, Clements, MO, Crossley, H, Ingham, E & Foster, SJ (2001). PerR controls oxidative stress resistance and iron storage proteins and is required for virulence in *Staphylococcus aureus*. *Infect Immun*, **69**:3744-3754.

Hyde, BB, Hodge, AJ, Kahn, A & Birnstiel, ML (1963). Studies on Phytoferritin: I. Identification and Localization. *J Ultrastruct Res*, **9**:248-258.

Ilari, A, Stefanini, S, Chiancone, E & Tsernoglou, D (2000). The dodecameric ferritin from *Listeria innocua* contains a novel intersubunit iron-binding site. *Nat Struct Biol*, **7**:38-43.

Johnson, E, Cascio, D, Sawaya, MR, Gingery, M & Schroder, I (2005). Crystal structures of a tetrahedral open pore ferritin from the hyperthermophilic archaeon *Archaeoglobus fulgidus*. *Structure*, **13**:637-648.

Keilin, D (1934). Cytochrome and the Supposed Direct Spectroscopic Observation of Oxidase. *Nature*, **133**:290-291.

Lee, JW & Helmann, JD (2007). Functional specialization within the Fur family of metalloregulators. *Biometals*, **20**:485-499.

Ma, JF, Ochsner, UA, Klotz, MG, Nanayakkara, VK, Howell, ML, Johnson, Z, Posey, JE, Vasil, ML, Monaco, JJ & Hassett, DJ (1999). Bacterioferritin A modulates catalase A (KatA) activity and resistance to hydrogen peroxide in *Pseudomonas aeruginosa*. *J Bacteriol*, **181**:3730-3742.

Macedo, S, Romão, CV, Mitchell, E, Matias, PM, Liu, MY, Xavier, AV, LeGall, J, Teixeira, M, Lindley, P & Carrondo, MA (2003). The nature of the di-iron site in the bacterioferritin from *Desulfovibrio desulfuricans*. *Nat Struct Biol*, **10**:285-290.

Masse, E & Gottesman, S (2002). A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. *Proc Natl Acad Sci U S A*, **99**:4620-4625.

Masse, E, Vanderpool, CK & Gottesman, S (2005). Effect of RyhB small RNA on global iron use in *Escherichia coli*. *J Bacteriol*, **187**:6962-6971.

Masse, E, Salvail, H, Desnoyers, G & Arguin, M (2007). Small RNAs controlling iron metabolism. *Curr Opin Microbiol*, **10**:140-145.

Miles, S, Carpenter, BM, Gancz, H & Merrell, DS (2010). *Helicobacter pylori* apo-Fur regulation appears unconserved across species. *J Microbiol*, **48**:378-386.

Nandal, A, Huggins, CC, Woodhall, MR, McHugh, J, Rodriguez-Quinones, F, Quail, MA, Guest, JR & Andrews, SC (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**:637-657.

Neilands, JB (1981). Iron absorption and transport in microorganisms. *Annu Rev Nutr*, **1**:27-46.

Patzer, SI & Hantke, K (1998). The ZnuABC high-affinity zinc uptake system and its regulator Zur in *Escherichia coli*. *Mol Microbiol*, **28**:1199-1210.

Pereira, AS, Timoteo, CG, Guilherme, M, Folgosa, F, Naik, SG, Duarte, AG, Huynh, BH & Tavares, P (2012). Spectroscopic evidence for and characterization of a trinuclear ferroxidase center in bacterial ferritin from *Desulfovibrio vulgaris* Hildenborough. *J Am Chem Soc*, **134**:10822-10832.

Prousek, J (2007). Fenton chemistry in biology and medicine. *Pure Appl Chem*, **79**:2325-2338.

Ratnayake, DB, Wai, SN, Shi, Y, Amako, K, Nakayama, H & Nakayama, K (2000). Ferritin from the obligate anaerobe *Porphyromonas gingivalis*: purification, gene cloning and mutant studies. *Microbiology*, **146 (Pt 5)**:1119-1127.

Ren, B, Tibbelin, G, Kajino, T, Asami, O & Ladenstein, R (2003). The multi-layered structure of Dps with a novel di-nuclear ferroxidase center. *J Mol Biol*, **329**:467-477.

Rocha, ER, Andrews, SC, Keen, JN & Brock, JH (1992). Isolation of a ferritin from *Bacteroides fragilis*. *FEMS Microbiol Lett*, **74**:207-212.

Rocha, ER & Smith, CJ (2004). Transcriptional regulation of the *Bacteroides fragilis* ferritin gene (*ftnA*) by redox stress. *Microbiology*, **150**:2125-2134.

Rodionov, DA, Dubchak, I, Arkin, A, Alm, E & Gelfand, MS (2004). Reconstruction of regulatory and metabolic pathways in metal-reducing delta-proteobacteria. *Genome Biol*, **5**:R90.

Romão, CV, Louro, R, Timkovich, R, Lubben, M, Liu, MY, LeGall, J, Xavier, AV & Teixeira, M (2000). Iron-coproporphyrin III is a natural cofactor in bacterioferritin from the anaerobic bacterium *Desulfovibrio desulfuricans*. *FEBS Lett*, **480**:213-216.

Smith, JM, Quirk, AV, Plank, RW, Diffin, FM, Ford, GC & Harrison, PM (1988). The identity of *Escherichia coli* bacterioferritin and cytochrome b_1. *Biochem J*, **255**:737-740.

Stiefel, EI & Watt, GD (1979). *Azotobacter* cytochrome $b_{557.5}$ is a bacterioferritin. *Nature*, **279**:81-83.

Stillman, TJ, Hempstead, PD, Artymiuk, PJ, Andrews, SC, Hudson, AJ, Treffry, A, Guest, JR & Harrison, PM (2001). The high-resolution X-ray crystallographic structure of the ferritin (EcFtnA) of *Escherichia coli*; comparison with human H ferritin (HuHF) and the structures of the Fe^{3+} and Zn^{2+} derivatives. *J Mol Biol*, **307**:587-603.

Tatur, J, Hagen, WR & Matias, PM (2007). Crystal structure of the ferritin from the hyperthermophilic archaeal anaerobe *Pyrococcus furiosus*. *J Biol Inorg Chem*, **12**:615-630.

Timóteo, CG, Guilherme, M, Penas, D, Folgosa, F, Tavares, P & Pereira, AS (2012). *Desulfovibrio vulgaris* bacterioferritin uses H(2)O(2) as a co-substrate for iron oxidation and reveals DPS-like DNA protection and binding activities. *Biochem J*, **446**:125-133.

Tong, Y & Guo, M (2009). Bacterial heme-transport proteins and their heme-coordination modes. *Arch Biochem Biophys*, **481**:1-15.

Ueshima, J, Shoji, M, Ratnayake, DB, Abe, K, Yoshida, S, Yamamoto, K & Nakayama, K (2003). Purification, gene cloning, gene expression, and mutants of Dps from the obligate anaerobe *Porphyromonas gingivalis*. *Infect Immun*, **71**:1170-1178.

Wai, SN, Nakayama, K, Umene, K, Moriya, T & Amako, K (1996). Construction of a ferritin-deficient mutant of *Campylobacter jejuni*: contribution of ferritin to iron storage and protection against oxidative stress. *Mol Microbiol*, **20**:1127-1134.

Wildschut, JD, Caffrey, SM, Voordouw, JK & Voordouw, G (2012). Oxygen exposure increases resistance of *Desulfovibrio vulgaris* Hildenborough to killing by hydrogen peroxide. *Antonie Van Leeuwenhoek*, **101**:303-311.

Wong, SG, Abdulqadir, R, Le Brun, NE, Moore, GR & Mauk, AG (2012). Fe-haem bound to *Escherichia coli* bacterioferritin accelerates iron core formation by an electron transfer mechanism. *Biochem J*, **444**:553-560.

Yasmin, S, Andrews, SC, Moore, GR & Le Brun, NE (2011). A new role for heme, facilitating release of iron from the bacterioferritin iron biomineral. *J Biol Chem*, **286**:3473-3483.

Zeth, K (2012). Dps biomineralizing proteins: multifunctional architects of nature. *Biochem J*, **445**:297-311.

Zhang, Y, Fu, J, Chee, SY, Ang, EX & Orner, BP (2011). Rational disruption of the oligomerization of the mini-ferritin *E. coli* DPS through protein-protein interface mutation. *Protein Sci*, **20**:1907-1917.

Zhao, G, Ceci, P, Ilari, A, Giangiacomo, L, Laue, TM, Chiancone, E & Chasteen, ND (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**:27689-27696.

Zhou, A, He, Z, Redding-Johanson, AM, Mukhopadhyay, A, Hemme, CL, Joachimiak, MP, Luo, F, Deng, Y, Bender, KS, He, Q, Keasling, JD, Stahl, DA, Fields, MW, Hazen, TC, Arkin, AP, Wall, JD & Zhou, J (2010). Hydrogen peroxide-induced oxidative stress responses in *Desulfovibrio vulgaris* Hildenborough. *Environ Microbiol*, **12**:2645-2657.

Part 1

Chapter 3

Desulfovibrio responses to oxidative and nitrosative stresses

Chapter 3

Desulfovibrio responses to oxidative and nitrosative stresses

3.1 - Oxidative and nitrosative stresses

3.1.1 - Oxidative stress

The toxicity of oxygen is largely related to the formation of partially reduced species. Oxygen is able to cross the biological membranes and, inside the cell, oxidizes cofactors like metal centers, flavins and respiratory quinones which are electron carrier in respiratory chain, with generation of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radical (Figure 3.1) (Imlay, 2003).

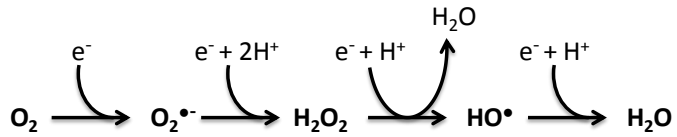


Figure 3.1 – Reactive oxygen species.
O_2 - molecular dioxygen; $O_2^{\bullet -}$ - superoxide anion; H_2O_2 - hydrogen peroxide; $HO^{\bullet}$ - hydroxyl radical; H_2O - water; e^- - electrons; H^+ - protons. Adapted from (Imlay, 2003).

The formation of ROS inside the bacterial cell occurs by sequential univalent reductions of molecular oxygen catalyzed by membrane-associated respiratory chain enzymes. Electrons from the bacterial electron transport chain can reduce oxygen molecules to superoxide anion, in a reaction similar to the one that occurs in eukaryotic mitochondria (Cabiscol *et al.*, 2000). Furthermore, both $O_2^{\bullet -}$ and H_2O_2 are generated by the autoxidation of non-respiratory flavoproteins (Imlay, 2013). In addition, a number of external agents can also trigger exogenous ROS

production, including ionizing radiation, ultraviolet light, chemotherapeutic drugs, environmental toxins and macrophages during the inflammatory response (Finkel & Holbrook, 2000).

The reaction of H_2O_2 with free intracellular ferrous iron (Fe^{2+}), leads to the formation of $OH^{\bullet}$ through the Fenton reaction. The hydroxyl radical can cause direct damage of several biomolecules, including DNA (Imlay, 2003; Imlay, 2008). Also, the negative charge of DNA molecules favors binding of metal ions, namely iron, generating further hydroxyl radicals that attack sugars, purines and pyrimidines of DNA, thus leading to cell death (Salmon *et al.*, 2004; Avery, 2011).

The degree of oxidation of proteins by ROS depends on factors like the protein content of oxidative-sensitive amino acid residues such as those containing aromatic side chain groups and sulfhydryl groups, the presence of metal binding sites, protein localization and solvent exposition (Avery, 2011). In particular, solvent exposed proteins containing iron-sulfur (Fe-S) clusters are very susceptible to oxidative damage due to the affinity of ROS to $[4Fe\text{-}4S]^{2+/1+}$. The binding of ROS to these centers results in the loss of one or more iron ions, thus causing partial or total degradation of the clusters and consequently inactivation of the enzymes. Moreover, the release of free iron from the enzymes contributes to increase the oxidative stress via the Fenton reaction (Flint *et al.*, 1993; Cabiscol *et al.*, 2000).

Lipids are also main targets of the oxidative stress. Polyunsaturated fatty acids in membranes are damaged by superoxide anion and hydroxyl radicals through initiation of autocatalytic lipid peroxidation. Apparently, this process only occurs in bacteria with ability to incorporate exogenous polyunsaturated fatty acids, such as the pathogens *Helicobacter pylori* and *Borrelia burgdorferi* (Hazell & Graham, 1990; Boylan *et al.*, 2008).

3.1.2 - Nitrosative stress

Nitric oxide (NO) was considered from many years a undesirable pollutant and potential carcinogen (Priestley, 1966). However, the molecule is enzymatically generated in mammalian systems, and the recognition that NO is involved in a wide range of biological processes, namely cell signaling, gave rise to a new era for NO biology (Ignarro, 2000; Bowman *et al.*, 2011). In fact, this molecule was considered "molecule of the year" in 1992 by journal Science (Koshland, 1992) due to the discovery of its role as a biological regulator and, since then, an intensive research surrounding NO and it metabolites has been performed.

To generate reactive nitrogen species (RNS) NO reacts with other radicals which are highly reactive and toxic (Figure 3.2). Nitric oxide combines with O_2 to form nitrogen dioxide (NO_2) that dimerises to give dinitrogen tetroxide (N_2O_4), which when dissolved in water forms nitrite (NO_2^-) and nitrate (NO_3^-). If the reaction occurs in water, the first product, NO_2, may react with another NO molecule forming N_2O_3, which is the anhydride form of nitrous acid, generating only nitrite (Hughes, 2008). The reaction of nitric oxide with superoxide anion ($O_2^{\bullet-}$) is extremely fast and results in the formation of peroxynitrite ($ONOO^-$), which is a powerful oxidizing and nitrating agent (Hughes, 2008; Bowman *et al.*, 2011).

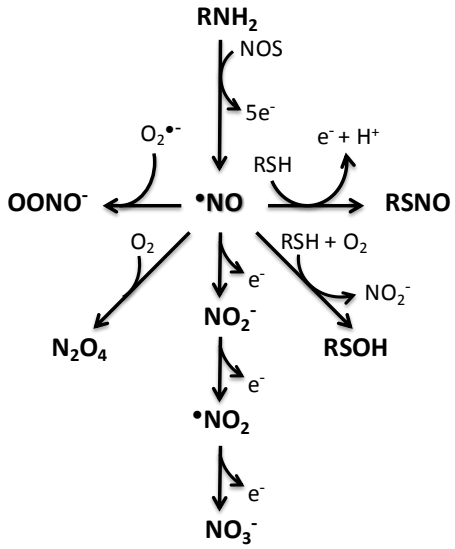


Figure 3.2 – Reactive nitrogen species formation in mammalian cells via NOS.
RNH_2 - guanidine nitrogen of L-arginine; NOS - nitric oxide synthase; $^{\bullet}NO$ - nitric oxide; RSNO - nitrosothiol; RSOH - sulfenic acid; $OONO^-$ - peroxynitrite; NO_2^- - nitrite; $^{\bullet}NO_2$ - nitrogen dioxide; NO_3^- - nitrate; N_2O_4 - dinitrogen tetroxide; $O_2^{\bullet-}$ - superoxide; O_2 - dioxygen; RSH - sulfhydril; e^- - electron; H^+ - proton. Adapted from (Nathan & Shiloh, 2000).

The production of nitric oxide in biological systems can occur by enzymatic or non-enzymatic ways. In humans, the mild acidification of nitrite is a source of NO, which is proposed to contribute to eradicate pathogens in stomach and urine (Weitzberg & Lundberg, 1998). Nitric oxide can also be enzymatically synthesized *in vivo* through two major processes: (i) NO is formed as an intermediate during the bacterial denitrification process and (ii) NO is generated from the oxidation of L-arginine to citrulline catalyzed by mammalian NO synthase (NOS).

Denitrification is one of the main steps of the nitrogen cycle sustained by bacteria, in which nitrate is enzymatically reduced via nitrite, NO and nitrous oxide (N_2O) to dinitrogen gas (N_2) (Figure 3.3). The nitric oxide generated by denitrifying bacteria can inhibit the growth of microbes present in the surrounding environment. For example, co-incubation of the denitrifier *Achromobacter cycloclastes* with *Bacilus subtilis* revealed the induction of the *Bacillus* flavohemoglobin (*hmp*) gene, suggesting that the NO produced by denitrifiers is freely diffusible and impacts on adjacent bacteria (Choi *et al.*, 2006). *Desulfovibrio* spp. are able to perform dissimilatory reduction of nitrate and nitrite (as mentioned in Part I - Chapter 1), but in this process the formation of intermediates such as NO was not yet shown (Einsle *et al.*, 2002; Lukat *et al.*, 2008; Silveira *et al.*, 2010).

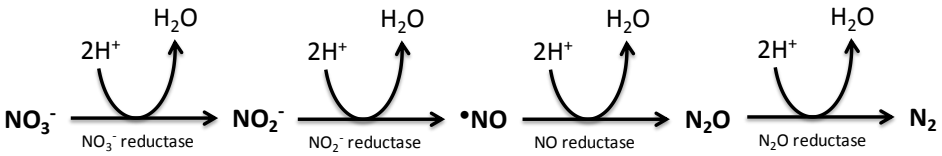


Figure 3.3 – Denitrification pathway.
Nitrate (NO_3^-) is reduced by nitrate reductase to nitrite (NO_2^-), which is reduced by nitrite reductase to nitric oxide ($^{\bullet}NO$), and undergo further reduction to nitrous oxide (N_2O) via NO reductase. The formation of dinitrogen gas (N_2) occurs though the reduction of nitrous oxide by N_2O reductase. Adapted from (Averill, 1996).

In mammalian cells, nitric oxide is synthesized by three types of nitric oxide synthase (NOS) isoforms: the endothelial (eNOS), the neuronal (nNOS), and the inducible (iNOS), all converting arginine into nitric oxide (L-arginine + O_2 + NADPH → NO + L-citrulline). The expression of iNOS is induced by several immunological or inflammatory signals, like interferon-gamma (IFN-γ) and the bacterial lipopolysaccharide (LPS) (MacMicking *et al.*, 1997; Stuehr, 1999). The iNOS enzyme is able of produce abundant quantities of NO (micromolar levels) when the inductive stimuli is present (*e.g.* cytokines, LPS) and the substrate L-arginine is not limited (Chakravortty & Hensel, 2003). Interestingly, the production of NO by iNOS-like proteins is also found in bacteria. These homologues occur mostly in Gram-positive bacteria, such as *Staphylococcus* spp. and *Bacillus* spp., also in the Gram-negative *Sorangium (Polyangium) cellulosum* and in the Archaeon *Natronomonas pharaonis* (Agapie *et al.*, 2009; Sudhamsu & Crane, 2009). In bacteria, the small amounts of NO produced are proposed to provide protection against oxidative and nitrosative stress, as it activates sensors that respond to nitric oxide (Shatalin *et al.*, 2008; Sudhamsu & Crane, 2009; Crane *et al.*, 2010).

3.2 - Stress environmental challenges

3.2.1 - *Desulfovibrio* spp. in oxidative habitats

The recognition of SRB as strict anaerobes started changing when sulfate reduction was found to occur in oxidized sediment layers (Jorgensen, 1977). The occurrence of SRB in oxic environments has been recognized in several oxic-anoxic interfaces of microbial mats or sediments as well as in periodically oxygenated zones of aquatic environments (Fukui & Takii, 1990; Teske *et al.*, 1998).

In the presence of oxygen *Desulfovibrio* spp. undergo several physiological alterations. Aggregation of cells or attachment to surfaces in association with oxygen-scavenging aerobic bacteria is one of the mechanisms that provides higher

tolerance to oxygen exposure as aggregates remain anaerobic (Sigalevich *et al.*, 2000).

Due to the aerotaxis primary defense mechanism in anaerobes, high oxygen concentrations are expected to function as a repellent; however, at low concentrations of oxygen *Desulfovibrio* spp. are reported to move towards the oxygen source and to reduce the oxygen to water (Johnson *et al.*, 1997). Sulfate reducing populations in opposition to that in microbial mats, avoid high oxygen concentrations through migration to deeper anoxic zones (Krekeler *et al.*, 1998). *In vitro* studies showed that *D. vulgaris*, *D. oxyclinae* and *D. desulfuricans* CSN behave differently. Indeed, *D. oxyclinae* and *D. desulfuricans* exposed to O_2 diffusion gradient did not swim into anoxic zones but accumulate in the oxic zone forming a ring-shaped band surrounding the O_2 bubbles (Eschemann *et al.*, 1999). Similar behavior was observed in *D. vulgaris* given that in a glass capillary with an oxygen gradient cells form a band near a oxygen concentration of 0.02 - 0.04% (Johnson *et al.*, 1997).

Consistent with association of aerotaxis with oxygen sensing mechanisms the genome of *D. vulgaris* contains a large family of 27 methyl-accepting chemotaxis proteins, including proteins like DcrA and DcrH (Heidelberg *et al.*, 2004). The DcrA transmembrane methyl-accepting protein contains a *c*-type heme in the periplasmic domain that functions as a redox and oxygen sensor (Fu *et al.*, 1994). Also, the cytoplasmic located hemerythrin domain (DrcH) is proposed to function as an O_2 sensor (Xiong *et al.*, 2000).

Interestingly, sulfate reducing bacteria can use molecular oxygen as a terminal electron acceptor and gain ATP from this process, but no growth using oxygen as the only electron acceptor was, so far, described (Dilling & Cypionka, 1990). Thus, the reduction of oxygen in SRB is most probably a protective mechanism instead of a growth linked energy coupled conservation mechanism (Lamrabet *et al.*, 2011). Depending on the species, sulfate reducing bacteria are

able to use at least one of the following electron donors to reduce oxygen: H_2, lactate, pyruvate, formate, acetate, butyrate, ethanol, sulfide, thiosulfate, and sulfite (Dannenberg *et al.*, 1992). Several studies revealed the different ability of several *Desulfovibrio* spp. to survive oxygen exposure. For example, exposure of *D. gigas* to approximately 0.5% of oxygen completely inhibited bacterial growth. Nevertheless, the bacterium was able to resume growth when the conditions switch again to anaerobiosis (Fareleira *et al.*, 2003). *D. desulfuricans* ATCC 27774 prove to be able to grow in the presence of nearly atmospheric oxygen levels (~ 18% O_2), while *D. desulfuricans* NCIB 8301 only sustained oxygen concentrations up to ~ 0.4%, and *D. desulfuricans* Essex and CSN display a strong growth decreased when submitted to an oxygen concentration of only ~ 2% (Abdollahi & Wimpenny, 1990; Marschall *et al.*, 1993; Lobo *et al.*, 2007).

3.2.2 - Mammalian immune system response against invading bacteria

Desulfovibrio spp. are found in humans as part of the microbial flora of the human intestine (Macfarlane *et al.*, 2007). However, in the last years these microorganisms have been proposed to be involved in several diseases, such as periodontitis, inflammatory bowel diseases and brain abscesses (Loubinoux *et al.*, 2000; Loubinoux *et al.*, 2002a; Loubinoux *et al.*, 2002b). As such *Desulfovibrio* are exposed to the immune system weapons against pathogens. The immune response is divided in anatomical and physiological barriers, which are the first line of defense, and in innate and adaptive defense mechanisms, that together eliminate pathogens and prevent disease (Medzhitov, 2007; Turvey & Broide, 2010). The adaptive immunity is mediated by antigen receptors and characterized by its high degree of specificity and the property of memory, while innate immunity is non-specific and employs a broad-acting anti-microbial strategy (Medzhitov, 2007; Turvey & Broide, 2010). During the first hours after a pathogen invasion, the innate mammalian immune system triggers a protective

inflammatory response mediated by pattern recognition receptors (*e.g.* Toll-like receptors), which bind components of the microbial cell wall like lipopolysaccharides, peptidoglicans and lipoteichoic acids. The innate immune response also triggers the activation of the adaptive immune response, which is initiated approximately 5 - 7 days post-infection by lymphocyte activation and proliferation (Radtke & O'Riordan, 2006; Medzhitov, 2007).

Phagocytes (*e.g*. macrophages and neutrophils) are key elements of the innate immunity responsible to eliminate both intracellular and extracellular bacteria (Medzhitov, 2007). The primary function of these cells is to engulf and destroy the pathogens and digest their remains. Phagocytes receptors recognize directly the pathogens coat triggering its engulfment in a phagocytic vacuole or phagosome, which fuses with intracellular granules (*e.g*. lysosome) to form a phagolysosome (Stuart & Ezekowitz, 2005). Compartmentalization of the pathogens provides an enclosed hostile environment, with low nutrient availability, low pH and iron limitation (Radtke & O'Riordan, 2006). Another potent antimicrobial defense mechanism within the phagolysosome is the production of ROS and RNS (Nathan & Shiloh, 2000). In mammals, the enzyme NADPH oxidase (Phox) is responsible for the production of superoxide, which is dismutated to H_2O_2 by the enzyme superoxide dismutase (Sod). The later product, that triggers the Fenton reaction to form $^{\bullet}OH$ and OH^-, is used by myeloperoxidase (MPO) to synthesize hypochlorous acid (HOCl) (Babior, 2000; Nathan & Shiloh, 2000).

The RNS generated in the phagolysosome are produced by the mammalian iNOS (Babior, 2000; Nathan & Shiloh, 2000). Due to its lipophilicity and small size the nitric oxide generated freely crosses and kills or inhibits a wide range of organisms such as bacteria, fungi, parasitic worms and protozoa. Generation of RNS occurs later then oxidative burst but last much longer (Chakravortty & Hensel, 2003; Hughes, 2008). ROS and RNS are considered key antimicrobial agents of the mammalian immune system, the abolition of ROS and RNS formation in mice

results in massive abscesses formed by spontaneous infections, which contain several commensal microorganisms (Shiloh *et al.*, 1999). Furthermore, they are considered to have non-redundant roles since the lack of either iNOS or Phox increased the susceptibility in mice to a variety of experimental infections (Jackson *et al.*, 1995; Pollock *et al.*, 1995; MacLean *et al.*, 1998; Nathan & Shiloh, 2000).

3.3 - *Desulfovibrio* systems putatively involved in oxygen and nitric oxide detoxification

In response to the hazardous effects of oxygen, nitric oxide and derivatives, bacteria activate different systems of defense, including O_2 reduction and ROS and RNS detoxification.

As mentioned in Chapter I - Part 1, the ability to reduce oxygen to water is widespread among SRB a protective mechanism. In *Desulfovibrio* spp., the oxygen reduction is accomplished by three systems: (i) membrane-bound oxygen reductases, (ii) periplasmic reduction involving hydrogenases and *c*-type cytochromes, and (iii) cytoplasmic reduction chain involving rubredoxin:oxygen oxidoreductase (Dolla *et al.*, 2007).

The first indication of a membrane-bound terminal oxygen reductase in *Desulfovibrio* resulted from the sequencing of *D. vulgaris* Mikazaki cytochrome c_{553} gene locus which showed to be located near a gene encoding a heme-copper oxygen reductase, sharing amino acid similarities to cytochrome *c* oxidase (Cox) family of proteins (Kitamura *et al.*, 1995). Subsequently, a canonical cytochrome *bd* quinol:oxygen oxidoreductase (CydAB) containing a heme *d* and two types of hemes *b* (b_{558} and b_{595}) was isolated from the membrane fractions of *D. gigas* (Lemos *et al.*, 2001). The genome of *D. vulgaris* Hildenborough also showed to contained *cydAB* and *cox* like genes (Heidelberg *et al.*, 2004). More recently, the characterization of Cox proved that it is composed of hemes type *c*, *o*, *b* and o_3 (Lamrabet *et al.*, 2011).

