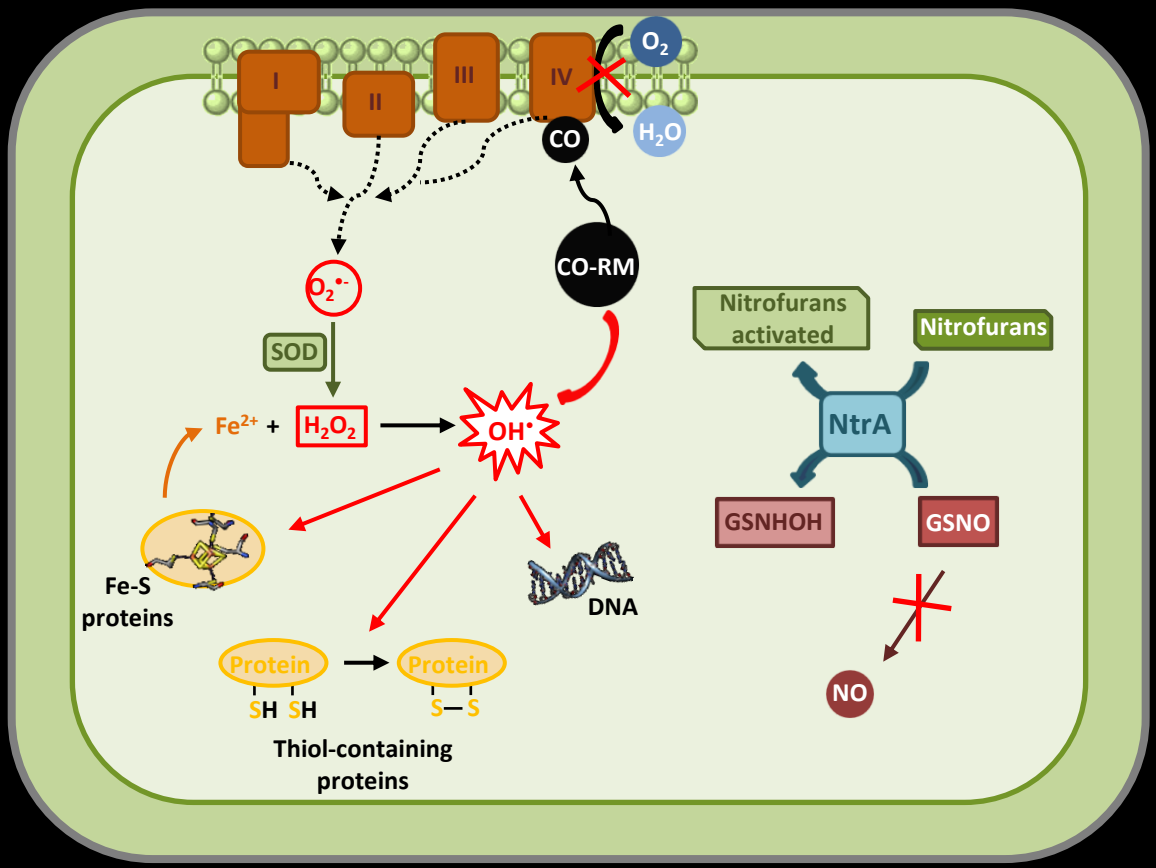
Novel insights into the action of antimicrobial agents against human pathogens

Ana Filipa Nogueira Tavares



Dissertation presented to obtain the Ph.D degree in Biochemistry

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
April, 2013

INSTITUTO DE TECNOLOGIA QUÍMICA E BIOLÓGICA /UNL

Knowledge Creation

Novel insights into the action of antimicrobial agents against human pathogens

Ana Filipa Nogueira Tavares

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

Oeiras, April 2013

FCT Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA EDUCAÇÃO E CIÊNCIA

INSTITUTO DE TECNOLOGIA QUÍMICA E BIOLÓGICA /UNL
Knowledge Creation

From left to the right: Maria de Fátima Lopes, Mónica Oleastro, Lígia Saraiva, Ivo Boneca, Mário Ramirez, Ana Filipa Tavares and Carlos Romão

2nd April 2013

Second Edition, April 2013

Molecular Genetics of Microbial Resistance Laboratory

Instituto de Tecnologia Química e Biológica

Universidade Nova de Lisboa

Av. da República (EAN)

Acknowledgements

I would like to formally express my sincere gratitude to the people that contributed and supported my work:

To Dr. **Lígia Saraiva** for receiving me in her laboratory and accepting to be my supervisor. Thank you for your constant support, encouragement and endless capacity to always see the bright side, even in hard and more challenging moments. For the trust and confidence in me, for your friendship and 24 h availability whenever I needed to ask a question or communicate the result of an experiment! Thank you for your great advices and for the careful revision of this thesis. A big thank you for everything!!!

To Prof. **Miguel Teixeira**, for the collaboration on the work presented in Chapter 4, especially for performing the EPR experiments, and for the very interesting and helpful discussions.

To Prof. **Carlos Romão**, for the collaboration on the work presented in Chapter 4, for the enthusiastic discussions and all the lessons about CO-Releasing Molecules chemistry.

To Dr. **Mónica Oleastro,** from Instituto Dr. Ricardo Jorge, for the collaboration on the work presented in Chapter 5, for receiving me in her laboratory and for the helpful advices. I would like also to thank **João Benoliel** for his help during my stay at this institute.

To Dr. **Mariana Pinho** and Dr. **Ilda Sanches** for being part of my scientific thesis commission, for their suggestions and helpful advices.

To Dr. **Colin McVey**, from Structural Genomics lab at ITQB for reviewing the abstract of this thesis, accordingly with ITQB intern rules.

To all past and present colleagues of MGMR lab´s for making it such an enjoyable and pleasant place to work. A very special thank you to **Lígia Nobre**, for all the help during the course of this work, for always have time to listen my new ideas or results, for the valuable discussions and for being a good friend. To **Marta Justino**, for the great discussions, all the help in statistical analyses and for always willing to give me new ideas and suggestions. To **Margarida Parente**, for the help provided in the last part of the experimental work. To **Susana Lobo,** for her support, friendship and also for the help with the cover of this thesis! To **Mafalda Figueiredo**, for her companionship and for being a great friend. To **Sara Sousa**, for her friendship and good moments. To **Joana Baptista**, for being a special friend, for all the funny moments inside and outside the lab, for being my "shoulder friend" when I need to laugh or cry!
To former members of the group, especially to **Cláudia Almeida**, for her help in my early days at the lab, and to **Vera Gonçalves**, for the good talks and laughs.
Thank you all for so many moments of happiness and so many experiences shared in the last years.

To everyone in the third floor of ITQB, past and present people, for contributing to the helpful and friendly environment.

To Alfama members for theirs pleasantness, good talks and for providing CO-Releasing Molecules. In special to **João Seixas**, for the collaboration on the work presented in Chapter 4.

To all my colleagues in other labs at the institute, especially to those from the 2008 class of the **ITQB PhD course** and from the **InTeraQB**. Thank you for providing me good moments, for the experiences changed and friendly words.

To my colleagues from University that became very especial friends: **Margarida Augusto, Joana Medeiros** and **Simão Luz**, for all the fun and friendship. To **Inês Lima**, for her comprehension and support, a true and special friendship full of precious and unforgotten moments throughout these 10 years!

Aos meus amigos de sempre e a todos os outros, muito obrigada por todo o apoio e amizade.

À minha família pelo vosso interminável e incondicional apoio e carinho e a quem devo aquilo que sou.
Aos **meus pais**, obrigada por acreditarem em mim e me darem força para ser sempre melhor e para tentar ir sempre mais longe.
À minha irmã **Xana** pela amizade, pelo apoio e por estares sempre presente em todos os momentos da minha vida!
E aos meus **avós**, por me acompanharem e ajudarem durante toda a vida.
OBRIGADA a todos por serem o meu suporte para tudo na vida.

Ao **Pedro**, por me teres sempre apoiado, pela paciência e compreensão inesgotável. Obrigada por estares sempre ao meu lado em todos os momentos e por me fazeres feliz!

Fundação para Ciência e Tecnologia for the financial support crucial for the accomplishment of this work and by awarding a PhD grant (SFRH/BD/38457/2007).

This thesis is dedicated to my family

Thesis Outline

This thesis comprises the research work 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.

The thesis is divided in three main parts: Part I consists of a general introduction organized in two chapters, one focusing on bacteria and their resistance to antibiotics and to the innate immune system, and a second one concerning an overview on the general aspects of carbon monoxide and its relation with biological systems and bacteria. Part II comprises the experimental results obtained during this work, which is divided in three chapters based on two original publications and one manuscript in preparation. Part III presents a general discussion of all the work performed in this thesis.

Thesis Publications

The work presented in this thesis is based on following original publications, listed by chronological order:

Tavares AFN, Nobre LS, Melo AM & Saraiva LM (2009) A Novel Nitroreductase of *Staphylococcus aureus* with S-Nitrosoglutathione Reductase Activity. *Journal of Bacteriology* **191**: 3403-3406.

Tavares AFN, Teixeira M, Romão CC, Seixas JD, Nobre LS & Saraiva LM (2011) Reactive oxygen species mediate bactericidal killing elicited by Carbon Monoxide-Releasing Molecules. *Journal of Biological Chemistry* **286**: 26708-26717.

Tavares AFN, Nobre LS & Saraiva LM (2012) A role for reactive oxygen species in the antibacterial properties of Carbon Monoxide-Releasing Molecules. *FEMS Microbiology Letters*, **336**:1-10.

Manuscript in preparation, based on results presented in Chapter 5:

Tavares AFN, Parente M, Justino MC**,** Oleastro M, Nobre LS & Saraiva LM (2013) *Fighting Helicobacter pylori* with Carbon Monoxide-Releasing Molecules.

Publications not included in this thesis:

Nobre LS, Todorovic S, **Tavares AFN**, Oldfield E, Hildebrandt P, Teixeira M & Saraiva LM (2010) Binding of azole antibiotics to *Staphylococcus aureus* flavohemoglobin increases intracellular oxidative stress. *Journal of Bacteriology* **192**: 1527-1533.

Abstract

Nowadays, the growing increase of antibiotic resistance represents a global public health concern. Hence, it is crucial to understand the mode of action of antimicrobial agents and to develop new strategies to control bacterial infections. The work presented in this thesis contributes with new insights into the mechanisms that underpin the action of antimicrobial agents against pathogenic bacteria through: (i) the study of a putative nitroreductase of *Staphylococcus aureus* and its involvement in nitrofurans activation; (ii) elucidation of the mechanisms that sustain the antibacterial activity of the recently discovered Carbon Monoxide-Releasing Molecules (CO-RMs); and (iii) evaluation of the bactericidal effect of CO-RMs on *Helicobacter pylori.*

The bactericidal effect of nitrofuran antibiotics is dependent on the reduction of their nitro group by bacterial nitroreductases. Sequence analysis of the *S. aureus* genome allowed identification of a novel putative nitroreductase, SA0UHSC_00833, herein named NtrA. To analyse the NtrA contribution to nitrofurans activation, a mutant strain lacking this gene was constructed and its resistance to nitrofurans was evaluated. Furthermore, the biochemical characterization of NtrA was performed. To achieve this, the *ntrA* gene was cloned, and the recombinant protein expressed, purified and analysed. The results revealed that: (i) the *S. aureus* strain lacking the *ntrA* nitroreductase gene is more resistant to nitrofurans; (ii) the increased resistance is related with a lower nitroreductase activity observed in mutant cell extracts; and (iii) the *S. aureus* nitroreductase NtrA is able to reduce nitrofurans, with specific activities of 20 and 15 $\mu mol.min^{-1}.mg\ protein^{-1}$ for nitrofurazone and nitrofurantoin, respectively. These values are within the range of activities usually observed for the canonical nitroreductases.

S. aureus nitroreductase NtrA was also found to be involved in the metabolism of S-Nitrosoglutathione (GSNO), a biological nitric oxide (NO) donor. Several lines of evidence supported this conclusion, namely: (i) the high induction of the *ntrA* gene transcription observed in cells of *S. aureus* exposed to GSNO; (ii) the higher susceptibility to GSNO killing of the *S. aureus* strain deleted in *ntrA* gene; and (iii) the ability of the recombinant NtrA to detoxify GSNO with a significant activity of 1.5 $\mu mol.min^{-1}.mg\ protein^{-1}$.

Altogether, *S. aureus* NtrA showed to be a bifunctional enzyme that besides promoting nitrofurans activation also protects bacteria from the GSNO deleterious effects. Importantly, a phylogenetic analysis based on the alignment of several nitroreductase amino acid sequences revealed that NtrA is member of a novel family of bacterial nitroreductases.

The second part of the work was devoted to the study of CO-RMs, which were previously reported to efficiently eliminate pathogens such as *Escherichia coli*, *S. aureus* and *Pseudomonas aeruginosa.* Until now, the antimicrobial effect of CO-RMs has been attributed only to the inhibition of the aerobic respiratory chain due to the expected binding of CO to terminal oxidases. However, CO-RMs were reported to be more potent bactericides than the CO gas, implying that other mechanisms support the antibacterial activity of these drugs. Previous DNA microarray experiments showed that CO-RMs alter the transcription of several genes involved in oxidative stress response, suggesting that this effect also played a role. The present work demonstrated that *E. coli* cells exposed to CO-RMs contain higher levels of intracellular reactive oxygen species (ROS). Furthermore, it was seen that: (i) supplementation of cell medium with the antioxidants glutathione and cysteine abolished the antimicrobial effect of CO-RMs; (ii) strains deleted in genes encoding ROS detoxifying systems, namely catalase and superoxide

dismutase, have increased susceptibility to CORM-2; (iii) CORM-2 induces DNA damage, and a strain deleted in *recA*, a gene product involved in DNA repair, exhibited higher susceptibility to these compounds; (iv) upon CORM-2 treatment, the intracellular levels of free iron increased by four times, with the iron-sulphur centres being the most probable source of iron; and (v) CORM-2 promoted oxidation of free thiols groups. Moreover, CO-RMs were shown to generate hydroxyl radicals *per se* in aqueous solution, which was abolished upon scavenging of the CO molecule. In conclusion, this work revealed that CO-RMs mediate bacterial cell death through ROS formation.

In the last part of this thesis, the bactericidal action of CO-RMs toward *H. pylori* was demonstrated. Indeed, CO-RMs were shown to cause a significant decrease in *H. pylori* viability. Moreover, this effect was not only observed for a laboratory strain but also for several clinical isolates. Interestingly, one of the mechanisms of CO-RMs bactericidal action on *H. pylori* was found to occur through inhibition of urease, an enzyme that is essential for survival and pathogenesis of this bacterium.

Additionally, CORM-2 was proven to enhance the *H. pylori* susceptibility to the antibiotics commonly used to eradicate the pathogen, namely, metronidazole, clarithromycin and amoxicillin. Actually, the minimal inhibitory and bactericidal concentration of these antibiotics to *H. pylori* strains significantly decreased in the presence of sub-lethal doses of CORM-2. Importantly, the viability of CORM-2-treated *H. pylori* in mammalian cells, such as macrophages, was found to be lower.

Overall, the work presented in this thesis has contributed to a better understanding of the molecular basis of action of nitrofurans and CO-RMs. In particular, the role of the *S. aureus* bifunctional nitroreductase NtrA in nitrofurans action and detoxification of GSNO was disclosed. In relation to CO-

RMs, ROS formation was demonstrated to be an important factor of their antibacterial properties. Finally, CO-RMs were shown, for the first time, to be able to eliminate *H. pylori,* which may represent a novel strategy to control these important human bacterial infections.

Resumo

Actualmente, o aumento de resistência por parte das bactérias aos antibióticos representa um problema de saúde pública global. Por isso, é fundamental entender o modo de acção dos agentes antimicrobianos e desenvolver novas estratégias para controlar as infecções bacterianas. O trabalho apresentado nesta tese contribuiu com novos dados sobre os mecanismos que sustentam a acção de agentes antimicrobianos contra bactérias patogénicas através de: (i) o estudo de uma nitroredutase hipotética de *Staphylococcus aureus* e do seu envolvimento na activação dos antibióticos nitrofuranos; (ii) a elucidação dos mecanismos que estão na base da actividade antibacteriana das recém-descobertas moléculas libertadoras de monóxido de carbono (CO-RMs); e (iii) a avaliação do efeito bactericida dos CO-RMs em *Helicobacter pylori*.

O efeito bactericida dos nitrofuranos é dependente da redução do seu grupo nitro por nitroredutases bacterianas. Ao analisar o genoma de *S. aureus*, identificou-se uma hipotética nitroredutase, SA0UHSC_00833, aqui designada NtrA. Para analisar a contribuição da NtrA para a activação dos nitrofuranos, construiu-se uma estirpe deletada neste gene e foi avaliada a sua resistência a estes antibióticos. Além disso, realizou-se também a caracterização bioquímica da NtrA. Para este fim, o gene *ntrA* foi clonado e a proteína recombinante expressa, purificada e analisada. Os resultados revelaram que: (i) a estirpe de *S. aureus*, cujo gene *ntrA* foi interrompido é mais resistente aos nitrofuranos; (ii) o aumento da resistência está relacionado com a menor actividade de nitroredutase observada em extractos de células mutadas; e (iii) a nitroredutase NtrA de *S. aureus* é capaz de reduzir nitrofuranos, com actividades específicas de 20 e 15

µmol.min^{-1}.mg proteina^{-1}, para a nitrofurazona e nitrofurantoina, respectivamente. Estes valores estão dentro da gama de actividades normalmente observados para as nitroredutases canónicas.

Foi ainda demonstrado que a nitroredutase NtrA de *S. aureus* está envolvida no metabolismo da S-nitrosoglutationa (GSNO), um dador biológico de óxido nítrico (NO). Várias evidências apoiam esta conclusão, nomeadamente: (i) a indução da transcrição do gene *ntrA* observada em células de *S. aureus* expostas ao GSNO; (ii) a maior susceptibilidade da estirpe mutada no gene *ntrA* ao tratamento com GSNO; e (iii) a capacidade da NtrA recombinante para destoxificar GSNO com uma actividade de 1.5 µmol.min^{-1}.mg proteina^{-1}.

Em conclusão, a nitroredutase NtrA de *S. aureus* mostrou ser uma enzima bifuncional que para além de promover a activação dos nitrofuranos também protege as bactérias contra os efeitos nocivos do GSNO. É importante notar que a análise filogenética feita com base no alinhamento de várias sequências de amino ácidos de nitroredutases revelou que a NtrA é membro de uma nova família de nitroredutases bacterianas.

A segunda parte do trabalho foi dedicada ao estudo dos CO-RMs, que foram anteriormente descritos como eficazes a eliminar agentes patogénicos, tais como *Escherichia coli*, *S. aureus* e *Pseudomonas aeruginosa.* Até agora, o efeito antimicrobiano dos CO-RMs foi atribuído apenas à inibição da cadeia respiratória aeróbica devido à afinidade do CO para as oxidases terminais. No entanto, os CO-RMs são bactericidas mais potentes do que o próprio CO gás, sugerindo que outros mecanismos são responsáveis pela actividade antibacteriana destes compostos. Experiências anteriores de DNA *microarrays* mostraram que os CO-RMs alteram a transcrição de vários genes envolvidos na resposta ao stress oxidativo,

sugerindo que este stress possa também ter um papel na acção destes compostos. O presente trabalho demonstrou que as células de *E. coli* expostas aos CO-RMs contêm níveis mais elevados de espécies reactivas de oxigénio (ERO) intracelulares. Além disso, mostrou-se que: (i) a suplementação do meio de cultura com os antioxidantes glutationa e cisteína aboliu o efeito bactericida dos CO-RMs; (ii) as estirpes mutadas nos genes que codificam sistemas de destoxificação de ERO, designadamente a catalase e a superóxido dismutase, têm maior susceptibilidade ao CORM-2; (iii) o CORM-2 induz danos no DNA, e uma estirpe mutada no gene *recA*, cujo produto está envolvido na reparação do DNA, exibiu maior susceptibilidade a estes compostos; (iv) após tratamento com CORM-2 os níveis intracelulares de ferro livre aumentaram quatro vezes, sendo os centros de ferro-enxofre a fonte mais provável de ferro; e (v) o CORM-2 promoveu a oxidação de grupos tióis livres. Além disso, em solução aquosa os CO-RMs provaram ser capazes de gerar *per se* o radical hidroxilo, o que não se observou quando o grupo CO não está presente na molécula. Este trabalho permitiu então concluir que os CO-RMs medeiam a morte celular bacteriana através da formação de ERO.

Na última parte desta tese, a acção bactericida do CO-RMs em *H. pylori* foi demonstrada. De facto, os CO-RMs causam uma redução significativa na viabilidade de *H. pylori*. Este efeito não foi somente observado para uma estirpe de laboratório mas também para os vários isolados clínicos que foram estudados. Foi interessante observar que um dos mecanismos da acção bactericida dos CO-RMs em *H. pylori* ocorre através da inibição da urease, uma enzima que é essencial para a sobrevivência e patogenicidade desta bactéria.

Adicionalmente, o CORM-2 aumentou a susceptibilidade de *H. pylori* aos antibióticos habitualmente utilizados para a sua erradicação, como o metronidazole, a claritromicina e a amoxicilina. De facto, a concentração

mínima inibitória e bactericida destes antibióticos para as estirpes de *H. pylori* diminui significativamente na presença de doses sub-letais de CORM-2. É importante notar que em células de mamíferos, tais como macrófagos, foi menor a viabilidade de *H. pylori* quando previamente tratada com CORM-2.

Em suma, o trabalho apresentado nesta tese contribuiu para uma melhor compreensão da base molecular de acção dos nitrofuranos e dos CO-RMs. Em particular, o papel da nitroredutase bifuncional NtrA de *S. aureus* na acção dos nitrofuranos e na destoxificação de GSNO foi desvendado. Em relação ao CO-RMs, a formação de ERO revelou ser um factor importante nas suas propriedades antibacterianas. Finalmente, foi mostrado, pela primeira vez, que os CO-RMs são capazes de eliminar *H. pylori*, o que pode vir a representar uma nova estratégia para controlar estas importantes infecções bacterianas em humanos.