The periplasmic oxygen reduction in *Desulfovibrio* spp. was first observed in *D. vulgaris* Marburg cells when using hydrogen as electron donor. This microorganism reduces oxygen to water in the periplasm through a mechanism proposed to involve electron transfer from hydrogenases to cytochromes c_3 (Baumgarten *et al.*, 2001). Indeed, the genome of *D. vulgaris* encodes four periplasmic hydrogenases, two cytoplasmic-facing membrane-bound hydrogenases, and seventeen putative *c*-type cytochromes that are periplasmic, periplasmically oriented and membrane-associated (Dolla *et al.*, 2007). The role of some of these proteins in oxidative protection in *D. vulgaris* was also suggested as exposure to oxygen increased the content of periplasmic cytochromes *c* and hydrogenases. Furthermore, a strain lacking the periplasmic [Fe] hydrogenase had decreased viability upon exposure to oxygen for 1 h. Under similar conditions, the transcription of the gene encoding [Fe] hydrogenase (DVU1769) was induced in *D. vulgaris* wild type cells (Fournier *et al.*, 2004; Fournier *et al.*, 2006).

In the presence of oxygen, *D. gigas* uses its polyglucose carbon reserves to produce ATP at the substrate level phosphorylation, leading to the formation of NADH, in a process that is combined with NADH re-oxidation by a cytoplasmic electron transfer chain (Santos *et al.*, 1993; Fareleira *et al.*, 1997). This chain is constituted by three cytoplasmic proteins: NADH:rubredoxin oxidoreductase (Nro), rubredoxin (Rd) and rubredoxin:oxygen oxidoreductase (Roo) (Chen *et al.*, 1993b; Chen *et al.*, 1993a). As proposed, Nro transfers the electrons resulting from NADH oxidation to rubredoxin, which acts as an intermediate carrier in the Roo-mediated reduction of oxygen to water (Gomes *et al.*, 1997).

D. gigas Roo was described as an O_2 reductase, but subsequent studies raised the possibility of an alternative/additional NO reductase function. Currently, Roo is considered a bifuntional protein that may function as an NO and/or O_2 reductase (Silaghi-Dumitrescu *et al.*, 2005; Wildschut *et al.*, 2006; Rodrigues *et al.*, 2006). The *Desulfovibrio* Roo was the first example of a flavodiiron containing protein (FDP)

family of proteins that is widespread among Archaea and Bacteria including anaerobes and facultative anaerobes and in protozoa (Saraiva *et al.*, 2004; Vicente *et al.*, 2008b). Several microorganisms contain more than one gene encoding putative flavodiiron proteins. For example, *Clostridia perfringens* has three *loci* coding for different FDPs (Shimizu *et al.*, 2002), and *Synechocystis* sp. PCC 6803 contains four putative FDP encoding genes (Zhang *et al.*, 2009). Two Roo homologues are present in *D. vulgaris* genome (*roo1*, DVU2014 and *roo2*, DVU3185), and their amino acid sequences share 29% identity (Heidelberg *et al.*, 2004; Figueiredo *et al.*, 2013). Analyses of strains lacking the two genes *roo1* and *roo2* lead to cells with lower resistance to oxygen (1% O_2) (Wildschut *et al.*, 2006; Johnston *et al.*, 2009; Yurkiw *et al.*, 2012). The crystal structure of *D. gigas* Roo showed that the protein is a functional homodimer, with each subunit containing two different domains: a β-lactamase-like domain with a diiron center where the reduction of both NO and O_2 occurs, and a flavodoxin-like domain containing a FMN (flavin mononucleotide) cofactor that transfers the electrons required for the dioxygen reduction (Figure 3.4) (Frazão *et al.*, 2000). The two domains are the structural core of FDPs, and are present in the simplest members of this family, named class A FDPs (Wasserfallen *et al.*, 1998; Saraiva *et al.*, 2004). Molecular dynamics studies of *D. gigas* Roo showed similar diffusion profiles for both O_2 and NO, but did not demonstrate significant preference to accommodate O_2 or NO (Victor *et al.*, 2009). Therefore, the physiological role of Roo in O_2 and NO detoxification in sulfate reducing bacteria is still under debate.

Cytochrome *c* nitrite reductase (NrfHA) is involved in the dissimilatory nitrate reduction (see Part I - Chapter 1) and proposed to confer resistance to nitrogen. Cytochrome *c* nitrite reductase in *D. desulfuricans* (originally described as hexaheme monomeric cytochrome) is capable of reducing NO to either ammonia or nitrous oxide (Costa *et al.*, 1990).

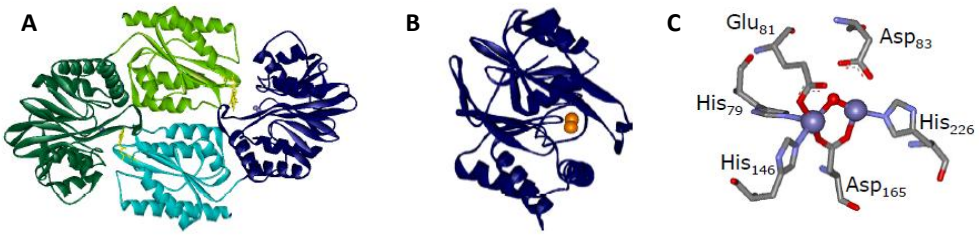


Figure 3.4 – Structural characteristics of *D. gigas* Roo.
A) Crystallographic structure of Roo illustrating its functional quaternary structure. The different colors represent different monomers, and different tones illustrate different modules. **B)** Metallo β-lactamase domain bearing the diiron centre; **C)** First coordination sphere of the diiron centre with ligands. Adapted from (Frazão *et al.*, 2000; Saraiva *et al.*, 2004).

Desulfovibrio spp. also encode enzymes specialized in detoxification of ROS. Iron-containing Sods (Fe-Sod) were isolated from *D. gigas, D. desulfuricans* and *D. vulgaris* (Hatchikian & Henry, 1977; Dos Santos *et al.*, 2000; Fournier *et al.*, 2003; Nakanishi *et al.*, 2003; Davydova *et al.*, 2006). Furthermore, a *D. vulgaris* mutant in *sodB* gene exhibited decreased viability when exposed to oxygen, indicating that SodB has an oxidative protective role (Fournier *et al.*, 2003). In anaerobes and microaerophiles, superoxide removal is performed by superoxide reductase (Sor), which converts superoxide into hydrogen peroxide (Pinto *et al.*, 2010). In *Desulfovibrio* spp., two Sor homologues have been identified: the rubredoxin oxidoreductase (Rbo, also known as desulfoferrodoxin (Dfx)) and neelaredoxin (Nlr) (Brumlik & Voordouw, 1989; Moura *et al.*, 1990; Chen *et al.*, 1994; Silva *et al.*, 2001). In *D. vulgaris*, Rbo acts as an oxygen defense protein causing the deletion of the gene an increase of the strain sensitivity to oxygen and superoxide stress (Voordouw & Voordouw, 1998; Lumppio *et al.*, 2001).

Heme catalases catalyze disproportionation of hydrogen peroxide to water and molecular oxygen ($2H_2O_2 \rightarrow O_2 + 2H_2O$) (Alfonso-Prieto *et al.*, 2009). These enzymes have also been isolated from *D. vulgaris* and *D. gigas* (Hatchikian *et al.*, 1977; Dos Santos *et al.*, 2000). Hydrogen peroxide is also removed in *Desulfovibrio*

by NAD(P)H dependent peroxidases such as rubrerythrin (Rbr) and nigerythrin (Ngr), which catalyze the two-electron reduction of hydrogen peroxide to water ($2e^- + 2H^+ + H_2O_2 \rightarrow 2H_2O$) (Coulter *et al.*, 1999; Coulter & Kurtz, 2001).

Although no peroxiredoxins have been yet isolated from *Desulfovibrio* spp., additional protection against hydrogen peroxide is proposed to be provided by thiol-specific peroxidases like thiol peroxidase (Tpx), bacterioferritin comigratory protein (Bcp), glutaredoxin (Glr), alkyl hydroperoxidases (AhpC and AhpF) and thioredoxin (Trx), all proteins that are encoded in the *Desulfovibrio* spp. genomes (Heidelberg *et al.*, 2004). *D. vulgaris* genes encoding Tpx and Bcp proteins exhibited up-regulation under oxygen exposure. Moreover, the protein level of Tpx, Bcp and Glr showed to be more abundant in oxygenated cells, suggesting that these thiol peroxidases are involved in defense of *D. vulgaris* against oxidative stress (Fournier *et al.*, 2006). Recent studies reinforced the role of *D. vulgaris tpx* and *ahp*C transcripts in hydrogen peroxide resistance by showing that these genes are regulated by H_2O_2 (Brioukhanov *et al.*, 2010).

The hybrid cluster protein (Hcp) of *D. desulfuricans* ATCC 27774 presents a peroxidase activity of the same order of magnitude to other peroxidases suggesting its involvement in oxidative stress defense (Almeida *et al.*, 2006). The three dimensional structure showed that *D. vulgaris* Hcp is composed of three domains: an N-terminal mainly α-helical domain and two similar domains comprising a central β-sheet flanked by α-helices (Figure 3.5) (Cooper *et al.*, 2000). Hcp contains two redox-active iron-sulfur clusters, one variable and present as either $[2Fe\text{-}2S]^{2+/1+}$ or $[4Fe\text{-}4S]^{2+/1+}$, and a second conserved cluster with an unusual asymmetric structure $[4Fe\text{-}2S\text{-}3O]^{2+/1+}$ type (van den Berg *et al.*, 2000). As the later was first thought to be a $[6Fe\text{-}6S]^{2+/1+}$ in a "prismane" configuration the protein was named prismane protein (Hagen *et al.*, 1989).

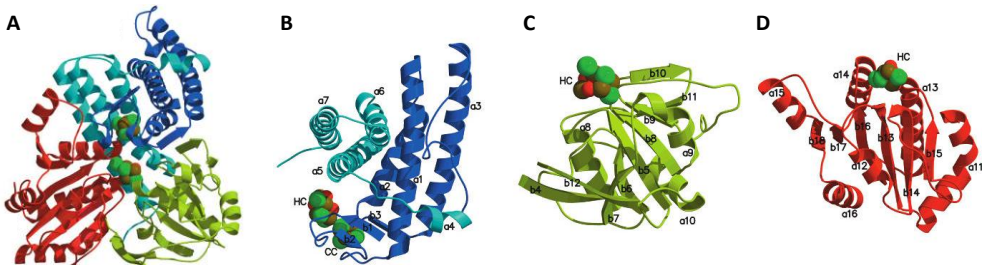


Figure 3.5 – Crystallographic structure of *D. vulgaris* Hcp.
A) Three dimensional structure of Hcp, different colors represent different domains, in light and dark blue (domain 1), in yellow (domain 2) and in red (domain 3). The iron, sulfur and oxygen atoms of the clusters are represented in brown, green and red spheres, respectively. **B)** The two α-helix bundles of domain 1. **C)** Domain 2 and **D)** domain 3 showing the central β-sheet flanked by α-helices. Adapted from (Cooper *et al.*, 2000).

In some microorganisms, such as *E. coli*, Hcp proved to be able to catalyze the hydroxylamine reduction using electrons from NADH or NADPH and therefore considered a hydroxylamine reductase enzyme able to protect against hydroxylamine generated during nitrite reduction to ammonia (Wolfe *et al.*, 2002; Cabello *et al.*, 2004; Overeijnder *et al.*, 2009). On the other hand, transcriptomic studies showed that *E. coli hcp* gene is strongly up-regulated under conditions of nitrosative stress (Flatley *et al.*, 2005). In *D. vulgaris*, which contains two genes coding for hybrid cluster proteins, namely Hcp1 (DVU2013) and Hcp2 (DVU2543), the up-regulation of *hpc2* gene is also induced by nitrite stress conditions. The lack of differential expression under nitrite and nitrate stress of *hcp1* gene suggested that this gene was constitutively expressed (Haveman *et al.*, 2004; He *et al.*, 2006; He *et al.*, 2010a). However, a different study reported up-regulation of *hcp1* gene in the presence of nitrite, albeit with a smaller fold-change when compared with that of the *hcp2* gene (Haveman *et al.*, 2005). Hence, the function of *Desulfovibrio* Hcp-like proteins in defense against oxygen and nitrogen stresses remains to be clarified.

Figure 3.6 represents a schematic model of some of the enzymes that participate in oxygen resistance in *Desulfovibrio*.

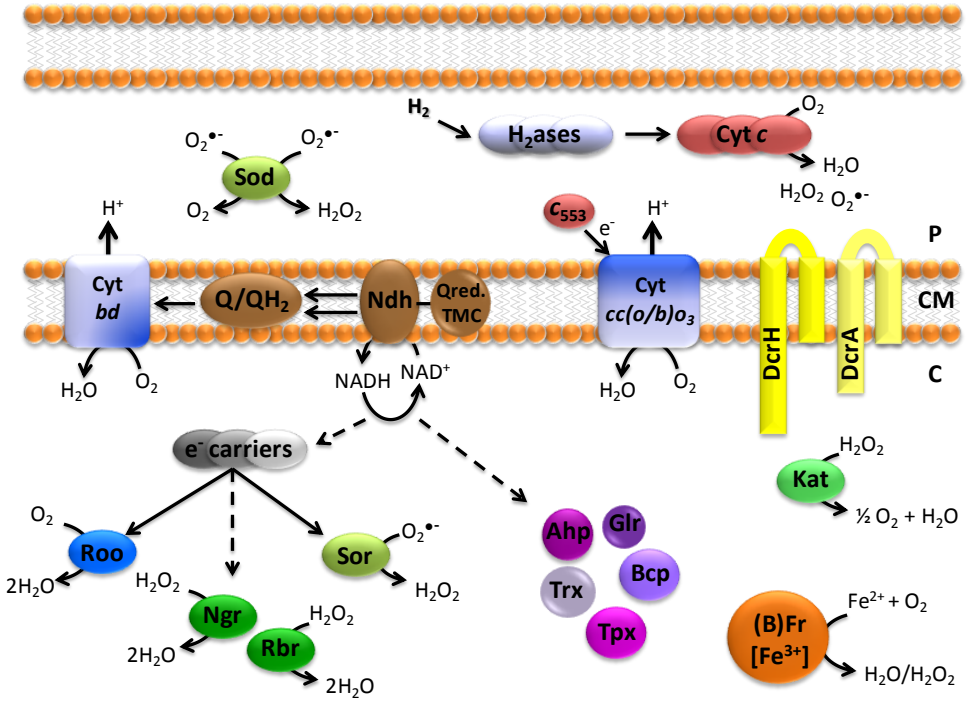


Figure 3.6 - Model for oxygen defense in *Desulfovibrio* spp.

Electrons for the reduction of oxygen and ROS can be generated by oxidation of lactate (cytoplasm), formate (periplasm) and chemical oxidation of sulfide. Blue proteins are involved in oxygen reduction: Roo (rubredoxin:oxygen oxidoreductase), Cyt *bd* (cytochrome *bd* quinol:oxygen oxidoreductase) and Cyt *cc(o/b)o*$_3$ (cytochrome *c* oxidase *cc*(*o*/*b*)*o*$_3$); green proteins are involved in ROS detoxification: Kat (catalase), Ngr (nigerytrin), Rbr (rubrerythrin), Sod (superoxide dismutase) and Sor (superoxide reductase); yellow proteins are involved in oxygen sensing: DcrA and DcrH (methyl-accepting chemotaxis proteins); brown proteins are involved in electron transfer across the membrane: Qred.TMC (menaquinones-reducing transmembrane complexes), Ndh (type II NADH dehydrogenase) and Q/QH$_2$ (menaquinones/menaquinol pool). H$_2$ases (hydrogenases); Cyt *c* (*c*-type cytochromes); c_{553} (monoheme cytochrome c_{553}); purple proteins are involved in repair systems: Ahp (alkyl hydroperoxidases), Glr (glutaredoxin), Trx (thioredoxin system), Bcp (bacterioferritin comigratory protein), Tpx (thiol peroxidase). (B)fr ((bacterio)ferritin); e^- (electron); P (periplasm); CM (cytoplasmic membrane); C (cytoplasm).

General model was adapted from (Dolla *et al.*, 2007), membrane-bound system was adapted from (Lamrabet *et al.*, 2011), DcrA topology was adapted from (Deckers & Voordouw, 1994) and DcrH topology adapted from (Xiong *et al.*, 2000).

3.4 - Regulators of *Desulfovibrio* for oxidative and nitrosative stress resistance

PerR is a homodimer with two metal binding sites per monomer; one site binds Zn^{2+} that probably has a structural role, and a second site that binds either Mn^{2+} or Fe^{2+}, which has a regulatory function (Herbig & Helmann, 2001).

In its active form, PerR is a transcriptional repressor that binds to the consensus box (TTATAAT(a/t)ATTATAA) blocking the transcription of the target genes (Mongkolsuk & Helmann, 2002). The PerR sensitivity to peroxides is dependent on the availability of Mn^{2+} and Fe^{2+} since the DNA-binding activity of Fe^{2+}-PerR-Zn^{2+} is abolished by exposure to H_2O_2 while Mn^{2+}-PerR-Zn^{2+} remains active. Upon exposure to H_2O_2, the iron atom is oxidized via a direct Fenton reaction that locally produces hydroxyl radical. The consequent metal dissociation generates apo-PerR-Zn^{2+} and its inability to bind DNA leads to the derepression and transcription of several genes (Herbig & Helmann, 2001; Lee & Helmann, 2006; Cornelis *et al.*, 2011). Since PerR regulates genes predicted to be involved in oxidative general stress response it is considered a peroxide regulator (Cornelis *et al.*, 2011). In *D. vulgaris*, the *perR* gene is predicted to be self regulated (*perR*, DVU3095), and to control several genes, namely: rubrerythrin (*rbr1*, DVU3094), rubredoxin-like protein (*rdl*, DVU3093), alkyl hydroperoxidase (*ahpC*, DVU2247), rubrerythrin 2 (*rbr2*, DVU2318) and a conserved hypothetical protein (DVU0772) (Figure 3.7).

Figure 3.7 – *D. vulgaris* predicted PerR regulon.
Candidates with potential PerR binding motifs; red squares represent the predicted *D. vulgaris* PerR consensus sequences. Adapted from (Rodionov *et al.*, 2004).

Desulfovibrio spp. contain a putative member of the CRP/FNR (cAMP receptor protein/fumarate and nitrate reduction regulator protein) family of transcriptional factors, namely the hybrid cluster protein regulator (HcpR), which regulon

comprise genes involved, for example, in sulfate reduction such as *sat* gene that encodes ATP sulfurylase, and the *aprsAB* genes encoding APS reductase (Rodionov *et al.*, 2004; Cadby *et al.*, 2011). Interestingly, the *hcpR* gene (DVU2547) is co-localized with *hcp2* in the chromosome (Figure 3.8) (Rodionov *et al.*, 2004). Regulators from the CRP/FNR family of global transcriptional regulators are able to both repress and activate gene expression. The analysis of the predicted promoter region of the *D. vulgaris hcp2-frdX* operon suggested a positive regulation by HcpR, since the HcpR binding site is located upstream the box of predicted promoters (Rodionov *et al.*, 2004). Moreover, a recent study showed significant down-regulation of the *hcp2* (DVU2543) gene expression in a *D. vulgaris hcpR* mutant strain demonstrating that HcpR activates the expression of *hcp2* (Zhou *et al.*, 2012).

HcpR is proposed to bind DNA sites similar to the general consensus sequence of CRP (TTGTGANNNNNNTCACAA) (Rodionov *et al.*, 2004). However, so far, no direct evidence of this binding has been demonstrated, and the signal that modulates the HcpR activity remains to be established (Zhou *et al.*, 2011).

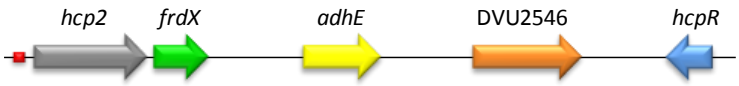


Figure 3.8 – Putative HcpR binding site upstream of the start codon of *hcp2*.
Genomic organization of *hcp2* (DVU2543) and *hcpR* (DVU2546) from *D. vulgaris* Hildenborough, the location of the putative HcpR binding site is represented by a red square; locus DVU2544: hypothetical ferredoxin (*frdX*), locus DVU2445: alcohol dehydrogenase (*adhE*), and locus DVU2546: histidine kinase. Adapted from (Rodionov *et al.*, 2004).

3.5 - *D. vulgaris* transcriptional response to stress conditions

The release of *D. vulgaris* genome originated several transcriptomic and proteomic studies focused on the discovering of genes involved in the response to stress conditions. These studies included the analysis of the *D. vulgaris* transcription in cells exposed to oxygen, (Zhang *et al.*, 2006; Fournier *et al.*, 2006;

Mukhopadhyay *et al.*, 2007; Pereira *et al.*, 2008), hydrogen peroxide (Zhou *et al.*, 2010; Brioukhanov *et al.*, 2010; Wildschut *et al.*, 2012), nitrate (He *et al.*, 2010a), nitrite (Haveman *et al.*, 2004; He *et al.*, 2006), NaCl and KCl (Mukhopadhyay *et al.*, 2006; He *et al.*, 2010b), heat shock (Chhabra *et al.*, 2006; Zhang *et al.*, 2006), alkaline pH (Stolyar *et al.*, 2007), and growth under depletion of electron donors (Clark *et al.*, 2006). In general, microorganisms exposed to stress conditions trigger the repression of genes that are involved in energy metabolism. Also, exposure of *D. vulgaris* to O_2, H_2O_2, starvation, nitrate and nitrite salts or heat shock triggered the repression of genes forming the operon coding for ATP synthesis (Chhabra *et al.*, 2006; Clark *et al.*, 2006; He *et al.*, 2006; Mukhopadhyay *et al.*, 2007; He *et al.*, 2010b; Zhou *et al.*, 2010). However, the response of *D. vulgaris* to high salinity caused an increase in the abundance of mRNA, and protein levels of genes involved in ATP synthesis pathway. This suggests an augment in the energy requirement which may result from the export and import of ions occurring in cells facing high salinity (Mukhopadhyay *et al.*, 2006; Stolyar *et al.*, 2007).

In the presence of oxygen, the expression of *D. vulgaris* genes proposed to be involved in detoxification of ROS, like superoxide dismutase and catalase did not change significantly, suggesting that the basal concentrations of these enzymes may be sufficient for responding to the oxidative stress (Zhang *et al.*, 2006; Mukhopadhyay *et al.*, 2007; Brioukhanov *et al.*, 2010). On the other hand, genes controlled by PerR, such as *ahpC*, *rbr1*, *rbr2* and *rdl* had a differential expression according to stress concentrations. In severe oxidative stress conditions (*e.g.* 21% O_2), genes from the predicted PerR regulon exhibited a significant down-regulation; in opposition, under lower oxidative stress conditions (*e.g.* 0.1%) genes undergo up-regulation (Zhang *et al.*, 2006; Fournier *et al.*, 2006; Mukhopadhyay *et al.*, 2007; Pereira *et al.*, 2008). Genes of the PerR regulon also appeared up-regulated in cells of *D. vulgaris* in other stress conditions like heavy metals, nitrate, nitrite and NaCl, suggesting that additional regulatory mechanisms may be active

(He *et al.*, 2006; Chhabra *et al.*, 2006; He *et al.*, 2010a; He *et al.*, 2010b; Zhou *et al.*, 2010).

In Part II - Chapter 2 of this thesis we provide evidences for a role of Hcp and Roo proteins in *D. vulgaris* survival against nitrosative stress, a stress to which pathogens are exposed when infecting mammalian cells imposed by the innate immune system.

3.6 - References

Abdollahi, H & Wimpenny, JWT (1990). Effects of oxygen on the growth of *Desulfovibrio desulfuricans*. *Microbiology* **136**:1025-1030.

Agapie, T, Suseno, S, Woodward, JJ, Stoll, S, Britt, RD & Marletta, MA (2009). NO formation by a catalytically self-sufficient bacterial nitric oxide synthase from *Sorangium cellulosum*. *Proc Natl Acad Sci U S A*, **106**:16221-16226.

Alfonso-Prieto, M, Biarnes, X, Vidossich, P & Rovira, C (2009). The molecular mechanism of the catalase reaction. *J Am Chem Soc*, **131**:11751-11761.

Almeida, CC, Romão, CV, Lindley, PF, Teixeira, M & Saraiva, LM (2006). The role of the hybrid cluster protein in oxidative stress defense. *J Biol Chem*, **281**:32445-32450.

Averill, BA (1996). Dissimilatory Nitrite and Nitric Oxide Reductases. *Chem Rev*, **96**:2951-2964.

Avery, SV (2011). Molecular targets of oxidative stress. *Biochem J*, **434**:201-210.

Babior, BM (2000). Phagocytes and oxidative stress. *Am J Med*, **109**:33-44.

Baumgarten, A, Redenius, I, Kranczoch, J & Cypionka, H (2001). Periplasmic oxygen reduction by *Desulfovibrio* species. *Arch Microbiol*, **176**:306-309.

Bowman, LA, McLean, S, Poole, RK & Fukuto, JM (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.

Boylan, JA, Lawrence, KA, Downey, JS & Gherardini, FC (2008). *Borrelia burgdorferi* membranes are the primary targets of reactive oxygen species. *Mol Microbiol*, **68**:786-799.

Brioukhanov, AL, Durand, MC, Dolla, A & Aubert, C (2010). Response of *Desulfovibrio vulgaris* Hildenborough to hydrogen peroxide: enzymatic and transcriptional analyses. *FEMS Microbiol Lett*, **310**:175-181.

Brumlik, MJ & Voordouw, G (1989). Analysis of the transcriptional unit encoding the genes for rubredoxin (*rub*) and a putative rubredoxin oxidoreductase (*rbo*) in *Desulfovibrio vulgaris* Hildenborough. *J Bacteriol*, **171**:4996-5004.

Cabello, P, Pino, C, Olmo-Mira, MF, Castillo, F, Roldan, MD & Moreno-Vivian, C (2004). Hydroxylamine assimilation by *Rhodobacter capsulatus* E1F1. requirement of the hcp gene (hybrid cluster protein) located in the nitrate assimilation nas gene region for hydroxylamine reduction. *J Biol Chem*, **279**:45485-45494.

Cabiscol, E, Tamarit, J & Ros, J (2000). Oxidative stress in bacteria and protein damage by reactive oxygen species. *Int Microbiol*, **3**:3-8.

Cadby, IT, Busby, SJ & Cole, JA (2011). An HcpR homologue from *Desulfovibrio desulfuricans* and its possible role in nitrate reduction and nitrosative stress. *Biochem Soc Trans*, **39**:224-229.

Chakravortty, D & Hensel, M (2003). Inducible nitric oxide synthase and control of intracellular bacterial pathogens. *Microbes Infect*, **5**:621-627.

Chen, L, Liu, MY, LeGall, J, Fareleira, P, Santos, H & Xavier, AV (1993a). Rubredoxin oxidase, a new flavo-hemo-protein, is the site of oxygen reduction to water by the "strict anaerobe" *Desulfovibrio gigas*. *Biochem Biophys Res Commun*, **193**:100-105.

Chen, L, Liu, MY, Legall, J, Fareleira, P, Santos, H & Xavier, AV (1993b). Purification and characterization of an NADH-rubredoxin oxidoreductase involved in the utilization of oxygen by *Desulfovibrio gigas*. *Eur J Biochem*, **216**:443-448.

Chen, L, Sharma, P, Le Gall, J, Mariano, AM, Teixeira, M & Xavier, AV (1994). A blue non-heme iron protein from *Desulfovibrio gigas*. *Eur J Biochem*, **226**:613-618.

Chhabra, SR, He, Q, Huang, KH, Gaucher, SP, Alm, EJ, He, Z, Hadi, MZ, Hazen, TC, Wall, JD, Zhou, J, Arkin, AP & Singh, AK (2006). Global analysis of heat shock response in *Desulfovibrio vulgaris* Hildenborough. *J Bacteriol*, **188**:1817-1828.

Choi, PS, Naal, Z, Moore, C, Casado-Rivera, E, Abruna, HD, Helmann, JD & Shapleigh, JP (2006). Assessing the impact of denitrifier-produced nitric oxide on other bacteria. *Appl Environ Microbiol*, **72**:2200-2205.

Clark, ME, He, Q, He, Z, Huang, KH, Alm, EJ, Wan, XF, Hazen, TC, Arkin, AP, Wall, JD, Zhou, JZ & Fields, MW (2006). Temporal transcriptomic analysis as *Desulfovibrio vulgaris* Hildenborough transitions into stationary phase during electron donor depletion. *Appl Environ Microbiol*, **72**:5578-5588.

Cooper, SJ, Garner, CD, Hagen, WR, Lindley, PF & Bailey, S (2000). Hybrid-cluster protein (HCP) from *Desulfovibrio vulgaris* (Hildenborough) at 1.6 Å resolution. *Biochemistry*, **39**:15044-15054.

Cornelis, P, Wei, Q, Andrews, SC & Vinckx, T (2011). Iron homeostasis and management of oxidative stress response in bacteria. *Metallomics*, **3**:540-549.

Costa, C, Macedo, A, Moura, I, Moura, JJ, Le Gall, J, Berlier, Y, Liu, MY & Payne, WJ (1990). Regulation of the hexaheme nitrite/nitric oxide reductase of *Desulfovibrio desulfuricans*, *Wolinella succinogenes* and *Escherichia coli*. A mass spectrometric study. *FEBS Lett*, **276**:67-70.

Coulter, ED, Shenvi, NV & Kurtz, DM, Jr. (1999). NADH peroxidase activity of rubrerythrin. *Biochem Biophys Res Commun*, **255**:317-323.

Coulter, ED & Kurtz, DM, Jr. (2001). A role for rubredoxin in oxidative stress protection in *Desulfovibrio vulgaris*: catalytic electron transfer to rubrerythrin and two-iron superoxide reductase. *Arch Biochem Biophys*, **394**:76-86.

Crane, BR, Sudhamsu, J & Patel, BA (2010). Bacterial nitric oxide synthases. *Annu Rev Biochem*, **79**:445-470.

Dannenberg, S, Kroder, M, Dilling, W & Cypionka, H (1992). Oxidation of H_2, organic compounds and inorganic sulfur compounds coupled to reduction of O_2 or nitrate by sulfate-reducing bacteria. *Archives of Microbiology*, **158**:93-99.

Davydova, MN, Gorshkov, OV & Tarasova, NB (2006). Periplasmic superoxide dismutase from *Desulfovibrio desulfuricans* 1388 is an iron protein. *Biochemistry (Mosc)*, **71**:68-72.

Deckers, HM & Voordouw, G (1994). Membrane topology of the methyl-accepting chemotaxis protein DcrA from *Desulfovibrio vulgaris* Hildenborough. *Antonie Van Leeuwenhoek*, **65**:7-12.

Dilling, W & Cypionka, H (1990). Aerobic respiration in sulfate-reducing bacteria. *FEMS Microbiology Letters*, **71**:123-127.

Dolla, A, Kurtz, DM, Teixeira, M & Voordouw, G (2007). Biochemical, proteomic and genetic characterization of oxygen survival mechanisms in sulphate-reducing bacteria of the genus *Desulfovibrio*. In *Sulphate-reducing Bacteria: Environmental and Engineered Systems*, pp. 185-213. Edited by L. L. Barton & W. A. Hamilton. New York: Cambridge University Press.

Dos Santos, WG, Pacheco, I, Liu, MY, Teixeira, M, Xavier, AV & LeGall, J (2000). Purification and characterization of an iron superoxide dismutase and a catalase from the sulfate-reducing bacterium *Desulfovibrio gigas*. *J Bacteriol*, **182**:796-804.

Einsle, O, Messerschmidt, A, Huber, R, Kroneck, PM & Neese, F (2002). Mechanism of the six-electron reduction of nitrite to ammonia by cytochrome *c* nitrite reductase. *J Am Chem Soc*, **124**:11737-11745.

Eschemann, A, Kuhl, M & Cypionka, H (1999). Aerotaxis in *Desulfovibrio*. *Environ Microbiol*, **1**:489-494.

Fareleira, P, Legall, J, Xavier, AV & Santos, H (1997). Pathways for utilization of carbon reserves in *Desulfovibrio gigas* under fermentative and respiratory conditions. *J Bacteriol*, **179**:3972-3980.

Fareleira, P, Santos, BS, Antonio, C, Moradas-Ferreira, P, LeGall, J, Xavier, AV & Santos, H (2003). Response of a strict anaerobe to oxygen: survival strategies in *Desulfovibrio gigas*. *Microbiology*, **149**:1513-1522.

Figueiredo, MC, Lobo, SA, Sousa, SH, Pereira, FP, Wall, JD, Nobre, LS & Saraiva, LM (2013). Hybrid Cluster Proteins and Flavodiiron Proteins Afford Protection to *Desulfovibrio vulgaris* upon Macrophage Infection. *J Bacteriol*, **195**:2684-2690.

Finkel, T & Holbrook, NJ (2000). Oxidants, oxidative stress and the biology of ageing. *Nature*, **408**:239-247.

Flatley, J, Barrett, J, Pullan, ST, Hughes, MN, Green, J & Poole, RK (2005). Transcriptional responses of *Escherichia coli* to S-nitrosoglutathione under defined chemostat conditions reveal major changes in methionine biosynthesis. *J Biol Chem*, **280**:10065-10072.

Flint, DH, Tuminello, JF & Emptage, MH (1993). The inactivation of Fe-S cluster containing hydro-lyases by superoxide. *J Biol Chem*, **268**:22369-22376.

Fournier, M, Zhang, Y, Wildschut, JD, Dolla, A, Voordouw, JK, Schriemer, DC & Voordouw, G (2003). Function of oxygen resistance proteins in the anaerobic, sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough. *J Bacteriol*, **185**:71-79.

Fournier, M, Dermoun, Z, Durand, MC & Dolla, A (2004). A new function of the *Desulfovibrio vulgaris* Hildenborough [Fe] hydrogenase in the protection against oxidative stress. *J Biol Chem*, **279**:1787-1793.

Fournier, M, Aubert, C, Dermoun, Z, Durand, MC, Moinier, D & Dolla, A (2006). Response of the anaerobe *Desulfovibrio vulgaris* Hildenborough to oxidative conditions: proteome and transcript analysis. *Biochimie*, **88**:85-94.

Frazão, C, Silva, G, Gomes, CM, Matias, P, Coelho, R, Sieker, L, Macedo, S, Liu, MY, Oliveira, S, Teixeira, M, Xavier, AV, Rodrigues-Pousada, C, Carrondo, MA & Le Gall, J (2000). Structure of a dioxygen reduction enzyme from *Desulfovibrio gigas*. *Nat Struct Biol*, **7**:1041-1045.

Fu, R, Wall, JD & Voordouw, G (1994). DcrA, a *c*-type heme-containing methyl-accepting protein from *Desulfovibrio vulgaris* Hildenborough, senses the oxygen concentration or redox potential of the environment. *J Bacteriol*, **176**:344-350.

Fukui, M & Takii, S (1990). Survival of sulfate-reducing bacteria in oxic surface sediment of a seawater lake. *FEMS Microbiology Letters*, **73**:317-322.

Gomes, CM, Silva, G, Oliveira, S, LeGall, J, Liu, MY, Xavier, AV, Rodrigues-Pousada, C & Teixeira, M (1997). Studies on the redox centers of the terminal oxidase from *Desulfovibrio gigas* and evidence for its interaction with rubredoxin. *J Biol Chem*, **272**:22502-22508.

Hagen, WR, Pierik, AJ & Veeger, C (1989). Novel electron paramagnetic resonance signals from an iron/sulfur protein containing six iron atoms. *J Chem Soc Faraday Trans*, **85**:4083-4090.

Hatchikian, EC & Henry, YA (1977). An iron-containing superoxide dismutase from the strict anaerobe *Desulfovibrio desulfuricans* (Norway 4). *Biochimie*, **59**:153-161.

Hatchikian, EC, LeGall, J & Bell, GR (1977). Significance of superoxide dismutase and catalase activities in the strict anaerobes, sulphate-reducing bacteria. In *Superoxide ans superoxide dismutase*, pp. 159-172. Edited by A. M. Michelson, J. M. McCord & I. Fridovich. New York: Academic Press.

Haveman, SA, Greene, EA, Stilwell, CP, Voordouw, JK & Voordouw, G (2004). Physiological and gene expression analysis of inhibition of *Desulfovibrio vulgaris* Hildenborough by nitrite. *J Bacteriol*, **186**:7944-7950.

Haveman, SA, Greene, EA & Voordouw, G (2005). Gene expression analysis of the mechanism of inhibition of *Desulfovibrio vulgaris* Hildenborough by nitrate-reducing, sulfide-oxidizing bacteria. *Environ Microbiol*, **7**:1461-1465.

Hazell, SL & Graham, DY (1990). Unsaturated fatty acids and viability of *Helicobacter (Campylobacter) pylori*. *J Clin Microbiol*, **28**:1060-1061.

He, Q, Huang, KH, He, Z, Alm, EJ, Fields, MW, Hazen, TC, Arkin, AP, Wall, JD & Zhou, J (2006). Energetic consequences of nitrite stress in *Desulfovibrio vulgaris* Hildenborough, inferred from global transcriptional analysis. *Appl Environ Microbiol*, **72**:4370-4381.

He, Q, He, Z, Joyner, DC, Joachimiak, M, Price, MN, Yang, ZK, Yen, HC, Hemme, CL, Chen, W, Fields, MM, Stahl, DA, Keasling, JD, Keller, M, Arkin, AP, Hazen, TC, Wall, JD & Zhou, J (2010a). Impact of elevated nitrate on sulfate-reducing bacteria: a comparative study of *Desulfovibrio vulgaris*. *Isme J*, **4**:1386-1397.

He, Z, Zhou, A, Baidoo, E, He, Q, Joachimiak, MP, Benke, P, Phan, R, Mukhopadhyay, A, Hemme, CL, Huang, K, Alm, EJ, Fields, MW, Wall, J, Stahl, D, Hazen, TC, Keasling, JD, Arkin, AP & Zhou, J (2010b). Global transcriptional, physiological, and metabolite analyses of the responses of *Desulfovibrio vulgaris* Hildenborough to salt adaptation. *Appl Environ Microbiol*, **76**:1574-1586.

Heidelberg, JF, Seshadri, R, Haveman, SA, Hemme, CL, Paulsen, IT, Kolonay, JF, Eisen, JA, Ward, N, Methe, B, Brinkac, LM, Daugherty, SC, Deboy, RT, Dodson, RJ, Durkin, AS, Madupu, R, Nelson, WC, Sullivan, SA, Fouts, D, Haft, DH, Selengut, J, Peterson, JD, Davidsen, TM, Zafar, N, Zhou, L, Radune, D, Dimitrov, G, Hance, M, Tran, K, Khouri, H, Gill, J, Utterback, TR, Feldblyum, TV, Wall, JD, Voordouw, G & Fraser, CM (2004). The genome sequence of the anaerobic, sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough. *Nat Biotechnol*, **22**:554-559.

Herbig, AF & Helmann, JD (2001). Roles of metal ions and hydrogen peroxide in modulating the interaction of the *Bacillus subtilis* PerR peroxide regulon repressor with operator DNA. *Mol Microbiol*, **41**:849-859.

Hughes, MN (2008). Chemistry of nitric oxide and related species. *Methods Enzymol*, **436**:3-19.

Ignarro, LJ (2000). *Nitric Oxide: Biology and Pathobiology*. California, USA: Academic Press.

Imlay, JA (2003). Pathways of oxidative damage. *Annu Rev Microbiol*, **57**:395-418.

Imlay, JA (2008). How obligatory is anaerobiosis? *Mol Microbiol*, **68**:801-804.

Imlay, JA (2013). The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium. *Nat Rev Microbiol*, **11**:443-454.

Jackson, SH, Gallin, JI & Holland, SM (1995). The $p47^{phox}$ mouse knock-out model of chronic granulomatous disease. *J Exp Med*, **182**:751-758.

Johnson, MS, Zhulin, IB, Gapuzan, ME & Taylor, BL (1997). Oxygen-dependent growth of the obligate anaerobe *Desulfovibrio vulgaris* Hildenborough. *J Bacteriol*, **179**:5598-5601.

Johnston, S, Lin, S, Lee, P, Caffrey, SM, Wildschut, J, Voordouw, JK, da Silva, SM, Pereira, IA & Voordouw, G (2009). A genomic island of the sulfate-reducing bacterium *Desulfovibrio vulgaris*

Hildenborough promotes survival under stress conditions while decreasing the efficiency of anaerobic growth. *Environ Microbiol*, **11**:981-991.

Jorgensen, BB (1977). Bacterial Sulfate Reduction within Reduced Microniches of Oxidized Marine Sediments. *Marine Biology*, **41**:7-17.

Kitamura, M, Mizugai, K, Taniguchi, M, Akutsu, H, Kumagai, I & Nakaya, T (1995). A gene encoding a cytochrome *c* oxidase-like protein is located closely to the cytochrome c-553 gene in the anaerobic bacterium, *Desulfovibrio vulgaris* (Miyazaki F). *Microbiol Immunol*, **39**:75-80.

Koshland, DE, Jr. (1992). The molecule of the year. *Science*, **258**:1861.

Krekeler, D, Teske, A & Cypionka, H (1998). Strategies of sulfate-reducing bacteria to escape oxygen stress in a cyanobacterial mat. *FEMS Microbiology Ecology*, **25**:89-96.

Lamrabet, O, Pieulle, L, Aubert, C, Mouhamar, F, Stocker, P, Dolla, A & Brasseur, G (2011). Oxygen reduction in the strict anaerobe *Desulfovibrio vulgaris* Hildenborough: characterization of two membrane-bound oxygen reductases. *Microbiology*, **157**:2720-2732.

Lee, JW & Helmann, JD (2006). The PerR transcription factor senses H_2O_2 by metal-catalysed histidine oxidation. *Nature*, **440**:363-367.

Lemos, RS, Gomes, CM, Santana, M, LeGall, J, Xavier, AV & Teixeira, M (2001). The 'strict' anaerobe *Desulfovibrio gigas* contains a membrane-bound oxygen-reducing respiratory chain. *FEBS Lett*, **496**:40-43.

Lobo, SA, Melo, AM, Carita, JN, Teixeira, M & Saraiva, LM (2007). The anaerobe *Desulfovibrio desulfuricans* ATCC 27774 grows at nearly atmospheric oxygen levels. *FEBS Lett*, **581**:433-436.

Loubinoux, J, Mory, F, Pereira, IA & Le Faou, AE (2000). Bacteremia caused by a strain of *Desulfovibrio* related to the provisionally named *Desulfovibrio fairfieldensis*. *J Clin Microbiol*, **38**:931-934.

Loubinoux, J, Bisson-Boutelliez, C, Miller, N & Le Faou, AE (2002a). Isolation of the provisionally named *Desulfovibrio fairfieldensis* from human periodontal pockets. *Oral Microbiol Immunol*, **17**:321-323.

Loubinoux, J, Bronowicki, JP, Pereira, IA, Mougenel, JL & Faou, AE (2002b). Sulfate-reducing bacteria in human feces and their association with inflammatory bowel diseases. *FEMS Microbiol Ecol*, **40**:107-112.

Lukat, P, Rudolf, M, Stach, P, Messerschmidt, A, Kroneck, PM, Simon, J & Einsle, O (2008). Binding and reduction of sulfite by cytochrome *c* nitrite reductase. *Biochemistry*, **47**:2080-2086.

Lumppio, HL, Shenvi, NV, Summers, AO, Voordouw, G & Kurtz, DM, Jr. (2001). Rubrerythrin and rubredoxin oxidoreductase in *Desulfovibrio vulgaris*: a novel oxidative stress protection system. *J Bacteriol*, **183**:101-108.

Macfarlane, GT, Cummings, JH & Macfarlane, S (2007). Sulphate-reducing bacteria and the human large intestine. In *Sulphate-reducing Bacteria: Environmental and Engineered Systems*, pp. 503-521. Edited by L. L. Barton & W. A. Hamilton. New York: Cambridge University Press.

MacLean, A, Wei, XQ, Huang, FP, Al-Alem, UA, Chan, WL & Liew, FY (1998). Mice lacking inducible nitric-oxide synthase are more susceptible to herpes simplex virus infection despite enhanced Th1 cell responses. *J Gen Virol*, **79 (Pt 4)**:825-830.

MacMicking, J, Xie, QW & Nathan, C (1997). Nitric oxide and macrophage function. *Annu Rev Immunol*, **15**:323-350.

Marschall, C, Frenzel, P & Cypionka, H (1993). Influence of oxygen on sulfate reduction and growth of sulfate-reducing bacteria. *Archives of Microbiology*, **159**:168-173.

Medzhitov, R (2007). Recognition of microorganisms and activation of the immune response. *Nature*, **449**:819-826.

Mongkolsuk, S & Helmann, JD (2002). Regulation of inducible peroxide stress responses. *Mol Microbiol*, **45**:9-15.

Moura, I, Tavares, P, Moura, JJ, Ravi, N, Huynh, BH, Liu, MY & LeGall, J (1990). Purification and characterization of desulfoferrodoxin. A novel protein from *Desulfovibrio desulfuricans* (ATCC 27774) and from *Desulfovibrio vulgaris* (strain Hildenborough) that contains a distorted rubredoxin center and a mononuclear ferrous center. *J Biol Chem*, **265**:21596-21602.

Mukhopadhyay, A, He, Z, Alm, EJ, Arkin, AP, Baidoo, EE, Borglin, SC, Chen, W, Hazen, TC, He, Q, Holman, HY, Huang, K, Huang, R, Joyner, DC, Katz, N, Keller, M, Oeller, P, Redding, A, Sun, J, Wall, J, Wei, J, Yang, Z, Yen, HC, Zhou, J & Keasling, JD (2006). Salt stress in *Desulfovibrio vulgaris* Hildenborough: an integrated genomics approach. *J Bacteriol*, **188**:4068-4078.

Mukhopadhyay, A, Redding, AM, Joachimiak, MP, Arkin, AP, Borglin, SE, Dehal, PS, Chakraborty, R, Geller, JT, Hazen, TC, He, Q, Joyner, DC, Martin, VJ, Wall, JD, Yang, ZK, Zhou, J & Keasling, JD (2007). Cell-wide responses to low-oxygen exposure in *Desulfovibrio vulgaris* Hildenborough. *J Bacteriol*, **189**:5996-6010.

Nakanishi, T, Inoue, H & Kitamura, M (2003). Cloning and expression of the superoxide dismutase gene from the obligate anaerobic bacterium *Desulfovibrio vulgaris* (Miyazaki F). *J Biochem*, **133**:387-393.

Nathan, C & Shiloh, MU (2000). Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc Natl Acad Sci U S A*, **97**:8841-8848.

Overeijnder, ML, Hagen, WR & Hagedoorn, PL (2009). A thermostable hybrid cluster protein from *Pyrococcus furiosus*: effects of the loss of a three helix bundle subdomain. *J Biol Inorg Chem*, **14**:703-710.

Pereira, PM, He, Q, Xavier, AV, Zhou, J, Pereira, IA & Louro, RO (2008). Transcriptional response of *Desulfovibrio vulgaris* Hildenborough to oxidative stress mimicking environmental conditions. *Arch Microbiol*, **189**:451-461.

Pinto, AF, Rodrigues, JV & Teixeira, M (2010). Reductive elimination of superoxide: Structure and mechanism of superoxide reductases. *Biochim Biophys Acta*, **1804**:285-297.

Pollock, JD, Williams, DA, Gifford, MA, Li, LL, Du, X, Fisherman, J, Orkin, SH, Doerschuk, CM & Dinauer, MC (1995). Mouse model of X-linked chronic granulomatous disease, an inherited defect in phagocyte superoxide production. *Nat Genet*, **9**:202-209.

Priestley, J (1966). *A scientific autobiography of Joseph Priestley, 1733-1804: selected scientific correspondence*. Cambridge, MA.: M.I.T. Press.

Radtke, AL & O'Riordan, MX (2006). Intracellular innate resistance to bacterial pathogens. *Cell Microbiol*, **8**:1720-1729.

Rodionov, DA, Dubchak, I, Arkin, A, Alm, E & Gelfand, MS (2004). Reconstruction of regulatory and metabolic pathways in metal-reducing delta-proteobacteria. *Genome Biol*, **5**:R90.

Rodrigues, R, Vicente, JB, Felix, R, Oliveira, S, Teixeira, M & Rodrigues-Pousada, C (2006). *Desulfovibrio gigas* flavodiiron protein affords protection against nitrosative stress in vivo. *J Bacteriol*, **188**:2745-2751.

Salmon, TB, Evert, BA, Song, B & Doetsch, PW (2004). Biological consequences of oxidative stress-induced DNA damage in *Saccharomyces cerevisiae*. *Nucleic Acids Res*, **32**:3712-3723.

Santos, H, Fareleira, P, Xavier, AV, Chen, L, Liu, MY & LeGall, J (1993). Aerobic metabolism of carbon reserves by the "obligate anaerobe" *Desulfovibrio gigas*. *Biochem Biophys Res Commun*, **195**:551-557.

Saraiva, LM, Vicente, JB & Teixeira, M (2004). The role of the flavodiiron proteins in microbial nitric oxide detoxification. *Adv Microb Physiol*, **49**:77-129.

Shatalin, K, Gusarov, I, Avetissova, E, Shatalina, Y, McQuade, LE, Lippard, SJ & 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**:1009-1013.

Shiloh, MU, MacMicking, JD, Nicholson, S, Brause, JE, Potter, S, Marino, M, Fang, F, Dinauer, M & Nathan, C (1999). Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase. *Immunity*, **10**:29-38.

Shimizu, T, Ohtani, K, Hirakawa, H, Ohshima, K, Yamashita, A, Shiba, T, Ogasawara, N, Hattori, M, Kuhara, S & Hayashi, H (2002). Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. *Proc Natl Acad Sci U S A*, **99**:996-1001.

Sigalevich, P, Meshorer, E, Helman, Y & Cohen, Y (2000). Transition from anaerobic to aerobic growth conditions for the sulfate-reducing bacterium *Desulfovibrio oxyclinae* results in flocculation. *Appl Environ Microbiol*, **66**:5005-5012.

Silaghi-Dumitrescu, R, Ng, KY, Viswanathan, R & Kurtz, DM, Jr. (2005). A flavo-diiron protein from *Desulfovibrio vulgaris* with oxidase and nitric oxide reductase activities. Evidence for an in vivo nitric oxide scavenging function. *Biochemistry*, **44**:3572-3579.

Silva, G, LeGall, J, Xavier, AV, Teixeira, M & Rodrigues-Pousada, C (2001). Molecular characterization of *Desulfovibrio gigas* neelaredoxin, a protein involved in oxygen detoxification in anaerobes. *J Bacteriol*, **183**:4413-4420.

Silveira, CM, Besson, S, Moura, I, Moura, JJ & Almeida, MG (2010). Measuring the cytochrome *c* nitrite reductase activity-practical considerations on the enzyme assays. *Bioinorg Chem Appl*.

Stolyar, S, He, Q, Joachimiak, MP, He, Z, Yang, ZK, Borglin, SE, Joyner, DC, Huang, K, Alm, E, Hazen, TC, Zhou, J, Wall, JD, Arkin, AP & Stahl, DA (2007). Response of *Desulfovibrio vulgaris* to alkaline stress. *J Bacteriol*, **189**:8944-8952.

Stuart, LM & Ezekowitz, RA (2005). Phagocytosis: elegant complexity. *Immunity*, **22**:539-550.

Stuehr, DJ (1999). Mammalian nitric oxide synthases. *Biochim Biophys Acta*, **1411**:217-230.

Sudhamsu, J & Crane, BR (2009). Bacterial nitric oxide synthases: what are they good for? *Trends Microbiol*, **17**:212-218.

Teske, A, Ramsing, NB, Habicht, K, Fukui, M, Kuver, J, Jorgensen, BB & Cohen, Y (1998). Sulfate-reducing bacteria and their activities in cyanobacterial mats of solar lake (Sinai, Egypt). *Appl Environ Microbiol*, **64**:2943-2951.

Turvey, SE & Broide, DH (2010). Innate immunity. *J Allergy Clin Immunol*, **125**:S24-32.

van den Berg, WA, Hagen, WR & van Dongen, WM (2000). The hybrid-cluster protein ('prismane protein') from *Escherichia coli*. Characterization of the hybrid-cluster protein, redox properties of the [2Fe-2S] and [4Fe-2S-2O] clusters and identification of an associated NADH oxidoreductase containing FAD and [2Fe-2S]. *Eur J Biochem*, **267**:666-676.

Vicente, JB, Justino, MC, Goncalves, VL, Saraiva, LM & Teixeira, M (2008). Biochemical, spectroscopic, and thermodynamic properties of flavodiiron proteins. *Methods Enzymol*, **437**:21-45.

Victor, BL, Baptista, AM & Soares, CM (2009). Dioxygen and nitric oxide pathways and affinity to the catalytic site of rubredoxin:oxygen oxidoreductase from *Desulfovibrio gigas*. *J Biol Inorg Chem*, **14**:853-862.

Voordouw, JK & Voordouw, G (1998). Deletion of the rbo gene increases the oxygen sensitivity of the sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough. *Appl Environ Microbiol*, **64**:2882-2887.

Wasserfallen, A, Ragettli, S, Jouanneau, Y & Leisinger, T (1998). A family of flavoproteins in the domains Archaea and Bacteria. *Eur J Biochem*, **254**:325-332.

Weitzberg, E & Lundberg, JO (1998). Nonenzymatic nitric oxide production in humans. *Nitric Oxide*, **2**:1-7.

Wildschut, JD, Lang, RM, Voordouw, JK & Voordouw, G (2006). Rubredoxin:oxygen oxidoreductase enhances survival of *Desulfovibrio vulgaris* Hildenborough under microaerophilic conditions. *J Bacteriol*, **188**:6253-6260.

Wildschut, JD, Caffrey, SM, Voordouw, JK & Voordouw, G (2012). Oxygen exposure increases resistance of *Desulfovibrio vulgaris* Hildenborough to killing by hydrogen peroxide. *Antonie Van Leeuwenhoek*, **101**:303-311.