Abbreviations

Δ	Deletion
ALF062	Pentacarbonyl bromide
ALF021	Bromo(pentacarbonyl)manganese
ALF492	Tricarbonyldichloro(thiogalactopyranoside) ruthenium(II)
AMX	Amoxicillin
BCA	Bicinchoninic acid method
BHI	Brain heart infusion
BMPO	5-tert-butoxycarbonyl 5-mwthyl-1-pyrroline N-oxide
ßCD	ß-cyclodextrin
CFU	Colony forming units
CH	Clarithromycin
CO	Carbon monoxide
CO_2	Carbon dioxide
$CO_3^{\bullet-}$	Carbonate
CoA	Coenzyme
CODH	Carbon Monoxide dehydrogenase
COHb	Carboxy-haemoglobin
CO-RM	Carbon Monoxide-Releasing Molecules
CORM-1	Dimanganese decacarbonyl
CORM-2	Tricarbonyldichloro ruthenium (II) dimer
CORM-3	Tricarbonyldichloro(glycinato) ruthenium (II)
CORM-A1	Sodium boranocarbonate
DCFH-DA	2´,7´-dichlorofluorescein diacetate
DMEM	Dulbecco´s Modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNIC	Dinitrosyl-iron-dithiol complexes
DTNB	5,5´Dithiobis-(2-nitrobenzoic acid)
e^-	Electron
eNOS	Endothelial nitric oxide synthase

EPR	Electron paramagnetic resonance
FAD	Flavin adenine dinucleotide
FALDH	Glutathione-dependent formaldehyde dehydrogenase
FCS	Fetal calf serum
FDP	Flavodiiron protein
Fe	Iron
Fe-S	Iron-sulphur cluster
FI	Fluorescence intensity
FMN	Flavin mononucleotide
g	EPR g-factor
GDH	Glutamate dehydrogenase
GOGAT	Glutamate synthase
GSH	Glutathione
GSNO	S-Nitrosoglutathione
GSNOR	GSNO reductase
H^+	Proton
Hb	Haemoglobin
HBA	Horse blood-agar
Hcp	Hybrid-cluster protein
Hmp	Flavohaemoglobin
HO	Haem oxygenase
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
HUS	Haemolytic uremic syndrome
I	Identity
IC_{50}	Half-maximal inhibitory concentration
iCO-RM	Inactive form of CO-RM (depletes of CO groups)
iCORM-2	Tetrakis(dimethylsulfoxide dichlororuthenium(II)
iNOS	Inducible nitric oxide synthase
IL-1	Interleukin 10
IL-6	Interleukin 6
INF-γ	Interferon-γ
IPTG	Isopropyl-ß-D-thiogalactopyranoside

Kat	Catalase
K_{Ca}	Large conductance calcium-activated potassium
k_{cat}	Catalytic constant
K_M	Michaelis-Menten constant
Da	Dalton
LB	Luria-Bertani
LPS	Lipopolysaccharides
M	Molar
MALT	Mucosa-associated lymphoid-tissue
MAPK	Mitogen-activated protein kinase
MBC	Minimal bactericidal concentration
MIC	Minimal inhibitory concentration
MCC	Metal carbonyl complexes
Mo	Molybdenum
MOI	Multiplicity of infection
MPO	Myeloperoxidase
MRSA	Methicillin-resisitant *S. aureus*
MS	Minimal salts
MTZ	Metronidazole
NAC	N-acetyl cysteine
NADH	ß-nicotinamide adenine dinucleotide, reduced form
NADP	ß-nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH	ß-nicotinamide adenine dinucleotide phosphate, reduced form
ND	Not determined
NH_3	Ammonia
NH_4^+	Ammonium ion
nNOS	Neural nitric oxide synthase
NO	Nitric oxide
NO_2^-	Nitrite
$^{\bullet}NO_2$	Nitrogen dioxide
N_2O_3	Nitrogen trioxide
NorV or FlRd	Flavorubredoxin
NOS	Nitric oxide synthase

Nrf	Pentahaem nitrite reductases
ns	non significant
NTR	Nitroreductase
O_2	Dyoxygen
$O_2^{\bullet-}$	Superoxide anion
$^{\bullet}OH$	Hydroxyl radical
$OHOO^-$	Peroxynitrite
OD	Optical density
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffer
PCR	Polymerase chain reaction
Phox	NADPH oxidase
PPI	Proton pump inhibitor
ppm	Parts per million
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase-polimerase chain reaction
Ru	Ruthenium
S	Similarity
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Standard error
sGC	Soluble guanylate cyclase
SMX	Sulfamethoxazole
SNAP	*S*-nitroso *N*-acetyl DL-penicillamine
Sod	Superoxide dismutase
SSTI	Skin and soft-tissue infection
$t_{1/2}$	Half-life
TMP	Trimethoprim
TNB	5-thio-2-nitrobenzoic acid
TNF-α	Tumor necrosis factor-α
TSA	TSB-agar
TSB	Tryptic soy broth

UTI	Urinary tract infection
UV	Ultraviolet
V_{max}	Maximum velocity
VISA	Vancomycin intermediate-resistant *S. aureus*
VRSA	Vancomycin resistant *S. aureus*
WHO	World Health Organization
wt	Wild type

Latin abbreviations

i.e.	*id est*, that is to say
e.g.	*exempli gratia*, for example
et al.	*et alia*, and other people
c.	*circa*, around

Strains

B. anthracis	*Bacillus anthracis*
B. subtilis	*Bacillus subtilis*
C. diphtheria	*Corynebacterium diphtheria*
C. jejuni	*Campylobacter jejuni*
C. tetani	*Clostridium tetani*
E. coli	*Escherichia coli*
H. pylori	*Helicobacter pylori*
M. tuberculosis	*Mycobacterium tuberculosis*
N. meningitidis	*Neisseria meningitidis*
P. aeruginosa	*Pseudomonas aeruginosa*
R. rubrum	*Rhodospirillum rubrum*
S. aureus	*Staphylococcus aureus*
S. typhimurium	*Salmonella typhimurium*

Aminoacids

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

Introduction

Results

Discussion

Introduction

Chapter 1|

Clinical relevance of bacteria and resistance to the innate immune system

“The microbes always have the last word” - Louis Pasteur

1.1 Pathogenic bacteria

The human body is exposed to millions of microorganisms, being estimated that it contains 10 times more bacterial than human cells (Shanahan, 2002; Tlaskalova-Hogenova *et al.*, 2004). The majority of bacteria are not harmful and some of them have developed beneficial and essential relationships with the host, constituting the normal microflora (Hooper & Gordon, 2001). However, some bacteria are able to cause damage or illness to the host, being considered as pathogens. The ability to cause disease results from the outcome of host-pathogen interactions, which will depend on the damage that microorganism will inflict to the host, and the defense mechanisms of both host and bacteria (Casadevall & Pirofski, 2000; Medzhitov *et al.*, 2012). When the host is not able to counteract a situation of bacterial infection, the use of antibiotics is required. However, the development and spread of antibiotic-resistant bacteria represents one of the most significant global health challenges for this century (Bush *et al.*, 2011).

1.1.1 *Escherichia coli*

The Gram-negative bacterium *E. coli* was first identified from feces of neonate and breast-fed infant by Theodor Escherich in 1885 (Escherich, 1989). This rod-shaped bacterium is by far the most well studied model organism and one of the first to have its genome completely sequenced, with approximately 4.6 million base pairs of DNA (Blattner *et al.*, 1997). Most *E. coli* strains are commonly regarded as non-pathogenic and along with other facultative anaerobe species have been identified in the normal microflora of the anaerobic environment of the colon (Eckburg *et al.*, 2005; Tenaillon *et al.*, 2010). In fact, *E. coli* cells typical colonize the gastrointestinal tract of human infants within a few hours after birth, and bacteria can coexist in the host with

mutual benefits for decades. *E. coli* strains may benefit from the dietary fibers degraded by anaerobes and on other hand, limit oxygen content of the intestines and produce vitamin K (Ramotar *et al.*, 1984; Kaper *et al.*, 2004; Jones *et al.*, 2007; Tenaillon *et al.*, 2010). Nevertheless, some *E. coli* strains have virulence factors, that can cause a broad spectrum of diseases that fall into two categories: intestinal pathologies, such as diarrhea, and extraintestinal pathologies including urinary tract infections (UTIs), haemolytic uremic syndrome (HUS), sepsis and meningitis (Finlay & Falkow, 1997; Russo & Johnson, 2003; Kaper *et al.*, 2004; Belanger *et al.*, 2011). It is estimated that pathogenic strains of *E. coli* are responsible for more than 2 million human deaths worldwide per year, particularly due to diarrhea among young children and septicemia, a bloodstream infection derived from urinary tract infections (Kosek *et al.*, 2003; Russo & Johnson, 2003). The most notorious pathogenic strain is the enterohaemorrhagic *E. coli* 0157:H7, which is a potentially fatal food-borne pathogen that causes haemorrhagic diarrhea and HUS, that may lead to acute renal failure (Karmali *et al.*, 1983; Riley *et al.*, 1983). According to the World Health Organization (WHO), up to 10% of patients with enterohaemorrhagic *E. coli* infections may develop HUS, with a fatality rate ranging from 3 to 5%. In 2011, an outbreak of haemorrhagic gastroenteritis and HUS caused by *E. coli* 0104:H4 occurred in Germany (Askar *et al.*, 2011). Data collected by WHO show that from May to end of June 2011, 896 patients (including 33 deaths) developed HUS and 3241 patients presented enterohaemorrhagic gastroenteritis worldwide (including 17 deaths) (Askar *et al.*, 2011; Wu *et al.*, 2011).

Urinary tract infections are among the most common humans bacterial infectious diseases, being in its majority (70-95%) caused by uropathogenic *E. coli* (Foxman, 2002; Russo & Johnson, 2003; Guay, 2008). For several decades,

the combination of trimethoprim and sulfamethoxazole antibiotics (TMP/SMX), which inhibits sequential steps in bacterial tetrahydrofolic acid synthesis, has been used as first-line drug of choice to treat UTIs (Warren *et al.*, 1999; Nicolle, 2003; Vouloumanou *et al.*, 2011). However, there is a concern about the emergence of *E. coli* resistance to TMP/SMX. In fact, increasing values of antibiotic resistance over 20% have been reported in several European countries and in United States of America (Huovinen *et al.*, 1995; Huovinen, 2001; Mazzei *et al.*, 2006). Hence, fluoroquinolone, an antibiotic that interferes with DNA replication, has been used as an alternative antimicrobial agent (Hooper, 1999; Guay, 2008). However, in some countries resistance to fluoroquinolones exceeds now 10-25%, and fosfomycin, an inhibitor of cell wall synthesis, and nitrofurantoin (see section 1.2) are used as alternative treatments (Guay, 2001, , 2008; Michalopoulos *et al.*, 2011). Some β-lactams drugs such as aminopenicillins and cephalosporins are also active against *E. coli* by blocking cell wall synthesis (Nickel, 2005). However, these antibacterial agents are not usually recommended because, in general, they are less effective than non-β-lactams such as TMP/SMX or fluoriquinolones. Furthermore, *E. coli* exhibits high levels of resistance to β-lactams antibiotics (Nicolle, 2002; Nickel, 2005; Guay, 2008; Gagliotti *et al.*, 2011). Accordingly with European Antimicrobial Resistance Surveillance System (www.ecdc.europa.eu/en/activities/surveillance/EARS-Net), the number of bloodstream infections caused by *E. coli* increased remarkably by 71% in the last years (2002 to 2009), associated with an alarming increase of antimicrobial resistance (Gagliotti *et al.*, 2011).

1.1.2 *Staphylococcus aureus*

S. aureus are Gram-positive spherical bacteria usually arranged in pairs, short chains or in clusters, which were first isolated from pus from an abscess by Alexander Ogston in the late 1800s (Ogston, 1882; Madigan *et al.*, 2000). This facultative anaerobic pathogen is an opportunistic commensal bacterium, being mainly found in upper respiratory tract, especially in nose and pharynx, and in skin surface of healthy individuals (Figure 1.1) (Wertheim *et al.*, 2005). The asymptomatic colonization provides a reservoir from which infection may occur when host defenses are breached (Kluytmans *et al.*, 1997; Wertheim *et al.*, 2005). Upon breaching the epithelium, this versatile pathogen is capable of causing from skin and soft-tissue infections (SSTIs), such as pimples, boils, acne and cellulites, most treatable with antibiotics, to life-threatening diseases including pneumonia, meningitis, arthritis, osteomyelitis, septicemia and toxic shock syndrome (Lowy, 1998; Stevens *et al.*, 2005; Klevens *et al.*, 2007; Gordon & Lowy, 2008). *S. aureus* infections have been recognized as a major worldwide health concern due to the emergence of antibiotic resistant strains. Actually, soon after the introduction in 1941 of the ß-lactam penicillin, antibiotic resistant strains rapidly spread, initially in hospitals and subsequently into the community (Rammelkamp & Maxon, 1942; Lowy, 2003). By the late of 1960s, more than 80% of both community and hospital-acquired

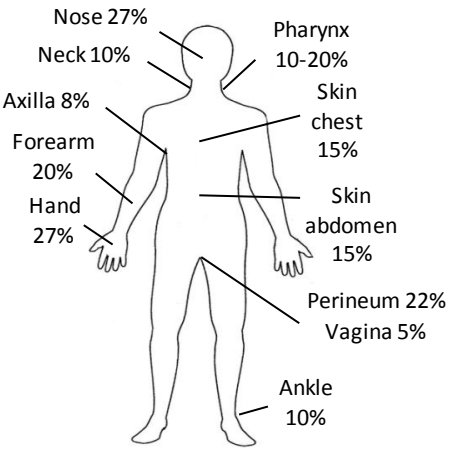


Figure 1.1| *S. aureus* carriage rates per body site in adults from general population. Adapted from (Wertheim *et al.*, 2005)

staphylococcal isolates were resistant to penicillin (Chambers, 2001; Lowy, 2003). Interestingly, the community-acquired isolates were often resistant only to penicillin, whereas hospital strains were typically resistant to several antibiotics (Chambers, 2001). In 1961, the penicillin derivative - methicillin was introduced in clinical practice to treat staphylococcal infections; again less than one year later, methicillin-resistant *S. aureus* strains (MRSA) were reported (Barber, 1961). Although until 1990s, MRSA remained a problem restricted to hospitals and other health care-associated settings, in the following years MRSA arose in patients without prior healthcare contact and became a widespread cause of community infections (Lyon *et al.*, 1984; Udo *et al.*, 1993; Herold *et al.*, 1998). Outbreaks of community acquired MRSA infections have been reported worldwide, and although usually described as a cause of superficial mild SSTIs (around 90% cases), some strains appear to be especially virulent, causing severe infections such sepsis, necrotizing fasciitis, necrotizing pneumonia and endocarditis (Chambers, 2001; Francis *et al.*, 2005; Gonzalez *et al.*, 2005; Seybold *et al.*, 2006; Chambers & Deleo, 2009; Kallen *et al.*, 2009). Fortunately, the prevalence of community acquired MRSA is still low in Portugal (Tavares *et al.*, 2010). Although methicillin is no longer in clinical use, the term MRSA remains to describe *S. aureus* strains resistant to β-lactam antibiotics (Foster, 2004; Grundmann *et al.*, 2010). Unlike healthcare-associated MRSA, which typically are resistant to multiple antibiotics as macrolides, tetracyclines, gentamicin and lincosamides, the community isolates are often resistant only to ß-lactam antibiotics (Chambers, 2001; Feng *et al.*, 2008; LaPlante *et al.*, 2008; Dryden *et al.*, 2010). In fact, antimicrobial compounds, such as TMP/SMX, clindamycin, tetracyclines, fluoroquinolones and linezoid have been used to successfully treat community acquired staphylococcal infections (Bhambri & Kim, 2009; Dryden *et al.*, 2010). The

severe nosocomial *S. aureus* infections have been treated with linezolid, daptomycin, and combinations of tetracycline plus rifampicin or fusidic acid and clindamycin plus rifampicin (Dryden *et al.*, 2010). The glycopeptide vancomycin has been shown to be the most effective therapeutic agent and is used as the "last resort" against infections caused by MRSA multidrug-resistant. Nevertheless in 1997, the first vancomycin intermediate-resistant *S. aureus* (VISA) was identified in Japan, and subsequently also reported in other countries, reveling that emergence of vancomycin resistance was globally spread (Hiramatsu *et al.*, 1997; Smith *et al.*, 1999). More recently, reports of complete vancomycin resistant *S. aureus* (VRSA) arose, which is even more alarming (Appelbaum, 2006). Combinations of vancomycin with aminoglycoside, fusicid acid or rifampicin have also been used to treat more serious infections caused by *S. aureus* (Dryden *et al.*, 2010). Figure 1.2 summarizes the target sites of the main antibiotics used against staphylococcal infections.

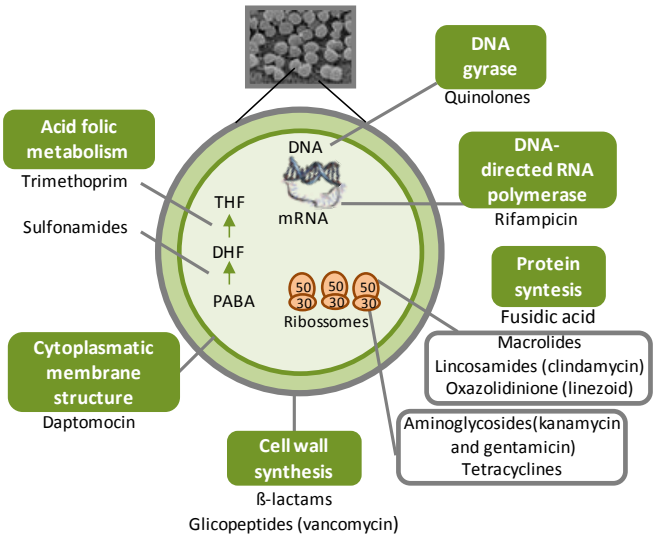


Figure 1.2| Target sites of the main antibiotics used against *S. aureus* infections. PABA (p-aminobenzoic acid), DHF (dihydrofolate) and THF (tetrahydrofolate).

1.1.3 *Helicobacter pylori*

H. pylori was first isolated from the gastric epithelium of a patient with chronic active gastritis by Warren and Marshall in the early 1980s (Warren & Marshall, 1983). In 2005, these two researchers won the Nobel Prize in Physiology or Medicine for the discovery and role of *H. pylori* in gastric diseases. *H. pylori* is a Gram-negative bacterium and usually presents a spiral-shaped morphology; however, after prolonged cultivation *in vitro* and in response to stresses such as starvation and antibiotic treatment, bacteria may be converted to a coccoid shape, which is described to be viable but nonculturable (Berry *et al.*, 1995; Kusters *et al.*, 1997; Enroth *et al.*, 1999; Kusters *et al.*, 2006).

This microaerophilic flagellated pathogen colonizes the stomach of more than half world´s population causing gastritis and being a predisposing condition to the development of severe upper gastrointestinal diseases including duodenal or gastric ulcers (1 to 10% of infected patients), gastric cancer (in 0.1 to 3%) and gastric mucosa-associated lymphoid-tissue (MALT) lymphoma (in <0.1%) (Dunn *et al.*, 1997a; Everhart, 2000; Farinha & Gascoyne, 2005; Houghton & Wang, 2005; McColl, 2010; Sachs & Scott, 2012). Hence, *H. pylori* is classified by the WHO as a class I carcinogen since 1994 (Conference, 1994).

H. pylori is unique in its capacity to efficiently colonize the acidic gastric environment and despite the host immune response, bacteria can persist for the lifetime of the host unless eradicated with antimicrobials. The diverse outcome of *H. pylori* infection is attributed to bacteria virulence factors, host genomic predisposition and environmental factors (Graham & Yamaoka, 2000; Clyne *et al.*, 2007; Wu *et al.*, 2008). In particular, several studies have reported that *H. pylori* strains mutated in the virulence factors, such as urease or

flagella, are unable to colonize animal models. These findings reveal the extreme importance of both virulence factors in enabling the adherence of pathogen to the gastric epithelium (Eaton *et al.*, 1991; Eaton *et al.*, 1992; Eaton & Krakowka, 1994; Tsuda *et al.*, 1994; Andrutis *et al.*, 1995). Indeed, urease allows bacterial survival in the acidic pH of the gastric lumen (described below), whereas flagella and the spiral-shaped morphology let bacteria bore into the gastric mucus to reach stomach's epithelial cell layer, where bacterial adhesins mediate close interaction with the host cells (Figure 1.3) (Marshall *et al.*, 1990; Montecucco & Rappuoli, 2001; Amieva & El-Omar, 2008).

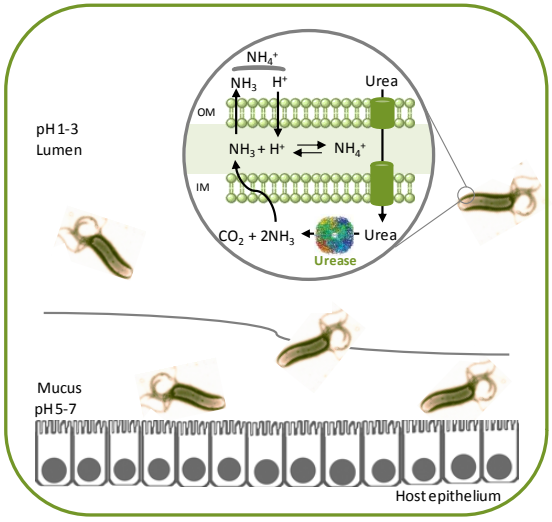


Figure 1.3| Colonization factors of *H. pylori.* During infection, the bacterium enters the gastric lumen; via urease bacteria survives the acidic environment by producing ammonia (NH_3), which is then converted to ammonium (NH_4^+) by accepting protons (H^+) that enter in the periplasm space. Ammonia is also able to diffuse through the outer membrane to neutralize the medium acidity. Cytoplasmatic ammonia can also neutralize protons entering the cytoplasm. Flagella and spiral-shaped morphology of the bacterium allow the penetration through the mucus layer to reach gastric epithelial cells, to which it sticks via specialized adhesins. OM- outer membrane and IM- inner membrane. Adapted from (Algood & Cover, 2006; Zanotti & Cendron, 2010; Scott *et al.*, 2007)

The role of urease in mediating *H. pylori* acid resistance is attributable to its ability to hydrolyze urea, present in gastric juice, to carbon dioxide (CO_2) and ammonia (NH_3) (Marshall *et al.*, 1990). The ammonia generated by urease activity consumes protons (H^+) neutralizing the gastric juice, enabling *H. pylori* to survive and multiply in the stomach (Figure 1.3) (Eaton *et al.*, 1991; Scott *et al.*, 2007; Zanotti & Cendron, 2010). Importantly, ammonia also has a cytotoxic effect on gastric epithelial cells (Smoot *et al.*, 1990).

H. pylori urease is a 1.1 MDa spherical assembly of 12 catalytic units, each composed of UreA-UreB subunits (Ha *et al.*, 2001). Besides these two structural subunits, four accessory proteins (UreEFGH) are involved in urease assembly by mediating incorporation of nickel into the apoenzyme (Cussac *et al.*, 1992; Burne & Chen, 2000; Zanotti & Cendron, 2010). This nickel-containing enzyme represents 6% of total *H. pylori* cell proteins and can be found in the cytoplasm as well as on bacterial surface upon autolysis of neighboring bacteria (Hu & Mobley, 1990; Phadnis *et al.*, 1996; Dunn *et al.*, 1997b; Ha *et al.*, 2001; Stingl & De Reuse, 2005). Although, it is well established that cytoplasmatic urease protects *H. pylori* against gastric acidity, the role of surface urease during *H. pylori* gastric colonization remains under debate. Some authors argue that extracellular urease does not contribute to acid resistance of *H. pylori* since the purified enzyme is irreversible inactivated at a pH<4. On the other hand, others reported that bacteria without extracellular urease activity are susceptible to an external pH of 3, and that urease supramolecular assembly plays a critical role in the activity of the enzyme as it protects the active sites, remaining the activity unaffected down to pH of 3 (Bauerfeind *et al.*, 1997; Krishnamurthy *et al.*, 1998; Ha *et al.*, 2001; Stingl & De Reuse, 2005).

Due to the key role of urease in host stomach´s colonization, urease inhibitors constitute potential drugs for treatment of gastric infections (Follmer, 2010; Kosikowska & Berlicki, 2011). In fact, new strategies to eliminate *H. pylori* infections are urgently needed since a dramatic fall in eradication rates has been reported worldwidely in the last years, mainly due to the increased emergence of antibiotic resistance (Selgrad & Malfertheiner, 2011).

H. pylori eradication is usually done by the so-called triple therapy, which combines two antibiotics (clarithromycin, amoxicillin and metronidazole) with a proton pump inhibitor (PPI, *e.g.* omeprazole) to reduce gastric acid production (Coelho *et al.*, 2000; Gisbert *et al.*, 2005; Chey & Wong, 2007; Malfertheiner *et al.*, 2007; Wolle & Malfertheiner, 2007). However, triple therapy has become progressively less effective with treatment success rates being, in most countries currently lower that 80%, mainly due to antibiotic resistance (Megraud, 2004; Wolle & Malfertheiner, 2007; Graham & Fischbach, 2010; Yakoob *et al.*, 2010; Rimbara *et al.*, 2011). Therefore, triple clarithromycin-based therapy should be avoided unless infection is known to be susceptible to clarithromycin or in areas with proven low macrolide resistance rates (*i.e.* ≤20%) (Chuah *et al.*, 2011; Rimbara *et al.*, 2011). Importantly, resistance to clarithromycin cannot be overcome by increasing the dose or duration of therapy (Rimbara *et al.*, 2011). Clarithromycin resistance is mainly associated with mutations in the 23S ribosomal RNA inside 50S ribosomal subunits, which results in conformational changes that leads to a decrease binding affinity of the drug to *H. pylori* ribossomes, allowing normal bacterial protein synthesis (Versalovic *et al.*, 1996; Occhialini *et al.*, 1997; Taylor *et al.*, 1997). In case of low resistance to clarithromycin and in populations with less than 40% of metronidazole resistance, PPI-

clarithromycin-metronidazole is recommended (Malfertheiner *et al.*, 2007). Nevertheless, metronidazole resistance is also problematic and although it can be partially overcome by increasing the dose of antibiotic and the duration of therapy, it may cause intolerable side effects and pill burden, which compromises treatment (Rimbara *et al.*, 2011). Metronidazole action is similar to that described to nitrofurans (see section 1.2 and 1.2.1), since this nitroimidazole antibiotic also needs to be converted to cytotoxic forms through reduction of the nitro group to exert antimicrobial effects (Lindmark & Muller, 1976). Several studies have shown that oxygen insensitive-nitroreductase *rdxA*, NAD(P)H flavin oxidoreductase *frxA* and ferredoxin-like protein *fdxB* contribute to the activation of metronidazole in *H. pylori.* In agreement, metronidazole resistance is usually associated with mutations in these genes (Goodwin *et al.*, 1998; Kwon *et al.*, 2000; Jenks & Edwards, 2002; Mendz & Megraud, 2002). On the contrary, amoxicillin resistance is not a major concern since the resistance rate remains so far low (Yakoob *et al.*, 2010).

Due to the increase inefficiency of the triple therapy, the use of four-drug regiments has been recommended. Such treatment include quadruple or sequential therapy comprising PPI plus three antibiotics (clarithromycin, metronidazole and amoxicillin), or a bismuth-containing quadruple therapy (PPI plus bismuth salts, metronidazole and tetracycline). So far, these treatments have provided better eradication rates (Fischbach & Evans, 2007; Chuah *et al.*, 2011; Rimbara *et al.*, 2011; Selgrad & Malfertheiner, 2011; Malfertheiner *et al.*, 2012; Megraud, 2012).