Wolfe, MT, Heo, J, Garavelli, JS & Ludden, PW (2002). Hydroxylamine reductase activity of the hybrid cluster protein from *Escherichia coli*. *J Bacteriol*, **184**:5898-5902.

Xiong, J, Kurtz, DM, Jr., Ai, J & Sanders-Loehr, J (2000). A hemerythrin-like domain in a bacterial chemotaxis protein. *Biochemistry*, **39**:5117-5125.

Yurkiw, MA, Voordouw, J & Voordouw, G (2012). Contribution of rubredoxin:oxygen oxidoreductases and hybrid cluster proteins of *Desulfovibrio vulgaris* Hildenborough to survival under oxygen and nitrite stress. *Environ Microbiol*, **14**:2711-2725.

Zhang, P, Allahverdiyeva, Y, Eisenhut, M & Aro, EM (2009). Flavodiiron proteins in oxygenic photosynthetic organisms: photoprotection of photosystem II by Flv2 and Flv4 in *Synechocystis* sp. PCC 6803. *PLoS One*, **4**:e5331.

Zhang, W, Culley, DE, Hogan, M, Vitiritti, L & Brockman, FJ (2006). Oxidative stress and heat-shock responses in *Desulfovibrio vulgaris* by genome-wide transcriptomic analysis. *Antonie Van Leeuwenhoek*, **90**:41-55.

Zhou, A, He, Z, Redding-Johanson, AM, Mukhopadhyay, A, Hemme, CL, Joachimiak, MP, Luo, F, Deng, Y, Bender, KS, He, Q, Keasling, JD, Stahl, DA, Fields, MW, Hazen, TC, Arkin, AP, Wall, JD & Zhou, J (2010). Hydrogen peroxide-induced oxidative stress responses in *Desulfovibrio vulgaris* Hildenborough. *Environ Microbiol*, **12**:2645-2657.

Zhou, A, Chen, YI, Zane, GM, He, Z, Hemme, CL, Joachimiak, MP, Baumohl, JK, He, Q, Fields, MW, Arkin, AP, Wall, JD, Hazen, TC & Zhou, J (2012). Functional characterization of Crp/Fnr-type global transcriptional regulators in *Desulfovibrio vulgaris* Hildenborough. *Appl Environ Microbiol*, **78**:1168-1177.

Zhou, J, He, Q, Hemme, CL, Mukhopadhyay, A, Hillesland, K, Zhou, A, He, Z, Van Nostrand, JD, Hazen, TC, Stahl, DA, Wall, JD & Arkin, AP (2011). How sulphate-reducing microorganisms cope with stress: lessons from systems biology. *Nat Rev Microbiol*, **9**:452-466.

Results

Part II

Chapter 1

Bacterioferritin protects the anaerobe *Desulfovibrio vulgaris* Hildenborough against oxygen

This work was published in an international peer review journal:

Figueiredo, M. C., Lobo, S. A., Carita, J. N., Nobre, L. S. & Saraiva, L. M. (2012). Bacterioferritin protects the anaerobe *Desulfovibrio vulgaris* Hildenborough against oxygen. *Anaerobe* 18:454-458.

Mafalda Figueiredo performed all the experimental work, with the exception of the mutant strains construction. Mafalda Figueiredo also participated in the writing of the manuscript.

Summary

Intracellular free iron, is under aerobic conditions and via the Fenton reaction a catalyst for the formation of harmful reactive oxygen species. In this article, we analyzed the relation between intracellular iron storage and oxidative stress response in the sulfate reducing bacterium *Desulfovibrio vulgaris* Hildenborough, an anaerobe that is often found in oxygenated niches. To this end, we investigated the role of the iron storage protein bacterioferritin using transcriptomic and physiological approaches. We observed that transcription of bacterioferritin is strongly induced upon exposure of cells to an oxygenated atmosphere. When grown in the presence of high concentrations of oxygen the *D. vulgaris* bacterioferritin mutant exhibited, in comparison with the wild type strain, lower viability and a higher content of intracellular reactive oxygen species. Furthermore, the bacterioferritin gene is under the control of the oxidative stress response regulator *D. vulgaris* PerR. Altogether the data revealed a previously unrecognized ability for the iron storage bacterioferritin to contribute to the oxygen tolerance exhibited by *D. vulgaris.*

1.1 - Introduction

Desulfovibrio species are anaerobic sulfate reducing bacteria (SRB) found in the environment, such as soil, water, and sewage, and in the human oral, intestinal and vaginal flora (Ichiishi *et al.*, 2010). These microbes are often found close to oxic habitats and contain several gene products that enable resistance to oxidative stress. *Desulfovibrio* spp. possess genes encoding membrane bound oxygen reductases of the heme-copper and cytochrome *bd* types, together with gene products typical of the aerobic organisms, like catalase (Kat), superoxide dismutase (Sod) and alkyl hydroperoxidase (AhpC) (Cypionka, 2000; Lemos *et al.*, 2001; Fournier *et al.*, 2003; Lamrabet *et al.*, 2011). Furthermore, *Desulfovibrios* express a set of unique proteins that are proposed to reinforce tolerance to reactive oxygen

species (ROS) namely, rubrerythrins (Rbr1 and Rbr2), rubredoxin:oxygen oxidoreductase (Roo), superoxide reductase (Sor) previously named rubredoxin oxidoreductase, and nygerythrin (Ngr). The oxidative stress-responsive regulator PerR, whose regulon includes *rbr1*, *rbr2*, *rd* encoding rubredoxin, and *aphC*, is also present in *Desulfovibrio* spp. (Rodionov *et al.*, 2004; Mukhopadhyay *et al.*, 2007; Dolla *et al.*, 2007).

One of the consequences of oxidative stress is the inactivation of key metabolic proteins through the disassembling and release of their metal centers. Moreover, free metals, and in particular iron, are important sources of ROS through triggering of Fenton reaction, which can be avoided by storage of the metal into specialized proteins. The genomes of *Desulfovibrio* species contain genes for iron storage proteins, such as ferritin and bacterioferritin. However, so far, the only one studied was the bacterioferritin from *Desulfovibrio desulfuricans* ATCC 27774 for which a comprehensive biochemical, spectroscopic and structural characterization was done (Romão *et al.*, 2000a; Romão *et al.*, 2000b; Macedo *et al.*, 2003). The *D. desulfuricans* ATCC 27774 bacterioferritin constituted the first example of an iron storage protein isolated from an anaerobic microorganism having unique features, namely an iron-coproporphyrin III as natural cofactor in replacement of the canonical iron-protoporphyrin IX (heme *b*) (Romão *et al.*, 2000a), and a native diiron center. The *D. desulfuricans* ATCC 27774 bacterioferritin gene was shown to form a di-cistronic unit with the rubredoxin 2 gene (da Costa *et al.*, 2001).

Since the function of bacterioferritins in anaerobes remains largely unclear, in this work we examined the role of bacterioferritin in relation to oxygen protection in *Desulfovibrio vulgaris* Hildenborough, an anaerobic organism widely used as a model for the study of the metabolism of sulfate reducing bacteria. We have analyzed how oxygen impacts on the gene expression of the *D. vulgaris* bacterioferritin and compare it with the transcription profile of genes encoding

proteins proposed to have a role in oxidative protection. Furthermore, physiological studies of the *D. vulgaris* bacterioferritin mutant strain under oxygen stress conditions were also performed.

1.2 - Materials and Methods

Bacterial strains and growth conditions

D. vulgaris Hildenborough wild type and derivative strains lacking the genes coding for bacterioferritin (*D. vulgaris* JW9101) and PerR (*D. vulgaris* JW708) (Table 1.1) were analyzed. All *D. vulgaris* strains were grown anaerobically in a 3L fermenter (Applikon, Biocontroler 4DI 1030), in Wall LS4 medium (Keller *et al.*, 2009), at 37 ºC, under a nitrogen flow rate of 150 ml min^{-1}. The pH of the culture medium was monitored during growth with a pH meter system (Mettler Toledo), and automatically maintained by the addition of KOH or HCl. When cells reached the mid-log phase ($OD_{600} \sim 0.4$), part of the nitrogen was replaced by an amount of air flux, by means of an Applikon gas mixer, so that concentrations of dissolved oxygen of 5, 10 and 21% were achieved. Cells were monitored for approximately 10 h, and samples for gene expression analysis were collected at 1 h and 3 h after the initial oxygen exposure.

Table 1.1 - Strains used in this study.

D. vulgaris Hildenborough strains	Description[a]	Source
Wild type	*D. vulgaris*, ATCC 29579	ATCC
JW708	Δ*perR* Km^r	J. D. Wall Laboratory
JW9101	Δ*bfr* Km^r	J. D. Wall Laboratory

[a] Km^r, kanamycin resistance

Viability assays and evaluation of ROS content

Cells of wild type and JW9101 Δ*bfr* mutant strain were grown anaerobically in Wall LS4 medium, until reaching an $OD_{600} \sim 1$. At this point (time zero), samples were taken and serially diluted, and aliquots of the 10^{-6}-fold diluted cultures spread on plates of Tryptic Soy Agar medium supplemented with 2.5 g/l $NaC_3H_5O_3$, 2.0 g/l $MgSO_4.7H_2O$ and 0.5 g/l $(NH_4)_2Fe(SO_4)_2$ and 20 mg sodium thioglycolate/ascorbic acid solution (TSAS) (van den Berg *et al.*, 1989). The remaining of the cells was diluted 1:4 into fresh medium contained in open flasks that were stirred, at 60 rpm, and grown at room temperature. Aliquots of these air exposed cultures were collected after 15 and 30 min, and used to evaluate cell viability and endogenous ROS content. The number of colony forming units (CFU) was determined by performing serial dilutions of cells, plated on TSAS medium and anaerobically incubated at 37 ºC, for 2 days. The percentage of survival was calculated dividing the number of colonies resulting from cultures exposed to oxygen by the number of colonies from cultures grown anaerobically.

To measure the endogenous ROS content, cells collected after air exposure for 15 min were washed and resuspended in phosphate buffered saline (PBS). The fluorophore 2',7'-dichlorofluorescein diacetate (DCFH-DA, 10 µM) was added and incubated up to 120 min, and the ROS content evaluated by means of a fluorometric assay (Myhre *et al.*, 2003). The fluorescence intensities (FI) were measured on a Varian Eclipse 96-well spectrofluorimeter (excitation at 485 nm and emission at 538 nm), and normalized in relation to the final OD_{600nm} of each culture.

Real-time quantitative RT-PCR

Cells exposed to 5% and 10% oxygen for 0, 1 and 3 h were collected and used to extract total RNA. In the case of exposure to 21% of oxygen, cell samples were only collected at time zero and 1 h after incubation, as a significant loss of viability

was observed for prolonged exposures. Cell pellets were then submitted to the phenol-chloroform method (Chomczynski & Sacchi, 1987), and once integrity was confirmed by gel agarose electrophoresis the RNA concentration was determined using a Nanodrop ND-100 spectrophotometer (NanoDrop Technologies).

RNA (1 µg) was used to generate cDNA in a reverse transcription reaction using the Transcriptor High Fidelity cDNA synthesis kit (Roche Diagnostics). Approximately 5 ng of cDNA was used in the real-time quantitative RT-PCR reactions together with specific oligonucleotides, which were designed to amplify an internal region of 200 to 300 bp for each target gene (Table 1.2). Reactions were prepared using the LightCycler Fast Start DNA Master SYBR Green I kit (Roche Diagnostics), according to the manufacturer's instructions, and performed in a LightCycler instrument. The expression ratio was normalized to the *D. vulgaris* 16S ribosomal RNA as standard reference gene (Table 1.2). Real-time quantitative RT-PCR experiments were performed using, at least, two biological independent samples that were analyzed in triplicate.

Table 1.2 - Oligonucleotides used in this work.

Protein	Gene	Oligonucleotide sequence (5' - 3')
Nigerythrin	*ngr*	CGCCAAGGCAGCCAGAGAAC CCTCCTCCTGCGCCTTCCTG
Bacterioferritin	*bfr*	CCAACGTGAAGCTCATCGCC CATGCTCACGCTGTCGCCAC
Ferritin	*ftn*	GGGTTTGCCAACTGGATGCG GGAAGATGTTCGTGGCGTGGTC
Cytochrome *c* oxidase, subunit II	*coxII*	CATCCTGTGCGCCGAATACTG CGATGATCTTGCGCTTGCTTC
Rubrerythrin 2	*rbr2*	GCAATGCGGGCAAAATCGG GGCCTTCGTGGGTGTATCCG
Superoxide dismutase	*sodB*	CTGCCAAGGCGGGGGTCTGTTC CTGCGTCATCGGGTTGTCTGC
Rubrerythrin 1	*rbr1*	CGCACGCGAAGAGGGCTAC GCCGAGGAGTTCGAAGTGCG
Superoxide reductase	*sor*	CTGACGGGGCCAAGGAAAAG GACCTTGTCGGCCTTGATGC
Rubredoxin:oxygen oxidoreductase 2	*roo2*	CCTGCCCGAACTGATAGCCC GCGTAGCGTTCGGTGGAGG
Cytochrome *bd* oxidase, subunit I	*cydA*	GGGGCAGTACTTCCACACCATC CCTTGCCGGTCTCCCAGTG
16S rRNA	*rrs*	CCTAGGGCTACACACGTACTACAA GAGCATGCTGATCTCGAATTACTA

1.3 - Results

Effect of oxygen on gene expression of iron storage proteins and ROS protective enzymes

D. vulgaris cells were exposed to increasing concentrations of oxygen, namely 5, 10 and 21% of oxygen, collected after one and three hours (Figure 1.1) and the change in mRNA synthesis of bacterioferritin gene (*bfr*, DVU1397) was evaluated. For comparison purposes, we also analyzed the alteration of the expression of ferritin (*ftn*, DVU1568) and of several other genes that are proposed to confer oxygen tolerance to *D. vulgaris*, namely *cydA* (DVU3271) and *coxII* (DVU1812), encoding subunit I of cytochrome *bd* oxidase and subunit II of cytochrome *c* oxidase, respectively, superoxide dismutase *sodB* (DVU2410), rubrerythrin 1 *rbr1* (DVU3094), rubrerythrin 2 *rbr2* (DVU2318), rubredoxin:oxygen oxidoreductase *roo2* (DVU3185), superoxide reductase *sor* (DVU3183) and nigerythrin *ngr* (DVU0019).

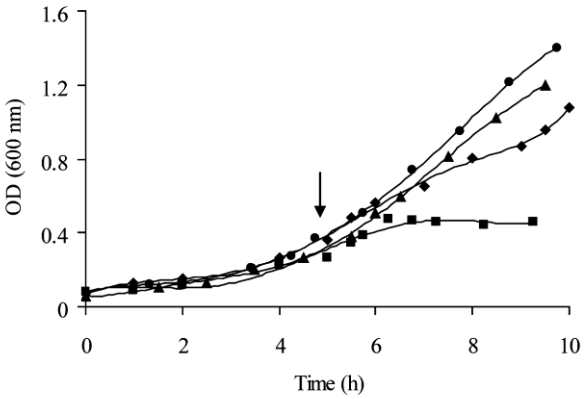


Figure 1.1 - Growth behavior of *D. vulgaris* exposed to oxygen.
Growth curves of *D. vulgaris* wild type cells grown under anaerobic conditions (●) and exposed, at the mid-log phase (OD_{600} ~ 0.4), to 5% (▲), 10% (♦) and 21% O_2 (■). Experiments were performed for two biological samples. The arrow indicates the start of air exposure.

Overall, the transcription profile was dependent on the time and amount of oxygen to which the cells were submitted (Figure 1.2, Table 1.3). For *D. vulgaris* cells exposed for 1 h to 5% O_2, *rbr1* exhibited the highest increase in transcription (~ 7-fold), while in cells treated with 10% O_2 *roo2* was the highest induced gene (~

5-fold). For cells submitted to 21% O_2, *bfr* was the gene that underwent the highest increase in expression (~ 7-fold) (Figure 1.2, Table 1.3).

Comparison of the data acquired for cells exposed to several oxygen concentrations (from 5% to 21% O_2) for the same length of time (1 h) showed that the gene expression tends to decrease with increasing concentrations of oxygen (Table 1.3).

The expression of ferritin didn't change significantly suggesting that its basal level is sufficient to cope with oxygen toxicity. In contrast, the transcription of the *bfr* gene was up-regulated by 10% and 21% O_2, while no fold variation was observed in cells treated with 5% O_2.

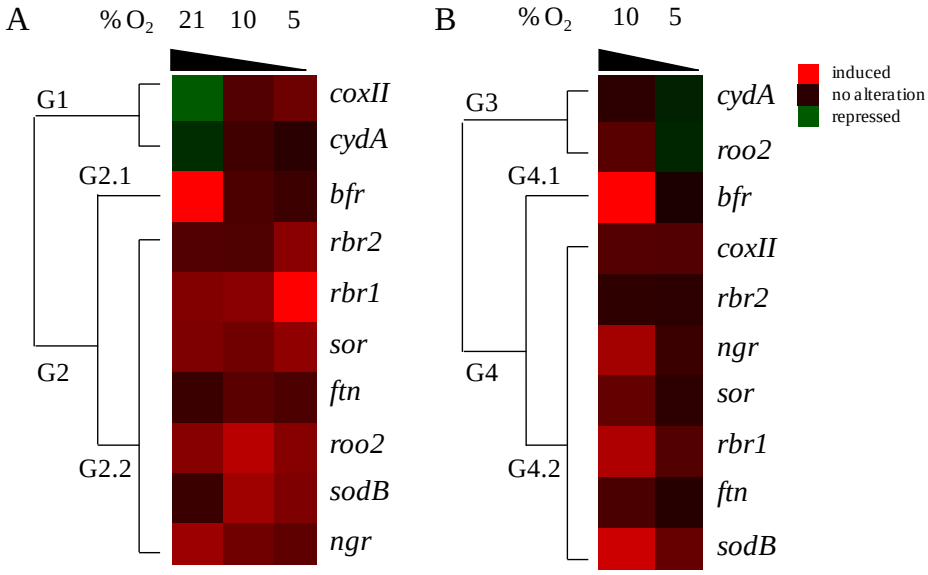


Figure 1.2 - Hierarchical cluster analysis of *D. vulgaris* gene transcription.
Cells growing under anaerobic conditions were exposed to the indicated oxygen concentrations for 1 h **(A)**, and 3 h **(B)**. The fold variation was determined in relation to the mRNA level of cells growing in the absence of oxygen. The color scale ranges from light to dark green for negative fold variation, and from dark to light red for positive fold variation. Intervals of fold variation: light to dark green (<1), dark to light red in A (2-7), and dark to light red in B (2-14). Black squares refer to genes with unchanged fold.

The hierarchical analysis performed with Cluster and TreeView Software (Eisen *et al.*, 1998) of the dataset acquired for cells exposed for 1 h to different oxygen concentrations divided the genes in two major groups (Figure 1.2A). The first group (G1) includes genes that were repressed when the cells were exposed to 21% O_2, namely *coxII* (-2.5-fold) and *cydA* (-1.3-fold). The second group (G2) appeared split into two subgroups: G2.1 that only contains *bfr*, the gene with the highest induction at 21% oxygen, and G2.2 with genes whose expression decreased with the increase of oxygen concentration (*sor*, *rbr1*, *rbr2*, *roo2*, *ngr*, *ftn*, and *sodB)*.

For the same oxygen concentration and different times of exposure (1 and 3 h, Figure 1.2A and 1.2B respectively) the expression folds measured at longer times were, in general, either similar or lower in comparison with those measured at 1 h. (Figure 1.2, Table 1.3). Exceptions to this trend were observed in *D. vulgaris* cells exposed to 10% O_2 for the *rbr1*, *sodB*, *ngr* and *bfr*, which exhibited up-regulation after 3 h of O_2 exposure.

In general, at longer times of exposure (3 h), the increase of O_2 concentration (from 5 to 10%) caused an augment of the gene expression (Figure 1.2B), with a *bfr* induction of $\sim$ 14-fold (Table 1.3). The hierarchical analysis of this dataset separated genes repressed upon exposure to 5% O_2 (G3 with *cydA* and *roo2*) from all other genes. Nevertheless, within the G4 group the *bfr* gene, which showed the highest induction upon exposure to 10% O_2, was differentiated from the other genes that displayed small changes in mRNA levels upon increase of O_2 concentration (*rbr2*, *coxII* , *roo2*, *ftn, rbr1*, *ngr* and *sodB*) (Figure 1.2B, Table 1.3).

The expression of ferritin was not significantly altered by the presence of oxygen, and for lower percentages of oxygen the variation of *rbr1* gene expression suggests that it plays the major protective role (5% O_2). However, the transcription of *rbr1* tends to decrease when cells are shifted to higher oxygen concentrations and longer exposures. Contrasting with this behavior, up-regulation of

bacterioferritin was observed in cells treated with high oxygen concentrations suggesting a role in oxygenated environments.

Table 1.3 - Effect of oxygen concentration on *D. vulgaris* gene expression.

			Fold Variation				
			5% O_2		10% O_2		21% O_2
ORF	Gene	Protein	1 h	3 h	1 h	3 h	1 h
DVU0019	*ngr*	Nigerythrin	2.6 ± 0.4	2.1 ± 0.4	3.1 ± 0.0	5.9 ± 0.5	4.3 ± 0.4
DVU1397	*bfr*	Bacterioferritin	1.7 ± 0.8	1.0 ± 0.6	2.1 ± 0.3	14.2 ± 2.7	7.5 ± 3.1
DVU1568	*ftn*	Ferritin	2.1 ± 0.0	1.4 ± 0.2	2.5 ± 0.4	2.7 ± 0.2	1.6 ± 0.5
DVU1812	*coxII*	Cytochrome *c* oxidase, subunit II	3.4 ± 0.9	3.7 ± 2.0	2.3 ± 0.3	3.1 ± 0.2	0.5 ± 0.1
DVU2318	*rbr2*	Rubrerythrin 2	3.8 ± 0.1	1.6 ± 0.9	2.2 ± 1.0	1.7 ± 0.2	2.3 ± 0.5
DVU2410	*sodB*	Superoxide dismutase B	3.5 ± 1.0	3.7 ± 2.7	4.4 ± 2.1	7.4 ± 0.8	1.6 ± 0.8
DVU3094	*rbr1*	Rubrerythrin 1	7.3 ± 0.8	3.0 ± 0.8	3.8 ± 1.1	6.3 ± 0.4	3.6 ± 1.6
DVU3183	*sor*	Superoxide reductase	4.0 ± 0.7	1.6 ± 0.5	3.1 ± 0.2	3.6 ± 1.0	3.5 ± 0.7
DVU3185	*roo2*	Rubredoxin:oxygen oxidoreductase 2	3.7 ± 0.1	0.7 ± 0.3	5.1 ± 0.0	3.2 ± 0.4	3.7 ± 1.2
DVU3271	*cydA*	Cytochrome *bd* oxidase, subunit I	1.2 ± 0.0	0.8 ± 0.3	1.8 ± 0.4	1.6 ± 0.3	0.8 ± 0.2

Two biological independent samples and, at least, three replicates were analyzed.
Fold variation resulting from the oxygen stress is calculated using the expression levels from treated cultures and from cultures immediately before oxygen exposure.

Bacterioferritin improves survival of *D. vulgaris* in the presence of oxygen

Based on the previous results we next tested if bacterioferritin protected *D. vulgaris* from oxygen stress by comparing the *D. vulgaris* viability of wild type and *bfr* mutant strains. In the absence of stress, the survival of the wild type and *bfr* mutant strains were similar (data not shown), indicating that *bfr* is not essential for the anaerobic sulfate reduction of *D. vulgaris*. In contrast, a significant decrease of the viability of the *bfr* mutant strain was observed after 15 min post-exposure of

cells to the air stream (Figure 1.3A), and no recover was seen up to 1 h (data not shown).

Bacterioferritin contributes to lowering the formation of ROS

We also examined whether the protection conferred by bacterioferritin was related to the intracellular content of reactive oxygen species by measuring the ROS level of wild type and *bfr* mutant cells after exposure to 21% oxygen. As expected, for both strains the ROS content increased upon exposure to oxygen but the ROS produced in *bfr* mutant cells were significantly higher than in wild type (Figure 1.3B). These results were consistent with the phenotypic data showing that the *bfr* mutant is more sensitive to oxygen (Figure 1.3A), and indicated that bacterioferritin contributes to avoid the formation of reactive oxygen species.

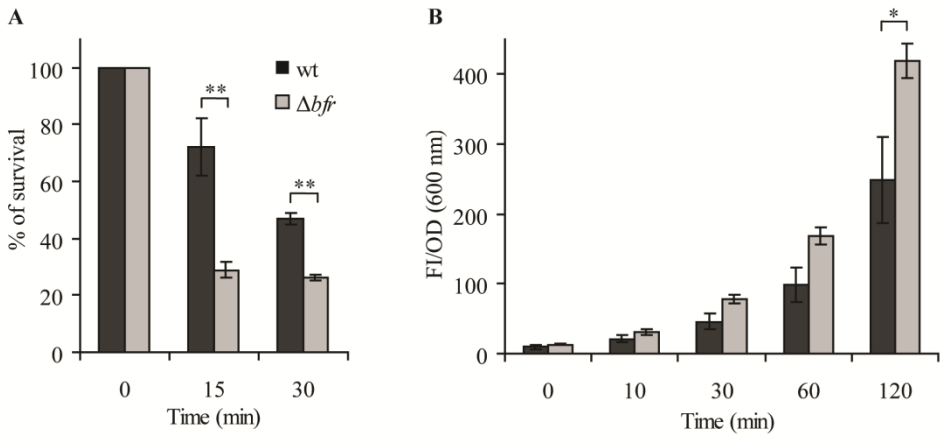


Figure 1.3 - Viability and ROS content of *D. vulgaris* exposed to oxygen.
(A) Effect of oxygen in the viability of *D. vulgaris* wild type (dark grey bars) and mutant Δ*bfr* (light grey bars). **(B)** Endogenous ROS content measured at the indicated times, of *D. vulgaris* wild type and Δ*bfr* mutant after exposure to 21% of oxygen for 15 min. The standard errors from at least two biological replicates are shown as error bars. A t-test was performed showing significant differences at the *, $P < 0.005$, **, $P < 0.01$ levels.

PerR regulates *bfr*

Genes encoding iron storage proteins are, in general, under the control of the major ferric uptake regulator, Fur. However, this is not the case of *bfr* as the transcription was similar in *D. vulgaris* wild type and *fur* mutant cells (data not shown). Since the *bfr* expression was induced by oxygen, we sought whether the PerR regulator, which is also encoded in the *D. vulgaris* genome (Zhou *et al.*, 2011), played a role in the modulation of the *bfr* expression. To this end, a *D. vulgaris perR* mutant was used to evaluate the level of *bfr* expression in comparison to the parental strain. We observed that the expression of *bfr* is repressed in the *D. vulgaris perR* mutant (fold variation = -3.5 ± 0.1; n= 4), thus showing that PerR regulates *bfr*. Consistent with PerR regulation of *bfr,* we identified upstream of the start codon of *bfr* (starting at -213 bp) a sequence (TAAACGAATCTTTACACAC) that matches by 15 out of 19 bases the PerR consensus motif (Novichkov *et al.*, 2010), therefore constituting a potential PerR binding site.

1.4 - Discussion

In this work we observed the significant induction of the expression of *bfr* in *D. vulgaris* cells exposed to oxygen. Furthermore, the two oxygen reductase encoding genes showed a different transcription behavior with *coxII* being always more up-regulated than *cydA*, except under high concentrations of oxygen. This suggests that cytochrome *c* oxidase confers to *D. vulgaris* cells higher protection than the *bd* oxidase.

The fact that all analyzed genes had their transcription altered upon exposure of cells to oxygen, albeit at different levels, indicates that they are not constitutive and that the lack of variation reported in previous studies is most probably due to the oxygen conditions utilized (Zhang *et al.*, 2006; Zhou *et al.*, 2011). Apart from nigerythrin and bacterioferritin, the other genes seem to be devoted to protect *D. vulgaris* from lower concentrations of oxygen, particularly *rbr1*. The high levels of

induction observed under all growth conditions for the *sor* and *roo2* genes imply that they play an important role in defending the *D. vulgaris* independently of the oxygen concentration to which cells are submitted.

We also observed that, in general, genes exhibited a lower fold induction in cells exposed to high levels of oxygen. Yet, the dependence on oxygen of the expression profile of bacterioferritin is different since this was the gene with the highest induction in cells exposed to 21% of air. Consistent with the high change in the *bfr* mRNA level upon air exposure, the strain lacking this gene had lower viability and increased intracellular content of reactive oxygen species. Altogether, the data suggest that this iron storage system may be one of the most relevant oxygen-related protective mechanisms for the anaerobic lifestyle of *D. vulgaris*.

Overall our results agree with previous proteomic and microarrays data of *D. vulgaris* cells submitted to high concentration of oxygen as lower fold induction of *cydA*, *rbr1*, and *rbr2* was also detected (Fournier *et al.*, 2006; Mukhopadhyay *et al.*, 2007; Pereira *et al.*, 2008). In opposition to our results, the induction of bacterioferritin was not observed in *Desulfovibrio* cells exposed to high concentrations of O_2/air (Fournier *et al.*, 2006; Dolla *et al.*, 2007; Mukhopadhyay *et al.*, 2007; Pereira *et al.*, 2008), which is most probably due to the different growth conditions utilized and to the severe loss of viability that exposure to air caused in almost all cases. More recently, a study performed in *Bacteroides fragilis* described the augment of the *bfr* mRNA levels in response to O_2 and reported that *bfr* null mutant had reduced viability (Gauss *et al.*, 2011), results that confirm the herein demonstrated contribution of Bfr to the aerotolerance of *D. vulgaris*.

The induction of bacterioferritin by oxygen indicates the need to regulate the iron cellular concentrations most probably to prevent the iron mediated formation of reactive oxygen species via the Fenton chemistry. Although in most bacteria Fur carries out this regulation, in *D. vulgaris* we observed that expression of bacterioferritin is independent of this major iron regulator. This result is in

agreement with a previous data study that didn't show regulation of bacterioferritin and ferritin by Fur (Bender *et al.*, 2007). The decreased expression of *bfr* in *D. vulgaris* cells lacking PerR and the identification of a putative PerR binding sequence in the DNA region upstream of the *bfr* start codon indicate that the transcription of *bfr* is mediated by PerR. The role of *D. vulgaris* PerR as activator was also reported for two other genes involved in iron transport (*feoA* and *feoB*) (Zhou *et al.*, 2010; Wildschut *et al.*, 2012). Although PerR-like binding sites are predicted in promoter regions of other PerR controlled genes, such as *ahpC* and *rbr2* (Rodionov *et al.*, 2004; Mukhopadhyay *et al.*, 2007), no experiments were until now reported confirming the binding of this regulator. In spite of repeated attempts, we also could not achieve the binding of PerR to the *bfr* promoter region.

The analysis of the available *Desulfovibrio* genomes revealed that the gene organization of *bfr* and its neighborhood is quite diverse and, in the majority of the genomes, *bfr* is surrounded by genes encoding hypothetical proteins putatively unrelated to PerR or to oxygen detoxification. Although in the *D. vulgaris* genome the *bfr* gene is distantly located from *perR*, in *D. desulfuricans* ATCC 27774 the gene coding for PerR is immediately upstream of the operon formed by *bfr* and rubredoxin *rd2*. Interestingly, the *D. desulfuricans* ATCC 27774 Bfr was shown to form an *in vitro* transient complex with Rd2, a protein whose gene, together with rubrerythrin, is proposed to be under the control of PerR in *D. vulgaris* (da Costa *et al.*, 2001; Wildschut *et al.*, 2012).

The regulation of *bfr* by PerR may allow *D. vulgaris*, whose metabolism is dependent on several iron-containing proteins, to simultaneously coordinate the level of iron and intracellular content of antioxidant proteins. The increase of the ROS level upon deletion of *bfr* in *D. vulgaris* indicates that besides serving as a reservoir from which an iron-starved cell could withdraw iron, bacterioferritin also contributes to scavenge free iron and mitigate deleterious reactions caused by

ROS. This apparent dual functionality of bacterioferritin seems to be restricted to this iron-storage protein as ferritin showed no ability to protect *D. vulgaris* cells against oxygen stress (data not shown).

Although the role of bacterioferritin in anaerobic bacteria has been proposed to be related to oxygen defense, to our knowledge, the herein work constitutes the first evidence of a direct link between bacterioferritin and oxygen protection of *Desulfovibrio*. Moreover, these data show that bacterioferritin contributes to survival of *D. vulgaris* in environments where oxidative stress and low iron availability usually reduces bacterial proliferation.

1.5 - Acknowledgements

We are grateful to Professor Judy D. Wall (University of Missouri, USA) for providing wild type and mutant strains of *D. vulgaris*. The work was funded by PEst-OE/EQB/LA0004/2011, FCT project PTDC/BIA-PRO/098224/2008 and by FCT studentships SFRH/BD/44140/2008 (MF), SFRH/ BPD/ 63944/ 2009 (SL) and SRFH/BPD/69325/2010 (LN).

1.6 - References

Bender, KS, Yen, HC, Hemme, CL, Yang, Z, He, Z, He, Q, Zhou, J, Huang, KH, Alm, EJ, Hazen, TC, Arkin, AP & Wall, JD (2007). Analysis of a ferric uptake regulator (Fur) mutant of *Desulfovibrio vulgaris* Hildenborough. *Appl Environ Microbiol*, **73**:5389-5400.

Chomczynski, P & Sacchi, N (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem*, **162**:156-159.

Cypionka, H (2000). Oxygen respiration by *Desulfovibrio* species. *Annu Rev Microbiol*, **54**:827-848.

da Costa, PN, Romao, CV, LeGall, J, Xavier, AV, Melo, E, Teixeira, M & Saraiva, LM (2001). The genetic organization of *Desulfovibrio desulphuricans* ATCC 27774 bacterioferritin and rubredoxin-2 genes: involvement of rubredoxin in iron metabolism. *Mol Microbiol*, **41**:217-227.

Dolla, A, Kurtz, DM, Teixeira, M & Voordouw, G (2007). Biochemical, proteomic and genetic characterization of oxygen survival mechanisms in sulphate-reducing bacteria of the genus *Desulfovibrio*. In *Sulphate-reducing Bacteria: Environmental and Engineered Systems*, pp. 185-213. Edited by L. L. Barton & W. A. Hamilton. New York: Cambridge University Press.

Eisen, MB, Spellman, PT, Brown, PO & Botstein, D (1998). Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A*, **95**:14863-14868.

Fournier, M, Zhang, Y, Wildschut, JD, Dolla, A, Voordouw, JK, Schriemer, DC & Voordouw, G (2003). Function of oxygen resistance proteins in the anaerobic, sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough. *J Bacteriol*, **185**:71-79.

Fournier, M, Aubert, C, Dermoun, Z, Durand, MC, Moinier, D & Dolla, A (2006). Response of the anaerobe *Desulfovibrio vulgaris* Hildenborough to oxidative conditions: proteome and transcript analysis. *Biochimie*, **88**:85-94.

Gauss, GH, Reott, MA, Rocha, ER, Young, MJ, Douglas, T, Smith, CJ & Lawrence, CM (2011). Characterization of the *Bacteroides fragilis bfr* gene product identifies a bacterial DPS-like protein and suggests evolutionary links in the ferritin superfamily. *J Bacteriol*, **194**:15-27.

Ichiishi, S, Tanaka, K, Nakao, K, Izumi, K, Mikamo, H & Watanabe, K (2010). First isolation of *Desulfovibrio* from the human vaginal flora. *Anaerobe*, **16**:229-233.

Keller, KL, Bender, KS & Wall, JD (2009). Development of a markerless genetic exchange system for *Desulfovibrio vulgaris* Hildenborough and its use in generating a strain with increased transformation efficiency. *Appl Environ Microbiol*, **75**:7682-7691.

Lamrabet, O, Pieulle, L, Aubert, C, Mouhamar, F, Stocker, P, Dolla, A & Brasseur, G (2011). Oxygen reduction in the strict anaerobe *Desulfovibrio vulgaris* Hildenborough: characterization of two membrane-bound oxygen reductases. *Microbiology*, **157**:2720-2732.

Lemos, RS, Gomes, CM, Santana, M, LeGall, J, Xavier, AV & Teixeira, M (2001). The 'strict' anaerobe *Desulfovibrio gigas* contains a membrane-bound oxygen-reducing respiratory chain. *FEBS Lett*, **496**:40-43.

Macedo, S, Romão, CV, Mitchell, E, Matias, PM, Liu, MY, Xavier, AV, LeGall, J, Teixeira, M, Lindley, P & Carrondo, MA (2003). The nature of the di-iron site in the bacterioferritin from *Desulfovibrio desulfuricans*. *Nat Struct Biol*, **10**:285-290.

Mukhopadhyay, A, Redding, AM, Joachimiak, MP, Arkin, AP, Borglin, SE, Dehal, PS, Chakraborty, R, Geller, JT, Hazen, TC, He, Q, Joyner, DC, Martin, VJ, Wall, JD, Yang, ZK, Zhou, J & Keasling, JD (2007). Cell-wide responses to low-oxygen exposure in *Desulfovibrio vulgaris* Hildenborough. *J Bacteriol*, **189**:5996-6010.

Myhre, O, Andersen, JM, Aarnes, H & Fonnum, F (2003). Evaluation of the probes 2',7'-dichlorofluorescin diacetate, luminol, and lucigenin as indicators of reactive species formation. *Biochem Pharmacol*, **65**:1575-1582.

Novichkov, PS, Laikova, ON, Novichkova, ES, Gelfand, MS, Arkin, AP, Dubchak, I & Rodionov, DA (2010). RegPrecise: a database of curated genomic inferences of transcriptional regulatory interactions in prokaryotes. *Nucleic Acids Res*, **38**:D111-118.

Pereira, PM, He, Q, Xavier, AV, Zhou, J, Pereira, IA & Louro, RO (2008). Transcriptional response of *Desulfovibrio vulgaris* Hildenborough to oxidative stress mimicking environmental conditions. *Arch Microbiol*, **189**:451-461.

Rodionov, DA, Dubchak, I, Arkin, A, Alm, E & Gelfand, MS (2004). Reconstruction of regulatory and metabolic pathways in metal-reducing delta-proteobacteria. *Genome Biol*, **5**:R90.

Romão, CV, Louro, R, Timkovich, R, Lubben, M, Liu, MY, LeGall, J, Xavier, AV & Teixeira, M (2000a). Iron-coproporphyrin III is a natural cofactor in bacterioferritin from the anaerobic bacterium *Desulfovibrio desulfuricans*. *FEBS Lett*, **480**:213-216.

Romão, CV, Regalla, M, Xavier, AV, Teixeira, M, Liu, MY & Le Gall, J (2000b). A bacterioferritin from the strict anaerobe *Desulfovibrio desulfuricans* ATCC 27774. *Biochemistry*, **39**:6841-6849.

van den Berg, WAM, Stokkermans, JPWG & van Dongen, WMAM (1989). Development of a plasmid transfer system for the anaerobic sulphate reducer, *Desulfovibrio vulgaris*. *Journal of Biotechnology*, **12**:173-184.

Wildschut, JD, Caffrey, SM, Voordouw, JK & Voordouw, G (2012). Oxygen exposure increases resistance of *Desulfovibrio vulgaris* Hildenborough to killing by hydrogen peroxide. *Antonie Van Leeuwenhoek*, **101**:303-311.

Zhang, W, Culley, DE, Hogan, M, Vitiritti, L & Brockman, FJ (2006). Oxidative stress and heat-shock responses in *Desulfovibrio vulgaris* by genome-wide transcriptomic analysis. *Antonie Van Leeuwenhoek*, **90**:41-55.

Zhou, A, He, Z, Redding-Johanson, AM, Mukhopadhyay, A, Hemme, CL, Joachimiak, MP, Luo, F, Deng, Y, Bender, KS, He, Q, Keasling, JD, Stahl, DA, Fields, MW, Hazen, TC, Arkin, AP, Wall, JD & Zhou, J (2010). Hydrogen peroxide-induced oxidative stress responses in *Desulfovibrio vulgaris* Hildenborough. *Environ Microbiol*, **12**:2645-2657.

Zhou, J, He, Q, Hemme, CL, Mukhopadhyay, A, Hillesland, K, Zhou, A, He, Z, Van Nostrand, JD, Hazen, TC, Stahl, DA, Wall, JD & Arkin, AP (2011). How sulphate-reducing microorganisms cope with stress: lessons from systems biology. *Nat Rev Microbiol*, **9**:452-466.

Part II

Chapter 2

Hybrid cluster proteins and flavodiiron proteins afford protection to *Desulfovibrio vulgaris* upon macrophage infection

This work was published in an international peer review journal:

Figueiredo, MC, Lobo, SA, Sousa, SH, Pereira, FP, Wall, JD, Nobre, LS & Saraiva, LM (2013). Hybrid Cluster Proteins and Flavodiiron Proteins Afford Protection to *Desulfovibrio vulgaris* upon Macrophage Infection. *J Bacteriol*, 195:2684-2690.

Mafalda Figueiredo performed all the experimental work, with the exception of the mutant strains construction and nitrite determination in macrophages. Mafalda Figueiredo also participated in the writing of the manuscript.

Summary

Desulfovibrio species are Gram-negative anaerobic sulfate reducing bacteria that colonize the human gut. Recently, *Desulfovibrio* spp. have been implicated in gastrointestinal diseases, and shown to stimulate the epithelial immune response leading to increased production of inflammatory cytokines by macrophages. Activated macrophages are key cells of the immune system that during phagocytosis impose nitrosative stress. Hence, we have analyzed the *in vitro* and *in vivo* response of *Desulfovibrio vulgaris* Hildenborough to nitric oxide (NO), and the role of the hybrid cluster proteins (Hcp1, Hcp2) and rubredoxin:oxygen oxidoreductases (Roo1, Roo2) in NO protection. Amongst the four genes, *hcp2* was the gene most highly induced by NO, and the *hcp2* transposon mutant exhibited the lowest viability under NO stress. Studies in murine macrophages revealed that *D. vulgaris* survives incubation with these phagocytes, and triggers NO production at levels similar to those stimulated by the cytokine interferon-gamma, IFN-γ. Furthermore, *D. vulgaris hcp* and *roo* mutants exhibited reduced viability when incubated with macrophages, revealing that these gene products contribute to the survival of *D. vulgaris* during macrophage infection.

2.1 - Introduction

Desulfovibrio spp. are anaerobic sulfate reducing bacteria (SRB) that occur in several environmental niches, like marine and freshwater sediments, as well as in humans as part of the normal oral cavity and gut flora. In particular, four *Desulfovibrio* spp. were detected in healthy humans, namely *D. fairfieldensis*, *D. desulfuricans*, *D. piger* and *D. vulgaris* (Nakao *et al.*, 2009; Ichiishi *et al.*, 2010; Jia *et al.*, 2012). Furthermore, *Desulfovibrio* spp. have also been implicated in gastrointestinal diseases, such as inflammatory bowel diseases and periondontitis, since *Desulfovibrio* strains were isolated from biopsy specimens of patients with

ulcerative colitis, brain, abdominal wall and liver abscesses, and appendicitis (Lozniewski *et al.*, 1999; Rowan *et al.*, 2010; Gaillard *et al.*, 2011).

Recently, *D. desulfuricans* and *D. fairfieldensis* were shown to be able to invade non-professional phagocytic cells like the oral epithelial cells and to stimulate the epithelial immune response by increasing the production of inflammatory interleukins (Bisson-Boutelliez *et al.*, 2010). Nonetheless, the ability of *Desulfovibrio* spp. to survive professional phagocytes, such as macrophages, remains to be evaluated.

Two of the main weapons of the innate immune system to eradicate pathogens are the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are derived from the superoxide and nitric oxide produced by the NADPH oxidase and the mammalian inducible nitric oxide synthase (iNOS), respectively (Nathan & Shiloh, 2000; Kidd, 2011). These chemicals inflict serious damage in bacteria, which is avoided through the expression of several detoxification systems (Kidd, 2011). The flavodiiron proteins (FDP) constitute a large family of enzymes widespread amongst Archaea and Bacteria, including in *Desulfovibrio* spp.. They are believed to contribute to bacterial survival under oxidative and nitrosative stress conditions (Vicente *et al.*, 2008a). FDPs are homodimeric proteins, with each monomer formed by a flavodoxin-like domain, containing a FMN cofactor, and a β-lactamase-like domain harboring a diiron centre (Saraiva *et al.*, 2004). The first *Desulfovibrio* FDP to be studied was that of *D. gigas*, the rubredoxin:oxygen oxidoreductase (Roo) (Frazão *et al.*, 2000), which was shown to reduce dioxygen to water with electrons from rubredoxin (Gomes *et al.*, 1997). Since subsequent studies reported that several prokaryotic FDPs have significant nitric oxide reductase activity, FDPs are currently believed to be either oxygen or NO reductases or even be bifunctional (Justino *et al.*, 2005; Vicente *et al.*, 2008a; Baptista *et al.*, 2012).

The hybrid cluster proteins (Hcp) constitute another family of bacterial proteins proposed to protect against ROS and RNS toxicity. Hcps contain two redox-active iron-sulfur clusters, namely a canonical $[4Fe\text{-}4S]^{2+/1+}$ or $[2Fe\text{-}2S]^{2+/1+}$ cluster and a hybrid iron-sulfur-oxygen cluster $[4Fe\text{-}2S\text{-}2O]^{2+/1+}$ (Macedo *et al.*, 2002; Aragão *et al.*, 2003). Previous work reported that an *E. coli* strain mutated in the *hcp* gene has lower resistance to hydrogen peroxide and S-nitrosoglutathione (GSNO) (Almeida *et al.*, 2006; Vine & Cole, 2011b; Seth *et al.*, 2012). Furthermore, the recombinant *E. coli* Hcp exhibited hydrogen peroxide and hydroxylamine reductase activities, the latter being also described for the Hcps of *Rhodobacter capsulatus* and *Pyrococcus furiosus* (Wolfe *et al.*, 2002; Cabello *et al.*, 2004; Almeida *et al.*, 2006; Overeijnder *et al.*, 2009). Hence, Hcps seem also to have more than one enzymatic function.

Like many other *Desulfovibrio* spp. with known genome sequences, *D. vulgaris* Hildenborough contains two homologues of the FDPs (Roo1 and Roo2) and of Hcps (Hcp1 and Hcp2). The genes encoding Roo1 (DVU2014) and Hcp1 (DVU2013) are adjacent, and within a genomic island (Johnston *et al.*, 2009), while the genes encoding Roo2 (DVU3185) and Hcp2 (DVU2543) are located elsewhere and are separated in the genome. In *Desulfovibrio* spp., these proteins are proposed to promote survival in oxygenated environments and to remove RNS generated by nitrite reduction (He *et al.*, 2006; Wildschut *et al.*, 2006; Johnston *et al.*, 2009; He *et al.*, 2010a; Yurkiw *et al.*, 2012).

Although several reports have implicated *Desulfovibrio* spp. in infectious processes, the behavior of these bacteria has not yet been evaluated when contacting cells of the immune system such as macrophages. Since infected macrophages produce NO that contributes to eradication of pathogens (Kidd, 2011), in this work we started by analyzing the expression of the four *D. vulgaris* Hildenborough *hcp* and *roo* genes under *in vitro* NO stress. The phenotype and NO consumption activity of *D. vulgaris* wild type and transposon mutants with

inactivated *roo* and *hcp* genes were evaluated in cells exposed to NO donors. Moreover, we also tested the ability of these strains for survival when exposed to macrophages.

2.2 - Materials and Methods

Bacterial strains and growth conditions

D. vulgaris Hildenborough strains used in this study are listed in Table 2.1. All *Desulfovibrio* strains were grown anaerobically, at 37ºC, in Wall LS4 medium (Keller *et al.*, 2009). For phenotype assays, cells were grown anaerobically until the stationary phase (approximately 48 h), collected by centrifugation, diluted in fresh medium to an OD_{600} ~ 0.2, and further incubated until reaching early exponential growth phase (OD_{600} ~ 0.3). At this stage, cells were left untreated or exposed to the NO releaser dipropylenetriamine NONOate (DPTA NONOate, 100 µM; half-life of 3 h, at 37ºC.) (Cayman), and growth was monitored for 8 h.

Table 2.1 – *D. vulgaris* Hildenborough strains used in this study.

Strain	Description	Source
Wild type	*D. vulgaris* ATCC 29579	ATCC
GZ6896	*hcp1*-398::Tn*5*-RL27; insertion at 398/1662 bp for the gene; Km^{r*}	J. D. Wall Laboratory
GZ11714	*hcp2*-173::Tn*5*-RL27; insertion at 173/1620 bp for the gene; Km^{r}	J. D. Wall Laboratory
GZ2505	*roo1*-164::Tn*5*-RL27; insertion at 164/1176 bp for the gene; Km^{r}	J. D. Wall Laboratory
GZ14874	*roo2*-134::Tn*5*-RL27; insertion at 134/1209 bp for the gene; Km^{r}	J. D. Wall Laboratory

* Km^{r}, kanamycin resistance.

Quantitative real-time PCR analysis

D. vulgaris wild type was grown anaerobically, at 37ºC in Wall LS4 medium, until an OD_{600} ~ 0.3 and treated with an NO releaser (100 µM). Cells were then

exposed during 1 h to the fast releaser spermine NONOate (Sigma) that decomposes with a half-life of 39 min, at T=37ºC. To analyze the gene transcription of *D. vulgaris* exposed to NO for 4 h, a slower NO releaser was used, namely the DPTA NONOate that decomposes with a half-life of 3 h, at 37ºC. Total RNA was isolated with the RNeasy Mini kit (Qiagen), quantified in a Nanodrop ND-100 spectrophotometer (NanoDrop technologies), and its integrity confirmed by gel agarose electrophoresis. cDNA was then synthesized from 2 µg total RNA with the Transcriptor High Fidelity cDNA Synthesis Kit following the manufacturer's protocol (Roche Applied Science). Quantitative real-time PCR reactions were performed in a LightCycler Instrument according to the LightCycler FastStart DNA Master SYBR Green I kit's instructions (Roche Applied Science). The amplification reactions were carried out with equal amounts of cDNA (10 ng) as the initial template, together with the specific pair of oligonucleotides, which were designed to amplify an internal region of 200-300 bp for each target gene (Table 2.2). The expression ratio of the target gene was determined relative to a *D. vulgaris* 16S rRNA reference gene whose transcription remains unchanged under all tested conditions. Quantitative real-time PCR experiments were performed for two biologically independent samples that were assayed in triplicate.

Table 2.2 – Oligonucleotides used in this study.

Protein	Locus/ Gene name	Oligonucleotide sequence
Hcp1	DVU2013/*hcp1*	Fw: 5´-GAACCCCGGCATCCTCATC Rv: 5´-GGATGGGGCCGTTGAAGG
Hcp2	DVU2543/*hcp2*	Fw: 5´-GGCGCTTCAGGACCTCACCATC Rv: 5´-CTGTGCCACCAGCCCGTCG
Roo1	DVU2014/*roo1*	Fw: 5´-GGGTACATGAAGCGGCAAAACG Rv: 5´-CGAAGGGAAAGGCCACCAGG
Roo2	DVU3185/*roo2*	Fw: 5'-CCTGCCCGAACTGATAGCCC Rv: 5´-GCGTAGCGTTCGGTGGAGG
16S rRNA	Dv16S/*rrs*	Fw: 5´-CCTAGGGCTACACACGTACTACAA Rv: 5´-GAGCATGCTGATCTCGAATTACTA

NO consumption assays

Wild type and mutant strains were grown anaerobically in Wall LS4 medium. When the cultures reached an OD_{600} ~ 0.3, the cells were left untreated or exposed to 100 µM DPTA NONOate for 4 h. *D. vulgaris* lysates were prepared by incubating the cells for 15 min with 0.1 mg lysozyme/mL and 0.01% (w/v) sodium deoxycholate. Assays were carried out anaerobically, at room temperature, in PBS buffer supplemented with 20 mM glucose, 130 U catalase/mL, 17 U glucose oxidase/mL, 0.2 mM NADPH, 0.2 mM NADH and 4 - 6 µM NO. NO was added to the samples by means of injection of an appropriate volume of a 2 mM NO-saturated water solution, prepared as previously described (Beckman *et al.*, 1996). Upon addition of the cell lysates, the NO consumption rate was monitored amperometrically with an NO electrode (ISO-NOP) connected to an APOLLO-4000 Free Radical Analyzer (WPI, Europe). Two biological samples were assayed in triplicate.

Macrophage assays and determination of nitrite

Murine macrophages RAW264.7 (ATCC Tib71) were inoculated (5×10^5 cells/ml) in 24-well plates containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (GIBCO), and 70 U penicillin/ml, and 70 µg streptomycin/ml (GIBCO) antibiotics, and cultured for 3 h, at 37°C, in 5% CO_2-air atmosphere. Prior to infection, macrophages were activated for 12 h with 0.3 µg/ml interferon-gamma IFN-γ (Sigma). When required, 400 µM N^G-monomethyl-L-arginine acetate salt (L-NMMA, Sigma) was added to inhibit the activity of the murine iNOS. Bacterial suspensions of *D. vulgaris* wild type and mutant strains were grown anaerobically in Wall LS4 medium with no antibiotics. When cultures reached the stationary growth phase, cells were collected, washed three times with phosphate buffered saline (PBS) and resuspended in DMEM to obtain an OD_{600} ~ 0.3, and the viability (CFU/ml) evaluated before incubation in

macrophages (time zero). Cells were then used to infect macrophages, at a multiplicity of infection (MOI) of 40, during 5, 8 and 24 h. Bacterial survival was evaluated by colony formation on plates loaded with serial dilutions of cultures in PBS. Briefly, 5-µl of, at least, two different dilutions was spread on Tryptic Soy Agar medium supplemented per liter with 2.5 g sodium lactate, 2.0 g magnesium sulfate, 0.5 g ammonium iron (II) sulfate and 20 mg sodium thioglycolate/ascorbic acid solution (van den Berg *et al.*, 1989). The plates were then incubated, at 37ºC, in a jar containing an anaerobic generator (GENbox anaer from Biomérieux) and after 2 days the number of colonies was evaluated. Four independent biological samples with three replicates each were analyzed.

To determine the number of cells phagocytized by the macrophages, *D. vulgaris* was firstly incubated with macrophages for 2 h. Next, the macrophages were washed and the non-internalized bacteria eliminated by incubation, for 5 min, with DMEM supplemented with 70 U penicillin/ml, and 70 µg streptomycin/ml. After addition of fresh DMEM medium, the infection proceeded for extra 3, 6 and 22 h, at which time macrophages were washed and lysed with saponin (2% w/v) and the intracellular bacterial content evaluated by counting of CFU.