1.2 Nitrofurans as antimicrobial agents

Nitrofurans are a class of synthetic antimicrobials that include nitrofurantoin and nitrofurazone. These compounds remain clinically effective against a wide range of bacteria, including those responsible for UTIs as *E. coli* (see section 1.1.1) (Chamberlain, 1976; Johnson *et al.*, 1993; Guay, 2001). Although, *S. aureus* is a relatively uncommon cause of UTIs in the general population (<1%), it is quite usual among patients submitted to urinary tract catheterization and to genitourinary procedures (Barrett *et al.*, 1999; Goldstein, 2000; Muder *et al.*, 2006; Guay, 2008). Importantly, the majority of staphylococcal isolates from patients with catheter-associated UTIs are MRSA (Muder *et al.*, 2006).

Nitrofurantoin is currently used as an oral antibiotic for the treatment of genitourinary infections and although, nitrofurazone is primarily utilized as a topical antibiotic in burns and skin grafts, a nitrofurazone-impregnated catheter can be utilized to prevent catheter-associated urinary tract infections (Johnson *et al.*, 1993; Guay, 2001). Although the bactericidal action of nitrofurans has yet to be fully elucidated, reduction of nitro group by nitroreductases (NTR, see section 1.2.1) is essential for its activation (McCalla *et al.*, 1970; Bryant & DeLuca, 1991; McOsker & Fitzpatrick, 1994). Therefore, mutations in genes encoding nitroreductases result in increased bacterial resistance to nitrofurans (McCalla *et al.*, 1978; Bryant *et al.*, 1981; Whiteway *et al.*, 1998). Following activation, the actual antimicrobial action of these prodrugs remains largely unknown, but it is believed to be due to the inhibition of bacterial

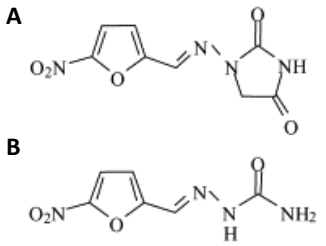


Figure 1.4| Chemical structures of nitrofurantoin (A) and nitrofurazone (B). Adapted from (Mottier *et al.*, 2005).

enzymes involved, at least, in DNA and RNA synthesis and carbohydrate metabolism (McCalla *et al.*, 1970; Streeter & Hoener, 1988; Shah & Wade, 1989; McOsker & Fitzpatrick, 1994). The multiple target sites of nitrofuran antibiotics may explain the reduced ability of bacteria to develop resistance toward these drugs (Guay, 2001). In particular, several reports have demonstrated that MRSA isolates from catheter-associated UTIs are susceptibility to both nitrofurantoin and nitrofurazone (Shah & Wade, 1989; Flournoy & Robinson, 1990; Johnson *et al.*, 1993; Johnson *et al.*, 1999). The observations that *S. aureus* are still susceptibility to nitrofurans even after several decades of use are of clinical importance (Chamberlain, 1976; McOsker & Fitzpatrick, 1994; Guay, 2001).

1.2.1 Nitroreductases

Nitroreductases are the enzymes responsible for nitrofurans activation (McCalla *et al.*, 1978; McCoy *et al.*, 1981; Roldan *et al.*, 2008). These enzymes that reduce the nitro group are divided into two groups based on oxygen sensitivity: type I-oxygen insensitive and type II-oxygen sensitive enzymes (Peterson *et al.*, 1979).

Type I-oxygen insensitive nitroreductases are the best-studied enzymes, and although usually found in bacteria, they rarely occur in eukaryotes (Knox *et al.*, 1993; Ueda *et al.*, 2003; Roldan *et al.*, 2008). This type of nitroreductases are FMN binding proteins and NAD(P)H-dependent enzymes. Nitroreductases catalyze the sequential reduction of nitro group through the addition of two electrons from NAD(P)H to yield a nitroso intermediate, which is reduced to amine via a hydroxilamine derivative. In itself, hydroxylamine has toxic, carcinogenic and mutagenic properties (Figure 1.5) (Peterson *et al.*, 1979; Bryant *et al.*, 1981; Roldan *et al.*, 2008).

Type II-oxygen sensitive nitroreductases are FAD- or FMN-containing enzymes that mediate one-electron reduction of the nitro group forming a nitro anion radical, which in the presence of oxygen produces superoxide anion in a futile cycle, regenerating the parental nitro-compound. Thus, these enzymes only mediate reduction of nitrofurans under anaerobic conditions (Figure 1.5) (Mason & Holtzman, 1975a, 1975b; Peterson *et al.*, 1979). The former type of nitroreductases is found in *E. coli* and several *Clostridium* strains (McCalla *et al.*, 1975; Peterson *et al.*, 1979; Angermaier & Simon, 1983). In general, nitrofuran reduction in eukaryotic cells is mediated by this type of nitoreductases (Wolpert *et al.*, 1973; Adams & Rickert, 1995).

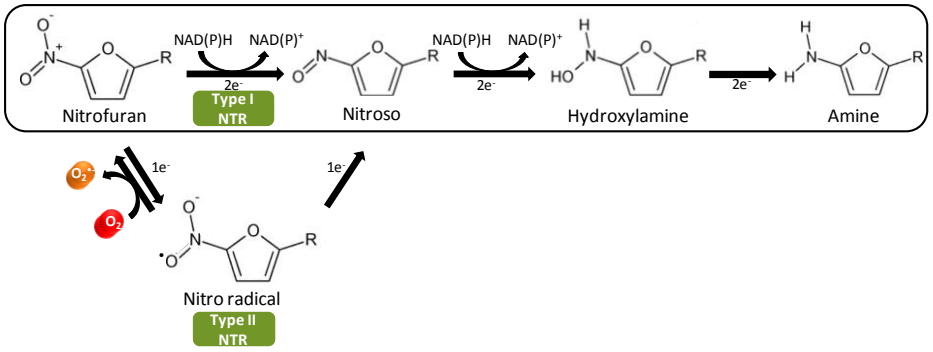


Figure 1.5| General scheme of nitrofurans reduction by nitroreductases (NTR). The oxygen-insensitive nitroreductases (type I) catalyze the reduction of the nitro group of nitrofurans by addition of a pair of electrons forming the nitroso and hydroxylamino intermediates and finally the amine formation. The oxygen-sensitive nitroreductases (type II) catalyze the single-electron reduction of the nitro group to produce a nitro anion radical, which can be reoxidized aerobically to the original structure with the concomitant production of superoxide anion ($O_2^{\bullet -}$) in a futile cycle. Adapted from (Koder *et al.*, 2002; Hall *et al.*, 2011; Roldan *et al.*, 2008).

Although bacteria may contain both types of nitroreductases, as is the case of *E. coli*, it seems to be those from type I that underpin nitrofurans action. In fact, bacterial resistance to nitrofurans is usually attributed to mutations in genes encoding oxygen insensitive nitroreductases (McCalla *et al.*, 1970; McCalla *et al.*, 1975; McCalla *et al.*, 1978; Sastry & Jayaraman, 1984; Whiteway *et al.*, 1998). Table 1.1 summarizes the most relevant bacterial oxygen-insensitive (type I) nitroreductases reported to promote reduction of nitrofuran antibiotics.

Table 1.1| Bacterial oxygen-insensitive nitroreductases (NTR) mediating nitrofuran reduction.

Bacteria	NTR	Monomer size (kDa)	Electron Donors	References
E. coli	**NfsA**	27	NADPH	(Bryant *et al.*, 1981) (Zenno *et al.*, 1996a) (Zenno *et al.*, 1998a)
	NfsB	24	NAD(P)H	(Bryant *et al.*, 1981) (Zenno *et al.*, 1996c) (Zenno *et al.*, 1996b) (Zenno *et al.*, 1996a)
Enterobacter cloacae	**NR**	27	NAD(P)H	(Bryant & DeLuca, 1991) (Koder & Miller, 1998)
Synechocystis sp.	**DrgA**	26	NAD(P)H	(Takeda *et al.*, 2007)
Bacillus subtilis	**NfrA1**	29	NADPH	(Zenno *et al.*, 1998b)
S. aureus	**NfrA**	29	NADPH	(Streker *et al.*, 2005)
H. pylori	**RdxA**	26	NAD(P)H	(Olekhnovich *et al.*, 2009)
	FrxA	25	NAD(P)H	(Sisson *et al.*, 2002)

Adapted from (Roldan *et al.*, 2008).

Bacterial oxygen insensitive nitroreductases are divided into two main groups according to their similarity with *E. coli* nitroreductases, NfsA and NfsB, which share very low identity (Zenno *et al.*, 1996a; Zenno *et al.*, 1996c, 1996b; Roldan *et al.*, 2008). The NfsA group, composed by *B. subtilis* NfrA1 and *S. aureus* NfrA is usually NADPH-dependent whereas NfsB group (Enterobacter NR, *Synechocystis* DrgA, and *H. pylori* RdxA and FrxA) may use both NADH and NADPH as electron donors (Table 1.1) (Bryant *et al.*, 1981; Zenno *et al.*, 1996a; Zenno *et al.*, 1996c, 1996b; Whiteway *et al.*, 1998; Zenno *et al.*, 1998a; Roldan *et al.*, 2008). In spite of low degree of amino acid sequence similarity, the two groups of bacterial insensitive nitroreductases share similar structure and biochemical properties as all occur as homodimers (24-30 kDa subunits) with a characteristic α+ß-fold, contain FMN as cofactor and catalyze, besides nitrofurans, the reduction of a broad range of substrates (Zenno *et al.*, 1996a; Zenno *et al.*, 1996c; Parkinson *et al.*, 2000; Kobori *et al.*, 2001; Haynes *et al.*, 2002; Race *et al.*, 2005; Roldan *et al.*, 2008).

More interestingly, several bacterial nitroreductases have been described to protect against oxidative stress, which together with nitrosative stress are imposed to bacteria by mammalian immune system (see section 1.3). In particular, it was shown that *E. coli nfsA* is induced by paraquat as a member of the *soxRS* regulon, which is involved in the control of oxidative stress response in *E. coli* (Liochev *et al.*, 1999). Moreover, *B. subtilis nfrA1* and *S. aureus nfrA* are also induced under oxidative stress conditions (Mostertz *et al.*, 2004; Streker *et al.*, 2005). Interestingly, *B. subtilis* NfrA1 is able to rapidly scavenge hydrogen peroxide (H_2O_2) (Cortial *et al.*, 2010). Furthermore, *S. aureus* NfrA nitroreductase exhibits disulfide reductase activity, which allows a thiol-disulfide balance that is quite important since oxidation of thiols resulting in disulfide bond formation is one major effect of oxidative stress on proteins

(see section 1.3) (Streker *et al.*, 2005). The study of a *S. aureus* nitroreductase, which is involved in nitrosative stress response, is presented in Chapter 3.

Besides their role in antibiotic activation and stress responses, nitroreductases have been suggested for medical applications as cancer therapy (Knox *et al.*, 1993; Connors & Knox, 1995; Green *et al.*, 2004; Searle *et al.*, 2004). In particular, several studies have been carried out with *E. coli* nitroreductases in order to promote activation of prodrugs in tumor cells (Searle *et al.*, 2004; Vass *et al.*, 2009).

Additionally, bacterial nitroreductases can be used for biodegradation and bioremediation of nitroaromatic pollutant compounds (Spain, 1995; Rieger *et al.*, 2002; Lewis *et al.*, 2004; Ramos *et al.*, 2005; Symons & Bruce, 2006).

1.3 Innate immune system response against bacteria

The mammalian immune system is constituted by innate and adaptive defense mechanisms, which cooperate to protect host cells against microbial infections (Medzhitov, 2007). The adaptive immunity is characterized by its high degree of specificity and the property of memory, being its response mediated by lymphocytes (B and T cells), which produce immune molecules such as antibodies, cytokines and antigen binding receptors (Medzhitov, 2007). The innate immunity is non-specific and provides the first line of defense during the critical period just after the host´s exposure to the pathogen. Innate immune recognition is mediated by pattern recognition receptor (*e.g.* Toll-like receptors), which bind to conserved microbial structures, the so-called pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS), peptidoglycans and lipoteichoic acids (Ishii *et al.*, 2008). Among the several constituents of the innate immunity system,

phagocytes (*e.g.* macrophages and neutrophiles) are key components of the antimicrobial response, being responsible for killing and clearance of invading bacteria through phagocytosis. On encountering bacteria, phagocytes engulf microbes into a phagosome that fuses with intracellular granules, *e.g.* lysosome, to form a phagolysosome. Within the phagolysosome, bacteria faces a hostile environment with low nutrient availability, iron limitation, low pH (~4.8) and several antimicrobial products, which include reactive oxygen species (ROS) and reactive nitrosative species (RNS) (Figure 1.6) (Underhill & Ozinsky, 2002; Mayer-Scholl *et al.*, 2004; Radtke & O'Riordan, 2006; Slauch, 2011). In particular, ROS and RNS are considered essential antimicrobial agents of the immune system. Indeed, simultaneous deletion of the nitric oxide synthase and NADPH oxidase, which produces nitric oxide and superoxide, respectively (described below), result in massive death of mice caused by spontaneous infections with commensal microorganisms (Shiloh *et al.*, 1999).

1.3.1 Oxidative stress

Oxidative stress in phagocytes is initiated by the NADPH oxidase (also designated as phagocyte oxidase, Phox). Activated upon phagocytosis, this multicomponent membrane-bound enzyme uses NADPH to catalyze the reduction of dioxygen to superoxide, leading to a burst of oxidative stress as schematized in Figure 1.6 (Babior, 2000). Briefly, superoxide dismutates spontaneously by reacting with itself or enzymatically via superoxide dismutase (SOD), leading to the formation of hydrogen peroxide and dioxygen (Babior, 2000). In eukaryotes two types of SODs have been identified: the citoplasmatic and constitutive Cu/Zn-SOD, and the mitochondrial and stress inducible Mn-SOD (Babior, 2000).

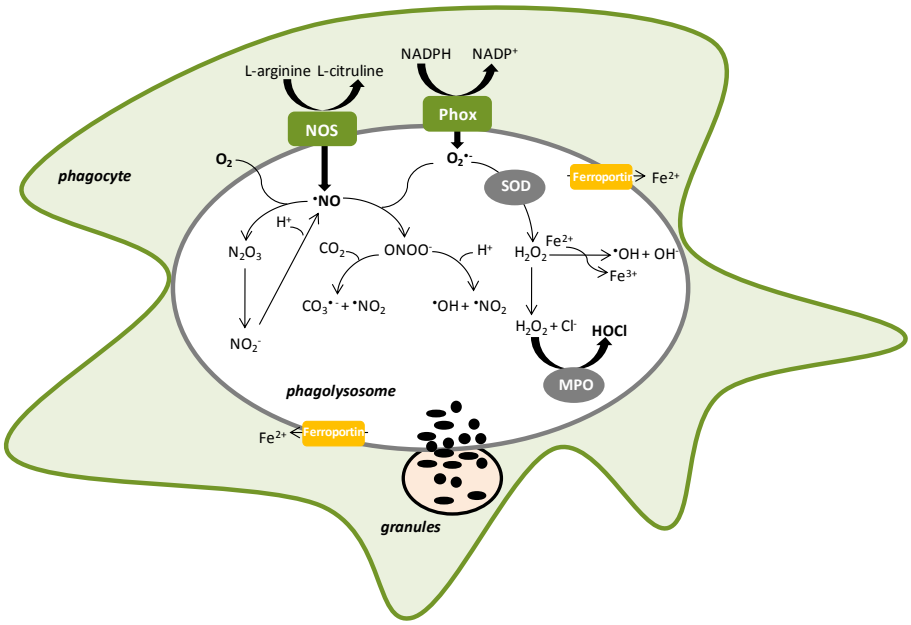


Figure 1.6| Schematic representation of a phagolysosome generating reactive oxygen and nitrosative species. NO synthase (NOS) and NADPH oxidase (Phox) produce nitric oxide ($^{\bullet}NO$) and superoxide ($O_2^{\bullet-}$), respectively. Superoxide is dismutated by superoxide dismutase (SOD) releasing hydrogen peroxide (H_2O_2). H_2O_2 is converted into radical hydroxil ($^{\bullet}OH$) and hydroxide ion (OH^-) by the Fenton reaction, and is used by myeloperoxidase (MPO) to synthesize hypochlorous acid (HOCl). Superoxide reacts with $^{\bullet}NO$ generating peroxynitrite ($OHOO^-$). Peroxynitrite is decomposed into $^{\bullet}OH$ and nitrogen dioxide ($^{\bullet}NO_2$) or reacts with carbon dioxide (CO_2) producing carbonate ($CO_3^{\bullet-}$) and $^{\bullet}NO_2$. $^{\bullet}NO$ also reacts with dioxygen (O_2) forming nitrogen trioxide (N_2O_3), which is rapidly converted to nitrite (NO_2^-) followed by reducion to NO. Ferroportin pumps Fe^{2+} out of the phagosomal lumen and granules release their content (*e.g.* lysozyme, cathepsins) into the phagossome. Adapted (Nobre, 2010).

In neutrophils, hydrogen peroxide reacts with chloride producing hypochlorous acid by the action of myeloperoxidase (MPO), an enzyme that is absent in macrophages. Hydrogen peroxide, which diffuses freely through membranes, may lead to the formation of the highly reactive hydroxyl radical

through the iron-catalyzed Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$). Although the major source of hydroxyl radical results from the Fenton reaction, the radical can also be produced by the reaction of superoxide with hypochlorous acid ($O_2^{\bullet-} + HOCl \rightarrow {}^{\bullet}OH + O_2 + Cl^-$) (Liochev, 1999; Babior, 2000; Bogdan *et al.*, 2000).

In phagocytes, the importance of antimicrobial properties of ROS against a broad range of pathogens has been comprehensively reported. For example, a Phox-deficient macrophage is unable to restrict *Salmonella typhimurium* infection and exhibit distinct disadvantage in the ability to eliminate infectious organisms as *S. aureus, E. coli, Aspergillus fumigates* and *Mycobacterium tuberculosis* (Jackson *et al.*, 1995; Pollock *et al.*, 1995; Adams *et al.*, 1997; Vazquez-Torres *et al.*, 2000; Gao *et al.*, 2002; Pizzolla *et al.*, 2012). Additionally, mice deficient in MPO display higher sensitivity to *Candida albicans* infection (Aratani *et al.*, 1999; Babior, 2000). In humans, the presence of a nonfunctional Phox leads to immunodeficiency characterized by recurrent bacterial and fungal infections (Baehner & Nathan, 1967). On the other hand, the deletion of bacterial defense mechanisms involved in ROS detoxification increases the susceptibility of pathogens to be killed in phagocytes (see section 1.3.3) (Basu *et al.*, 2004; La Carbona *et al.*, 2007). In bacteria, the cellular oxidative stress arises from an imbalance between ROS inflicted from the external environment and the antioxidant bacterial defenses, which results in damage of the major cellular macromolecules - DNA, lipids and proteins (Cabiscol *et al.*, 2000; Scandalios, 2002; Avery, 2011; Fang, 2011).

Although, superoxide and hydrogen peroxide do not react directly with DNA at physiological levels, the hydroxyl radical is able to attack sugars and DNA bases (purines and pyrimidines), generating a multitude of products. Among the products formed, one of the most well-known is the highly

mutagenic 8-hydroxyguanine, generated by guanine oxidation (Imlay & Linn, 1988; Michaels & Miller, 1992; Fang, 2004; Avery, 2011). In general, oxidative DNA damages include bases modifications, and DNA single and double strand breaks (Fang, 2011).

Hydroxyl radical is also able to initiate the process of lipid peroxidation in membranes, which alters the membrane proprieties by disrupting the membrane-bound proteins and lowering the membrane fluidity, which leads to the loss of membrane integrity (Cabiscol *et al.*, 2000; Avery, 2011; Pradenas *et al.*, 2012).

Proteins can undergo a range of oxidative modifications of the amino acid side chains. In particular, cysteine amino acid residues are prone to be damaged by ROS, as thiols group are highly susceptible to be attack generating disulphide bonds (2RSH -> RS-SR + 2 H^+ + 2e^-). Aromatic amino acid such as tyrosine, phenylalanine and tryptophan are also among the most oxidation-prone residues together with methionine, which is oxidized to methionine sulfoxide (Cabiscol *et al.*, 2000; Fang, 2004; Avery, 2011). Other targets of ROS are the iron-sulphur (Fe-S) clusters present in several proteins that have a key role in many cellular processes. In particular, solvent exposed Fe-S clusters, such as those of the [4Fe-4S] family of dehydratases, which are present in key enzymes of the citric acid cycle (*e.g.* aconitase and fumarase), are highly susceptible to oxidative damage. Moreover, the dismantling of these clusters results in an increase of free iron, which reacts with hydrogen peroxide via the Fenton reaction, exacerbating the effects of oxidative stress (Brzoska *et al.*, 2006; Imlay, 2006; Jang & Imlay, 2007). Overall, these oxidative modifications lead to protein structure alteration and, consequently, to functional changes that disturb the bacterial cellular metabolism.

1.3.2 Nitrosative stress

Within the phagossome, NO is generated by NO synthase (NOS) through the oxidation of L-arginine to citruline in the presence of oxygen and NADPH (Figure 1.6) (MacMicking *et al.*, 1997; Stuehr, 1999). Mammals contain three types of NOS isoforms: the endothelial (eNOS), the neural (nNOS) and the inducible (iNOS). Although, the first two isoforms are constitutively expressed, the iNOS is inducible by several immunological or inflammatory signals, like cytokines (*e.g.* IFN-γ) and the bacterial LPS (MacMicking *et al.*, 1997; Alderton *et al.*, 2001). NO is a small (30 Da) non-polar uncharged molecule and a free radical with a high diffusion coefficient, which allows rapid diffusion across the biological membranes (Fang, 1997). NO reacts with other molecules giving rise to species with higher reactivity and toxicity, the so-called reactive nitrogen species (RNS). In particular, the powerful oxidizing peroxynitrite is formed from the reaction of NO with superoxide. This reaction is extremely fast, occurring at a higher rate than the decomposition of superoxide by SOD (Huie & Padmaja, 1993; Hughes, 2008). Peroxynitrite can be then decomposed to form nitrogen dioxide and $^{\bullet}OH$ radicals, and in the presence of carbon dioxide also produces nitrogen dioxide and carbonate ion radicals. NO also reacts rapidly with dioxygen yielding nitrogen dioxide (Figure 1.6) (Goldstein & Merenyi, 2008; Ferrer-Sueta & Radi, 2009).

The antimicrobial activity of RNS has been demonstrated by a variety of approaches, in strong analogy with studies bearing on the role of ROS. In animal models, it has been shown the increased expression of iNOS and NO production at sites of infection. In accordance, NOS inhibitors contribute to increase bacteria survival and mice deficient in iNOS are more susceptible to microbial infection (Fang, 1997; MacMicking *et al.*, 1997; Shiloh & Nathan, 2000; Chakravortty & Hensel, 2003). Other evidence for the role of RNS in

reducing bacterial proliferation comes from the correlation of microbial resistance with the expression of NO-detoxifying enzymes (see section 1.3.3), similar to what happens to ROS (MacMicking *et al.*, 1997). The antimicrobial action of NO as well as the NO-derivates products consists in damaging several cellular components, like DNA, lipids and proteins.

Concerning DNA, although NO *per se* does not react with this macromolecule, nitrogen dioxide, nitrogen trioxide or peroxynitrite derivatives can lead to nitrosative deamination of DNA bases such as guanine and cytosine. Peroxynitrite and nitrogen dioxide also damage DNA resulting in abasic sites and strand breaks (Fang, 1997).

Another important biological target of RNS are lipids. Although NO is not a strong oxidant and does not initiate lipid peroxidation, peroxynitrite and nitrogen dioxide are powerful oxidizing species capable of initiating and propagating lipid peroxidation (Radi *et al.*, 1991; Hogg & Kalyanaraman, 1999).

In relation to proteins, RNS may react with multiple targets including thiols, aromatic amino acid residues and metal centres (*e.g.* haem groups, Fe-S clusters and non-haem iron) (Hughes, 2008). In particular, thiols are among the most important protein targets, in which the end product is the formation of S-nitrosothiols, a modification of the sulphur atom of cysteine residues, also called S-nitrosation or S-nitrosylation. Although NO itself cannot act as a nitrosating agent, its congeners, such as nitrogen dioxide, nitrogen trioxide or peroxinitrite are particularly potent in this context. S-nitrosoglutathione (GSNO) is the most abundant S-nitrosothiol in eukaryotic cells in a concentration range of μM. GSNO is formed by the nitrosylation of glutathione (GSH), a tripeptide with potent antioxidant properties that is composed by glutamate, cysteine and glycine (Fang, 1997; Gaston, 1999). Most bacteria encounter GSNO intracellularly at the sites of infections, since glutathione and

NO are present in host cells at high concentrations (Singh *et al.*, 1996). The GSNO formed is able to participate in transnitrosylation reactions with thiol-containing proteins unless it is decomposed by GSNO reductases (Arnelle & Stamler, 1995). Although cysteine residues are in general the preferred targets, aromatic amino acid residues (such as histidine, tyrosine, phenylalanine and tryptophan) and methionine may also react with RNS like peroxynitrite or nitrogen dioxide (Fang, 1997). Among the reactions of NO with metal-containing proteins, the reactions with iron are by far the most studied, especially those involving haem groups and Fe-S centres. For example, the binding of NO to the ferrous haem iron of cytochrome oxidase and to the ferric haem of catalase has been reported to inhibit the activity of both enzymes (Brown, 1995; Giuffre *et al.*, 1996; Cooper, 1999; Hughes, 2008). The reaction of NO with Fe-S centres leads to the formation of dinitrosyl-iron-dithiol complexes (DNIC), which results in the inactivation of the enzymes with important metabolic functions (*e.g.* aconitase, NADH and succinate dehydrogenase) (Cooper, 1999; Hughes, 2008).