The amount of NO produced by macrophages was measured as the nitrite accumulated in the supernatants of murine macrophage cell cultures grown in DMEM and activated either by 0.3 µg IFN-γ /mL or infected with *D. vulgaris* (MOI ~ 40), and in the absence and presence of the inhibitor L-NMMA (400 µM). The microtiter plate colorimetric assay (Multiskan GO, Thermo Scientific) was performed by reading the absorbance at 540 nm of 1:1 mixtures of supernatants (100 µl) and Griess reagent (1% (w/v) sulfanilamide, 0.1% (w/v) naphthylene diamine dihydrochloride, 2% (v/v) phosphoric acid). Sodium nitrite was used as standard.

2.3 - Results

Transcriptional response of *hcp* and *roo* genes to nitrosative stress

D. vulgaris Hildenborough, a model organism in the study of SRB, was used to assess the function of Roo and Hcp proteins in *in vitro* protection against NO. To this end, we first analyzed the expression of genes DVU2013 (*hcp1*), DVU2543 (*hcp2*), DVU2014 (*roo1)* and DVU3185 (*roo2*) in *D. vulgaris* grown to early exponential growth phase and exposed to an NO donor (Figure 2.1). Exposure of *D. vulgaris* to NONOates caused no significant change in the transcription of the *roo* genes, but slightly lowered the mRNA abundance of the *hcp1* after treatment for 1 h (Figure 2.1). Importantly, high increases of the *hcp2* gene expression of approximately 1900-fold and 400-fold were observed in cells treated with nitrosative stress for 1 h and 4 h, respectively (Figure 2.1).

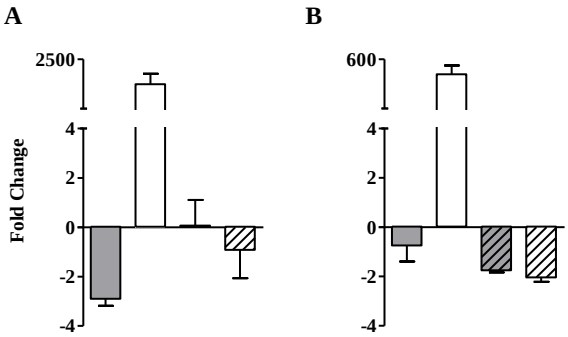


Figure 2.1 - Effect of NO stress on the transcription of *D. vulgaris hcp* and *roo* genes.
The fold variations of the expression of the genes *hcp1* (gray bars), *hcp2* (white bars), *roo1* (gray striped bars), and *roo2* (white striped bars) upon exposure of the *D. vulgaris* wild type strain to 100 µM spermine NONOate for 1 h **(A)** and 100 µM DPTA NONOate for 4 h **(B)** are indicated. Fold change values represent the ratio of the expression level of treated culture to that of untreated culture and were considered significant when they exceeded ± 2-fold. Values are means ± standard errors (n = 6).

Sensitivity of *D. vulgaris* wild type and mutant strains to nitric oxide donors

Next, the *in vitro* susceptibility of *D. vulgaris* and transposon mutants of *roo* and *hcp* to nitrosative stress was evaluated by monitoring the anaerobic growth behavior of untreated cells and of cells exposed to 100 µM DPTA NONOate. Under these conditions, *D. vulgaris* transposon mutants of *roo1* and *roo2* were slightly more resistant to NO than the wild type strain; whereas, the mutant lacking *hcp2* stopped growing immediately after the introduction of the stress (Figure 2.2). These results revealed that inactivation of *hcp2* resulted in a *D. vulgaris* strain with a lower ability to cope with NO stress.

Since the *roo* mutants did not show reduced susceptibility to NO when compared with the wild type, we further tested the effect of another NO source, on their growth behavior, namely GSNO. For all strains, 10 µM GSNO had a moderate inhibitory effect on growth; whereas, 50 µM GSNO caused strong growth impairment (Figure S1 in Supplementary Material). However, in all cases no significant differences were observed between the growth of the wild type and *roo* mutant strains.

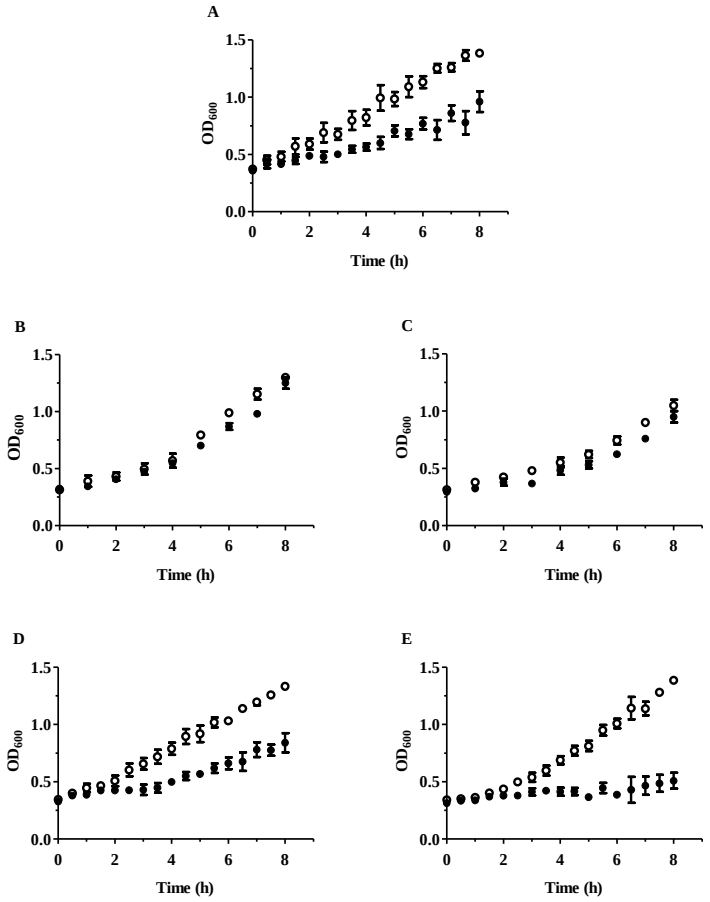


Figure 2.2 - Growth of *D. vulgaris* wild type and mutant strains in the presence of NO.
Growth curves of *D. vulgaris* wild type **(A)**, and strains mutated in *roo1* **(B)**, *roo2* **(C)**, *hcp1* **(D)** and *hcp2* **(E)**, which were left untreated (o) and treated with 100 µM DPTA NONOate (●). Three biological samples were analyzed and values are means ± standard error.

NO consumption activity of *D. vulgaris* wild type and mutant strains

The cellular NO reductase activity of *D. vulgaris* wild type was also evaluated by measuring the NO consumption of lysates prepared from cells grown anaerobically and left untreated or exposed to 100 µM DPTA NONOate. We observed that the activity of the wild type is slightly higher in NO-treated cells (91.5 ± 24.1 and 140.8 ± 21.7 pmol NO/min/mg protein total for untreated and NO-treated cell, respectively) (Figure 2.3). Analysis of the mutants showed that in

untreated cells, inactivation of either of the *roo* or *hcp* genes did not change the NO consumption rates (Figure 2.3A). However, for NO-treated cells, the strains interrupted in *roo1* or *roo2* exhibited lower NO consumption compared to the NO-treated wild type, of approximately 85% and 25% for *roo1* and *roo2* transposon mutants, respectively (Figure 2.3B). These results revealed that, among the four proteins, Roo1 is the major contributor for the NO reduction capability of *D. vulgaris*.

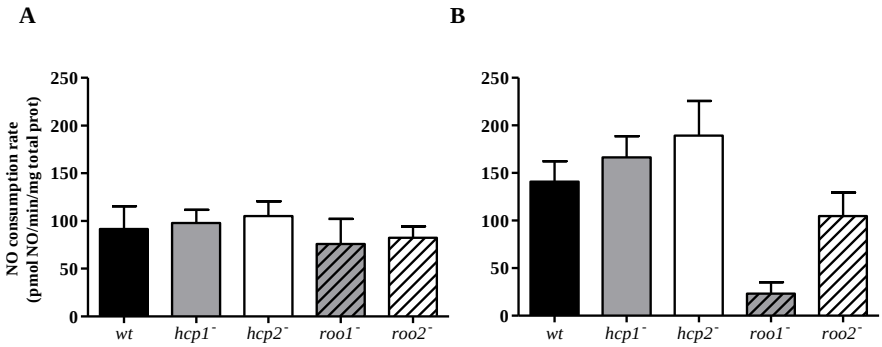


Figure 2.3 - NO reduction activity.
NO consumption activity determined amperometrically in *D. vulgaris* cell lysates prepared from untreated cultures **(A)** and cultures exposed for 4 h to 100 µM DPTA NONOate **(B)**. *D. vulgaris* wild type (black bars); *hcp1*$^{-}$ (grey bars); *hcp2*$^{-}$ (white bars); *roo1*$^{-}$ (grey striped bars); and *roo2*$^{-}$ (white striped bars). Values are means ± standard error (*n* = 6). Prot, protein.

Infection of macrophages with *D. vulgaris*

Although it has been proposed that *Desulfovibrio* spp. are involved in infectious processes, so far, no data are available on the viability of *Desulfovibrio* within key cells of the innate immune system, such as macrophages. Hence, in this work the *D. vulgaris* wild type was cultured anaerobically until the late exponential growth phase and incubated with murine macrophages RAW264.7. After 2 h of infection, the extracellular bacteria were eliminated by addition of the standard antibiotics used in macrophage culturing (see Methods Section). After periods of 5 h, 8 h and 24 h, the macrophages were lysed and the intracellular bacterial content

determined. Under all conditions no viable bacterial cells were detected (data not shown), suggesting that *D. vulgaris* is not capable of intracellular replication in macrophages. However, *D. vulgaris* was able to survive extracellularly in DMEM (Figure S2 in Supplementary Material), and upon co-culture with macrophages *D. vulgaris* wild type suffered an approximately 30% decrease of its survival (Figure 2.4). Similar experiments done in the presence of L-NMMA, an inhibitor of the mammalian iNOS, showed that inhibition of macrophage NO production allows the recovery of *D. vulgaris* wild type, particularly after 24 h infection (Figure 2.4). At this time of *D. vulgaris* viable cells in macrophages that do not produce NO is similar to that observed in DMEM medium, indicating a ~ 100% viability recovery of the wild type strain (Figure 2.4 and S2 in Supplementary Material).

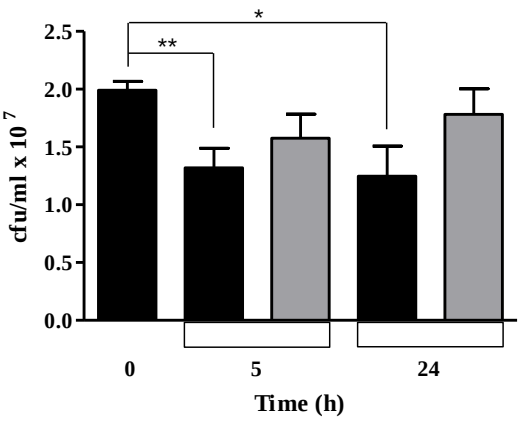


Figure 2.4 - Survival of *D. vulgaris* upon interaction with macrophages.
Activated murine macrophages RAW264.7 were infected with *D. vulgaris* in the absence and in the presence of the iNOS inhibitor L-NMMA (black and grey bars, respectively). The bacterial survival was determined by CFU counting immediately before *D. vulgaris* was incubated with macrophages (time zero), and after 5 h and 24 h post-infection. Values are means ± standard error (n = 12) with t-test (* $p < 0.005$ and ** $p < 0.01$).

To determine whether *D. vulgaris* was able to activate the production of NO by the mammalian iNOS, cultures of macrophages were infected with *D. vulgaris* wild type for 14 h and the nitrite content measured in the supernatants (Figure 2.5). For comparison purposes, similar assays but in which the activation of iNOS was achieved by addition of the macrophage-activator interferon-gamma INF-γ (MacMicking *et al.*, 1997) were also performed. The results showed that *D. vulgaris* activates the production of NO in macrophages to a level similar to that

stimulated by INF-γ (Figure 2.5). Moreover, experiments done in the presence of L-NMMA inhibitor caused a significant decrease of the nitrite content in the supernatants (Figure 2.5).

Altogether, these results revealed that *D. vulgaris* is able to trigger the induction of iNOS in macrophages.

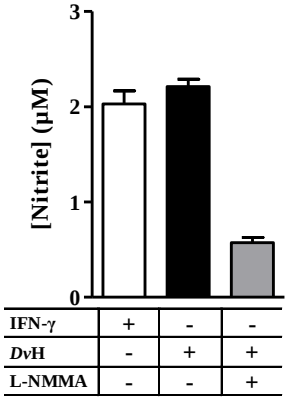


Figure 2.5 – Nitrite production by *D. vulgaris* infected macrophages.
Nitrite concentration accumulated, during 14 h, in macrophages pre-activated with IFN-γ (white bar) or infected with *D. vulgaris*, in the absence (black bar) and in the presence of L-NMMA (grey bar). Values represent means and the corresponding standard error (*n* = 8).

Survival of *D. vulgaris* mutant strains upon contact with macrophages

The contribution of the Hcp and Roo proteins to the survival of *D. vulgaris* in macrophages was also investigated. For this purpose, macrophages were infected with *D. vulgaris* wild type and mutant strains for 5 h, 8 h and 24 h. While no differences between the wild type and mutants were seen up to 5 h and 8 h of incubation (data not shown), the results after 24 h of infection were distinct (Figure 2.6). At this time, the survival of all mutant strains was reduced by approximately 90% relative to the wild type, while the viability of the parental strain decreased only 30% (Figure 2.6). Impairment of the macrophage NO production by L-NMMA resulted in an increase in the survival of the four mutant strains. Nevertheless, in the absence of NO production the recovery of viability of the mutant strains was still lower than that observed in the absence of macrophages (time zero in Figure 2.6). These data suggest that the compromised

viability of the mutant strains upon incubation with macrophages is related, albeit partially, to the lower nitrosative stress resistance of these strains.

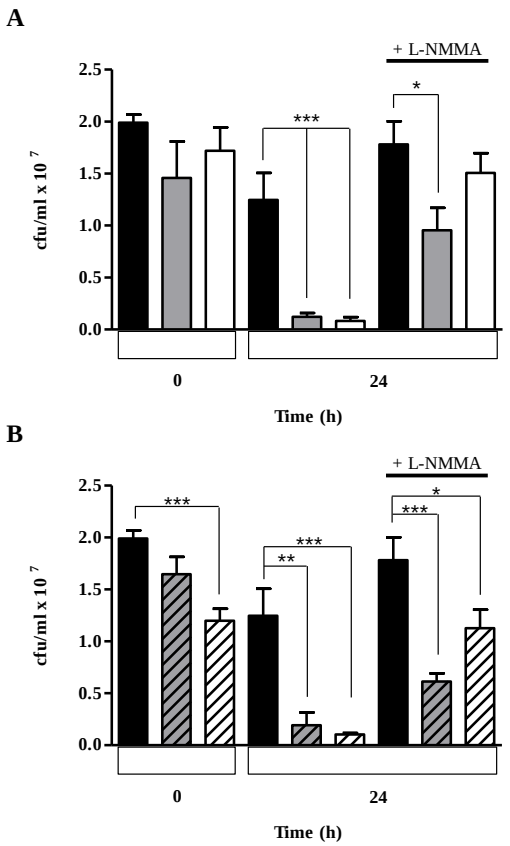


Figure 2.6 - Survival of *D. vulgaris* wild type and mutant strains upon interaction with macrophages.
Activated macrophages were infected with the *D. vulgaris* wild type strain (black bars) and with the following transposon mutants: the *hcp1* mutant (gray bars) and the *hcp2* mutant (white bars) **(A)** and the *roo1* mutant (gray striped bars) and the *roo2* mutant (white striped bars) **(B)**. The bacterial survival was determined by CFU counting immediately before *D. vulgaris* was incubated with macrophages or had the iNOS inhibitor L-NMMA added (time zero). Again, CFU were determined after 24 h of exposure to either macrophages or inhibitor. Values are means ± standard errors (n = 12) determined with a t test (*, P 0.005; **, P 0.01; ***, P 0.001).

2.4 - Discussion

In this work, it was demonstrated that among the four studied genes the *hcp*2 gene was the highest up-regulated gene under NO stress. A strong induction of *hcp2* was also seen in *D. vulgaris* cells exposed to nitrate and nitrite stress conditions (Haveman *et al.*, 2004; Haveman *et al.*, 2005). Moreover, only the *hcp2* transposon mutant generated a *D. vulgaris* strain with high susceptibility to NO. Hence, Hcp2 seems to primarily contribute to the *in vitro* survival of *D. vulgaris* under NO stress.

Recent data from Voordouw and co-workers reported that *D. vulgaris* Hcp1 and Hcp2 are required to maintain the high rates of nitrite reduction by the nitrite reductase NrfHA, and therefore the authors proposed a role for Hcps in protection from nitrite derived products (Yurkiw *et al.*, 2012). Although, our phenotypic data indicate that Hcp2 participates in NO defense, the NO consumption rates of *D. vulgaris* remained unaltered upon deletion of *hcp2*. In agreement, none of the Hcp proteins studied exhibited NO reductase activity (Wolfe *et al.*, 2002; Cabello *et al.*, 2004; Almeida *et al.*, 2006; Overeijnder *et al.*, 2009).

Concerning the *D. vulgaris roo* genes, we observed no significant transcriptional alterations under nitrosative stress which fully agrees with earlier gene expression studies (Haveman *et al.*, 2004; Haveman *et al.*, 2005; He *et al.*, 2006; He *et al.*, 2010a). Furthermore, the growth behavior of the *D. vulgaris roo1* and *roo2* mutants upon exposure to the NO donor was similar to the wild type. Since earlier work with disk diffusion assays indicated that *D. vulgaris roo*2 mutant was moderately more sensitive to GSNO (Wildschut *et al.*, 2006), it is possible that these divergent results are related to the growth conditions and sources of NO used in each case. Nevertheless, we cannot exclude that in the presence of other *D. vulgaris* RNS protecting enzymes/proteins the contribution of *roo2* to the overall growth is not discernible, a situation that recalls that of *E. coli* which encodes several NO detoxifying enzymes (Justino *et al.*, 2005; Vine & Cole, 2011a).

Interestingly, we observed that the NO reduction rate of the strain lacking *roo1* is significantly lower, suggesting that Roo1 is the major contributor for the NO consumption activity in *D. vulgaris*. In agreement, the *D. gigas* Roo was previously shown to be able to rescue an *E. coli* strain deleted in the NO-reductase flavodiiron gene and to have a significant *in vitro* NO reductase activity (Rodrigues *et al.*, 2006).

While the results herein support the previously proposed role of Roo as a NO detoxifier (Rodrigues *et al.*, 2006; Wildschut *et al.*, 2006) a more complex

mechanism may explain the protection conferred by Hcp. So far, Hcps have been described as having peroxidase activity (*E. coli* and *D. desulfuricans* ATCC 27774) (Almeida *et al.*, 2006) and hydroxylamine reductase activity (*E. coli*, *Pyrococcus furiosus*, and *Rhodobacter capsulatus* E1F1) (Wolfe *et al.*, 2002; Cabello *et al.*, 2004; Overeijnder *et al.*, 2009). However, hydroxylamine was not assumed to be the natural substrate because of the low catalytic efficiency of the reaction (Wolfe *et al.*, 2002; Cabello *et al.*, 2004; Overeijnder *et al.*, 2009). Here, also the direct involvement of Hcp in NO detoxification could not be inferred. Although the *hcp2* mutant was more sensitive to the NO donor than the wild type strain, the NO consumption rate of *hcp2*$^{-}$ was similar to the consumption rate measured for the wild type strain. Hence, other roles for Hcp need to be considered. For example, Vine and Cole have proposed that Hcp may be involved in the repair of damage caused by nitrosative stress (Vine & Cole, 2011a).

Since *Desulfovibrio* spp. are proposed to act as opportunistic pathogens, in this study we addressed the ability of these bacteria to survive when contacting macrophages. Our results suggest that *D. vulgaris* is not capable of intracellular replication in macrophages but survives extracellularly. This is consistent with the capacity of *Desulfovibrio* spp. to replicate outside host cells in the gastrointestinal tract (Macfarlane *et al.*, 2007). Furthermore, this bacterium stimulates NO production in macrophages at levels similar to those induced by IFN-γ. The fact that *D. vulgaris* triggers NO production is consistent with the observed induction of IL-8 and IL-6 cytokines in HeLA cells upon infection with *D. fairfieldensis* and *D. desulfuricans* (Bisson-Boutelliez *et al.*, 2010). Moreover, the NO released lowers *Desulfovibrio* survival as the viability of the wild type and mutant strains was inversely related to the NO generated.

We have also found that Hcp and Roo proteins contribute to survival of *D. vulgaris* when infecting macrophages. Interestingly, the herein observed lack of correlation between the no increased susceptibility of the *hcp1* and the two *roo*

mutants upon *in vitro* exposure to NO and the positive contribution of all strains to survival in animal cells was also seen in *Salmonella enterica*. Indeed, although the *hcp* mutant of the *S. enterica* serovar Typhimurium (Δ*nipA*) displayed no defects under *in vitro* stress conditions, the NipA protein did contribute to the virulence in mice (Kim *et al.*, 2003).

The increased viability of *D. vulgaris* mutants in macrophages that do not produce NO, may be interpreted to mean that Roo and Hcp proteins are related to NO defense mechanisms. However, the incomplete recovery of the mutant strains observed upon inhibition of iNOS suggests that they also participate in protection against other stresses imposed by the macrophages such as the oxidative stress. Studies in oxygenated environments showing that the *roo* and *hcp* mutations decrease the *D. vulgaris* survival under oxidative stress corroborate this hypothesis (Wildschut *et al.*, 2006; Almeida *et al.*, 2006; Johnston *et al.*, 2009; Figueiredo *et al.*, 2012; Yurkiw *et al.*, 2012).

Another interesting question relates to the presence in *D. vulgaris* of two Hcps and Roos that share high amino acid sequence identities (Roo1 and Roo2, 29% identity; Hcp1 and Hcp2, 42% identity), including the conservation of the ligands for the diiron centers and iron-sulfur centers, respectively. This conservation suggests that each pair of homologues shares similar functional roles. However, our results indicate otherwise, as the *hcp2* transposon mutant, contrary to that of *hcp1*, is more susceptible to elimination by NO, and Roo1 contributes significantly more than Roo2 to cellular NO consumption. It is possible that these apparently distinct roles result from different gene regulation mechanisms. While in *D. vulgaris*, HcpR, a Cpr/Fnr-like global regulator that responds to nitrosative stress (Zhou *et al.*, 2012), has been proposed to control the *hcp2* expression, the regulation of *roo2* and that of the gene cluster *hcp1-roo1*, present in an isolated *D. vulgaris* genomic island (Johnston *et al.*, 2009), remains unknown. Noteworthy in *E. coli*, the *hcp* gene is regulated by the peroxide-sensing transcriptional factor

OxyR and the recombinant protein exhibits peroxidase activity (Almeida *et al.*, 2006; Seth *et al.*, 2012). Therefore, we speculate that in each case the *in vivo* function is controlled by transcriptional factors that respond to either oxidative or nitrosative stress, and their involvement in NO defenses may occur as RNS/ROS detoxifiers or indirectly in a not yet recognized way. Hence, the finding that the four gene products contribute, independently, to bacterial protection against macrophages may be rationalized by considering that macrophages expose *D. vulgaris* to both oxidative and nitrosative stress conditions.

In summary, we have shown for the first time that *D. vulgaris* triggers macrophage effectors and that the Hcp and Roo proteins contribute to the resistance of the bacterium during macrophage infection.

2.5 - Acknowledgements

We thank Grant Zane for the generation and confirmation of the transposon mutants of *D. vulgaris* Hildenborough used in this work. The work was funded by PEst-OE/EQB/LA0004/2011, FCT project PTDC/BIA-PRO/098224/2008 and by the following FCT student fellowships: SFRH/BD/44140/2008 (MF), SFRH/ BPD/ 63944/ 2009 (SL), SRFH/BPD/69325/2010 (LN), and BI/001/BI-BI/2012 (SS). JDW was funded by ENIGMA- Ecosystems and Networks Integrated with Genes and Molecular Assemblies (http://enigma.lbl.gov), a Scientific Focus Area Program at Lawrence Berkeley National Laboratory, supported by the Office of Science, Office of Biological and Environmental Research, of the U. S. Department of Energy under Contract No. DE-AC02-05CH11231.

2.6 - References

Almeida, CC, Romão, CV, Lindley, PF, Teixeira, M & Saraiva, LM (2006). The role of the hybrid cluster protein in oxidative stress defense. *J Biol Chem*, **281**:32445-32450.

Aragão, D, Macedo, S, Mitchell, EP, Romao, CV, Liu, MY, Frazao, C, Saraiva, LM, Xavier, AV, LeGall, J, van Dongen, WM, Hagen, WR, Teixeira, M, Carrondo, MA & Lindley, P (2003). Reduced hybrid cluster proteins (HCP) from *Desulfovibrio desulfuricans* ATCC 27774 and *Desulfovibrio vulgaris* (Hildenborough): X-ray structures at high resolution using synchrotron radiation. *J Biol Inorg Chem*, **8**:540-548.

Baptista, JM, Justino, MC, Melo, AM, Teixeira, M & Saraiva, LM (2012). Oxidative stress modulates the nitric oxide defense promoted by *Escherichia coli* flavorubredoxin. *J Bacteriol*, **194**:3611-3617.

Beckman, JS, Wink, DA & Crow, JP (1996). *Methods in Nitric Oxide Research*. Chichester, UK: John Wiley & Sons Ltd.

Bisson-Boutelliez, C, Massin, F, Dumas, D, Miller, N & Lozniewski, A (2010). *Desulfovibrio* spp. survive within KB cells and modulate inflammatory responses. *Mol Oral Microbiol*, **25**:226-235.

Cabello, P, Pino, C, Olmo-Mira, MF, Castillo, F, Roldan, MD & Moreno-Vivian, C (2004). Hydroxylamine assimilation by *Rhodobacter capsulatus* E1F1. requirement of the hcp gene (hybrid cluster protein) located in the nitrate assimilation nas gene region for hydroxylamine reduction. *J Biol Chem*, **279**:45485-45494.

Figueiredo, MC, Lobo, SA, Carita, JN, Nobre, LS & Saraiva, LM (2012). Bacterioferritin protects the anaerobe *Desulfovibrio vulgaris* Hildenborough against oxygen. *Anaerobe*, **18**:454-458.

Frazão, C, Silva, G, Gomes, CM, Matias, P, Coelho, R, Sieker, L, Macedo, S, Liu, MY, Oliveira, S, Teixeira, M, Xavier, AV, Rodrigues-Pousada, C, Carrondo, MA & Le Gall, J (2000). Structure of a dioxygen reduction enzyme from *Desulfovibrio gigas*. *Nat Struct Biol*, **7**:1041-1045.

Gaillard, T, Pons, S, Darles, C, Beausset, O, Monchal, T & Brisou, P (2011). [*Desulfovibrio fairfieldensis* bacteremia associated with acute sigmoiditis]. *Med Mal Infect*, **41**:267-268.

Gomes, CM, Silva, G, Oliveira, S, LeGall, J, Liu, MY, Xavier, AV, Rodrigues-Pousada, C & Teixeira, M (1997). Studies on the redox centers of the terminal oxidase from *Desulfovibrio gigas* and evidence for its interaction with rubredoxin. *J Biol Chem*, **272**:22502-22508.

Haveman, SA, Greene, EA, Stilwell, CP, Voordouw, JK & Voordouw, G (2004). Physiological and gene expression analysis of inhibition of *Desulfovibrio vulgaris* Hildenborough by nitrite. *J Bacteriol*, **186**:7944-7950.

Haveman, SA, Greene, EA & Voordouw, G (2005). Gene expression analysis of the mechanism of inhibition of *Desulfovibrio vulgaris* Hildenborough by nitrate-reducing, sulfide-oxidizing bacteria. *Environ Microbiol*, **7**:1461-1465.

He, Q, Huang, KH, He, Z, Alm, EJ, Fields, MW, Hazen, TC, Arkin, AP, Wall, JD & Zhou, J (2006). Energetic consequences of nitrite stress in *Desulfovibrio vulgaris* Hildenborough, inferred from global transcriptional analysis. *Appl Environ Microbiol*, **72**:4370-4381.

He, Q, He, Z, Joyner, DC, Joachimiak, M, Price, MN, Yang, ZK, Yen, HC, Hemme, CL, Chen, W, Fields, MM, Stahl, DA, Keasling, JD, Keller, M, Arkin, AP, Hazen, TC, Wall, JD & Zhou, J (2010). Impact of elevated nitrate on sulfate-reducing bacteria: a comparative study of *Desulfovibrio vulgaris*. *Isme J*, **4**:1386-1397.

Ichiishi, S, Tanaka, K, Nakao, K, Izumi, K, Mikamo, H & Watanabe, K (2010). First isolation of *Desulfovibrio* from the human vaginal flora. *Anaerobe*, **16**:229-233.

Imlay, JA, Hassett, DJ, Wong, SMS, Akerley, BJ, Spiro, S, Ulett, G & er, AP (2011). Oxidative and Nitrosative Stress. In *Stress Response in Pathogenic Bacteria*, pp. 3-79. Edited by S. P. Kidd. Oxfordshire, UK: CAB International.

Jia, W, Whitehead, RN, Griffiths, L, Dawson, C, Bai, H, Waring, RH, Ramsden, DB, Hunter, JO, Cauchi, M, Bessant, C, Fowler, DP, Walton, C, Turner, C & Cole, JA (2012). Diversity and distribution of sulphate-reducing bacteria in human faeces from healthy subjects and patients with inflammatory bowel disease. *FEMS Immunol Med Microbiol*, **65**:55-68.

Johnston, S, Lin, S, Lee, P, Caffrey, SM, Wildschut, J, Voordouw, JK, da Silva, SM, Pereira, IA & Voordouw, G (2009). A genomic island of the sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough promotes survival under stress conditions while decreasing the efficiency of anaerobic growth. *Environ Microbiol*, **11**:981-991.

Justino, MC, Vicente, JB, Teixeira, M & Saraiva, LM (2005). New genes implicated in the protection of anaerobically grown *Escherichia coli* against nitric oxide. *J Biol Chem*, **280**:2636-2643.

Keller, KL, Bender, KS & Wall, JD (2009). Development of a markerless genetic exchange system for *Desulfovibrio vulgaris* Hildenborough and its use in generating a strain with increased transformation efficiency. *Appl Environ Microbiol*, **75**:7682-7691.

Kim, CC, Monack, D & Falkow, S (2003). Modulation of virulence by two acidified nitrite-responsive loci of *Salmonella enterica* serovar Typhimurium. *Infect Immun*, **71**:3196-3205.

Lozniewski, A, Maurer, P, Schuhmacher, H, Carlier, JP & Mory, F (1999). First isolation of *Desulfovibrio* species as part of a polymicrobial infection from a brain abscess. *Eur J Clin Microbiol Infect Dis*, **18**:602-603.

Macedo, S, Mitchell, EP, Romão, CV, Cooper, SJ, Coelho, R, Liu, MY, Xavier, AV, LeGall, J, Bailey, S, Garner, DC, Hagen, WR, Teixeira, M, Carrondo, MA & Lindley, P (2002). Hybrid cluster proteins (HCPs) from *Desulfovibrio desulfuricans* ATCC 27774 and *Desulfovibrio vulgaris* (Hildenborough): X-ray structures at 1.25 Å resolution using synchrotron radiation. *J Biol Inorg Chem*, **7**:514-525.

MacMicking, J, Xie, QW & Nathan, C (1997). Nitric oxide and macrophage function. *Annu Rev Immunol*, **15**:323-350.

Nakao, K, Tanaka, K, Ichiishi, S, Mikamo, H, Shibata, T & Watanabe, K (2009). Susceptibilities of 23 *Desulfovibrio* isolates from humans. *Antimicrob Agents Chemother*, **53**:5308-5311.

Nathan, C & Shiloh, MU (2000). Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc Natl Acad Sci U S A*, **97**:8841-8848.

Overeijnder, ML, Hagen, WR & Hagedoorn, PL (2009). A thermostable hybrid cluster protein from *Pyrococcus furiosus*: effects of the loss of a three helix bundle subdomain. *J Biol Inorg Chem*, **14**:703-710.

Rodrigues, R, Vicente, JB, Felix, R, Oliveira, S, Teixeira, M & Rodrigues-Pousada, C (2006). *Desulfovibrio gigas* flavodiiron protein affords protection against nitrosative stress in vivo. *J Bacteriol*, **188**:2745-2751.

Rowan, F, Docherty, NG, Murphy, M, Murphy, B, Calvin Coffey, J & O'Connell, PR (2010). *Desulfovibrio* bacterial species are increased in ulcerative colitis. *Dis Colon Rectum*, **53**:1530-1536.

Saraiva, LM, Vicente, JB & Teixeira, M (2004). The role of the flavodiiron proteins in microbial nitric oxide detoxification. *Adv Microb Physiol*, **49**:77-129.

Seth, D, Hausladen, A, Wang, YJ & Stamler, JS (2012). Endogenous protein S-Nitrosylation in *E. coli*: regulation by OxyR. *Science*, **336**:470-473.

van den Berg, WAM, Stokkermans, JPWG & van Dongen, WMAM (1989). Development of a plasmid transfer system for the anaerobic sulphate reducer, *Desulfovibrio vulgaris*. *Journal of Biotechnology*, **12**:173-184.

Vicente, JB, Carrondo, MA, Teixeira, M & Frazão, C (2008). Flavodiiron Proteins: Nitric Oxide and/or Oxygen Reductases. In *Handbook of Metalloproteins*. Edited by A. Messerschmidt. Chichester, UK: John Wiley & Sons, Ltd.

Vine, CE & Cole, JA (2011a). Nitrosative stress in *Escherichia coli*: reduction of nitric oxide. *Biochem Soc Trans*, **39**:213-215.

Vine, CE & Cole, JA (2011b). Unresolved sources, sinks, and pathways for the recovery of enteric bacteria from nitrosative stress. *FEMS Microbiol Lett*, **325**:99-107.

Wildschut, JD, Lang, RM, Voordouw, JK & Voordouw, G (2006). Rubredoxin:oxygen oxidoreductase enhances survival of *Desulfovibrio vulgaris* Hildenborough under microaerophilic conditions. *J Bacteriol*, **188**:6253-6260.

Wolfe, MT, Heo, J, Garavelli, JS & Ludden, PW (2002). Hydroxylamine reductase activity of the hybrid cluster protein from *Escherichia coli*. *J Bacteriol*, **184**:5898-5902.

Yurkiw, MA, Voordouw, J & Voordouw, G (2012). Contribution of rubredoxin:oxygen oxidoreductases and hybrid cluster proteins of *Desulfovibrio vulgaris* Hildenborough to survival under oxygen and nitrite stress. *Environ Microbiol*, **14**:2711-2725.

Zhou, A, Chen, YI, Zane, GM, He, Z, Hemme, CL, Joachimiak, MP, Baumohl, JK, He, Q, Fields, MW, Arkin, AP, Wall, JD, Hazen, TC & Zhou, J (2012). Functional characterization of Crp/Fnr-type global transcriptional regulators in *Desulfovibrio vulgaris* Hildenborough. *Appl Environ Microbiol*, **78**:1168-1177.

2.7 - Supplemetary data

Figure S1: Growth of *D. vulgaris* wild type and *roo1* and *roo2* mutated strains upon exposure to GSNO.

D. vulgaris wild type **(A)**, and strains mutated in *roo1* **(B)** and *roo2* **(C)** that reach the early exponential growth phase (OD_{600} ~ 0.3) were left untreated (o), treated with 10 µM GSNO (●) and with 50 µM GSNO (♦).

Experiments were performed for two biological independent samples with two replicates. Values represent means ± standard error.

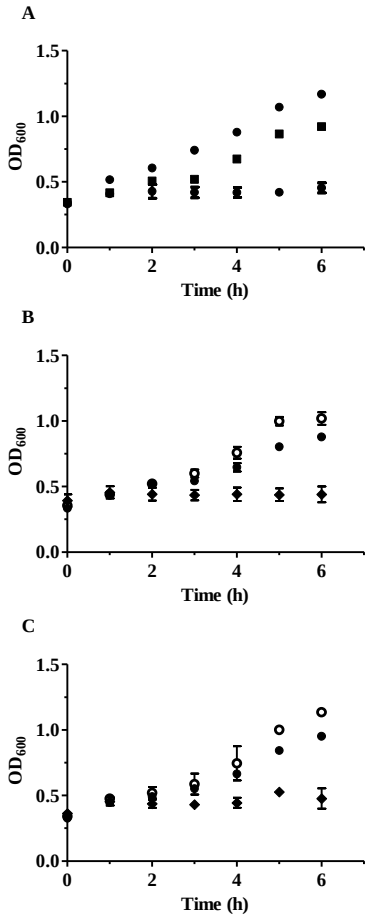
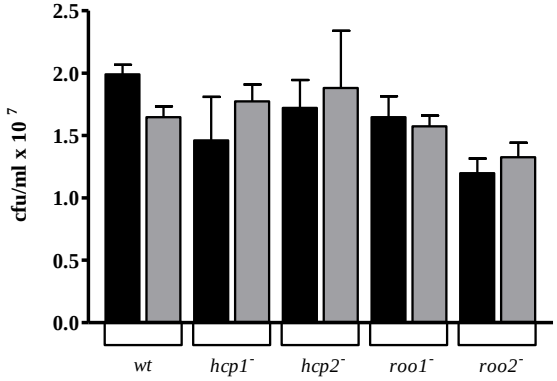


Figure S2: Viability of *D. vulgaris* cells in DMEM

Viability of *D. vulgaris* cells inoculated in well plates containing DMEM medium (time zero, black bars) and after incubated with 5% CO_2, at 37 ºC for 24 h (grey bars). Experiments were performed in triplicate and values represent means ± standard error.



General Discussion

Part III

Chapter 1

General discussion

Chapter 1

General Discussion

1.1 - General *D. vulgaris* response to oxidative stress

For many years *Desulfovibrio* spp. have been known as strict anaerobes. However, several studies demonstrated that these bacteria may also be active in oxic zones revealing that these microorganisms are aerotolerant (Fukui & Takii, 1990; Teske *et al.*, 1998). In the work presented in this thesis, we studied the behavior of *D. vulgaris* when exposed to oxygen, and showed that these bacteria are able to grow in an atmosphere containing oxygen in a concentration up to 10% (~ 100 µM) (Figure 1.1, Part II - Chapter 1) (Figueiredo *et al.*, 2012). Moreover, under these conditions no lag-phase was observed indicating that *D. vulgaris* is able to a rapid adaptation to oxygen. Previous work also demonstrated that *D. desulfuricans* ATCC 27774 sustains high levels of oxygen as it grows at nearly atmospheric oxygen levels (18% O_2, ~ 170 µM) (Lobo *et al.*, 2007). Nevertheless, under atmospheric concentrations of oxygen (21%, ~ 200 µM) *D. vulgaris* stops growing and its viability decreases approximately 50% only 30 min after oxygen exposure (Figure 1.3, Part II - Chapter 1) (Figueiredo *et al.*, 2012). In other studies, oxygen also reduces the viability of *D. vulgaris* cells. For example, a decline in viability of approximately 30% was observed for *D. vulgaris* exposed to 1 mM oxygen for 1 h (Pereira *et al.*, 2008). Furthermore, a severe lowering of survival was seen in cells exposed to pure oxygen during 1 h (~ 80%) (Fournier *et al.*, 2004) and for *D. vulgaris* exposed to air for 4 h (~ 90%) (Mukhopadhyay *et al.*, 2007). In contrast, Zhang and co-workers reported no significant alteration of the *D. vulgaris* viability when submitted to a continuous bubbling with air for 2 h (Zhang *et al.*, 2006). Altogether, these divergent results suggested that the ability of *D. vulgaris*

to face oxygen stress depends on conditions such as growth media, type of oxygen source and length of the exposure.

The availability of *D. vulgaris* genome, in 2004, allowed several transcriptomic studies to assess the genetic response of these microorganisms to stress conditions. The different transcriptomic studies suggest that some *D. vulgaris* genes are dedicated to detoxification at low oxygen concentrations. Table 1.1 resumes the transcriptional profile of some of the genes currently proposed to be involved in the oxygen detoxification.

Table 1.1 - Effect of oxygen in the expression of *D. vulgaris* genes.

	sodB	*bfr*	*ftn*	*ngr*	*coxII*	*rbr2*	*rbr1*	*sor*	*roo2*	*cydA*
Pure O_2 1h [(1)]	ND	ND	ND	ND	ND	↓	↓	↓	ND	ND
21% O_2 1 h [(2)]	=	ND	ND	ND	ND	↑	ND	=	ND	ND
0.1% O_2 4 h [(3)]	ND	=	ND	=	ND	↑	↑	=	=	=
21% O_2 4 h [(3)]	ND	=	ND	↓	ND	↓	↓	↓	=	=
1 mM pure O_2 1 h [(4)]	ND	ND	ND	=	ND	↓	=	=	=	↓
5% O_2 1 h [(5)]	↑	=	=	↑	↑	↑	↑	↑	↑	=
21% O_2 1 h [(5)]	=	↑	=	↑	↓	↑	↑	↑	↑	=

sodB: superoxide dismutase; *bfr*: bacterioferritin; *ftn*: ferritin; *ngr*: nigerythrin; *coxII*: cytochrome *c* oxidase subunit II; *rbr2*: rubrerythrin 2; *rbr1*: rubrerythrin 1; *sor*: superoxide reductase; *roo2*: rubredoxin:oxygen oxidoreductase 2; *cydA*: cytochrome *bd* oxidase subunit I.
(↑) up-regulation; (↓) down-regulation; (=) no changes; (ND) not determined.
(1) (Fournier *et al.*, 2006); (2) (Zhang *et al.*, 2006); (3) (Mukhopadhyay *et al.*, 2007); (4) (Pereira *et al.*, 2008); (5) (Figueiredo *et al.*, 2012).

The genes proposed to belong to the PerR regulon (*rbr1* and *rbr2*), had their expression increased in cells treated with 0.1% and 5% O_2, but when submitted to higher concentrations their transcription is repressed. Hence, it suggests that these proteins are dedicated to the protection of *D. vulgaris* under low oxygen concentration (Zhang *et al.*, 2006; Fournier *et al.*, 2006; Pereira *et al.*, 2008; Figueiredo *et al.*, 2012). The gene coding for *sodB* is only up-regulated when cells are exposed to mild-oxygen concentrations, such as 5% or 10% (Table 1.3, Part II -

Chapter 1), indicating they are not involved in the protection of *D. vulgaris* against higher oxygen concentrations. The transcriptional profile of *coxII* under oxidative conditions was only investigated in our study. We observed that expression of *coxII* is up-regulated in cells submitted to low oxygen levels and down-regulated under higher concentrations, suggesting a role only under small oxygen concentrations (Figueiredo *et al.*, 2012). *D. vulgaris roo2* is considered a oxygen protective detoxifier (Silaghi-Dumitrescu *et al.*, 2005; Wildschut *et al.*, 2006; Johnston *et al.*, 2009; Yurkiw *et al.*, 2012). In agreement, our results demonstrated that the gene coding Roo2 had its transcription elevated in similar extent (~ 4-fold) in both low and high oxygen concentrations (5% and 21% O_2) (Figueiredo *et al.*, 2012). The lack of induction seen in previous studies is possible related with the conditions used in each study that did not allow the induction of the gene translation.

The results of this thesis work further revealed the role of bacterioferritin in protecting *D. vulgaris* from oxidative damage under high concentrations of oxygen. In fact, *bfr* is the analyzed gene with the highest up-regulation (7.5-fold) in cells exposed to atmospheric oxygen levels (Part II - Chapter 1). Moreover, we show that the transcriptional regulator which responds to peroxide stress (PerR) induces the expression of *bfr*, and a PerR potential binding site located upstream of the start codon of *bfr* was detected (Figure 1.1) (Figueiredo *et al.*, 2012). Altogether, these data reveal a previously unrecognized ability of the iron storage bacterioferritin to protect *D. vulgaris* against oxygen (Figure 1.2).

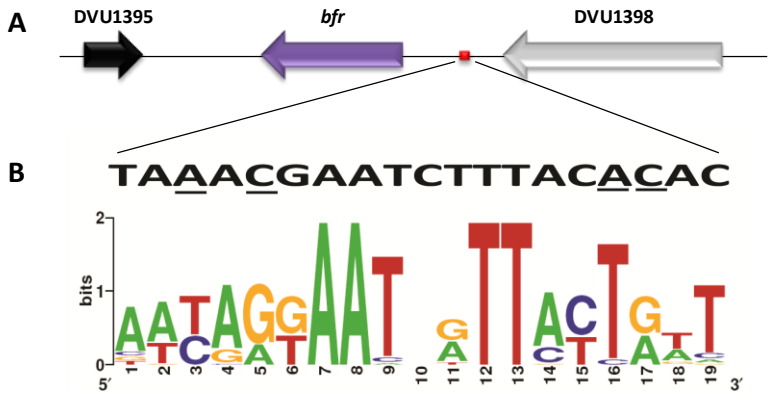


Figure 1.1 - Putative PerR binding site upstream of start codon of *D. vulgaris bfr*.
A) Genomic organization of *bfr* (DVU1397) from *D. vulgaris* Hildenborough, the location of the putative PerR binding site is represented by a red square; locus DVU1395: C4-type zinc finger protein (DksA/TraR family) and locus DVU1398: transposase OrfB (ISDvu2). Adapted from (Dehal *et al.*, 2009). **B)** Alignment of 39 *D. vulgaris* predicted PerR boxes (Novichkov *et al.*, 2010) were used to generate a sequence logo using WebLogo (Schneider & Stephens, 1990; Crooks *et al.*, 2004). The letters above the logo show the sequence of *D. vulgaris* putative PerR binding site of the *bfr* gene, located between -213 bp and - 194 bp upstream of the start codon of the *bfr* gene. The underlined bases represent mismatches from the consensus bases obtained when using all *Desulfovibrio* predicted PerR boxes.

The role of bacterioferritin in oxygen detoxification herein demonstrated was also proposed for bacterioferritin of *Bacteroides fragilis*. In this anaerobe, the increased expression of the *bfr* transcript in response to oxygen stress and the decreased oxygen tolerance of the *bfr* deletion strain proved that the *bfr* gene product protects against oxidative stress. Furthermore, the protein protects DNA against oxidative stress *in vitro* and exhibits a dodecameric structure which led to the proposal that this protein and the bacterial ortologs constitute a new class of miniferritin proteins similar to Dps-like proteins (Gauss *et al.*, 2011). Timóteo and colleagues also proposed for *D. vulgaris* Bfr a Dps-like function, as the recombinant protein was able to bind supercoiled plasmid DNA and showed lower affinity to short double-stranded DNA fragments. However, *D. vulgaris* recombinant Bfr binds DNA in a different manner of the one proposed for *E. coli* Dps which self-aggregates and condenses DNA. This is probably due to the fact that *D. vulgaris* Bfr does not contain the mobile amino acid terminal extension responsible for this

feature in the Dps proteins (see Figure 2.2 C Part I - Chapter 2). Therefore, authors proposed that the DNA-binding ability of *D. vulgaris* Bfr may be related to the presence of positively charged amino acids in N- and C- terminal regions. The same work also revealed that bacterioferritin uses H_2O_2 to reduce free Fe^{2+} avoiding the Fenton reaction (Timóteo *et al.*, 2012). These studies corroborate the proposal that bacterioferritin contributes to the oxygen resistance of the anaerobic sulfate reducer *D. vulgaris.*

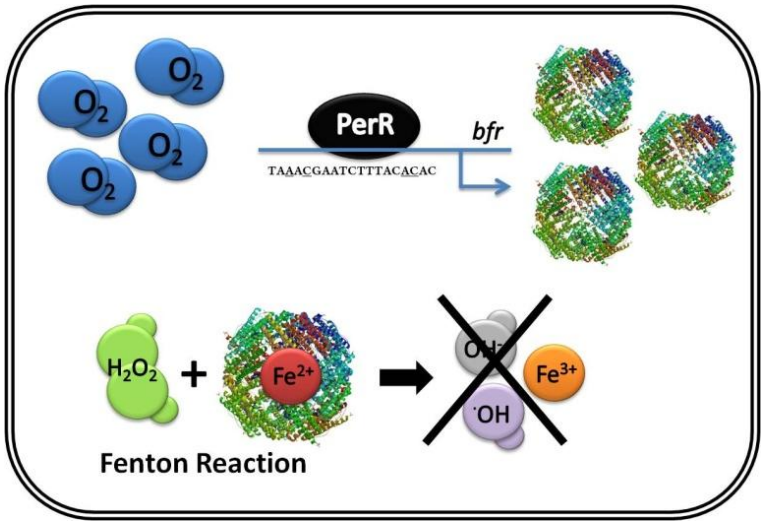


Figure 1.2 - Schematic representation of *D. vulgaris* bacterioferritin action in the presence of high concentrations of oxygen.
In the presence of high concentrations of oxygen the peroxide regulator PerR induces the expression of bacterioferritin gene. Bacterioferritin reduces the level of ferrous ions present in the cell and therefore prevents the occurrence of the Fenton reaction.

Interestingly, the expression of *ftn* did not suffer significant alteration upon exposure of *D. vulgaris* to high oxygen concentrations. While exposure to air of the *D. vulgaris bfr* mutant strain reduced the growth and viability, under similar conditions *D. vulgaris ftn* mutant did not show altered viability (Figueiredo *et al.*, 2012). This suggests that contrary to bacterioferritin, ferritin is not involved in the oxygen resistance of *D. vulgaris*. Differential expression between iron storage proteins has also been seen in other organisms such as *Bacteroides fragilis* when

subjected to air stress conditions, *Pseudomonas putida* and *Salmonella enterica* serovar Typhimurium under different iron levels, and *Bacillus subtilis* in the presence of H_2O_2 (Chen *et al.*, 1995; Antelmann *et al.*, 1997; Velayudhan *et al.*, 2007; Chen *et al.*, 2009; Gauss *et al.*, 2011).

As mentioned in Part I - Chapter 2, bacterioferritins and ferritins are distantly related in evolution. Like in other organisms such as *E. coli*, comparison of *D. vulgaris* amino acid sequences of Bfr and Ftn reveals low degree of similarity (~ 15% identity). The amino acid sequence alignment of Bfr and Ftn of *D. vulgaris* and *E. coli* show that the ligands of the ferroxidase center in Bfr and Ftn are different (Figure 1.3). Moreover, Ftn has a trinuclear ferroxidase center while Bfr contains a diiron site where the oxidation of Fe^{2+} occurs.

So far, the results indicate that ferritins are dedicated to iron homeostasis. *E. coli* FtnA is a iron storage protein providing a source of iron during iron deficient conditions, since a *ftn*A mutant strain resulted in approximately 50% reduction of the intracellular iron in iron-rich media and displayed a reduced growth rate under iron-restricted conditions. (Abdul-Tehrani *et al.*, 1999). In *H. pylori* ferritin proved to have a role in the survival of these pathogens during gastric colonization. The inactivation of *ferritin (pfr)* gene during iron starvation limited the growth of this microbe and lowered the intracellular iron content (Waidner *et al.*, 2002). It remais to be seen if Ftn plays a similar role in *D. vulgaris*.

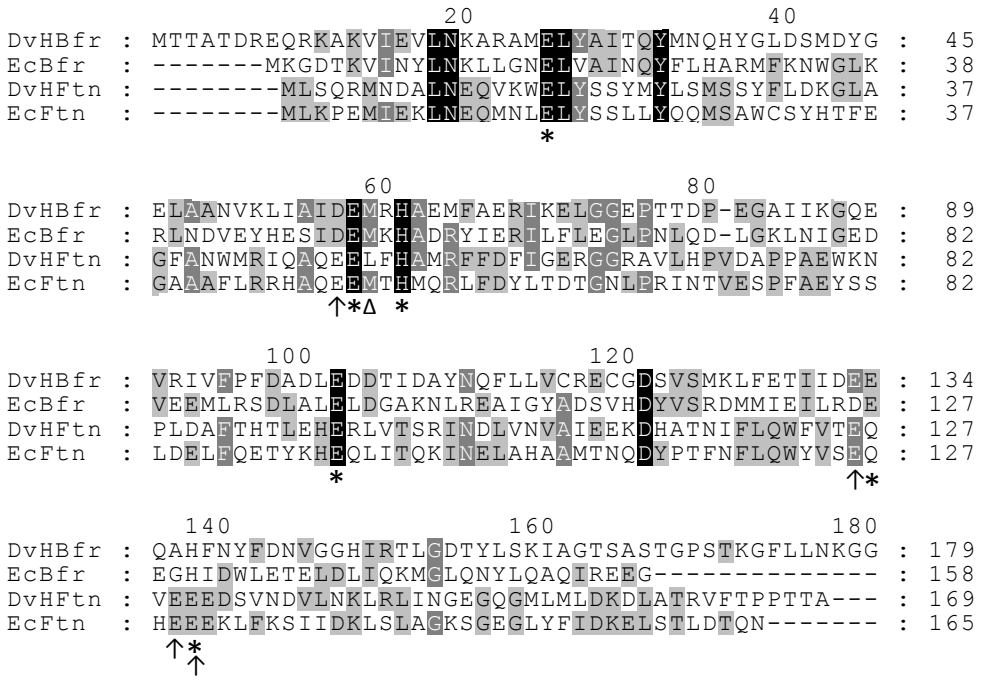


Figure 1.3 - Amino acid sequence alignment of Bfr and Ftn from *D. vulgaris* and *E. coli*.
DvHBfr corresponds to *D. vulgaris* Hildenborough Bacterioferritin (locus: DVU1397, NCBI - GI: 46579808), EcBfr corresponds to *E. coli* str. K-12 substr. MG1655 Bacterioferritin (locus: b3336, NCBI-GI: 16131215), DvHFtn corresponds to *D. vulgaris* Hildenborough Ferritin (locus: DVU1568, NCBI - GI: 46579979), and EcFtn corresponds to *E. coli* str. K-12 substr. MG1655 Ferritin A (locus: b1905, NCBI-GI: 16129855). (∗) represent the ligands to iron sites A and B from Bfr and Ftn, (↑) indicates residues that are ligands to iron site C in Ftn, (Δ) indicates the Bfr heme methionine ligand. Black boxes represent conserved residues, and grey boxes represent almost strictly conserved residues. The location of the Fe binding sites are represented according to (da Costa *et al.*, 2001; Pereira *et al.*, 2012).