1.3.3 Bacterial defense mechanisms

To counteract the toxic effects of ROS and RNS, bacteria posse protective detoxification systems, besides DNA and protein repairing systems. The major detoxification systems present in bacteria used in this study are described on Tables 1.2 and 1.3.

Regarding oxidative stress, bacteria comprise superoxide dismutase (SOD) that catalyses the dismutation of superoxide to dioxygen and hydrogen peroxide ($2O_2^{\bullet-} + 2H^+ \rightarrow O_2 + H_2O_2$). Four types of SODs have been identified in prokaryotes, which differ by the metal centre present in the active site, namely manganese, iron, copper-zinc and nickel (Fridovich, 1995). The Fe-SOD

and Mn-SOD are both cytoplasmatic, but while the first is constitutively expressed the second is induced by dioxygen and other stress agents (*e.g.* metals) (Geslin *et al.*, 2001). Bacterial Cu/Zn-SOD is periplasmatic and seems to be critical for survival during the stationary phase of growth (St John & Steinman, 1996). In addition, the periplasmic SOD has also been reported to play an essential role in several bacterial pathogens, by withstanding the oxidative burst imposed by the host macrophages (De Groote *et al.*, 1997; Rushing & Slauch, 2011). The Ni-SOD has been described in *Streptomyces,* and is proposed to be regulated by nickel (Youn *et al.*, 1996; Kim *et al.*, 1998).

In order to scavenge hydrogen peroxide, bacteria use catalases and peroxidases, which are ubiquitous oxidoreductases that catalyze the following reactions, $2H_2O_2$ -> O_2 + $2H_2O$ and RH_2 + H_2O_2 -> R + $2H_2O$, respectively (Imlay, 2008). In addition, peroxidases such as alkyl hydroperoxidase reductase (Ahp) are described to reduce peroxynitrite, and toxic organic peroxides to the corresponding alcohols (Chen *et al.*, 1998; Bryk *et al.*, 2000; Poole, 2005). The hybrid-cluster protein (Hcp) of *E. coli* has been also reported to have peroxidase activity, thus conferring protection against oxidative stress (Almeida *et al.*, 2006).

Hydroxyl radical has been considered as extremely toxic and acutely lethal, not only due to the damages that it inflict on bacteria, but also because no enzyme able to detoxify this ROS is apparently present in bacteria.

Table 1.2 summarizes the major defense mechanisms used by bacteria studied in this thesis against oxidative stress.

The bacterial response to oxidative stress is controlled by two major transcriptional regulators, OxyR and SoxRS. The first responds to increased levels of hydrogen peroxide, while the second senses superoxide, hydrogen peroxide and redox-cycling drugs (*e.g.* paraquat and menadione) (Zheng *et al.*,

2001; Gu & Imlay, 2011; Lushchak, 2011). These regulators are well studied in *E. coli*, with *katG, ahpC* and *hcp* belonging to the OxyR regulon, and *sodA* controlled by SoxRS (Imlay, 2008; Lushchak, 2011; Seth *et al.*, 2012). Interestingly, none of these regulators are apparently present in *S. aureus* and *H. pylori.*

Table 1.2| Major defense mechanisms used by bacteria studied in this thesis against oxidative stress.

Bacteria	Oxidative stress	References
E. coli	Mn-SOD (SodA)	(Keele *et al.*, 1970)
	Fe-SOD (SodB)	(Yost & Fridovich, 1973)
	Cu/Zn-SOD (SodC)	(Benov & Fridovich, 1994)
	KatG (HPI)	(Triggs-Raine & Loewen, 1987)
	KatE (HPII)	(Loewen, 1996)
	AhpC	(Seaver & Imlay, 2001)
	Hcp	(Almeida *et al.*, 2006)
S. aureus	Mn-Sod (SodA)	(Clements *et al.*, 1999)
	Mn-Sod (SodM)	(Valderas & Hart, 2001)
	KatA	(Cosgrove *et al.*, 2007)
	AhpC	(Cosgrove *et al.*, 2007)
H. pylori	Fe-SOD (SodB)	(Spiegelhalder *et al.*, 1993)
	KatA	(Hazell *et al.*, 1991)
	AhpC	(Lundstrom & Bolin, 2000)

Concerning nitrosative stress, the three major known bacterial NO detoxification systems are the membrane-bound respiratory NO reductases,

the flavodiiron proteins and microbial globins (Hendriks *et al.*, 2000; Saraiva *et al.*, 2004).

The membrane-bound respiratory bacterial NO reductases catalyzes the reducing of NO to nitrous oxide ($2NO \rightarrow N_2O + H_2O$) as part of the denitrification pathway (*e.g. Pseudomonas stutzeri* and *Paracoccus denitrificans*) (Zumft, 2005; Tavares *et al.*, 2006). However, their role in the protection against NO produced by macrophages is well established also in non-denitrifying organisms (*e.g. Neisseria meningitidis* and *Pseudomonas aeruginosa*) (Anjum *et al.*, 2002; Stevanin *et al.*, 2005; Kakishima *et al.*, 2007).

The flavodiiron proteins (FDPs) are considered a prominent family of NO detoxifying enzymes for their ability to reduce NO. In particular, flavorubredoxins (NorV) were the first member of the FDPs to be assigned as an NO reductase (Gomes *et al.*, 2002; Saraiva *et al.*, 2004).

Microbial globins belong to the haemoglobin family of proteins and are considered one of first line of microbial defense against NO. In particular, flavohaemoglobins (Hmp), which is the best-studied class, are able to detoxify NO either aerobically by oxidizing NO to nitrate or anaerobically by reducing NO to nitrous oxide (Gardner *et al.*, 1998; Hausladen *et al.*, 1998; Kim *et al.*, 1999).

In *E. coli*, other bacterial proteins have also been implicated in resistance to nitric oxide, such as the pentahaem nitrite reductases (Nrf) and Hcp (van Wonderen *et al.*, 2008; Vine & Cole, 2011; Seth *et al.*, 2012).

Interestingly, *H. pylori* is an extremely peculiar bacteria since it can counteract host NO production by expressing an arginase that competes with iNOS for the same substrate. In fact, inactivation of arginase restored NO production by macrophages (Gobert *et al.*, 2001). Recently, our laboratory

identified the first system of NO detoxification in *H. pylori*, which belongs to a novel family of microbial NO reductases (Justino *et al.*, 2012).

Table 1.3 summarizes the major defense mechanisms used by bacteria studied in this thesis to fight nitrosative stress.

Table 1.3| Major defense mechanisms used by bacteria studied in this thesis against nitrosative stress.

Bacteria	Nitrosative stress	References
E. coli	Hmp	(Poole *et al.*, 1996)
	FlRd (NorV)	(Gomes *et al.*, 2002)
	Hcp	(Seth *et al.*, 2012)
	Nfr	(van Wonderen *et al.*, 2008)
S. aureus	Hmp	(Goncalves *et al.*, 2006)
H. pylori	NorH	(Justino *et al.*, 2012)

The knowledge of microbial defense mechanisms against phagocytic cells is quite important to design new strategies to fight pathogens. In the Chapter 3, it will be presented a novel GSNO detoxifying system active in *S. aureus*, which was studied during this PhD work.

1.4 References

Adams LB, Dinauer MC, Morgenstern DE & Krahenbuhl JL (1997) Comparison of the roles of reactive oxygen and nitrogen intermediates in the host response to Mycobacterium tuberculosis using transgenic mice. *Tuber Lung Dis* **78**: 237-246.

Adams PC & Rickert DE (1995) Metabolism of [14C]1,3-dinitrobenzene by rat small intestinal mucosa in vitro. *Drug Metab Dispos* **23**: 982-987.

Alderton WK, Cooper CE & Knowles RG (2001) Nitric oxide synthases: structure, function and inhibition. *Biochem J* **357**: 593-615.

Algood HM & Cover TL (2006) Helicobacter pylori persistence: an overview of interactions between H. pylori and host immune defenses. *Clin Microbiol Rev* **19**: 597-613.

Almeida CC, Romao 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.

Amieva MR & El-Omar EM (2008) Host-bacterial interactions in Helicobacter pylori infection. *Gastroenterology* **134**: 306-323.

Andrutis KA, Fox JG, Schauer DB, Marini RP, Murphy JC, Yan L & Solnick JV (1995) Inability of an isogenic urease-negative mutant stain of Helicobacter mustelae to colonize the ferret stomach. *Infect Immun* **63**: 3722-3725.

Angermaier L & Simon H (1983) On nitroaryl reductase activities in several Clostridia. *Hoppe Seylers Z Physiol Chem* **364**: 1653-1663.

Anjum MF, Stevanin TM, Read RC & Moir JW (2002) Nitric oxide metabolism in Neisseria meningitidis. *J Bacteriol* **184**: 2987-2993.

Appelbaum PC (2006) The emergence of vancomycin-intermediate and vancomycin-resistant Staphylococcus aureus. *Clin Microbiol Infect* **12 Suppl 1**: 16-23.

Aratani Y, Koyama H, Nyui S, Suzuki K, Kura F & Maeda N (1999) Severe impairment in early host defense against Candida albicans in mice deficient in myeloperoxidase. *Infect Immun* **67**: 1828-1836.

Arnelle DR & Stamler JS (1995) NO+, NO, and NO- donation by S-nitrosothiols: implications for regulation of physiological functions by S-nitrosylation and acceleration of disulfide formation. *Arch Biochem Biophys* **318**: 279-285.

Askar M, Faber MS, Frank C, Bernard H, Gilsdorf A, Fruth A, Prager R, Hohle M, Suess T, Wadl M, Krause G, Stark K & Werber D (2011) Update on the ongoing outbreak of haemolytic uraemic syndrome due to Shiga toxin-producing Escherichia coli (STEC) serotype O104, Germany, May 2011. *Euro Surveill* **16**.

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.

Baehner RL & Nathan DG (1967) Leukocyte oxidase: defective activity in chronic granulomatous disease. *Science* **155**: 835-836.

Barber M (1961) Methicillin-resistant staphylococci. *J Clin Pathol* **14**: 385-393.

Barrett SP, Savage MA, Rebec MP, Guyot A, Andrews N & Shrimpton SB (1999) Antibiotic sensitivity of bacteria associated with community-acquired urinary tract infection in Britain. *J Antimicrob Chemother* **44**: 359-365.

Basu M, Czinn SJ & Blanchard TG (2004) Absence of catalase reduces long-term survival of Helicobacter pylori in macrophage phagosomes. *Helicobacter* **9**: 211-216.

Bauerfeind P, Garner R, Dunn BE & Mobley HL (1997) Synthesis and activity of Helicobacter pylori urease and catalase at low pH. *Gut* **40**: 25-30.

Belanger L, Garenaux A, Harel J, Boulianne M, Nadeau E & Dozois CM (2011) Escherichia coli from animal reservoirs as a potential source of human extraintestinal pathogenic E. coli. *FEMS Immunol Med Microbiol* **62**: 1-10.

Benov LT & Fridovich I (1994) Escherichia coli expresses a copper- and zinc-containing superoxide dismutase. *J Biol Chem* **269**: 25310-25314.

Berry V, Jennings K & Woodnutt G (1995) Bactericidal and morphological effects of amoxicillin on Helicobacter pylori. *Antimicrob Agents Chemother* **39**: 1859-1861.

Bhambri S & Kim G (2009) Use of Oral Doxycycline for Community-acquired Methicillin-resistant Staphylococcus aureus (CA-MRSA) Infections. *J Clin Aesthet Dermatol* **2**: 45-50.

Blattner FR, Plunkett G, 3rd, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B & Shao Y (1997) The complete genome sequence of Escherichia coli K-12. *Science* **277**: 1453-1462.

Bogdan C, Rollinghoff M & Diefenbach A (2000) Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. *Curr Opin Immunol* **12**: 64-76.

Brown GC (1995) Reversible binding and inhibition of catalase by nitric oxide. *Eur J Biochem* **232**: 188-191.

Bryant C & DeLuca M (1991) Purification and characterization of an oxygen-insensitive NAD(P)H nitroreductase from Enterobacter cloacae. *J Biol Chem* **266**: 4119-4125.

Bryant DW, McCalla DR, Leeksma M & Laneuville P (1981) Type I nitroreductases of Escherichia coli. *Can J Microbiol* **27**: 81-86.

Bryk R, Griffin P & Nathan C (2000) Peroxynitrite reductase activity of bacterial peroxiredoxins. *Nature* **407**: 211-215.

Brzoska K, Meczynska S & Kruszewski M (2006) Iron-sulfur cluster proteins: electron transfer and beyond. *Acta Biochim Pol* **53**: 685-691.

Burne RA & Chen YY (2000) Bacterial ureases in infectious diseases. *Microbes Infect* **2**: 533-542.

Bush K, Courvalin P, Dantas G, Davies J, Eisenstein B, Huovinen P, Jacoby GA, Kishony R, Kreiswirth BN, Kutter E, Lerner SA, Levy S, Lewis K, Lomovskaya O, Miller JH, Mobashery S, Piddock LJ, Projan S, Thomas CM, Tomasz A, Tulkens PM, Walsh TR, Watson JD, Witkowski J, Witte W, Wright G, Yeh P & Zgurskaya HI (2011) Tackling antibiotic resistance. *Nat Rev Microbiol* **9**: 894-896.

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

Casadevall A & Pirofski LA (2000) Host-pathogen interactions: basic concepts of microbial commensalism, colonization, infection, and disease. *Infect Immun* **68**: 6511-6518.

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

Chamberlain RE (1976) Chemotherapeutic properties of prominent nitrofurans. *J Antimicrob Chemother* **2**: 325-336.

Chambers HF (2001) The changing epidemiology of Staphylococcus aureus? *Emerg Infect Dis* **7**: 178-182.

Chambers HF & Deleo FR (2009) Waves of resistance: Staphylococcus aureus in the antibiotic era. *Nat Rev Microbiol* **7**: 629-641.

Chen L, Xie QW & Nathan C (1998) Alkyl hydroperoxide reductase subunit C (AhpC) protects bacterial and human cells against reactive nitrogen intermediates. *Mol Cell* **1**: 795-805.

Chey WD & Wong BC (2007) American College of Gastroenterology guideline on the management of Helicobacter pylori infection. *Am J Gastroenterol* **102**: 1808-1825.

Chuah SK, Tsay FW, Hsu PI & Wu DC (2011) A new look at anti-Helicobacter pylori therapy. *World J Gastroenterol* **17**: 3971-3975.

Clements MO, Watson SP & Foster SJ (1999) Characterization of the major superoxide dismutase of Staphylococcus aureus and its role in starvation survival, stress resistance, and pathogenicity. *J Bacteriol* **181**: 3898-3903.

Clyne M, Dolan B & Reeves EP (2007) Bacterial factors that mediate colonization of the stomach and virulence of Helicobacter pylori. *FEMS Microbiol Lett* **268**: 135-143.

Coelho LG, Leon-Barua R & Quigley EM (2000) Latin-American Consensus Conference on Helicobacter pylori infection. Latin-American National Gastroenterological Societies affiliated with the Inter-American Association of Gastroenterology (AIGE). *Am J Gastroenterol* **95**: 2688-2691.

Conference NC (1994) Helicobacter pylori in peptic ulcer disease. NIH Consensus Development Panel on Helicobacter pylori in Peptic Ulcer Disease. *Jama* **272**: 65-69.

Connors TA & Knox RJ (1995) Prodrugs in cancer chemotherapy. *Stem Cells* **13**: 501-511.

Cooper CE (1999) Nitric oxide and iron proteins. *Biochim Biophys Acta* **1411**: 290-309.

Cortial S, Chaignon P, Iorga BI, Aymerich S, Truan G, Gueguen-Chaignon V, Meyer P, Morera S & Ouazzani J (2010) NADH oxidase activity of Bacillus subtilis nitroreductase NfrA1: insight into its biological role. *FEBS Lett* **584**: 3916-3922.

Cosgrove K, Coutts G, Jonsson IM, Tarkowski A, Kokai-Kun JF, Mond JJ & Foster SJ (2007) Catalase (KatA) and alkyl hydroperoxide reductase (AhpC) have compensatory roles in peroxide stress resistance and are required for survival, persistence, and nasal colonization in Staphylococcus aureus. *J Bacteriol* **189**: 1025-1035.

Cussac V, Ferrero RL & Labigne A (1992) Expression of Helicobacter pylori urease genes in Escherichia coli grown under nitrogen-limiting conditions. *J Bacteriol* **174**: 2466-2473.

De Groote MA, Ochsner UA, Shiloh MU, Nathan C, McCord JM, Dinauer MC, Libby SJ, Vazquez-Torres A, Xu Y & Fang FC (1997) Periplasmic superoxide dismutase protects Salmonella from products of phagocyte NADPH-oxidase and nitric oxide synthase. *Proc Natl Acad Sci U S A* **94**: 13997-14001.

Dryden M, Andrasevic AT, Bassetti M, Bouza E, Chastre J, Cornaglia G, Esposito S, French G, Giamarellou H, Gyssens IC, Nathwani D, Unal S & Voss A (2010) A European survey of antibiotic management of methicillin-resistant Staphylococcus aureus infection: current clinical opinion and practice. *Clin Microbiol Infect* **16 Suppl 1**: 3-30.

Dunn BE, Cohen H & Blaser MJ (1997a) Helicobacter pylori. *Clin Microbiol Rev* **10**: 720-741.

Dunn BE, Vakil NB, Schneider BG, Miller MM, Zitzer JB, Peutz T & Phadnis SH (1997b) Localization of Helicobacter pylori urease and heat shock protein in human gastric biopsies. *Infect Immun* **65**: 1181-1188.

Eaton KA, Brooks CL, Morgan DR & Krakowka S (1991) Essential role of urease in pathogenesis of gastritis induced by Helicobacter pylori in gnotobiotic piglets. *Infect Immun* **59**: 2470-2475.

Eaton KA & Krakowka S (1994) Effect of gastric pH on urease-dependent colonization of gnotobiotic piglets by Helicobacter pylori. *Infect Immun* **62**: 3604-3607.

Eaton KA, Morgan DR & Krakowka S (1992) Motility as a factor in the colonisation of gnotobiotic piglets by Helicobacter pylori. *J Med Microbiol* **37**: 123-127.

Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE & Relman DA (2005) Diversity of the human intestinal microbial flora. *Science* **308**: 1635-1638.

Enroth H, Wreiber K, Rigo R, Risberg D, Uribe A & Engstrand L (1999) In vitro aging of Helicobacter pylori: changes in morphology, intracellular composition and surface properties. *Helicobacter* **4**: 7-16.

Escherich T (1989) The intestinal bacteria of the neonate and breast-fed infant. 1885. *Rev Infect Dis* **11**: 352-356.

Everhart JE (2000) Recent developments in the epidemiology of Helicobacter pylori. *Gastroenterol Clin North Am* **29**: 559-578.

Fang FC (1997) Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. *J Clin Invest* **99**: 2818-2825.

Fang FC (2004) Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nat Rev Microbiol* **2**: 820-832.

Fang FC (2011) Antimicrobial actions of reactive oxygen species. *MBio* **2**.

Farinha P & Gascoyne RD (2005) Helicobacter pylori and MALT lymphoma. *Gastroenterology* **128**: 1579-1605.

Feng Y, Chen CJ, Su LH, Hu S, Yu J & Chiu CH (2008) Evolution and pathogenesis of Staphylococcus aureus: lessons learned from genotyping and comparative genomics. *FEMS Microbiol Rev* **32**: 23-37.

Ferrer-Sueta G & Radi R (2009) Chemical biology of peroxynitrite: kinetics, diffusion, and radicals. *ACS Chem Biol* **4**: 161-177.

Finlay BB & Falkow S (1997) Common themes in microbial pathogenicity revisited. *Microbiol Mol Biol Rev* **61**: 136-169.

Fischbach L & Evans EL (2007) Meta-analysis: the effect of antibiotic resistance status on the efficacy of triple and quadruple first-line therapies for Helicobacter pylori. *Aliment Pharmacol Ther* **26**: 343-357.

Flournoy DJ & Robinson MC (1990) In vitro antimicrobial susceptibilities of 349 methicillin-resistant Staphylococcus aureus isolates from veterans. *Methods Find Exp Clin Pharmacol* **12**: 541-544.

Follmer C (2010) Ureases as a target for the treatment of gastric and urinary infections. *J Clin Pathol* **63**: 424-430.

Foster TJ (2004) The Staphylococcus aureus "superbug". *J Clin Invest* **114**: 1693-1696.

Foxman B (2002) Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. *Am J Med* **113 Suppl 1A**: 5S-13S.

Francis JS, Doherty MC, Lopatin U, Johnston CP, Sinha G, Ross T, Cai M, Hansel NN, Perl T, Ticehurst JR, Carroll K, Thomas DL, Nuermberger E & Bartlett JG (2005) Severe community-onset pneumonia in healthy adults caused by methicillin-resistant Staphylococcus aureus carrying the Panton-Valentine leukocidin genes. *Clin Infect Dis* **40**: 100-107.

Fridovich I (1995) Superoxide radical and superoxide dismutases. *Annu Rev Biochem* **64**: 97-112.

Gagliotti C, Balode A, Baquero F, Degener J, Grundmann H, Gur D, Jarlier V, Kahlmeter G, Monen J, Monnet DL, Rossolini GM, Suetens C, Weist K & Heuer O (2011) Escherichia coli and Staphylococcus aureus: bad news and good news from the European Antimicrobial Resistance Surveillance Network (EARS-Net, formerly EARSS), 2002 to 2009. *Euro Surveill* **16**.

Gao XP, Standiford TJ, Rahman A, Newstead M, Holland SM, Dinauer MC, Liu QH & Malik AB (2002) Role of NADPH oxidase in the mechanism of lung neutrophil sequestration and microvessel injury induced by Gram-negative sepsis: studies in p47phox-/- and gp91phox-/- mice. *J Immunol* **168**: 3974-3982.

Gardner PR, Gardner AM, Martin LA & Salzman AL (1998) Nitric oxide dioxygenase: an enzymic function for flavohemoglobin. *Proc Natl Acad Sci U S A* **95**: 10378-10383.

Gaston B (1999) Nitric oxide and thiol groups. *Biochim Biophys Acta* **1411**: 323-333.

Geslin C, Llanos J, Prieur D & Jeanthon C (2001) The manganese and iron superoxide dismutases protect Escherichia coli from heavy metal toxicity. *Res Microbiol* **152**: 901-905.

Gisbert JP, Calvet X, Gomollon F & Mones J (2005) [Eradication treatment of Helicobacter pylori. Recommendations of the II Spanish Consensus Conference]. *Med Clin (Barc)* **125**: 301-316.

Giuffre A, Sarti P, D'Itri E, Buse G, Soulimane T & Brunori M (1996) On the mechanism of inhibition of cytochrome c oxidase by nitric oxide. *J Biol Chem* **271**: 33404-33408.

Gobert AP, McGee DJ, Akhtar M, Mendz GL, Newton JC, Cheng Y, Mobley HL & Wilson KT (2001) Helicobacter pylori arginase inhibits nitric oxide production by eukaryotic cells: a strategy for bacterial survival. *Proc Natl Acad Sci U S A* **98**: 13844-13849.

Goldstein FW (2000) Antibiotic susceptibility of bacterial strains isolated from patients with community-acquired urinary tract infections in France. Multicentre Study Group. *Eur J Clin Microbiol Infect Dis* **19**: 112-117.

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

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.

Goncalves VL, Nobre LS, Vicente JB, Teixeira M & Saraiva LM (2006) Flavohemoglobin requires microaerophilic conditions for nitrosative protection of Staphylococcus aureus. *FEBS Lett* **580**: 1817-1821.

Gonzalez BE, Hulten KG, Dishop MK, Lamberth LB, Hammerman WA, Mason EO, Jr. & Kaplan SL (2005) Pulmonary manifestations in children with invasive community-acquired Staphylococcus aureus infection. *Clin Infect Dis* **41**: 583-590.

Goodwin A, Kersulyte D, Sisson G, Veldhuyzen van Zanten SJ, Berg DE & Hoffman PS (1998) Metronidazole resistance in Helicobacter pylori is due to null mutations in a gene (rdxA) that encodes an oxygen-insensitive NADPH nitroreductase. *Mol Microbiol* **28**: 383-393.

Gordon RJ & Lowy FD (2008) Pathogenesis of methicillin-resistant Staphylococcus aureus infection. *Clin Infect Dis* **46 Suppl 5**: S350-359.

Graham DY & Fischbach L (2010) Helicobacter pylori treatment in the era of increasing antibiotic resistance. *Gut* **59**: 1143-1153.

Graham DY & Yamaoka Y (2000) Disease-specific Helicobacter pylori virulence factors: the unfulfilled promise. *Helicobacter* **5 Suppl 1**: S3-9; discussion S27-31.

Green NK, Kerr DJ, Mautner V, Harris PA & Searle PF (2004) The nitroreductase/CB1954 enzyme-prodrug system. *Methods Mol Med* **90**: 459-477.

Grundmann H, Aanensen DM, van den Wijngaard CC, Spratt BG, Harmsen D & Friedrich AW (2010) Geographic distribution of Staphylococcus aureus causing invasive infections in Europe: a molecular-epidemiological analysis. *PLoS Med* **7**: e1000215.

Gu M & Imlay JA (2011) The SoxRS response of Escherichia coli is directly activated by redox-cycling drugs rather than by superoxide. *Mol Microbiol* **79**: 1136-1150.

Guay DR (2001) An update on the role of nitrofurans in the management of urinary tract infections. *Drugs* **61**: 353-364.

Guay DR (2008) Contemporary management of uncomplicated urinary tract infections. *Drugs* **68**: 1169-1205.

Ha NC, Oh ST, Sung JY, Cha KA, Lee MH & Oh BH (2001) Supramolecular assembly and acid resistance of Helicobacter pylori urease. *Nat Struct Biol* **8**: 505-509.

Hall BS, Bot C & Wilkinson SR (2011) Nifurtimox activation by trypanosomal type I nitroreductases generates cytotoxic nitrile metabolites. *J Biol Chem* **286**: 13088-13095.

Hausladen A, Gow AJ & Stamler JS (1998) Nitrosative stress: metabolic pathway involving the flavohemoglobin. *Proc Natl Acad Sci U S A* **95**: 14100-14105.

Haynes CA, Koder RL, Miller AF & Rodgers DW (2002) Structures of nitroreductase in three states: effects of inhibitor binding and reduction. *J Biol Chem* **277**: 11513-11520.

Hazell SL, Evans DJ, Jr. & Graham DY (1991) Helicobacter pylori catalase. *J Gen Microbiol* **137**: 57-61.

Hendriks J, Oubrie A, Castresana J, Urbani A, Gemeinhardt S & Saraste M (2000) Nitric oxide reductases in bacteria. *Biochim Biophys Acta* **1459**: 266-273.

Herold BC, Immergluck LC, Maranan MC, Lauderdale DS, Gaskin RE, Boyle-Vavra S, Leitch CD & Daum RS (1998) Community-acquired methicillin-resistant Staphylococcus aureus in children with no identified predisposing risk. *Jama* **279**: 593-598.

Hiramatsu K, Hanaki H, Ino T, Yabuta K, Oguri T & Tenover FC (1997) Methicillin-resistant Staphylococcus aureus clinical strain with reduced vancomycin susceptibility. *J Antimicrob Chemother* **40**: 135-136.

Hogg N & Kalyanaraman B (1999) Nitric oxide and lipid peroxidation. *Biochim Biophys Acta* **1411**: 378-384.

Hooper DC (1999) Mode of action of fluoroquinolones. *Drugs* **58 Suppl 2**: 6-10.

Hooper LV & Gordon JI (2001) Commensal host-bacterial relationships in the gut. *Science* **292**: 1115-1118.

Houghton J & Wang TC (2005) Helicobacter pylori and gastric cancer: a new paradigm for inflammation-associated epithelial cancers. *Gastroenterology* **128**: 1567-1578.

Hu LT & Mobley HL (1990) Purification and N-terminal analysis of urease from Helicobacter pylori. *Infect Immun* **58**: 992-998.

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

Huie RE & Padmaja S (1993) The reaction of no with superoxide. *Free Radic Res Commun* **18**: 195-199.

Huovinen P (2001) Resistance to trimethoprim-sulfamethoxazole. *Clin Infect Dis* **32**: 1608-1614.

Huovinen P, Sundstrom L, Swedberg G & Skold O (1995) Trimethoprim and sulfonamide resistance. *Antimicrob Agents Chemother* **39**: 279-289.

Imlay JA (2006) Iron-sulphur clusters and the problem with oxygen. *Mol Microbiol* **59**: 1073-1082.

Imlay JA (2008) Cellular defenses against superoxide and hydrogen peroxide. *Annu Rev Biochem* **77**: 755-776.

Imlay JA & Linn S (1988) DNA damage and oxygen radical toxicity. *Science* **240**: 1302-1309.

Ishii KJ, Koyama S, Nakagawa A, Coban C & Akira S (2008) Host innate immune receptors and beyond: making sense of microbial infections. *Cell Host Microbe* **3**: 352-363.

Jackson SH, Gallin JI & Holland SM (1995) The p47phox mouse knock-out model of chronic granulomatous disease. *J Exp Med* **182**: 751-758.

Jang S & Imlay JA (2007) Micromolar intracellular hydrogen peroxide disrupts metabolism by damaging iron-sulfur enzymes. *J Biol Chem* **282**: 929-937.

Jenks PJ & Edwards DI (2002) Metronidazole resistance in Helicobacter pylori. *Int J Antimicrob Agents* **19**: 1-7.

Johnson JR, Berggren T & Conway AJ (1993) Activity of a nitrofurazone matrix urinary catheter against catheter-associated uropathogens. *Antimicrob Agents Chemother* **37**: 2033-2036.

Johnson JR, Delavari P & Azar M (1999) Activities of a nitrofurazone-containing urinary catheter and a silver hydrogel catheter against multidrug-resistant bacteria characteristic of catheter-associated urinary tract infection. *Antimicrob Agents Chemother* **43**: 2990-2995.

Jones SA, Chowdhury FZ, Fabich AJ, Anderson A, Schreiner DM, House AL, Autieri SM, Leatham MP, Lins JJ, Jorgensen M, Cohen PS & Conway T (2007) Respiration of Escherichia coli in the mouse intestine. *Infect Immun* **75**: 4891-4899.

Justino MC, Ecobichon C, Fernandes AF, Boneca IG & Saraiva LM (2012) Helicobacter pylori Has an Unprecedented Nitric Oxide Detoxifying System. *Antioxid Redox Signal*.

Kakishima K, Shiratsuchi A, Taoka A, Nakanishi Y & Fukumori Y (2007) Participation of nitric oxide reductase in survival of Pseudomonas aeruginosa in LPS-activated macrophages. *Biochem Biophys Res Commun* **355**: 587-591.

Kallen AJ, Brunkard J, Moore Z, Budge P, Arnold KE, Fosheim G, Finelli L, Beekmann SE, Polgreen PM, Gorwitz R & Hageman J (2009) Staphylococcus aureus community-acquired pneumonia during the 2006 to 2007 influenza season. *Ann Emerg Med* **53**: 358-365.

Kaper JB, Nataro JP & Mobley HL (2004) Pathogenic Escherichia coli. *Nat Rev Microbiol* **2**: 123-140.

Karmali MA, Petric M, Lim C, Fleming PC & Steele BT (1983) Escherichia coli cytotoxin, haemolytic-uraemic syndrome, and haemorrhagic colitis. *Lancet* **2**: 1299-1300.

Keele BB, Jr., McCord JM & Fridovich I (1970) Superoxide dismutase from escherichia coli B. A new manganese-containing enzyme. *J Biol Chem* **245**: 6176-6181.

Kim EJ, Chung HJ, Suh B, Hah YC & Roe JH (1998) Transcriptional and post-transcriptional regulation by nickel of sodN gene encoding nickel-containing superoxide dismutase from Streptomyces coelicolor Muller. *Mol Microbiol* **27**: 187-195.

Kim SO, Orii Y, Lloyd D, Hughes MN & Poole RK (1999) Anoxic function for the Escherichia coli flavohaemoglobin (Hmp): reversible binding of nitric oxide and reduction to nitrous oxide. *FEBS Lett* **445**: 389-394.

Klevens RM, Morrison MA, Nadle J, Petit S, Gershman K, Ray S, Harrison LH, Lynfield R, Dumyati G, Townes JM, Craig AS, Zell ER, Fosheim GE, McDougal LK, Carey RB & Fridkin SK (2007) Invasive methicillin-resistant Staphylococcus aureus infections in the United States. *Jama* **298**: 1763-1771.

Kluytmans J, van Belkum A & Verbrugh H (1997) Nasal carriage of Staphylococcus aureus: epidemiology, underlying mechanisms, and associated risks. *Clin Microbiol Rev* **10**: 505-520.

Knox RJ, Friedlos F & Boland MP (1993) The bioactivation of CB 1954 and its use as a prodrug in antibody-directed enzyme prodrug therapy (ADEPT). *Cancer Metastasis Rev* **12**: 195-212.

Kobori T, Sasaki H, Lee WC, Zenno S, Saigo K, Murphy ME & Tanokura M (2001) Structure and site-directed mutagenesis of a flavoprotein from Escherichia coli that reduces nitrocompounds: alteration of pyridine nucleotide binding by a single amino acid substitution. *J Biol Chem* **276**: 2816-2823.

Koder RL, Haynes CA, Rodgers ME, Rodgers DW & Miller AF (2002) Flavin thermodynamics explain the oxygen insensitivity of enteric nitroreductases. *Biochemistry* **41**: 14197-14205.

Koder RL & Miller AF (1998) Overexpression, isotopic labeling, and spectral characterization of Enterobacter cloacae nitroreductase. *Protein Expr Purif* **13**: 53-60.

Kosek M, Bern C & Guerrant RL (2003) The global burden of diarrhoeal disease, as estimated from studies published between 1992 and 2000. *Bull World Health Organ* **81**: 197-204.

Kosikowska P & Berlicki L (2011) Urease inhibitors as potential drugs for gastric and urinary tract infections: a patent review. *Expert Opin Ther Pat* **21**: 945-957.

Krishnamurthy P, Parlow M, Zitzer JB, Vakil NB, Mobley HL, Levy M, Phadnis SH & Dunn BE (1998) Helicobacter pylori containing only cytoplasmic urease is susceptible to acid. *Infect Immun* **66**: 5060-5066.

Kusters JG, Gerrits MM, Van Strijp JA & Vandenbroucke-Grauls CM (1997) Coccoid forms of Helicobacter pylori are the morphologic manifestation of cell death. *Infect Immun* **65**: 3672-3679.

Kusters JG, van Vliet AH & Kuipers EJ (2006) Pathogenesis of Helicobacter pylori infection. *Clin Microbiol Rev* **19**: 449-490.

Kwon DH, El-Zaatari FA, Kato M, Osato MS, Reddy R, Yamaoka Y & Graham DY (2000) Analysis of rdxA and involvement of additional genes encoding NAD(P)H flavin oxidoreductase (FrxA) and ferredoxin-like protein (FdxB) in metronidazole resistance of Helicobacter pylori. *Antimicrob Agents Chemother* **44**: 2133-2142.

La Carbona S, Sauvageot N, Giard JC, Benachour A, Posteraro B, Auffray Y, Sanguinetti M & Hartke A (2007) Comparative study of the physiological roles of three peroxidases (NADH peroxidase, Alkyl hydroperoxide reductase and Thiol peroxidase) in oxidative stress response, survival inside macrophages and virulence of Enterococcus faecalis. *Mol Microbiol* **66**: 1148-1163.

LaPlante KL, Leonard SN, Andes DR, Craig WA & Rybak MJ (2008) Activities of clindamycin, daptomycin, doxycycline, linezolid, trimethoprim-sulfamethoxazole, and vancomycin against community-associated methicillin-resistant Staphylococcus aureus with inducible clindamycin resistance in murine thigh infection and in vitro pharmacodynamic models. *Antimicrob Agents Chemother* **52**: 2156-2162.

Lewis TA, Newcombe DA & Crawford RL (2004) Bioremediation of soils contaminated with explosives. *J Environ Manage* **70**: 291-307.

Lindmark DG & Muller M (1976) Antitrichomonad action, mutagenicity, and reduction of metronidazole and other nitroimidazoles. *Antimicrob Agents Chemother* **10**: 476-482.

Liochev SI (1999) The mechanism of "Fenton-like" reactions and their importance for biological systems. A biologist's view. *Met Ions Biol Syst* **36**: 1-39.

Liochev SI, Hausladen A & Fridovich I (1999) Nitroreductase A is regulated as a member of the soxRS regulon of Escherichia coli. *Proc Natl Acad Sci U S A* **96**: 3537-3539.

Loewen P (1996) Probing the structure of catalase HPII of Escherichia coli--a review. *Gene* **179**: 39-44.

Lowy FD (1998) Staphylococcus aureus infections. *N Engl J Med* **339**: 520-532.

Lowy FD (2003) Antimicrobial resistance: the example of Staphylococcus aureus. *J Clin Invest* **111**: 1265-1273.

Lundstrom AM & Bolin I (2000) A 26 kDa protein of helicobacter pylori shows alkyl hydroperoxide reductase (AhpC) activity and the mono-cistronic transcription of the gene is affected by pH. *Microb Pathog* **29**: 257-266.

Lushchak VI (2011) Adaptive response to oxidative stress: Bacteria, fungi, plants and animals. *Comp Biochem Physiol C Toxicol Pharmacol* **153**: 175-190.

Lyon BR, Iuorio JL, May JW & Skurray RA (1984) Molecular epidemiology of multiresistant Staphylococcus aureus in Australian hospitals. *J Med Microbiol* **17**: 79-89.

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

Madigan MT, Martinko JM & Parker J, (2000) *Brock Biology of Microorganisms*. Prentice Hall International, Inc, New Jersey.

Malfertheiner P, Megraud F, O'Morain C, Bazzoli F, El-Omar E, Graham D, Hunt R, Rokkas T, Vakil N & Kuipers EJ (2007) Current concepts in the management of Helicobacter pylori infection: the Maastricht III Consensus Report. *Gut* **56**: 772-781.

Malfertheiner P, Megraud F, O'Morain CA, Atherton J, Axon AT, Bazzoli F, Gensini GF, Gisbert JP, Graham DY, Rokkas T, El-Omar EM & Kuipers EJ (2012) Management of Helicobacter pylori infection--the Maastricht IV/ Florence Consensus Report. *Gut* **61**: 646-664.

Marshall BJ, Barrett LJ, Prakash C, McCallum RW & Guerrant RL (1990) Urea protects Helicobacter (Campylobacter) pylori from the bactericidal effect of acid. *Gastroenterology* **99**: 697-702.

Mason RP & Holtzman JL (1975a) The mechanism of microsomal and mitochondrial nitroreductase. Electron spin resonance evidence for nitroaromatic free radical intermediates. *Biochemistry* **14**: 1626-1632.

Mason RP & Holtzman JL (1975b) The role of catalytic superoxide formation in the O2 inhibition of nitroreductase. *Biochem Biophys Res Commun* **67**: 1267-1274.

Mayer-Scholl A, Averhoff P & Zychlinsky A (2004) How do neutrophils and pathogens interact? *Curr Opin Microbiol* **7**: 62-66.

Mazzei T, Cassetta MI, Fallani S, Arrigucci S & Novelli A (2006) Pharmacokinetic and pharmacodynamic aspects of antimicrobial agents for the treatment of uncomplicated urinary tract infections. *Int J Antimicrob Agents* **28 Suppl 1**: S35-41.

McCalla DR, Kaiser C & Green MH (1978) Genetics of nitrofurazone resistance in Escherichia coli. *J Bacteriol* **133**: 10-16.

McCalla DR, Olive P, Tu Y & Fan ML (1975) Nitrofurazone-reducing enzymes in E. coli and their role in drug activation in vivo. *Can J Microbiol* **21**: 1484-1491.

McCalla DR, Reuvers A & Kaiser C (1970) Mode of action of nitrofurazone. *J Bacteriol* **104**: 1126-1134.

McColl KE (2010) Clinical practice. Helicobacter pylori infection. *N Engl J Med* **362**: 1597-1604.

McCoy EC, Rosenkranz HS & Mermelstein R (1981) Evidence for the existence of a family of bacterial nitroreductases capable of activating nitrated polycyclics to mutagens. *Environ Mutagen* **3**: 421-427.

McOsker CC & Fitzpatrick PM (1994) Nitrofurantoin: mechanism of action and implications for resistance development in common uropathogens. *J Antimicrob Chemother* **33 Suppl A**: 23-30.

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

Medzhitov R, Schneider DS & Soares MP (2012) Disease tolerance as a defense strategy. *Science* **335**: 936-941.

Megraud F (2004) H pylori antibiotic resistance: prevalence, importance, and advances in testing. *Gut* **53**: 1374-1384.

Megraud F (2012) The challenge of Helicobacter pylori resistance to antibiotics: the comeback of bismuth-based quadruple therapy. *Therap Adv Gastroenterol* **5**: 103-109.

Mendz GL & Megraud F (2002) Is the molecular basis of metronidazole resistance in microaerophilic organisms understood? *Trends Microbiol* **10**: 370-375.

Michaels ML & Miller JH (1992) The GO system protects organisms from the mutagenic effect of the spontaneous lesion 8-hydroxyguanine (7,8-dihydro-8-oxoguanine). *J Bacteriol* **174**: 6321-6325.

Michalopoulos AS, Livaditis IG & Gougoutas V (2011) The revival of fosfomycin. *Int J Infect Dis* **15**: e732-739.

Montecucco C & Rappuoli R (2001) Living dangerously: how Helicobacter pylori survives in the human stomach. *Nat Rev Mol Cell Biol* **2**: 457-466.

Mostertz J, Scharf C, Hecker M & Homuth G (2004) Transcriptome and proteome analysis of Bacillus subtilis gene expression in response to superoxide and peroxide stress. *Microbiology* **150**: 497-512.

Mottier P, Khong SP, Gremaud E, Richoz J, Delatour T, Goldmann T & Guy PA (2005) Quantitative determination of four nitrofuran metabolites in meat by isotope dilution liquid chromatography-electrospray ionisation-tandem mass spectrometry. *J Chromatogr A* **1067**: 85-91.

Muder RR, Brennen C, Rihs JD, Wagener MM, Obman A, Stout JE & Yu VL (2006) Isolation of Staphylococcus aureus from the urinary tract: association of isolation with

symptomatic urinary tract infection and subsequent staphylococcal bacteremia. *Clin Infect Dis* **42**: 46-50.

Nickel JC (2005) Management of urinary tract infections: historical perspective and current strategies: Part 2--Modern management. *J Urol* **173**: 27-32.

Nicolle LE (2002) Urinary tract infection: traditional pharmacologic therapies. *Am J Med* **113 Suppl 1A**: 35S-44S.

Nicolle LE (2003) Urinary tract infection: traditional pharmacologic therapies. *Dis Mon* **49**: 111-128.

Nobre LS (2010) Unravelling novel modes of antimicrobial action. ITQB - Universidade Nova de Lisboa, PhD Thesis.

Occhialini A, Urdaci M, Doucet-Populaire F, Bebear CM, Lamouliatte H & Megraud F (1997) Macrolide resistance in Helicobacter pylori: rapid detection of point mutations and assays of macrolide binding to ribosomes. *Antimicrob Agents Chemother* **41**: 2724-2728.

Ogston A (1882) Micrococcus Poisoning. *J Anat Physiol* **17**: 24-58.

Olekhnovich IN, Goodwin A & Hoffman PS (2009) Characterization of the NAD(P)H oxidase and metronidazole reductase activities of the RdxA nitroreductase of Helicobacter pylori. *Febs J* **276**: 3354-3364.

Parkinson GN, Skelly JV & Neidle S (2000) Crystal structure of FMN-dependent nitroreductase from Escherichia coli B: a prodrug-activating enzyme. *J Med Chem* **43**: 3624-3631.

Peterson FJ, Mason RP, Hovsepian J & Holtzman JL (1979) Oxygen-sensitive and -insensitive nitroreduction by Escherichia coli and rat hepatic microsomes. *J Biol Chem* **254**: 4009-4014.

Phadnis SH, Parlow MH, Levy M, Ilver D, Caulkins CM, Connors JB & Dunn BE (1996) Surface localization of Helicobacter pylori urease and a heat shock protein homolog requires bacterial autolysis. *Infect Immun* **64**: 905-912.

Pizzolla A, Hultqvist M, Nilson B, Grimm MJ, Eneljung T, Jonsson IM, Verdrengh M, Kelkka T, Gjertsson I, Segal BH & Holmdahl R (2012) Reactive oxygen species produced by the NADPH oxidase 2 complex in monocytes protect mice from bacterial infections. *J Immunol* **188**: 5003-5011.

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.

Poole LB (2005) Bacterial defenses against oxidants: mechanistic features of cysteine-based peroxidases and their flavoprotein reductases. *Arch Biochem Biophys* **433**: 240-254.

Poole RK, Anjum MF, Membrillo-Hernandez J, Kim SO, Hughes MN & Stewart V (1996) Nitric oxide, nitrite, and Fnr regulation of hmp (flavohemoglobin) gene expression in Escherichia coli K-12. *J Bacteriol* **178**: 5487-5492.

Pradenas GA, Paillavil BA, Reyes-Cerpa S, Perez-Donoso JM & Vasquez CC (2012) Reduction of the monounsaturated fatty acid content of Escherichia coli results in increased resistance to oxidative damage. *Microbiology* **158**: 1279-1283.

Race PR, Lovering AL, Green RM, Ossor A, White SA, Searle PF, Wrighton CJ & Hyde EI (2005) Structural and mechanistic studies of Escherichia coli nitroreductase with the antibiotic nitrofurazone. Reversed binding orientations in different redox states of the enzyme. *J Biol Chem* **280**: 13256-13264.

Radi R, Beckman JS, Bush KM & Freeman BA (1991) Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch Biochem Biophys* **288**: 481-487.

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

Rammelkamp CH & Maxon T (1942) Resistance of Staphylococcus aureus to the Action of Penicillin. *Experimental Biology and Medicine* **51**: 386-389

Ramos JL, Gonzalez-Perez MM, Caballero A & van Dillewijn P (2005) Bioremediation of polynitrated aromatic compounds: plants and microbes put up a fight. *Curr Opin Biotechnol* **16**: 275-281.

Ramotar K, Conly JM, Chubb H & Louie TJ (1984) Production of menaquinones by intestinal anaerobes. *J Infect Dis* **150**: 213-218.

Rieger PG, Meier HM, Gerle M, Vogt U, Groth T & Knackmuss HJ (2002) Xenobiotics in the environment: present and future strategies to obviate the problem of biological persistence. *J Biotechnol* **94**: 101-123.

Riley LW, Remis RS, Helgerson SD, McGee HB, Wells JG, Davis BR, Hebert RJ, Olcott ES, Johnson LM, Hargrett NT, Blake PA & Cohen ML (1983) Hemorrhagic colitis associated with a rare Escherichia coli serotype. *N Engl J Med* **308**: 681-685.

Rimbara E, Fischbach LA & Graham DY (2011) Optimal therapy for Helicobacter pylori infections. *Nat Rev Gastroenterol Hepatol* **8**: 79-88.

Roldan MD, Perez-Reinado E, Castillo F & Moreno-Vivian C (2008) Reduction of polynitroaromatic compounds: the bacterial nitroreductases. *FEMS Microbiol Rev* **32**: 474-500.

Rushing MD & Slauch JM (2011) Either periplasmic tethering or protease resistance is sufficient to allow a SodC to protect Salmonella enterica serovar Typhimurium from phagocytic superoxide. *Mol Microbiol* **82**: 952-963.

Russo TA & Johnson JR (2003) Medical and economic impact of extraintestinal infections due to Escherichia coli: focus on an increasingly important endemic problem. *Microbes Infect* **5**: 449-456.

Sachs G & Scott DR (2012) Helicobacter pylori: Eradication or Preservation. *F1000 Med Rep* **4**: 7.

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

Sastry SS & Jayaraman R (1984) Nitrofurantoin-resistant mutants of Escherichia coli: isolation and mapping. *Mol Gen Genet* **196**: 379-380.

Scandalios JG (2002) Oxidative stress responses--what have genome-scale studies taught us? *Genome Biol* **3**: REVIEWS1019.

Scott DR, Marcus EA, Wen Y, Oh J & Sachs G (2007) Gene expression in vivo shows that Helicobacter pylori colonizes an acidic niche on the gastric surface. *Proc Natl Acad Sci U S A* **104**: 7235-7240.

Searle PF, Chen MJ, Hu L, Race PR, Lovering AL, Grove JI, Guise C, Jaberipour M, James ND, Mautner V, Young LS, Kerr DJ, Mountain A, White SA & Hyde EI (2004) Nitroreductase: a prodrug-activating enzyme for cancer gene therapy. *Clin Exp Pharmacol Physiol* **31**: 811-816.