1.2 - Role of flavodiiron and hybrid cluster proteins in *D. vulgaris*

Flavodiiron proteins are a large superfamily of enzymes proposed to have two possible roles, namely in nitric oxide and oxygen detoxification. Nevertheless, the conditions under which each enzymatic function occurs remain to be elucidated. *D. gigas* Roo was the first enzyme of the flavodiiron family shown to be able to reduce oxygen, and this O_2 scavenging function was proposed to allow this anaerobic microorganism to survive transient oxygen exposures (Chen *et al.*, 1993b; Chen *et al.*, 1995). This protein also has a significant role under nitrosative

conditions, as *D. gigas roo* mutant strain has decreased viability under nitrosative stress and the protein is able to restore the NO-sensitive phenotype of the *E. coli* strain mutated in *norV* gene (the gene coding flavorubredoxin (FlRd) in *E. coli*) (Rodrigues *et al.*, 2006). Several studies with other bacterial FDPs also raised the hypothesis that these enzymes may have a bifunctional role, as below described.

E. coli flavorubredoxin binds NO to the diirion center and possesses nitric oxide reductase activity (Gomes *et al.*, 2000; Gomes *et al.*, 2002). Its role in nitric oxide detoxification was also suggested by the highly increased transcription levels of *norV* under nitrosative stress and by *in vitro* studies in which the strain mutated of this gene had increased sensitivity to NO gas and NO-releasing compounds (Gardner *et al.*, 2002; Hutchings *et al.*, 2002; Mukhopadhyay *et al.*, 2004; Justino *et al.*, 2005).

D. vulgaris genome encodes two FDP proteins, Roo1 (DVU2014) and Roo2 (DVU3185). *In vitro* experiments with *D. vulgaris* Roo2 established that this enzyme displays both O_2 and NO reductase activities. Moreover, Roo2 was also able to *in vivo* protection of an NO-sensitive *E. coli* Δ*norV* strain (Silaghi-Dumitrescu *et al.*, 2005). Physiological studies corroborate these results, since Roo proteins from *D. vulgaris* proved to be able to promote the survival of *D. vulgaris* under microaerobic and nitrite stress conditions (Wildschut *et al.*, 2006; Johnston *et al.*, 2009; Yurkiw *et al.*, 2012). FDP from *Moorella thermoacetica* is also able of both NO and O_2 reductase activities (Vicente *et al.*, 2008a). On the contrary, the majority of FDPs studied reduce exclusively oxygen to water as is the case of the metanogenic Archaea *Methanothermobacter marburgensis* and *Methanobrevibacter arboriphilus* (Seedorf *et al.*, 2004; Seedorf *et al.*, 2007). Table 1.2 summarizes the nitric oxide and oxygen reductase activities proposed for several flavodiiron proteins.

Table 1.2 - Oxygen and nitric oxide reductase activities of some flavodiiron proteins.

Organism	O_2 reductase activity	NO reductase activity	Reference
Desulfovibrio gigas	+	+	(Chen *et al.*, 1993a; Gomes *et al.*, 1997; Rodrigues *et al.*, 2006)
Desulfovibrio vulgaris	+	+	(Silaghi-Dumitrescu *et al.*, 2005)
Entamoeba histolytica	+	-	(Vicente *et al.*, 2012)
Escherichia coli	-	+	(Gardner *et al.*, 2002; Gomes *et al.*, 2002; Hutchings *et al.*, 2002)
Giardia intestinalis	+	-	(Di Matteo *et al.*, 2008)
Methanobrevibacter arboriphilus	+	-	(Seedorf *et al.*, 2004)
Methanothermobacter marburgensis	+	-	(Seedorf *et al.*, 2007)
Moorella thermoacetica	+	+	(Silaghi-Dumitrescu *et al.*, 2003; Silaghi-Dumitrescu *et al.*, 2005)
Synechocystis spp.	+	-	(Vicente *et al.*, 2002)
Thermotoga maritima	+	-	(Hayashi *et al.*, 2010)
Trichomonas vaginalis	+	-	(Smutná *et al.*, 2009)

(+) presence of reductase activity; (-) absence of reductase activity.

Nitric oxide and oxygen detoxification is also crucial for microbes that face the cytotoxic effects of RNS and ROS synthesized in activated macrophages as part of the host immune response to invading pathogens (Nathan & Shiloh, 2000; Baptista *et al.*, 2012). The work of this thesis showed that both *roo1* and *roo2* genes contribute for the survival of *D. vulgaris* upon contact with mammalian macrophages. Furthermore, the behavior of *D. vulgaris* strains deleted in these genes revealed that they are related with detoxification of nitric oxide, as when the production of NO by the macrophage was inhibited the mutant strains resume growth. However, the survival recovery was not complete suggesting that *roo* genes are also protecting from the oxidative stress imposed by macrophages (Figueiredo *et al.*, 2013). These findings are consistent with the previous proposed role of FDPs in the protection of microorganisms against oxidative and nitrosative stresses (Saraiva *et al.*, 2004; Vicente *et al.*, 2008a).

Interestingly, the location of *D. vulgaris roo1* gene is 75 base pairs upstream of the start codon for a gene encoding the hybrid cluster protein (Hcp1)

(Heidelberg *et al.*, 2004). *D. vulgaris* also contains two Hcp homologs, namely Hcp1 (DVU2013) and Hcp2 (DVU2543). Although the role of hybrid cluster proteins has been the subject of intensive studies performed in several organisms, their function remains unclear. Hcp proteins are annotated in databases as hydroxylamine reductase enzymes due to studies in *E. coli*, *Rhodobacter capsulatus* E1F1 and *Pyrococcus furiosus*, which reported that Hcp proteins reduce hydroxylamine *in vitro* generating NH_3 and H_2O (Wolfe *et al.*, 2002; Cabello *et al.*, 2004; Overeijnder *et al.*, 2009). However, Hcp has an extremely low catalytic efficiency which makes unlikely that hydroxylamine is its physiological substrate.

Several transcriptional studies on bacterial response to nitrosative stress have consistently implicated Hcp in nitric oxide detoxification. In 2005, Flatley and colleagues reported that *hcp* is highly up-regulated (30-fold) after 5 min of GSNO treatment (Flatley *et al.*, 2005). Subsequent studies, discovered that *E. coli hcp-hcr* operon (*hcr* stands for NADH oxidoreductase) is regulated by nitrite-sensitive repressor NsrR, which plays a key role in bacterial nitrosative stress resistance (Filenko *et al.*, 2007). A recent investigation established that *E. coli* Hcp protects against nitrosative stress, as inactivation of the gene resulted in a strain more sensitive to GSNO than the parental, with a growth inhibition comparable to that occurring when the strains lacks the canonical nitrosative detoxifying enzyme, flavohemoglobin (Hmp) (Seth *et al.*, 2012). Moreover, the *E. coli* Δ*hcp* suffers growth impairment in the presence of activated macrophages, and the phenotype is rescued upon blockage of macrophage-NO production. Similar results were obtained for the facultative intracellular pathogen *Salmonella enterica* serovar Typhimurium, in which the *hcp-hcr* operon is under the control of NsrR regulator and displays a supportive role in NO detoxification under aerobic conditions (Karlinsey *et al.*, 2012).

In *D. vulgaris* the expression of the hybrid cluster proteins genes (*hcp1* and *hcp2*) is induced by nitrite and nitrate stresses (Haveman *et al.*, 2004; Haveman *et*

al., 2005; He *et al.*, 2006; He *et al.*, 2010a). Moreover, these gene products have been associated with the survival of this microorganism to oxygen and nitrite stress (Johnston *et al.*, 2009; Yurkiw *et al.*, 2012). Johnston and co-workers proposed that these enzymes remove RNS using the reducing power from lactate and H_2; however the exact mechanism by which this reduction occurs was not yet been disclosed (Yurkiw *et al.*, 2012). In our study, we showed that the deletion of these gene products do not result in alteration of the NO reduction rate (Figure 2.3, Part II - Chapter 2), revealing that Hcp1 and Hcp2 do not direct detoxify NO. However, *hcp1* and *hcp*2 gene products are required to *D. vulgaris* survival upon contact with activated murine macrophages, suggesting a role in both nitrosative and oxidative stress resistance (Figueiredo *et al.*, 2013). In agreement, the role of Hcp proteins in oxidative stress detoxification was proposed based on the observation that *E. coli hcp* gene is up-regulated by hydrogen peroxide, and the gene is controlled by the redox-sensitive transcriptional activator OxyR (Almeida *et al.*, 2006). Furthermore, the *E. coli* and *D. desulfuricans* ATCC 27774 Hcps exhibited peroxidase activity with values that are within the range of other peroxidases (Almeida *et al.*, 2006). Therefore, the mechanism whereby these enzymes confer resistance to these stresses in *D. vulgaris* is currently unknown and needs further investigation.

Altogether, the work performed during this PhD dissertation contributed to clarify the underlying mechanisms in *D. vulgaris* that permit these bacteria to sustain and overcome the oxidative and nitrosative stresses. A transcriptional and physiological approach allowed the recognition, for the first time, of the bacterioferritin role in the anaerobe *D. vulgaris* when exposed to an oxygen atmosphere. The present work also provides evidences of how *D. vulgaris* is able to survive against nitrosative stress imposed by the immune system, being the initial step to the comprehension of the role of these putative pathogenic bacteria

in mammalian cells. Moreover, we proved that Hcp and Roo proteins contribute to survival of *D. vulgaris* upon interaction with mammalian macrophages, showing their participation in resistance against the mammalian immune system.

1.3 - References

Abdul-Tehrani, H, Hudson, AJ, Chang, YS, Timms, AR, Hawkins, C, Williams, JM, Harrison, PM, Guest, JR & Andrews, SC (1999). Ferritin mutants of *Escherichia coli* are iron deficient and growth impaired, and fur mutants are iron deficient. *J Bacteriol*, **181**:1415-1428.

Almeida, CC, Romão, CV, Lindley, PF, Teixeira, M & Saraiva, LM (2006). The role of the hybrid cluster protein in oxidative stress defense. *J Biol Chem*, **281**:32445-32450.

Antelmann, H, Engelmann, S, Schmid, R, Sorokin, A, Lapidus, A & Hecker, M (1997). Expression of a stress- and starvation-induced *dps/pexB*-homologous gene is controlled by the alternative sigma factor σ^B in *Bacillus subtilis*. *J Bacteriol*, **179**:7251-7256.

Baptista, JM, Justino, MC, Melo, AM, Teixeira, M & Saraiva, LM (2012). Oxidative stress modulates the nitric oxide defense promoted by Escherichia coli flavorubredoxin. *J Bacteriol*, **194**:3611-3617.

Cabello, P, Pino, C, Olmo-Mira, MF, Castillo, F, Roldan, MD & Moreno-Vivian, C (2004). Hydroxylamine assimilation by *Rhodobacter capsulatus* E1F1. requirement of the hcp gene (hybrid cluster protein) located in the nitrate assimilation nas gene region for hydroxylamine reduction. *J Biol Chem*, **279**:45485-45494.

Chen, L, Liu, MY, LeGall, J, Fareleira, P, Santos, H & Xavier, AV (1993a). Rubredoxin oxidase, a new flavo-hemo-protein, is the site of oxygen reduction to water by the "strict anaerobe" *Desulfovibrio gigas*. *Biochem Biophys Res Commun*, **193**:100-105.

Chen, L, Liu, MY, Legall, J, Fareleira, P, Santos, H & Xavier, AV (1993b). Purification and characterization of an NADH-rubredoxin oxidoreductase involved in the utilization of oxygen by *Desulfovibrio gigas*. *Eur J Biochem*, **216**:443-448.

Chen, L, Keramati, L & Helmann, JD (1995). Coordinate regulation of *Bacillus subtilis* peroxide stress genes by hydrogen peroxide and metal ions. *Proc Natl Acad Sci U S A*, **92**:8190-8194.

Chen, S, Bleam, WF & Hickey, WJ (2009). Simultaneous analysis of bacterioferritin gene expression and intracellular iron status in *Pseudomonas putida* KT2440 by using a rapid dual luciferase reporter assay. *Appl Environ Microbiol*, **75**:866-868.

Crooks, GE, Hon, G, Chandonia, JM & Brenner, SE (2004). WebLogo: a sequence logo generator. *Genome Res*, **14**:1188-1190.

da Costa, PN, Romao, CV, LeGall, J, Xavier, AV, Melo, E, Teixeira, M & Saraiva, LM (2001). The genetic organization of *Desulfovibrio desulphuricans* ATCC 27774 bacterioferritin and rubredoxin-2 genes: involvement of rubredoxin in iron metabolism. *Mol Microbiol*, **41**:217-227.

Dehal, PS, Joachimiak, MP, Price, MN, Bates, JT, Baumohl, JK, Chivian, D, Friedland, GD, Huang, KH, Keller, K, Novichkov, PS, Dubchak, IL, Alm, EJ & Arkin, AP (2009). MicrobesOnline: an integrated portal for comparative and functional genomics. *Nucleic Acids Res*, **38**:D396-400.

Di Matteo, A, Scandurra, FM, Testa, F, Forte, E, Sarti, P, Brunori, M & Giuffre, A (2008). The O_2-scavenging flavodiiron protein in the human parasite *Giardia intestinalis*. *J Biol Chem*, **283**:4061-4068.

Figueiredo, MC, Lobo, SA, Carita, JN, Nobre, LS & Saraiva, LM (2012). Bacterioferritin protects the anaerobe *Desulfovibrio vulgaris* Hildenborough against oxygen. *Anaerobe*, **18**:454-458.

Figueiredo, MC, Lobo, SA, Sousa, SH, Pereira, FP, Wall, JD, Nobre, LS & Saraiva, LM (2013). Hybrid Cluster Proteins and Flavodiiron Proteins Afford Protection to *Desulfovibrio vulgaris* upon Macrophage Infection. *J Bacteriol*, **195**:2684-2690.

Filenko, N, Spiro, S, Browning, DF, Squire, D, Overton, TW, Cole, J & 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**:4410-4417.

Flatley, J, Barrett, J, Pullan, ST, Hughes, MN, Green, J & Poole, RK (2005). Transcriptional responses of *Escherichia coli* to S-nitrosoglutathione under defined chemostat conditions reveal major changes in methionine biosynthesis. *J Biol Chem*, **280**:10065-10072.

Fournier, M, Dermoun, Z, Durand, MC & Dolla, A (2004). A new function of the *Desulfovibrio vulgaris* Hildenborough [Fe] hydrogenase in the protection against oxidative stress. *J Biol Chem*, **279**:1787-1793.

Fournier, M, Aubert, C, Dermoun, Z, Durand, MC, Moinier, D & Dolla, A (2006). Response of the anaerobe *Desulfovibrio vulgaris* Hildenborough to oxidative conditions: proteome and transcript analysis. *Biochimie*, **88**:85-94.

Fukui, M & Takii, S (1990). Survival of sulfate-reducing bacteria in oxic surface sediment of a seawater lake. *FEMS Microbiology Letters*, **73**:317-322.

Gardner, AM, Helmick, RA & Gardner, PR (2002). Flavorubredoxin, an inducible catalyst for nitric oxide reduction and detoxification in *Escherichia coli*. *J Biol Chem*, **277**:8172-8177.

Gauss, GH, Reott, MA, Rocha, ER, Young, MJ, Douglas, T, Smith, CJ & Lawrence, CM (2011). Characterization of the *Bacteroides fragilis bfr* gene product identifies a bacterial DPS-like protein and suggests evolutionary links in the ferritin superfamily. *J Bacteriol*, **194**:15-27.

Gomes, CM, Silva, G, Oliveira, S, LeGall, J, Liu, MY, Xavier, AV, Rodrigues-Pousada, C & Teixeira, M (1997). Studies on the redox centers of the terminal oxidase from *Desulfovibrio gigas* and evidence for its interaction with rubredoxin. *J Biol Chem*, **272**:22502-22508.

Gomes, CM, Vicente, JB, Wasserfallen, A & 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**:16230-16237.

Gomes, CM, Giuffre, A, Forte, E, Vicente, JB, Saraiva, LM, Brunori, M & Teixeira, M (2002). A novel type of nitric-oxide reductase. *Escherichia coli* flavorubredoxin. *J Biol Chem*, **277**:25273-25276.

Haveman, SA, Greene, EA, Stilwell, CP, Voordouw, JK & Voordouw, G (2004). Physiological and gene expression analysis of inhibition of *Desulfovibrio vulgaris* Hildenborough by nitrite. *J Bacteriol*, **186**:7944-7950.

Haveman, SA, Greene, EA & Voordouw, G (2005). Gene expression analysis of the mechanism of inhibition of *Desulfovibrio vulgaris* Hildenborough by nitrate-reducing, sulfide-oxidizing bacteria. *Environ Microbiol*, **7**:1461-1465.

Hayashi, T, Caranto, JD, Wampler, DA, Kurtz, DM & Moenne-Loccoz, P (2010). Insights into the nitric oxide reductase mechanism of flavodiiron proteins from a flavin-free enzyme. *Biochemistry*, **49**:7040-7049.

He, Q, Huang, KH, He, Z, Alm, EJ, Fields, MW, Hazen, TC, Arkin, AP, Wall, JD & Zhou, J (2006). Energetic consequences of nitrite stress in *Desulfovibrio vulgaris* Hildenborough, inferred from global transcriptional analysis. *Appl Environ Microbiol*, **72**:4370-4381.

He, Q, He, Z, Joyner, DC, Joachimiak, M, Price, MN, Yang, ZK, Yen, HC, Hemme, CL, Chen, W, Fields, MM, Stahl, DA, Keasling, JD, Keller, M, Arkin, AP, Hazen, TC, Wall, JD & Zhou, J (2010). Impact of elevated nitrate on sulfate-reducing bacteria: a comparative study of *Desulfovibrio vulgaris*. *Isme J*, **4**:1386-1397.

Heidelberg, JF, Seshadri, R, Haveman, SA, Hemme, CL, Paulsen, IT, Kolonay, JF, Eisen, JA, Ward, N, Methe, B, Brinkac, LM, Daugherty, SC, Deboy, RT, Dodson, RJ, Durkin, AS, Madupu, R, Nelson, WC, Sullivan, SA, Fouts, D, Haft, DH, Selengut, J, Peterson, JD, Davidsen, TM, Zafar, N, Zhou, L, Radune, D, Dimitrov, G, Hance, M, Tran, K, Khouri, H, Gill, J, Utterback, TR, Feldblyum, TV, Wall, JD, Voordouw, G & Fraser, CM (2004). The genome sequence of the anaerobic, sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough. *Nat Biotechnol*, **22**:554-559.

Hutchings, MI, Mandhana, N & 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**:4640-4643.

Johnston, S, Lin, S, Lee, P, Caffrey, SM, Wildschut, J, Voordouw, JK, da Silva, SM, Pereira, IA & Voordouw, G (2009). A genomic island of the sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough promotes survival under stress conditions while decreasing the efficiency of anaerobic growth. *Environ Microbiol*, **11**:981-991.

Justino, MC, Vicente, JB, Teixeira, M & Saraiva, LM (2005). New genes implicated in the protection of anaerobically grown *Escherichia coli* against nitric oxide. *J Biol Chem*, **280**:2636-2643.

Karlinsey, JE, Bang, IS, Becker, LA, Frawley, ER, Porwollik, S, Robbins, HF, Thomas, VC, Urbano, R, McClelland, M & Fang, FC (2012). The NsrR regulon in nitrosative stress resistance of *Salmonella enterica* serovar Typhimurium. *Mol Microbiol*, **85**:1179-1193.

Lobo, SA, Melo, AM, Carita, JN, Teixeira, M & Saraiva, LM (2007). The anaerobe *Desulfovibrio desulfuricans* ATCC 27774 grows at nearly atmospheric oxygen levels. *FEBS Lett*, **581**:433-436.

Mukhopadhyay, A, Redding, AM, Joachimiak, MP, Arkin, AP, Borglin, SE, Dehal, PS, Chakraborty, R, Geller, JT, Hazen, TC, He, Q, Joyner, DC, Martin, VJ, Wall, JD, Yang, ZK, Zhou, J & Keasling, JD (2007). Cell-wide responses to low-oxygen exposure in *Desulfovibrio vulgaris* Hildenborough. *J Bacteriol*, **189**:5996-6010.

Mukhopadhyay, P, Zheng, M, Bedzyk, LA, LaRossa, RA & 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**:745-750.

Nathan, C & Shiloh, MU (2000). Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc Natl Acad Sci U S A*, **97**:8841-8848.

Novichkov, PS, Laikova, ON, Novichkova, ES, Gelfand, MS, Arkin, AP, Dubchak, I & Rodionov, DA (2010). RegPrecise: a database of curated genomic inferences of transcriptional regulatory interactions in prokaryotes. *Nucleic Acids Res*, **38**:D111-118.

Overeijnder, ML, Hagen, WR & Hagedoorn, PL (2009). A thermostable hybrid cluster protein from *Pyrococcus furiosus*: effects of the loss of a three helix bundle subdomain. *J Biol Inorg Chem*, **14**:703-710.

Pereira, AS, Timoteo, CG, Guilherme, M, Folgosa, F, Naik, SG, Duarte, AG, Huynh, BH & Tavares, P (2012). Spectroscopic evidence for and characterization of a trinuclear ferroxidase center in bacterial ferritin from *Desulfovibrio vulgaris* Hildenborough. *J Am Chem Soc*, **134**:10822-10832.

Pereira, PM, He, Q, Xavier, AV, Zhou, J, Pereira, IA & Louro, RO (2008). Transcriptional response of *Desulfovibrio vulgaris* Hildenborough to oxidative stress mimicking environmental conditions. *Arch Microbiol*, **189**:451-461.

Rodrigues, R, Vicente, JB, Felix, R, Oliveira, S, Teixeira, M & Rodrigues-Pousada, C (2006). *Desulfovibrio gigas* flavodiiron protein affords protection against nitrosative stress in vivo. *J Bacteriol*, **188**:2745-2751.

Saraiva, LM, Vicente, JB & Teixeira, M (2004). The role of the flavodiiron proteins in microbial nitric oxide detoxification. *Adv Microb Physiol*, **49**:77-129.

Schneider, TD & Stephens, RM (1990). Sequence logos: a new way to display consensus sequences. *Nucleic Acids Res*, **18**:6097-6100.

Seedorf, H, Dreisbach, A, Hedderich, R, Shima, S & Thauer, RK (2004). $F_{420}H_2$ oxidase (FprA) from *Methanobrevibacter arboriphilus*, a coenzyme F_{420}-dependent enzyme involved in O_2 detoxification. *Arch Microbiol*, **182**:126-137.

Seedorf, H, Hagemeier, CH, Shima, S, Thauer, RK, Warkentin, E & Ermler, U (2007). Structure of coenzyme $F_{420}H_2$ oxidase (FprA), a di-iron flavoprotein from methanogenic Archaea catalyzing the reduction of O_2 to H_2O. *Febs J*, **274**:1588-1599.

Seth, D, Hausladen, A, Wang, YJ & Stamler, JS (2012). Endogenous protein S-Nitrosylation in *E. coli*: regulation by OxyR. *Science*, **336**:470-473.

Silaghi-Dumitrescu, R, Coulter, ED, Das, A, Ljungdahl, LG, Jameson, GN, Huynh, BH & Kurtz, DM, Jr. (2003). A flavodiiron protein and high molecular weight rubredoxin from *Moorella thermoacetica* with nitric oxide reductase activity. *Biochemistry*, **42**:2806-2815.

Silaghi-Dumitrescu, R, Ng, KY, Viswanathan, R & Kurtz, DM, Jr. (2005). A flavo-diiron protein from *Desulfovibrio vulgaris* with oxidase and nitric oxide reductase activities. Evidence for an in vivo nitric oxide scavenging function. *Biochemistry*, **44**:3572-3579.

Smutná, T, Gonçalves, VL, Saraiva, LM, Tachezy, J, Teixeira, M & Hrdy, I (2009). Flavodiiron protein from *Trichomonas vaginalis* hydrogenosomes: the terminal oxygen reductase. *Eukaryot Cell*, **8**:47-55.

Teske, A, Ramsing, NB, Habicht, K, Fukui, M, Kuver, J, Jorgensen, BB & Cohen, Y (1998). Sulfate-reducing bacteria and their activities in cyanobacterial mats of solar lake (Sinai, Egypt). *Appl Environ Microbiol*, **64**:2943-2951.

Timóteo, CG, Guilherme, M, Penas, D, Folgosa, F, Tavares, P & Pereira, AS (2012). *Desulfovibrio vulgaris* bacterioferritin uses H(2)O(2) as a co-substrate for iron oxidation and reveals DPS-like DNA protection and binding activities. *Biochem J*, **446**:125-133.

Velayudhan, J, Castor, M, Richardson, A, Main-Hester, KL & Fang, FC (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**:1495-1507.

Vicente, JB, Gomes, CM, Wasserfallen, A & Teixeira, M (2002). Module fusion in an A-type flavoprotein from the cyanobacterium *Synechocystis* condenses a multiple-component pathway in a single polypeptide chain. *Biochem Biophys Res Commun*, **294**:82-87.

Vicente, JB, Carrondo, MA, Teixeira, M & Frazão, C (2008). Flavodiiron Proteins: Nitric Oxide and/or Oxygen Reductases. In *Handbook of Metalloproteins*. Edited by A. Messerschmidt. Chichester, UK: John Wiley & Sons, Ltd.

Vicente, JB, Tran, V, Pinto, L, Teixeira, M & Singh, U (2012). A detoxifying oxygen reductase in the anaerobic protozoan *Entamoeba histolytica*. *Eukaryot Cell*, **11**:1112-1118.

Waidner, B, Greiner, S, Odenbreit, S, Kavermann, H, Velayudhan, J, Stahler, F, Guhl, J, Bisse, E, van Vliet, AH, Andrews, SC, Kusters, JG, Kelly, DJ, Haas, R, Kist, M & Bereswill, S (2002). Essential role of ferritin Pfr in *Helicobacter pylori* iron metabolism and gastric colonization. *Infect Immun*, **70**:3923-3929.

Wildschut, JD, Lang, RM, Voordouw, JK & Voordouw, G (2006). Rubredoxin:oxygen oxidoreductase enhances survival of *Desulfovibrio vulgaris* Hildenborough under microaerophilic conditions. *J Bacteriol*, **188**:6253-6260.

Wolfe, MT, Heo, J, Garavelli, JS & Ludden, PW (2002). Hydroxylamine reductase activity of the hybrid cluster protein from *Escherichia coli*. *J Bacteriol*, **184**:5898-5902.

Yurkiw, MA, Voordouw, J & Voordouw, G (2012). Contribution of rubredoxin:oxygen oxidoreductases and hybrid cluster proteins of *Desulfovibrio vulgaris* Hildenborough to survival under oxygen and nitrite stress. *Environ Microbiol*, **14**:2711-2725.

Zhang, W, Culley, DE, Hogan, M, Vitiritti, L & Brockman, FJ (2006). Oxidative stress and heat-shock responses in *Desulfovibrio vulgaris* by genome-wide transcriptomic analysis. *Antonie Van Leeuwenhoek*, **90**:41-55.