Seaver LC & Imlay JA (2001) Alkyl hydroperoxide reductase is the primary scavenger of endogenous hydrogen peroxide in Escherichia coli. *J Bacteriol* **183**: 7173-7181.

Selgrad M & Malfertheiner P (2011) Treatment of Helicobacter pylori. *Curr Opin Gastroenterol* **27**: 565-570.

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

Seybold U, Kourbatova EV, Johnson JG, Halvosa SJ, Wang YF, King MD, Ray SM & Blumberg HM (2006) Emergence of community-associated methicillin-resistant Staphylococcus

aureus USA300 genotype as a major cause of health care-associated blood stream infections. *Clin Infect Dis* **42**: 647-656.

Shah RR & Wade G (1989) Reappraisal of the risk/benefit of nitrofurantoin: review of toxicity and efficacy. *Adverse Drug React Acute Poisoning Rev* **8**: 183-201.

Shanahan F (2002) The host-microbe interface within the gut. *Best Pract Res Clin Gastroenterol* **16**: 915-931.

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.

Shiloh MU & Nathan CF (2000) Reactive nitrogen intermediates and the pathogenesis of Salmonella and mycobacteria. *Curr Opin Microbiol* **3**: 35-42.

Singh SP, Wishnok JS, Keshive M, Deen WM & Tannenbaum SR (1996) The chemistry of the S-nitrosoglutathione/glutathione system. *Proc Natl Acad Sci U S A* **93**: 14428-14433.

Sisson G, Goodwin A, Raudonikiene A, Hughes NJ, Mukhopadhyay AK, Berg DE & Hoffman PS (2002) Enzymes associated with reductive activation and action of nitazoxanide, nitrofurans, and metronidazole in Helicobacter pylori. *Antimicrob Agents Chemother* **46**: 2116-2123.

Slauch JM (2011) How does the oxidative burst of macrophages kill bacteria? Still an open question. *Mol Microbiol* **80**: 580-583.

Smith TL, Pearson ML, Wilcox KR, Cruz C, Lancaster MV, Robinson-Dunn B, Tenover FC, Zervos MJ, Band JD, White E & Jarvis WR (1999) Emergence of vancomycin resistance in Staphylococcus aureus. Glycopeptide-Intermediate Staphylococcus aureus Working Group. *N Engl J Med* **340**: 493-501.

Smoot DT, Mobley HL, Chippendale GR, Lewison JF & Resau JH (1990) Helicobacter pylori urease activity is toxic to human gastric epithelial cells. *Infect Immun* **58**: 1992-1994.

Spain JC (1995) Biodegradation of nitroaromatic compounds. *Annu Rev Microbiol* **49**: 523-555.

Spiegelhalder C, Gerstenecker B, Kersten A, Schiltz E & Kist M (1993) Purification of Helicobacter pylori superoxide dismutase and cloning and sequencing of the gene. *Infect Immun* **61**: 5315-5325.

St John G & Steinman HM (1996) Periplasmic copper-zinc superoxide dismutase of Legionella pneumophila: role in stationary-phase survival. *J Bacteriol* **178**: 1578-1584.

Stevanin TM, Moir JW & Read RC (2005) Nitric oxide detoxification systems enhance survival of Neisseria meningitidis in human macrophages and in nasopharyngeal mucosa. *Infect Immun* **73**: 3322-3329.

Stevens DL, Bisno AL, Chambers HF, Everett ED, Dellinger P, Goldstein EJ, Gorbach SL, Hirschmann JV, Kaplan EL, Montoya JG & Wade JC (2005) Practice guidelines for the diagnosis and management of skin and soft-tissue infections. *Clin Infect Dis* **41**: 1373-1406.

Stingl K & De Reuse H (2005) Staying alive overdosed: how does Helicobacter pylori control urease activity? *Int J Med Microbiol* **295**: 307-315.

Streeter AJ & Hoener BA (1988) Evidence for the involvement of a nitrenium ion in the covalent binding of nitrofurazone to DNA. *Pharm Res* **5**: 434-436.

Streker K, Freiberg C, Labischinski H, Hacker J & Ohlsen K (2005) Staphylococcus aureus NfrA (SA0367) is a flavin mononucleotide-dependent NADPH oxidase involved in oxidative stress response. *J Bacteriol* **187**: 2249-2256.

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

Symons ZC & Bruce NC (2006) Bacterial pathways for degradation of nitroaromatics. *Nat Prod Rep* **23**: 845-850.

Takeda K, Iizuka M, Watanabe T, Nakagawa J, Kawasaki S & Niimura Y (2007) Synechocystis DrgA protein functioning as nitroreductase and ferric reductase is capable of catalyzing the Fenton reaction. *Febs J* **274**: 1318-1327.

Tavares DA, Sa-Leao R, Miragaia M & de Lencastre H (2010) Large screening of CA-MRSA among Staphylococcus aureus colonizing healthy young children living in two areas (urban and rural) of Portugal. *BMC Infect Dis* **10**: 110.

Tavares P, Pereira AS, Moura JJ & Moura I (2006) Metalloenzymes of the denitrification pathway. *J Inorg Biochem* **100**: 2087-2100.

Taylor DE, Ge Z, Purych D, Lo T & Hiratsuka K (1997) Cloning and sequence analysis of two copies of a 23S rRNA gene from Helicobacter pylori and association of clarithromycin resistance with 23S rRNA mutations. *Antimicrob Agents Chemother* **41**: 2621-2628.

Tenaillon O, Skurnik D, Picard B & Denamur E (2010) The population genetics of commensal Escherichia coli. *Nat Rev Microbiol* **8**: 207-217.

Tlaskalova-Hogenova H, Stepankova R, Hudcovic T, Tuckova L, Cukrowska B, Lodinova-Zadnikova R, Kozakova H, Rossmann P, Bartova J, Sokol D, Funda DP, Borovska D, Rehakova Z, Sinkora J, Hofman J, Drastich P & Kokesova A (2004) Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases. *Immunol Lett* **93**: 97-108.

Triggs-Raine BL & Loewen PC (1987) Physical characterization of katG, encoding catalase HPI of Escherichia coli. *Gene* **52**: 121-128.

Tsuda M, Karita M, Morshed MG, Okita K & Nakazawa T (1994) A urease-negative mutant of Helicobacter pylori constructed by allelic exchange mutagenesis lacks the ability to colonize the nude mouse stomach. *Infect Immun* **62**: 3586-3589.

Udo EE, Pearman JW & Grubb WB (1993) Genetic analysis of community isolates of methicillin-resistant Staphylococcus aureus in Western Australia. *J Hosp Infect* **25**: 97-108.

Ueda O, Kitamura S, Ohashi K, Sugihara K & Ohta S (2003) Xanthine oxidase-catalyzed metabolism of 2-nitrofluorene, a carcinogenic air pollutant, in rat skin. *Drug Metab Dispos* **31**: 367-372.

Underhill DM & Ozinsky A (2002) Phagocytosis of microbes: complexity in action. *Annu Rev Immunol* **20**: 825-852.

Valderas MW & Hart ME (2001) Identification and characterization of a second superoxide dismutase gene (sodM) from Staphylococcus aureus. *J Bacteriol* **183**: 3399-3407.

van Wonderen JH, Burlat B, Richardson DJ, Cheesman MR & Butt JN (2008) The nitric oxide reductase activity of cytochrome c nitrite reductase from Escherichia coli. *J Biol Chem* **283**: 9587-9594.

Vass SO, Jarrom D, Wilson WR, Hyde EI & Searle PF (2009) E. coli NfsA: an alternative nitroreductase for prodrug activation gene therapy in combination with CB1954. *Br J Cancer* **100**: 1903-1911.

Vazquez-Torres A, Jones-Carson J, Mastroeni P, Ischiropoulos H & Fang FC (2000) Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages in vitro. *J Exp Med* **192**: 227-236.

Versalovic J, Shortridge D, Kibler K, Griffy MV, Beyer J, Flamm RK, Tanaka SK, Graham DY & Go MF (1996) Mutations in 23S rRNA are associated with clarithromycin resistance in Helicobacter pylori. *Antimicrob Agents Chemother* **40**: 477-480.

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

Vouloumanou EK, Karageorgopoulos DE, Rafailidis PI, Michalopoulos A & Falagas ME (2011) Trimethoprim/sulfametrole: evaluation of the available clinical and pharmacokinetic/pharmacodynamic evidence. *Int J Antimicrob Agents* **38**: 197-216.

Warren JR & Marshall BJ (1983) Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet* **1**: 1273-1275.

Warren JW, Abrutyn E, Hebel JR, Johnson JR, Schaeffer AJ & Stamm WE (1999) Guidelines for antimicrobial treatment of uncomplicated acute bacterial cystitis and acute pyelonephritis in women. Infectious Diseases Society of America (IDSA). *Clin Infect Dis* **29**: 745-758.

Wertheim HF, Melles DC, Vos MC, van Leeuwen W, van Belkum A, Verbrugh HA & Nouwen JL (2005) The role of nasal carriage in Staphylococcus aureus infections. *Lancet Infect Dis* **5**: 751-762.

Whiteway J, Koziarz P, Veall J, Sandhu N, Kumar P, Hoecher B & Lambert IB (1998) Oxygen-insensitive nitroreductases: analysis of the roles of nfsA and nfsB in development of resistance to 5-nitrofuran derivatives in Escherichia coli. *J Bacteriol* **180**: 5529-5539.

Wolle K & Malfertheiner P (2007) Treatment of Helicobacter pylori. *Best Pract Res Clin Gastroenterol* **21**: 315-324.

Wolpert MK, Althaus JR & Johns DG (1973) Nitroreductase activity of mammalian liver aldehyde oxidase. *J Pharmacol Exp Ther* **185**: 202-213.

Wu CJ, Hsueh PR & Ko WC (2011) A new health threat in Europe: Shiga toxin-producing Escherichia coli O104:H4 infections. *J Microbiol Immunol Infect* **44**: 390-393.

Wu MS, Chow LP, Lin JT & Chiou SH (2008) Proteomic identification of biomarkers related to Helicobacter pylori-associated gastroduodenal disease: challenges and opportunities. *J Gastroenterol Hepatol* **23**: 1657-1661.

Yakoob J, Abid S, Abbas Z & Jafri SN (2010) Antibiotic susceptibility patterns of Helicobacter pylori and triple therapy in a high-prevalence area. *Br J Biomed Sci* **67**: 197-201.

Yost FJ, Jr. & Fridovich I (1973) An iron-containing superoxide dismutase from Escherichia coli. *J Biol Chem* **248**: 4905-4908.

Youn HD, Kim EJ, Roe JH, Hah YC & Kang SO (1996) A novel nickel-containing superoxide dismutase from Streptomyces spp. *Biochem J* **318 (Pt 3)**: 889-896.

Zanotti G & Cendron L (2010) Functional and structural aspects of Helicobacter pylori acidic stress response factors. *IUBMB Life* **62**: 715-723.

Zenno S, Kobori T, Tanokura M & Saigo K (1998a) Conversion of NfsA, the major Escherichia coli nitroreductase, to a flavin reductase with an activity similar to that of Frp, a flavin reductase in Vibrio harveyi, by a single amino acid substitution. *J Bacteriol* **180**: 422-425.

Zenno S, Kobori T, Tanokura M & Saigo K (1998b) Purification and characterization of NfrA1, a Bacillus subtilis nitro/flavin reductase capable of interacting with the bacterial luciferase. *Biosci Biotechnol Biochem* **62**: 1978-1987.

Zenno S, Koike H, Kumar AN, Jayaraman R, Tanokura M & Saigo K (1996a) Biochemical characterization of NfsA, the Escherichia coli major nitroreductase exhibiting a high amino acid sequence homology to Frp, a Vibrio harveyi flavin oxidoreductase. *J Bacteriol* **178**: 4508-4514.

Zenno S, Koike H, Tanokura M & Saigo K (1996b) Conversion of NfsB, a minor Escherichia coli nitroreductase, to a flavin reductase similar in biochemical properties to FRase I, the major flavin reductase in Vibrio fischeri, by a single amino acid substitution. *J Bacteriol* **178**: 4731-4733.

Zenno S, Koike H, Tanokura M & Saigo K (1996c) Gene cloning, purification, and characterization of NfsB, a minor oxygen-insensitive nitroreductase from Escherichia

coli, similar in biochemical properties to FRase I, the major flavin reductase in Vibrio fischeri. *J Biochem* **120**: 736-744.

Zheng M, Wang X, Templeton LJ, Smulski DR, LaRossa RA & Storz G (2001) DNA microarray-mediated transcriptional profiling of the Escherichia coli response to hydrogen peroxide. *J Bacteriol* **183**: 4562-4570.

Zumft WG (2005) Nitric oxide reductases of prokaryotes with emphasis on the respiratory, heme-copper oxidase type. *J Inorg Biochem* **99**: 194-215.

Chapter 2|

Carbon Monoxide:

the new face of an old molecule

“Science progresses best when observations force us to alter our preconceptions” - Vera Rubin

2.1 Chemical, physical and biological properties of carbon monoxide

The discovery of carbon monoxide (CO) is usually attributed to Joseph Priestley in the late 18th century. Even so, the existence of a "poisonous gas" resultant from burning coals has been known since Greek and Roman times. Interestingly, CO is thought to be one of the oldest molecules on Earth that might have participated in the origin of life along with oxygen and nitrogen (Schlesinger & Miller, 1983; Miyakawa *et al.*, 2002). This colourless, odourless and tasteless gas results in nature from the incomplete oxidation of organic materials such as wood, natural gas, coal and tobacco.

The CO molecule has ten valence electrons, four from carbon and six from oxygen, distributed among the three bonds and one lone pair on each atom. CO is considered a chemically stable molecule due to the high strength of the triple bond. Thus, chemical reduction of CO requires temperatures well above 100ºC and the oxidation of CO to carbon dioxide ($CO + H_2O \rightarrow CO_2 + H_2$) only occurs at high temperatures via the metal-catalyzed water-gas-shift reaction (Greenwood & Earnshaw, 1997; Wu & Wang, 2005). Albeit CO is sparingly soluble in water, at standard temperature and pressure, it dissolves in organic solvents such as chloroform, benzene, ethanol and methanol (Von Berg, 1999). CO does not readily react with the most common reducing agent – hydrogen, and the reaction with dioxygen is slow and has a high energy of activation (213 kJ mol^{-1}) (Wu & Wang, 2005). Nevertheless, CO

Table 2.1| Properties of Carbon Monoxide.

Properties of CO	
Molecular weight (g/mol)	**28.01**
Melting point (ºC)	**-205**
Boiling point (ºC)	**-191.5**
Density (g/L)	**1.25**
Interatomic distance (pm)	**112.8**
Dissociation energy (kJ/mol)	**1072**
Solubility in water (mM)	**1***

*at room temperature

Adapted from (Greenwood NN & Earnshaw A, 1997).

reacts with transition metals in low oxidation states as molybdenum, manganese, ruthenium, nickel, iron or cobalt (Boczkowski *et al.*, 2006). Hence, CO readily forms relatively stable metal carbonyls complexes through the donation of a pair of electrons from the lone pair on the carbon atom (Greenwood & Earnshaw, 1997; Piantadosi, 2002; Romão *et al.*, 2012).

In biological systems, CO binds preferentially and almost exclusively to transition metals that are present in structural and functional proteins, particularly to ferrous haem (Boczkowski *et al.*, 2006). In fact, due to the high affinity of CO to haemoglobin of red blood cells, the harmful action of CO in mammals has been extensively documented. In 1857, Claude Bernard first described the physiological effects of CO on the human body through its reversible binding to haemoglobin, forming carboxyl-haemoglobin (COHb) and leading to asphyxia (Bernard, 1857). After inhalation via the lungs, CO reaches the blood stream where it competes with dioxygen for binding to the four haem iron centres of haemoglobin (Haldane, 1927; Wu & Wang, 2005). The toxicity of CO is attributed to its much higher affinity (200–230 times) for haemoglobin when compared with that of dioxygen. This results in lower number of dioxygen molecules transported and released in the tissues, leading to hypoxia and, ultimately, to cell death and organ failure (Haldane, 1927; Wu & Wang, 2005). In particular, the brain and the heart are the most vulnerable to CO toxicity due to their high demand for dioxygen (Wu & Wang, 2005; Prockop & Chichkova, 2007).

The “silent killer” CO has been recognized as one of the most common causes of accidental and suicidal poisoning, being responsible for a great number of deaths worldwide (Raub *et al.*, 2000; Prockop & Chichkova, 2007). The adverse health outcome of CO intoxication depends on the concentration and duration of exposure since the binding of CO to haemoglobin is reversible,

as once ceased CO dissociates from haemoglobin and is eliminated through the lungs. Therefore, dioxygen is the "natural antidote" and a therapy with 100% dioxygen should be undertaken in order to hasten CO elimination (Raub *et al.*, 2000; Prockop & Chichkova, 2007; Weaver, 2009).

The atmosphere contains approximately 10 parts per million (ppm) of CO, but it can reach 50 ppm in metropolitan areas (Godin *et al.*, 1972; Otterbein, 2009). Currently, USA entities have stipulated a limit of 50 ppm for 8h/day, which generate a safe COHb level of 8-10% (Chin & Otterbein, 2009). While the basal level of COHb in normal tissues is less than 1%, it can reach 3% in non-smokers and may be as high as 10-15% in smokers (Von Berg, 1999; Ryter & Otterbein, 2004). COHb levels up to 10% caused by CO inhalation are usually asymptomatic. As COHb increases above 20%, symptoms of CO poisoning as headache, dizziness and nausea begin to appear, whilst coma and seizures due to cerebral edema are common for levels greater that 40%, and death is likely above 60% (Figure 2.1) (Romão *et al.*, 2012).

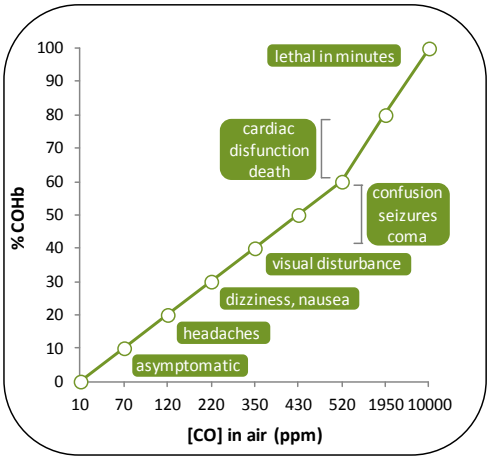


Figure 2.1| Symptoms after CO inhalation in humans. COHb levels obtained after inhalation of air with increasing concentrations of CO, and the consequently symptoms observed. Adapted (Romão CC *et al.*, 2012).

Besides CO binding to haemoglobin, which represents 80% of CO loaded in the body, CO also binds and compromises the activities of other mammalian haemoproteins, such as cytochrome *c* oxidase and cytochrome

P450. Nonetheless, the contribution of such mechanisms to the overall toxicity of CO remains controversial, since inhalation of sufficient CO to inhibit respiration and xenobiotic metabolism may likely be preceded by death due to tissue hypoxia (Piantadosi, 2002; Wu & Wang, 2005; Otterbein, 2009).

2.2 Endogenous production and physiological role of carbon monoxide

Paradoxically to the well-known toxicity associated to CO, it was found around 1950s that CO is endogenously generated as a normal cellular function in humans, and more importantly, it is involved in crucial physiological functions (Sjostrand, 1949). The major source of endogenous CO production is through the haem oxygenase (HO) activity, which accounts for about 86% of total CO produced in humans. The remaining CO arises from a mixture of sources, which includes photo-oxidation, lipid peroxidation and xenobiotics (Rodgers *et al.*, 1994; Ryter & Otterbein, 2004; Mann & Motterlini, 2007). Interestingly, the daily production of CO measured in exhaled air of adults can reach 6 mL, and this value may increase in patients with diseases such as asthma, bronchiectasis, cystic fibrosis and diabetes (Mann & Motterlini, 2007).

HO is the rate-limiting enzyme in haem degradation, catalyzing the oxidation of the α-methene bridge carbon of haem, and generating equimolar quantities of three biological active products: CO, ferrous iron and biliverdin (Figure 2.2) (Tenhunen *et al.*, 1970). Biliverdin is, in turn, reduced to bilirubin by biliverdin reductase (Figure 2.2), and both metabolites have been reported to possess strong antioxidant potential, by scavenging peroxyl radicals and inhibiting lipid peroxidation (Baranano *et al.*, 2002; Abraham & Kappas, 2005; Bilban *et al.*, 2008). More recently, bilirubin was shown to have a potent antiviral activity (Santangelo *et al.*, 2012). The iron formed is rapidly sequestered into the iron-storage protein - ferritin, thus preventing Fenton

reaction and conferring cytoprotection against oxidative stress (Bilban *et al.*, 2008). Contrary to the common perception that CO is a harmful molecule, several reports had demonstrated that CO is a primarily signaling molecule with many physiological roles in the neuronal, cardiovascular, and immune systems, as well as in the respiratory, reproductive, gastrointestinal and urogenital apparatus (Wu & Wang, 2005; Kim *et al.*, 2006; Gullotta *et al.*, 2012a; Gullotta *et al.*, 2012b). Indeed, CO confers cytoprotection by modulating signaling pathways, such as the soluble guanylate cyclase (sGS), mitogen-activated protein kinases (MAPKs) and calcium-activated potassium (K_{ca}) (Ryter *et al.*, 2006). In turn, these pathways mediate the vasoregulatory, anti-inflammatory, anti-apoptotic and anti-proliferative effects of CO (Ryter & Otterbein, 2004).

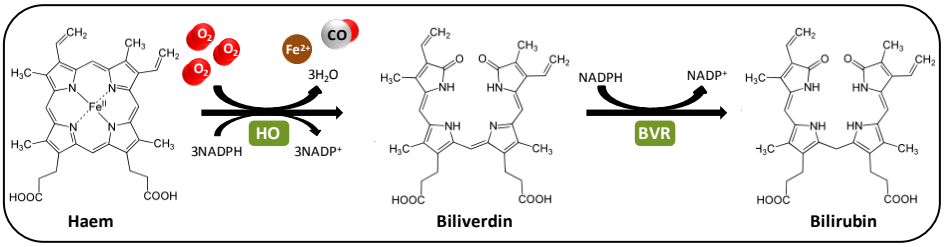


Figure 2.2| Oxidative haem degradation. Haem oxygenase (HO) catalyses the oxidation of haem, in the presence of three molecules of dioxygen and NADPH, yielding biliverdin, ferrous iron and CO. Biliverdin is subsequently converted to bilirubin by biliverdin reductase (BVR).

In mammals, three HO isoforms have been identified, namely HO-1, HO-2, and HO-3 (Maines, 1988; McCoubrey *et al.*, 1992; McCoubrey *et al.*, 1997; Ryter *et al.*, 2006). The inducible HO-1 presents low percentage of sequence identity and similarity with the two constitutively isoforms (HO-2 and HO-3), which share about 90% of amino acid similarity (Wagener *et al.*, 2003). HO-1 isoform occurs at undetectable levels in most tissues until being

stimulated by stress conditions that threaten cellular homeostasis, such as hypoxia, oxidative stress (*e.g.* hydrogen peroxide), UV radiation, bacterial endotoxins (*e.g.* LPS) and pro-inflammatory cytokines (*e.g.* IL-1, IL-6 and INF-γ) (Ryter *et al.*, 2002; Wagener *et al.*, 2003; Wu & Wang, 2005). The central role of HO-1 in the re-establishment of homeostasis has been demonstrated in models in which deletion of this isoform worsens the pathologic outcome of diseases, such as severe sepsis, atherosclerosis and myocardial infarction (Yet *et al.*, 2003; Chung *et al.*, 2008; Soares & Bach, 2009). Importantly, a human case of HO-1 deficiency exhibited severe growth retardation, persistent haemolytic anemia, abnormal coagulation/fibrinolysis system, and a persistent endothelial damage, that lead to patient death (Yachie *et al.*, 1999). Notably, several reports reveal that exogenous administration of CO gas exert beneficial effect that to some extent mimics the cytoprotective action elicited by HO-1 stimulation (Kim *et al.*, 2006). In fact, CO administration seems to be able to rescue HO-1 deficiency by suppressing the inflammatory response that otherwise would lead to enhanced pathologic progression of disease (Ryter *et al.*, 2006; Bilban *et al.*, 2008; Chung *et al.*, 2008; Chin & Otterbein, 2009; Soares & Bach, 2009). In HO-1-deficient mice challenged with endotoxin, a component of the Gram-negative cell wall, results in exaggerated organ injury and increased mortality (Wiesel *et al.*, 2000). The exogenous administration of CO to animals previously treated with endotoxin improves survival, and suggests the importance of CO in mediating protection against bacterial infections (Otterbein *et al.*, 2000).

The HO-2 is present under normal conditions in organs and tissues, such as brain, liver and endothelium, and its function is associated with neurotransmission and regulation of vascular tone (Maines, 1997; Foresti & Motterlini, 1999). Although HO-2 is considered a constitutive isoform, its

transcription is responsive to opiates and adrenal glucocorticoids (Maines & Panahian, 2001; Wagener *et al.*, 2003). Nevertheless, HO-2 has been pointed out to be a less likely candidate than HO-1 to have a central role in protection against disease, with a maximal activity of less than 10% of that reported to HO-1 (Maines & Panahian, 2001; Wagener *et al.*, 2003).

The biological function of HO-3, which is found in most tissues, remains to be elucidated as it is not able to degrade haem, and its role seems to be limited to haem binding and/or sensing (McCoubrey *et al.*, 1997; Wagener *et al.*, 2003). It is proposed that HO-3 derives from the retrotransposition of HO-2 as HO-3 does not contain introns (McCoubrey *et al.*, 1997).

2.3 Carbon Monoxide-Releasing Molecules

The discovery that CO endogenously produced has an essential role in biological systems triggered great interest in the potential pharmacological application of this molecule (Wu & Wang, 2005). However, the therapeutic use of CO gas seems unlikely, since inhalation of CO exhibited some drawbacks related to the manipulation and direct delivery of this gas to a specific tissue in an accurate, safe and measurable way (Figure 2.3) (Foresti *et al.*, 2008; Motterlini & Otterbein, 2010). In order to overcome these limitations, Motterlini and co-workers developed a novel class of compounds capable of safely storing and releasing controlled amounts of CO in biological systems, the so-called Carbon Monoxide-Releasing Molecules (CO-RMs) (Figure 2.3) (Motterlini *et al.*, 2002). In particular, metal carbonyl complexes (MCC) seem to be the most suitable class of compounds acting as carriers of CO (Romão *et al.*, 2012). These complexes contain a transition metal, such as manganese, molybdenum, ruthenium or iron, coordinated to carbonyl groups, and have

the ability to release CO to myoglobin/haemoglobin as indicated by the formation of carboxy-myoglobin/haemoglobin (Motterlini *et al.*, 2002; Choi *et al.*, 2003; Stanford *et al.*, 2003; Motterlini *et al.*, 2005a; Taille *et al.*, 2005; Chen *et al.*, 2009).

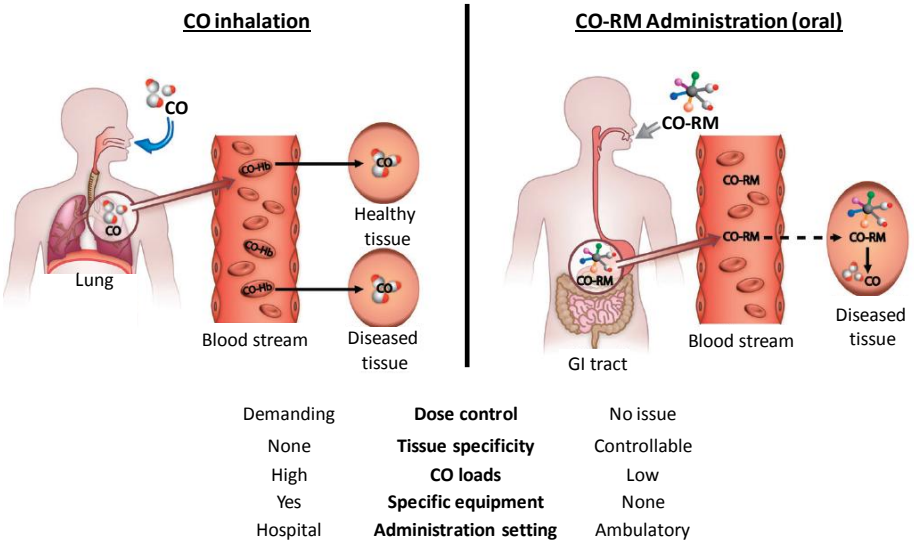


Figure 2.3| Advantages and disadvantages of CO gas and CO-RM for the therapeutic delivery of CO to diseased tissues. Adapted (Romão CC *et al.*, 2012).

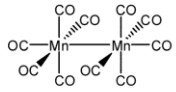
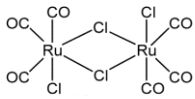
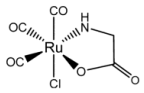
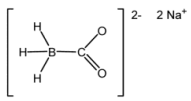
Several CO-RMs confirmed the beneficial effects of CO gas in different animal models of disease. Importantly, the biological properties of CO-RMs are attributed to the release of CO since addition of reduced myoglobin, and/or the substitution of CO-RM by an inactive form (iCO-RM), which does not contain carbonyl (CO) groups, impairs its bioactivity (Motterlini *et al.*, 2005a). Interestingly, CO-RMs seem to be able to avoid the biological trapping by haemoglobin, since the intravenous application of CO-RMs on mice does not increase COHb to dangerous levels (COHb levels < 10%) (Vera *et al.*, 2005; Foresti *et al.*, 2008).

The first CO-RMs to be produced were the diamanganese decacarbonyl [$Mn_2(CO)_{10}$] molecule and the tricarbonyldichloro ruthenium(II) dimer ([$Ru(CO)_3Cl_2]_2$), subsequently termed CORM-1 and CORM-2, respectively (Motterlini *et al.*, 2002). These CO-RMs are insoluble in water, but while CORM-1 requires light to release CO, CORM-2 rapidly promotes carboxy-myoglobin formation upon dissolution in dimethyl sulfoxide (DMSO) (Motterlini *et al.*, 2002; Motterlini *et al.*, 2003). Once dissolved in DMSO, CORM-2 converts from a dimer to a mixture of two distinct monomers, [$Ru(CO)_3Cl_2(DMSO)$] and [$Ru(CO)_2Cl_2(DMSO)_2$], with a ratio of 40:60, respectively (Motterlini *et al.*, 2002). Upon stimulation with light, CORM-1 induces vasodilatation of cerebral arterioles and attenuates the coronary vasoconstriction in isolated rat hearts perfused (Fiumana *et al.*, 2003; Xi *et al.*, 2004; Motterlini *et al.*, 2005a). This manganese-based carbonyl has also been described to play a protective role in acute renal failure by increasing renal blood flow and glomerular filtration rate in rats (Arregui *et al.*, 2004). Furthermore, in mice CORM-1 reduced the migration, rolling and adhesion of neutrophils to the endothelium in the inflammatory site (Freitas *et al.*, 2006). CORM-2 has been extensively used in several approaches and shown to mimic the known physiological functions of CO. In particular, CORM-2 elicits vasodilatation of aortic rings pre-contracted, attenuates coronary vasoconstriction in hearts, and prevents the increase of the mean arterial pressure in a rat model of acute hypertension (Motterlini *et al.*, 2002). Importantly, administration of CORM-2 to rats did not apparently change the levels of oxy-haemoglobin (Motterlini *et al.*, 2002). Interestingly, aortic transplantation in HO-1 deficient mice treated with CORM-2 significantly improved survival (Chen *et al.*, 2009). In addition, this ruthenium-based carbonyl has been demonstrated to have an anti-inflammatory effect as it is

able to reduce the production of ROS and NO by LPS-stimulated macrophages through inhibition of the haemoproteins activity, NADPH oxidase and iNOS, respectively (Sawle *et al.*, 2005; Srisook *et al.*, 2006). Moreover, CORM-2 impairs neutrophil adhesion to human umbilical vein endothelial cells (Cepinskas *et al.*, 2008; Sun *et al.*, 2008b). Furthermore, treatment of thermally injured mice with CORM-2 significantly attenuated neutrophils accumulation in kidney, liver and small intestine of burned mice (Sun *et al.*, 2007; Liu *et al.*, 2008; Sun *et al.*, 2008a). The beneficial role of this CO-releasing molecule in cell proliferation was also reported, with CORM-2 inhibiting the proliferation of both human airway and pulmonary artery smooth muscle cells *in vitro* (Stanford *et al.*, 2003; Taille *et al.*, 2005). The reno-protective, anti-carcinogenic and anti-apoptotic effects of CORM-2 have been also reported in several studies (Choi *et al.*, 2003; Vera *et al.*, 2005; Li *et al.*, 2006; Allanson & Reeve, 2007).

To improve CO-RMs compatibility with biological system, water soluble CO-releasing molecules have been synthesized and their biological activities assessed in various experimental models (Motterlini *et al.*, 2005a). The most promising results were obtained with the tricarbonylchloro(glycinate) ruthenium (II) ($Ru(CO)_3Cl$(glycinate)) and the sodium borancarbonate $Na_2[H_3BCO_2]$, known as CORM-3 and CORM-A1, respectively. CORM-3 is, like CORM-2, a ruthenium-based carbonyl and a CO releaser by ligand substitution, to which the presence of a glycine coordinated to the metal centre confers water solubility (Table 2.2). CORM-A1 is a boron-containing carboxylic acid that releases CO upon hydrolysis. This non-transition metal-based compound liberates CO at a slow rate, which is dependent on pH and temperature (Table 2.2). In fact, the CO release is accelerated by decrease of pH and increase of temperature (Motterlini *et al.*, 2003; Motterlini *et al.*, 2005b).

Table 2.2| Properties and physiological functions of the most relevant CO-releasing molecules (CO-RMs).

CO-RM	Chemical structure	Solubility	CO release mode	Physiological functions
CORM-1		Ethanol DMSO	Light-dependent $t_{1/2}$ < 1min	Vasodilator Reno-protective Anti-inflammatory
CORM-2		Ethanol DMSO	Ligand substitution $t_{1/2}$ ~1min	Vasodilator Reno-protective Anti-inflammatory Anti-carcinogenic Anti-apoptotic Anti-proliferative
CORM-3		Water	Ligand substitution $t_{1/2}$ ~ 1min	Vasodilator Reno-protective Cardio-protective Anti-inflammatory Anti-ischemic Anti-platelet aggregation
CORM-A1		Water	pH and temperature dependent $t_{1/2}$ ~ 21min (pH=7.4, 37ºC)	Vasodilator Reno-protective Anti-ischemic Anti-apoptotic

Adapted from (Motterlini *et al.*, 2005a; Gullotta *et al.*, 2012a).

The chemical differences of the two water soluble CO-RMs dictate the way CO causes vasorelaxation and hypotension: CORM-3 elicits a prompt and rapid vasodilatory effect whereas CORM-A1 promotes mild vasorelaxation and hypotension (Foresti *et al.*, 2004). Nevertheless, both CO-RMs are associated with anti-ischaemic effects that have been observed primarily in cardiac and renal tissues of rats and rabbits (Musameh *et al.*, 2006; Sandouka *et al.*, 2006). CORM-3 has also been shown to prevent hypoxia-reoxygenation damage in rat cardiomyocytes and protect against ischaemia/reperfusion injury in isolated hearts of rats (Clark *et al.*, 2003; Varadi *et al.*, 2007). In addition, administration of CORM-3 to mice reduced the infarct size and prolonged the

viability of cardiac allografts following transplantation (Clark *et al.*, 2003; Guo *et al.*, 2004). Reno-protective effects of CORM-3 were observed in mice following ischaemia-induced renal failure (Vera *et al.*, 2005). Additionally, CORM-3 contributed to mesenteric vasodilatation in cirrhotic rats (Bolognesi *et al.*, 2007). Notably, CORM-3 induced inhibition of human platelet aggregation *in vitro* (Chlopicki *et al.*, 2006). CORM-3 also has an anti-inflammatory action by decreasing the level of NO production and reducing the TNF-α release in microglia and macrophage cells (Sawle *et al.*, 2005; Bani-Hani *et al.*, 2006a, 2006b). Moreover, intravenous injection of CORM-3 in mice lowered the number of neutrophil extravasation and attenuated acute inflammation (Urquhart *et al.*, 2007). Finally, CORM-3 was shown to have anti-proliferative and anti-apoptotic effects in porcine aortic endothelial cells and in primate peripheral blood mononuclear cells (Motterlini *et al.*, 2005a). Unlike CORM-3, the biological activities of CORM-A1 remain largely unexplored. This boron-containing compound has been described to produce vasodilatation in isolated vessels from rats, and to promote reduction in arterial pressure *in vivo* at a rate that correlates with the CO release. Moreover, the inactive form (sodium borate) does not promote any beneficial effect (Motterlini *et al.*, 2005b). Moreover, administration of CORM-A1 causes an increase in renal blood flow and a decrease in vascular resistance in mice (Ryan *et al.*, 2006). Recently, CORM-A1 has been suggested as a novel strategy for the treatment of multiple sclerosis, since it was able to prevent the development of clinical and histological signs of the disease in rats (Fagone *et al.*, 2011).

Overall, CO-RMs seem to be able to mimic the beneficial effects of CO gas (Table 2.2), representing so far the safest way to delivery CO to tissues and organ. The range of pathological conditions, in which these compounds may be beneficial, is shown in Figure 2.4 (Motterlini & Otterbein, 2010; Gullotta *et al.*, 2012a).

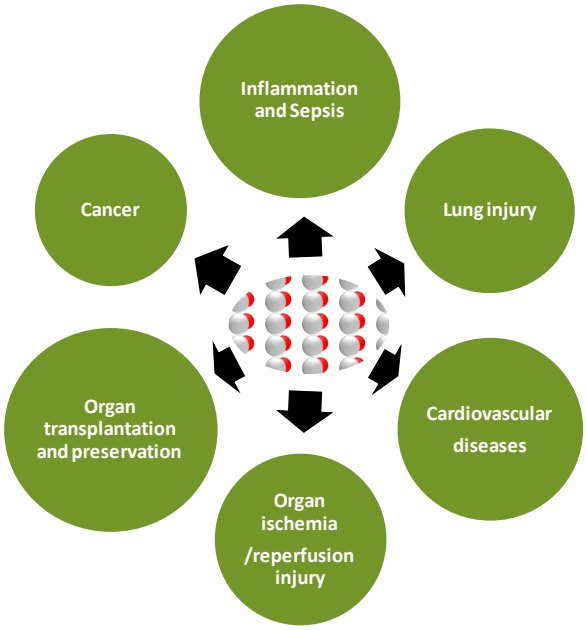


Figure 2.4| Potential medical applications of CO-RMs.

2.4 Carbon monoxide and bacteria

2.4.1 Carbon monoxide metabolism

The use of CO as a source of carbon and energy for growth has been reported for several bacteria (Ragsdale, 2004; Oelgeschlager & Rother, 2008). In both aerobic and anaerobic CO-metabolizing bacteria, the oxidation of CO to carbon dioxide is done by CO dehydrogenase (CODH) ($CO + H_2O \rightarrow CO_2 + 2H^+ + 2e^-$), which also catalyses the reverse reaction. CODH contains iron and either molybdenum (aerobes) or nickel (anaerobes) in its active site (Oelgeschlager & Rother, 2008). Interestingly, CO oxidation is coupled to several respiratory processes in which the reducing equivalents generated are received by electron acceptors into a respiratory chain, leading to the reduction of the terminal electron acceptor (King & Weber, 2007; Oelgeschlager & Rother, 2008). The best studied aerobic CO-oxidizing bacteria

are *Oligotropha carboxidovorans and Pseudomonas thermocarboxydovorans,* which contain a CO-insensitive respiratory chain and the reducing equivalents from CO oxidation are used for oxygen reduction or, in some cases, nitrate reduction (Jacobitz & Meyer, 1989; King & Weber, 2007; Oelgeschlager & Rother, 2008). Under anaerobic conditions, the respiratory processes that are coupled to CO metabolism distinguish three groups of CO-utilizing bacteria: (i) sulphate reducers (*e.g. Desulfovibrio vulgaris*), in which CO oxidation yields carbon dioxide and molecular hydrogen that is subsequently utilized for sulphate reduction ($4CO + SO_4^{2-} + H^+ \rightarrow 4CO_2 + HS^+$); (ii) hydrogenogens, such as the well characterized *Rhodospirillum rubrum*, which oxidize CO and reduce the protons derived from water to hydrogen. This process occurs in the absence of an electron acceptor and is analogous to the water-gas-shift reaction ($CO + H_2O \rightarrow CO_2 + H_2$); and (iii) acetogens, such as *Moorella thermoacetica*, that contain a bifunctional CODH/acetyl-coenzyme A synthase, which produces acetyl-CoA from a methyl group, coenzyme A and CO ($4CO + 2H_2O \rightarrow CH_3COO^- + 2CO_2 + H^+$) (Ragsdale, 2004; Drake *et al.*, 2008; Oelgeschlager & Rother, 2008; Gullotta *et al.*, 2012b).

2.4.2 Carbon monoxide sensing

The ability of bacteria to sense small gaseous molecules is a key attribute that allows adaptation to different environments. Since CO binds virtually all haem-containing proteins in ferrous state, haem-based transcriptional regulators might be considered potential CO sensors. The best studied CO sensor is CooA from *R. rubrum*, a haem-containing transcriptional factor that in the presence of exogenous CO binds to DNA and promotes the CODH gene expression (Bonam *et al.*, 1989; Aono, 2003). An interesting feature of this CO-sensing protein is its ability to discriminate between

gaseous molecules, as in spite of binding both CO and NO, only the CO-CooA promotes CODH expression (Reynolds *et al.*, 2000). Other CO-sensor is RcoM, a haem-containing protein present in both aerobic and anaerobic bacteria. The best studied is RcoM from *Burkholderia xenovorans,* that binds both CO and NO but not oxygen, and respond to exogenous CO by promoting transcription of several genes involved in CO oxidation (Kerby *et al.*, 2008; Marvin *et al.*, 2008). The two-component system DosST from *M. tuberculosis* is another example of a CO-sensor. In this case, CO is primarily sensed through DosS that induces the Dos regulon, whereas DosT plays a less prominent role. Binding of CO to the haem-containing sensors DosST induces the dormancy regulon shifting the bacteria to a latent state, characterized to be unresponsive to drug therapy (Kumar *et al.*, 2007; Kumar *et al.*, 2008). As usually observed for bacterial sensors, DosS and DosT are not specific as CO sensors, since they are also responsive to dioxygen and NO (Kumar *et al.*, 2007; Kumar *et al.*, 2008).

Other haem-based proteins have also been reported to bind CO, albeit its role in responding to CO is still unknown. This is the case of the dioxygen sensors FixL of *Sinorhizobium meliloti,* EcDos of *E. coli* and AxPDEA1 of *Acetobacter xylinum* (Gilles-Gonzalez *et al.*, 1994; Delgado-Nixon *et al.*, 2000; Chang *et al.*, 2001; Sasakura *et al.*, 2002).

2.4.3 Production of Carbon Monoxide

Like humans (see section 2.2), several bacteria encompass haem oxygenases that upon haem degradation produce CO along with iron and biliverdin. When compared with the eukaryotic counterparts, bacterial HOs are smaller and soluble enzymes that lack the C-terminal membrane anchor (Frankenberg-Dinkel, 2004). The first bacterial HO was identified in

Corynebacterium diphtheria due to the high homology with the human HO-1 (70% similarity) (Schmitt, 1997), and later recognized in several other bacteria (Table 2.3). The major role of bacterial HOs has been attributed to acquisition of iron, which is essential for growth and particularly important for pathogens during the infection process (Frankenberg-Dinkel, 2004). Indeed, bacterial HOs promote degradation of the haem imported from the external environment, via the haem acquisition systems, providing iron to the cell (Wandersman & Stojiljkovic, 2000; Andrews *et al.*, 2003). Hence, the use of HOs as a target for antimicrobials has been explored. Several compounds were shown to have high affinity to HOs of *P. aeruginosa* and *N. meningitidis* with some of them being able to inhibit bacterial growth when using haemoglobin as the only iron source (Furci *et al.*, 2007).

In addition to the role in iron acquisition, HOs seem also to protect bacterial cells against haem toxicity. In particular, strains of *B. anthracis* and *N. meningitidis* deleted in HOs genes exhibit growth impairment in the presence of haemin (Zhu *et al.*, 2000a; Skaar *et al.*, 2006). Furthermore, HO in the anaerobic bacterium *C. tetani* has been proposed to act as dioxygen scavenger in the process of wound colonization (Bruggemann *et al.*, 2004).

Besides iron, the fate of other HO-products remains elusive. Due to the lack of bacterial genes encoding homologues of mammalian biliverdin reductase, it seems unlikely that, biliverdin is metabolized to bilirubin. It has been suggested that in bacteria, biliverdin acts as antioxidant such as in mammalians or is only a waste product (Wilks, 2002; Frankenberg-Dinkel, 2004). The relevance of the CO produced by bacteria remains so far unclear. Whether CO in bacteria is able to participate in signaling pathways as it occurs in eukaryotes remains to be investigated (Frankenberg-Dinkel, 2004).

Table 2.3| Bacterial haem oxygenases.

Bacteria	HO	References
Bacillus anthracis	IsdG	(Skaar *et al.*, 2006)
B. subtillis	HmoA HmoB	(Gaballa & Helmann, 2012)
Bradyrhizobium japonicum	HmuQ HmuD	(Puri & O'Brian, 2006)
Brucella abortus	BhuQ	(Ojeda *et al.*, 2012)
Campylobacter jejuni	ChuZ	(Ridley *et al.*, 2006)
Clostridium perfringens	HemO	(Hassan *et al.*, 2010)
Clostridium tetani	HemT	(Bruggemann *et al.*, 2004)
C. diphtheria	HmuO	(Schmitt, 1997)
E. coli O157:H7	ChuS	(Suits *et al.*, 2005)
H. pylori	HugZ	(Guo *et al.*, 2008)
N. meningitidis	HemO	(Zhu *et al.*, 2000a; Zhu *et al.*, 2000b)
P. aeruginosa	PigA BphO	(Ratliff *et al.*, 2001) (Wegele *et al.*, 2004)
Shigella dysenteriae	ShuS	(Wyckoff *et al.*, 2005)
S. aureus	IsdG IsdH	(Skaar *et al.*, 2004)
Synechocystis	HO-1 HO-2	(Migita *et al.*, 2003) (Zhang *et al.*, 2005)
Vibrio cholerae	HutZ	(Wyckoff *et al.*, 2004)

Adapted from (Tavares *et al.*, 2012).

2.5 Carbon monoxide and Carbon Monoxide-Releasing Molecules as bactericides

The first indication that CO could affect bacteria viability came out in the early 1970s, when it was observed that CO administration to *E. coli* cultures caused a decrease in DNA replication (Weigel & Englund, 1975). Another indirect evidence was the report that CO administration to packing

systems on meat preservation resulted in less bacterial growth (Brashears & Brooks, 2006). An interesting work performed by Otterbein and collaborators found that exogenous treatment of macrophages with CO gas enhances the phagocytic clearance of *E. coli*. However, in this work the results obtained were associated with an increase of surface expression of Toll-like receptor 4 in macrophages, and not directly with the antimicrobial activity of CO (Otterbein *et al.*, 2005).

More recently, administration of both CO gas and CO-RMs was shown to strongly decrease the cell viability of the Gram-negative *E. coli* and Gram-positive *S. aureus* (Nobre *et al.*, 2007; Nobre, 2010). In particular, ruthenium based carbonyls CORM-2 and CORM-3 revealed to have an efficient bactericidal activity, as only after 30 min of treatment 50-80% of bacteria were killed, and after 4 h cells were unable to resume growth (Nobre *et al.*, 2007). Other CO-RMs, as ALF021 and ALF062, which contain manganese and molybdenum, respectively, also reduced significantly the viability of the two bacterial species. Importantly, cultures supplemented with haemoglobin, a CO scavenger, abolished the bactericidal effect of all CO-RMs. In addition, inactive forms, *i.e.* compounds devoid of CO display any antimicrobial effect. Hence, the antimicrobial action of these compounds seems to be dependent on the CO-released (Nobre *et al.*, 2007). Interestingly, CO-releasers have the ability to get inside bacteria cells as demonstrated by the increase of metal content in bacterial cells exposed to CO-RMs (Nobre *et al.*, 2007; Davidge *et al.*, 2009).

An interesting feature reported in the initial study performed by Nobre *et al.* is the higher effectiveness of CO-RMs as bactericides under anaerobic conditions. This fact was rationalized considering the preferential binding of CO to the ferrous state of proteins, which are predominant under anoxic conditions. Noteworthy that pathogen colonization occurs in nearly-anaerobic

environments. Furthermore, these results also reveal that other targets of CO-RMs action should be considered as inhibition of cell growth is not to be restricted to impairment of aerobic respiratory chain, which is the classic target of CO in bacteria (Nobre *et al.*, 2007).

Davidge and collaborators also showed that CORM-3 impairs *E. coli* growth, but they do not observe the same effect for the CO gas (Davidge *et al.*, 2009). The authors reported that CORM-3-derived CO binds to the terminal oxidases of the *E. coli* respiratory chain, leading to *c.* 50s% inhibition of respiration after 10 min (Davidge *et al.*, 2009). Studies on *P. aeruginosa* also show that both CORM-3 and CO gas are able to impair bacterial growth, even in antibiotic-resistant strains (Desmard *et al.*, 2009). In this study, CORM-3 treated *P. aeruginosa* exhibited lower dioxygen consumption, in accordance with the inhibition of the respiratory chain by the CO-releasing molecule (Desmard *et al.*, 2009). Interestingly, Ru-based compounds (CORM-2 and CORM-3) have been reported to exhibit higher bactericidal activity than the borocarbonates (CORM-A1) towards *P. aeruginosa* (Desmard *et al.*, 2011).

More recently, it was reported that the microaerophilic pathogen *C. jejuni* was not able to be eradicated by CORM-3, although its respiratory chain was inhibited (Smith *et al.*, 2011).

The work presented in Chapter 5 of this thesis demonstrates that CO-RMs are also able to eliminate *H. pylori*.

The potential antimicrobial action of CO-RMs has also been explored in infection models. In particular, Chung *et al.* demonstrated that administration of CORM-2 to HO-1 deficient mice, which display enhanced susceptibility to polymicrobial infections, decreases the number of viable bacteria and rescues mice from sepsis-induced lethality (Chung *et al.*, 2008). Moreover, administration of CORM-3 to *P. aeruginosa* infected mice prolongs their

survival and decrease bacterial cell viability in the host spleen (Desmard *et al.*, 2009). More recently, a Ru-based compound (ALF492) was shown to protect mice against malaria infection caused by the parasite *Plasmodium falciparum* (Pena *et al.*, 2012).

Altogether, the data so far available clearly demonstrated that CO-RMs have antimicrobial properties.

2.5.1 Transcriptional response of bacteria to Carbon Monoxide-Releasing Molecules

The discovery that CO and particularly CO-RMs have a potent bactericidal effect triggered the interest to find bacterial CO targets, besides the respiratory chain. Therefore, the transcriptional response of *E. coli* to Ru-based carbonyls (CORM-2 and CORM-3) was analysed by DNA microarray experiments (Davidge *et al.*, 2009; Nobre *et al.*, 2009). In agreement with the higher susceptibility of *E. coli* to CORM-2 under anaerobic conditions, a higher number of genes were altered under these conditions (Nobre *et al.*, 2007). In contrast, cells treated with CORM-3 are more sensitive under aerobic conditions, and consequently a higher number of genes were affected when *E. coli* cells were grown in the presence of oxygen (Davidge *et al.*, 2009). This discrepancy could be related to different experimental conditions utilized to performed microarray experiments. Nevertheless, both microarray experiments reveal several potential targets for CO-RMs antimicrobial action. Figure 2.5 summarizes the metabolic pathways affected by CO-releasing molecules under aerobic and anaerobic conditions.

Under aerobic conditions most of genes involved in the citric acid cycle were repressed (*sdhCD*, *fumA* and *sucAB*), indicating that the energy production is strongly compromised in the presence of CO-RMs (Davidge *et*

al., 2009; Nobre *et al.*, 2009). Additionally, a marked decreased in *cyo* operon expression, that encodes cytochrome oxidase, observed in cells growing in the presence of CORM-3 indicates that respiration is impaired (Davidge *et al.*, 2009).

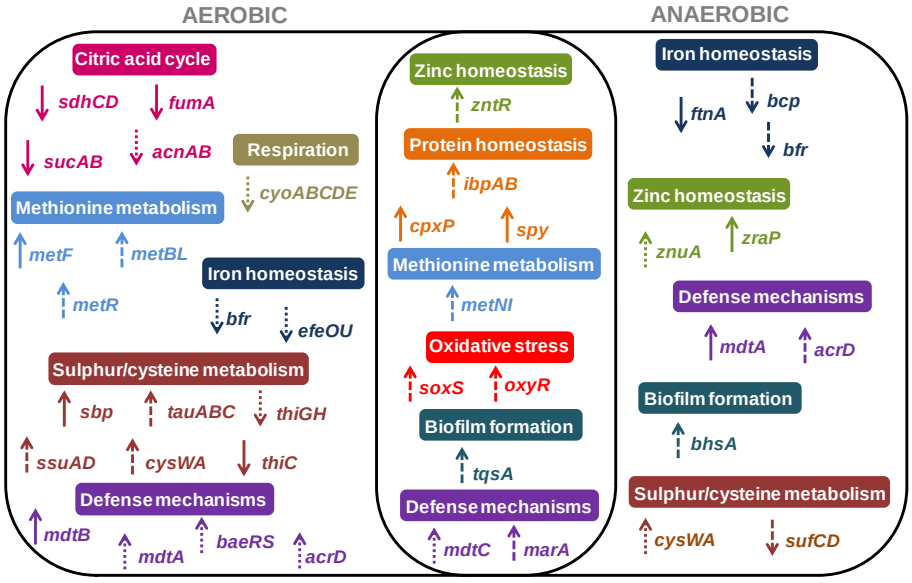


Figure 2.5| Metabolic pathways affected by CO-RMs. *E. coli* genes whose transcription was modified by CORM-2 and CORM-3 in cells grown aerobically (left side) or anaerobically (right side). The middle panel depicts genes commonly altered by both oxygen growth conditions. Arrows pointing up used for genes that were induced, and arrows pointing down for genes repressed by CORM-2 (dashed line), CORM-3 (dotted line) and by both COR-Ms (solid line). Adapted (Tavares *et al.*, 2012).

CO-RMs were found to interfere with the methionine biosynthetic pathway. Indeed, both CO-RMs induce the expression of *metF,* and CORM-2 up-regulated other methionine related genes as *metNI*, *metBL* and *metR,* which regulates the transcription of genes for methionine biosynthesis (Davidge *et al.*, 2009; Nobre *et al.*, 2009). Additionally, *E. coli* strains deleted in

metR, *metI* and *metN* exhibited enhanced susceptibility to CORM-2 (Nobre *et al.*, 2009).

Alterations in the sulphate and sulphur metabolism were also observed upon CO-RMs treatment. In fact, transcription of *sbp*, involved in sulphate uptake, which is crucial to cysteine biosynthesis, was induced by both CO-releasing molecules under aerobic conditions (Davidge *et al.*, 2009; Nobre *et al.*, 2009). Moreover, CORM-2 up-regulated genes involved in sulphur metabolism, such as *tauABC*, *ssuAD* and *cysWA* (Nobre *et al.*, 2009). In addition, *thiC* which is involved in thiamine biosynthesis, where cysteine is required, was repressed in *E. coli* cultures exposed to both CO-releasing molecules (Davidge *et al.*, 2009; Nobre *et al.*, 2009). In the presence of CORM-3, expression of other *E. coli* genes involved in thiamine biosynthesis were repressed namely, *thiG* and *thiH* (Davidge *et al.*, 2009).

Interestingly, genes related to metal homeostasis were altered upon CO-RMs treatment. In fact, *ftnA*, encoding an iron storage protein was repressed by both CO-releasing molecules under anaerobic conditions (Davidge *et al.*, 2009; Nobre *et al.*, 2009). Moreover, the transcription of *bfr* and *bcp*, coding for bacterioferritin and bacterioferritin comigratory protein, respectively, were repressed by CORM-2 (Nobre *et al.*, 2009). Under aerobic conditions, *bfr* was also down regulated by CORM-3 (Davidge *et al.*, 2009). Genes involved with zinc homeostasis were also altered, such as the zinc binding protein *zraP,* which was induced by both CO-RMs under anaerobic conditions (Davidge *et al.*, 2009; Nobre *et al.*, 2009). CORM-3 also induces the expression of other zinc-related gene, *znuA* under anaerobic conditions, and CORM-2 up-regulated the transcription of *zntR*, a zinc-responsive transcriptional regulator under both oxygen growth conditions (Davidge *et al.*,

2009; Nobre *et al.*, 2009). However, no alteration of the intracellular zinc content was detected in CORM-3 treated cells (Davidge *et al.*, 2009).

Microarray data also revealed that several genes involved in protein folding, namely, heat-shock proteins, chaperons and proteases were up-regulated by CO-RMs. In particular, the genes *ibpA* and *ibpB*, two heat-shock proteins that are directly connected with protein stability, were highly induced by CORM-2 in cells grown under both oxygen conditions (Nobre *et al.*, 2009). Accordingly, the deletion of these genes decreased the resistance of *E. coli* cells to CORM-2. In addition, CO-RMs seem to affect the integrity of cell envelope proteins as periplasmic combact stress gene *cpxP,* and an envelope stress induced periplasmic gene *spy* were up-regulated by both CO-RMs under both oxygen growth conditions (Davidge *et al.*, 2009; Nobre *et al.*, 2009).

Further studies also revealed that CORM-2 interferes with biofilm formation. In fact, genes related to biofilm formation such as *tqsA* and *bhsA* were up-regulated by CORM-2, and its deletion increased bacteria resistance to CO releaser (Nobre *et al.*, 2009). Genes related to defense mechanism as the multidrug efflux system (*mdtABC*) were also induced by CO-releasing molecules (Davidge *et al.*, 2009; Nobre *et al.*, 2009). Interestingly, genes directly related to oxidative stress such as *oxyR* and *soxS* were up-regulated by CORM-2 under both oxic and anoxic conditions, suggesting a possible involvement of ROS in CO-RMs action (Nobre *et al.*, 2009).

Although, CO and CO-releasing molecules potential for managing microbial infections is now recognized, further studies need to be performed in order understand the CO-RMs action and their targets in bacteria. Chapter 4 of this thesis will present recent findings that unravel the mode of CO-RMs action, and which changes the perception that CO-RMs are only CO-delivers.

2.6 References

Abraham NG & Kappas A (2005) Heme oxygenase and the cardiovascular-renal system. *Free Radic Biol Med* **39**: 1-25.

Allanson M & Reeve VE (2007) Carbon monoxide signalling reduces photocarcinogenesis in the hairless mouse. *Cancer Immunol Immunother* **56**: 1807-1815.

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

Aono S (2003) Biochemical and biophysical properties of the CO-sensing transcriptional activator CooA. *Acc Chem Res* **36**: 825-831.

Arregui B, Lopez B, Garcia Salom M, Valero F, Navarro C & Fenoy FJ (2004) Acute renal hemodynamic effects of dimanganese decacarbonyl and cobalt protoporphyrin. *Kidney Int* **65**: 564-574.

Bani-Hani MG, Greenstein D, Mann BE, Green CJ & Motterlini R (2006a) A carbon monoxide-releasing molecule (CORM-3) attenuates lipopolysaccharide- and interferon-gamma-induced inflammation in microglia. *Pharmacol Rep* **58 Suppl**: 132-144.

Bani-Hani MG, Greenstein D, Mann BE, Green CJ & Motterlini R (2006b) Modulation of thrombin-induced neuroinflammation in BV-2 microglia by carbon monoxide-releasing molecule 3. *J Pharmacol Exp Ther* **318**: 1315-1322.

Baranano DE, Rao M, Ferris CD & Snyder SH (2002) Biliverdin reductase: a major physiologic cytoprotectant. *Proc Natl Acad Sci U S A* **99**: 16093-16098.

Bernard C, (1857) *Lecons sur les effets des substances toxiques et medicamenteuses*. Bailliere, Paris.

Bilban M, Haschemi A, Wegiel B, Chin BY, Wagner O & Otterbein LE (2008) Heme oxygenase and carbon monoxide initiate homeostatic signaling. *J Mol Med (Berl)* **86**: 267-279.

Boczkowski J, Poderoso JJ & Motterlini R (2006) CO-metal interaction: Vital signaling from a lethal gas. *Trends Biochem Sci* **31**: 614-621.

Bolognesi M, Sacerdoti D, Piva A, Di Pascoli M, Zampieri F, Quarta S, Motterlini R, Angeli P, Merkel C & Gatta A (2007) Carbon monoxide-mediated activation of large-conductance calcium-activated potassium channels contributes to mesenteric vasodilatation in cirrhotic rats. *J Pharmacol Exp Ther* **321**: 187-194.

Bonam D, Lehman L, Roberts GP & Ludden PW (1989) Regulation of carbon monoxide dehydrogenase and hydrogenase in Rhodospirillum rubrum: effects of CO and oxygen on synthesis and activity. *J Bacteriol* **171**: 3102-3107.

Brashears MM & Brooks MM (2006) Effect of meat packaging techologies on the safety and spoilage-indicating characteristics of ground beef - phase 1:safety characteristics.

Bruggemann H, Bauer R, Raffestin S & Gottschalk G (2004) Characterization of a heme oxygenase of Clostridium tetani and its possible role in oxygen tolerance. *Arch Microbiol* **182**: 259-263.

Cepinskas G, Katada K, Bihari A & Potter RF (2008) Carbon monoxide liberated from carbon monoxide-releasing molecule CORM-2 attenuates inflammation in the liver of septic mice. *Am J Physiol Gastrointest Liver Physiol* **294**: G184-191.

Chang AL, Tuckerman JR, Gonzalez G, Mayer R, Weinhouse H, Volman G, Amikam D, Benziman M & Gilles-Gonzalez MA (2001) Phosphodiesterase A1, a regulator of cellulose synthesis in Acetobacter xylinum, is a heme-based sensor. *Biochemistry* **40**: 3420-3426.

Chen B, Guo L, Fan C, Bolisetty S, Joseph R, Wright MM, Agarwal A & George JF (2009) Carbon monoxide rescues heme oxygenase-1-deficient mice from arterial thrombosis in allogeneic aortic transplantation. *Am J Pathol* **175**: 422-429.

Chin BY & Otterbein LE (2009) Carbon monoxide is a poison... to microbes! CO as a bactericidal molecule. *Curr Opin Pharmacol* **9**: 490-500.

Chlopicki S, Olszanecki R, Marcinkiewicz E, Lomnicka M & Motterlini R (2006) Carbon monoxide released by CORM-3 inhibits human platelets by a mechanism independent of soluble guanylate cyclase. *Cardiovasc Res* **71**: 393-401.

Choi BM, Pae HO & Chung HT (2003) Nitric oxide priming protects nitric oxide-mediated apoptosis via heme oxygenase-1 induction. *Free Radic Biol Med* **34**: 1136-1145.

Chung SW, Liu X, Macias AA, Baron RM & Perrella MA (2008) Heme oxygenase-1-derived carbon monoxide enhances the host defense response to microbial sepsis in mice. *J Clin Invest* **118**: 239-247.

Clark JE, Naughton P, Shurey S, Green CJ, Johnson TR, Mann BE, Foresti R & Motterlini R (2003) Cardioprotective actions by a water-soluble carbon monoxide-releasing molecule. *Circ Res* **93**: e2-8.

Davidge KS, Sanguinetti G, Yee CH, Cox AG, McLeod CW, Monk CE, Mann BE, Motterlini R & Poole RK (2009) Carbon monoxide-releasing antibacterial molecules target respiration and global transcriptional regulators. *J Biol Chem* **284**: 4516-4524.

Delgado-Nixon VM, Gonzalez G & Gilles-Gonzalez MA (2000) Dos, a heme-binding PAS protein from Escherichia coli, is a direct oxygen sensor. *Biochemistry* **39**: 2685-2691.

Desmard M, Davidge KS, Bouvet O, Morin D, Roux D, Foresti R, Ricard JD, Denamur E, Poole RK, Montravers P, Motterlini R & Boczkowski J (2009) A carbon monoxide-releasing molecule (CORM-3) exerts bactericidal activity against Pseudomonas aeruginosa and improves survival in an animal model of bacteraemia. *Faseb J* **23**: 1023-1031.

Desmard M, Foresti R, Morin D, Dagouassat M, Berdeaux A, Denamur E, Crook SH, Mann BE, Scapens D, Montravers P, Boczkowski J & Motterlini R (2011) Differential antibacterial activity against Pseudomonas aeruginosa by carbon monoxide-releasing molecules. *Antioxid Redox Signal* **16**: 153-163.

Drake HL, Gossner AS & Daniel SL (2008) Old acetogens, new light. *Ann N Y Acad Sci* **1125**: 100-128.

Fagone P, Mangano K, Quattrocchi C, Motterlini R, Di Marco R, Magro G, Penacho N, Romao CC & Nicoletti F (2011) Prevention of clinical and histological signs of proteolipid protein (PLP)-induced experimental allergic encephalomyelitis (EAE) in mice by the water-soluble carbon monoxide-releasing molecule (CORM)-A1. *Clin Exp Immunol* **163**: 368-374.

Fiumana E, Parfenova H, Jaggar JH & Leffler CW (2003) Carbon monoxide mediates vasodilator effects of glutamate in isolated pressurized cerebral arterioles of newborn pigs. *Am J Physiol Heart Circ Physiol* **284**: H1073-1079.

Foresti R, Bani-Hani MG & Motterlini R (2008) Use of carbon monoxide as a therapeutic agent: promises and challenges. *Intensive Care Med* **34**: 649-658.

Foresti R, Hammad J, Clark JE, Johnson TR, Mann BE, Friebe A, Green CJ & Motterlini R (2004) Vasoactive properties of CORM-3, a novel water-soluble carbon monoxide-releasing molecule. *Br J Pharmacol* **142**: 453-460.

Foresti R & Motterlini R (1999) The heme oxygenase pathway and its interaction with nitric oxide in the control of cellular homeostasis. *Free Radic Res* **31**: 459-475.

Frankenberg-Dinkel N (2004) Bacterial heme oxygenases. *Antioxid Redox Signal* **6**: 825-834.

Freitas A, Alves-Filho JC, Secco DD, Neto AF, Ferreira SH, Barja-Fidalgo C & Cunha FQ (2006) Heme oxygenase/carbon monoxide-biliverdin pathway down regulates neutrophil rolling, adhesion and migration in acute inflammation. *Br J Pharmacol* **149**: 345-354.

Furci LM, Lopes P, Eakanunkul S, Zhong S, MacKerell AD, Jr. & Wilks A (2007) Inhibition of the bacterial heme oxygenases from Pseudomonas aeruginosa and Neisseria meningitidis: novel antimicrobial targets. *J Med Chem* **50**: 3804-3813.

Gaballa A & Helmann JD (2012) Bacillus subtilis Fur represses one of two paralogous haem-degrading monooxygenases. *Microbiology* **157**: 3221-3231.

Gilles-Gonzalez MA, Gonzalez G, Perutz MF, Kiger L, Marden MC & Poyart C (1994) Heme-based sensors, exemplified by the kinase FixL, are a new class of heme protein with distinctive ligand binding and autoxidation. *Biochemistry* **33**: 8067-8073.

Godin G, Wright G & Shephard RJ (1972) Urban exposure to carbon monoxide. *Arch Environ Health* **25**: 305-313.

Greenwood NN & Earnshaw A, (1997) *Chemistry of the Elements*. Butterworth Heinemann.

Gullotta F, di Masi A & Ascenzi P (2012a) Carbon monoxide: an unusual drug. *IUBMB Life* **64**: 378-386.

Gullotta F, di Masi A, Coletta M & Ascenzi P (2012b) CO metabolism, sensing, and signaling. *Biofactors* **38**: 1-13.

Guo Y, Guo G, Mao X, Zhang W, Xiao J, Tong W, Liu T, Xiao B, Liu X, Feng Y & Zou Q (2008) Functional identification of HugZ, a heme oxygenase from Helicobacter pylori. *BMC Microbiol* **8**: 226.

Guo Y, Stein AB, Wu WJ, Tan W, Zhu X, Li QH, Dawn B, Motterlini R & Bolli R (2004) Administration of a CO-releasing molecule at the time of reperfusion reduces infarct size in vivo. *Am J Physiol Heart Circ Physiol* **286**: H1649-1653.

Haldane JB (1927) Carbon Monoxide as a Tissue Poison. *Biochem J* **21**: 1068-1075.

Hassan S, Ohtani K, Wang R, Yuan Y, Wang Y, Yamaguchi Y & Shimizu T (2010) Transcriptional regulation of hemO encoding heme oxygenase in Clostridium perfringens. *J Microbiol* **48**: 96-101.

Jacobitz S & Meyer O (1989) Removal of CO dehydrogenase from Pseudomonas carboxydovorans cytoplasmic membranes, rebinding of CO dehydrogenase to depleted membranes, and restoration of respiratory activities. *J Bacteriol* **171**: 6294-6299.

Kerby RL, Youn H & Roberts GP (2008) RcoM: a new single-component transcriptional regulator of CO metabolism in bacteria. *J Bacteriol* **190**: 3336-3343.

Kim HP, Ryter SW & Choi AM (2006) CO as a cellular signaling molecule. *Annu Rev Pharmacol Toxicol* **46**: 411-449.

King GM & Weber CF (2007) Distribution, diversity and ecology of aerobic CO-oxidizing bacteria. *Nat Rev Microbiol* **5**: 107-118.

Kumar A, Deshane JS, Crossman DK, Bolisetty S, Yan BS, Kramnik I, Agarwal A & Steyn AJ (2008) Heme oxygenase-1-derived carbon monoxide induces the Mycobacterium tuberculosis dormancy regulon. *J Biol Chem* **283**: 18032-18039.

Kumar A, Toledo JC, Patel RP, Lancaster JR, Jr. & Steyn AJ (2007) Mycobacterium tuberculosis DosS is a redox sensor and DosT is a hypoxia sensor. *Proc Natl Acad Sci U S A* **104**: 11568-11573.

Li MH, Cha YN & Surh YJ (2006) Carbon monoxide protects PC12 cells from peroxynitrite-induced apoptotic death by preventing the depolarization of mitochondrial transmembrane potential. *Biochem Biophys Res Commun* **342**: 984-990.

Liu DM, Sun BW, Sun ZW, Jin Q, Sun Y & Chen X (2008) Suppression of inflammatory cytokine production and oxidative stress by CO-releasing molecules-liberated CO in the small intestine of thermally-injured mice. *Acta Pharmacol Sin* **29**: 838-846.

Maines MD (1988) Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. *Faseb J* **2**: 2557-2568.

Maines MD (1997) The heme oxygenase system: a regulator of second messenger gases. *Annu Rev Pharmacol Toxicol* **37**: 517-554.

Maines MD & Panahian N (2001) The heme oxygenase system and cellular defense mechanisms. Do HO-1 and HO-2 have different functions? *Adv Exp Med Biol* **502**: 249-272.

Mann BE & Motterlini R (2007) CO and NO in medicine. *Chem Commun (Camb)*: 4197-4208.

Marvin KA, Kerby RL, Youn H, Roberts GP & Burstyn JN (2008) The transcription regulator RcoM-2 from Burkholderia xenovorans is a cysteine-ligated hemoprotein that undergoes a redox-mediated ligand switch. *Biochemistry* **47**: 9016-9028.

McCoubrey WK, Jr., Ewing JF & Maines MD (1992) Human heme oxygenase-2: characterization and expression of a full-length cDNA and evidence suggesting that the two HO-2 transcripts may differ by choice of polyadenylation signal. *Arch Biochem Biophys* **295**: 13-20.

McCoubrey WK, Jr., Huang TJ & Maines MD (1997) Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. *Eur J Biochem* **247**: 725-732.

Migita CT, Zhang X & Yoshida T (2003) Expression and characterization of cyanobacterium heme oxygenase, a key enzyme in the phycobilin synthesis. Properties of the heme complex of recombinant active enzyme. *Eur J Biochem* **270**: 687-698.

Miyakawa S, Yamanashi H, Kobayashi K, Cleaves HJ & Miller SL (2002) Prebiotic synthesis from CO atmospheres: implications for the origins of life. *Proc Natl Acad Sci U S A* **99**: 14628-14631.

Motterlini R, Clark JE, Foresti R, Sarathchandra P, Mann BE & Green CJ (2002) Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities. *Circ Res* **90**: E17-24.

Motterlini R, Mann BE & Foresti R (2005a) Therapeutic applications of carbon monoxide-releasing molecules. *Expert Opin Investig Drugs* **14**: 1305-1318.

Motterlini R, Mann BE, Johnson TR, Clark JE, Foresti R & Green CJ (2003) Bioactivity and pharmacological actions of carbon monoxide-releasing molecules. *Curr Pharm Des* **9**: 2525-2539.

Motterlini R & Otterbein LE (2010) The therapeutic potential of carbon monoxide. *Nat Rev Drug Discov* **9**: 728-743.

Motterlini R, Sawle P, Hammad J, Bains S, Alberto R, Foresti R & Green CJ (2005b) CORM-A1: a new pharmacologically active carbon monoxide-releasing molecule. *Faseb J* **19**: 284-286.

Musameh MD, Fuller BJ, Mann BE, Green CJ & Motterlini R (2006) Positive inotropic effects of carbon monoxide-releasing molecules (CO-RMs) in the isolated perfused rat heart. *Br J Pharmacol* **149**: 1104-1112.

Nobre LS (2010) Unravelling novel modes of antimicrobial action. ITQB - Universidade Nova de Lisboa, PhD Thesis.

Nobre LS, Al-Shahrour F, Dopazo J & Saraiva LM (2009) Exploring the antimicrobial action of a carbon monoxide-releasing compound through whole-genome transcription profiling of Escherichia coli. *Microbiology* **155**: 813-824.

Nobre LS, Seixas JD, Romao CC & Saraiva LM (2007) Antimicrobial action of carbon monoxide-releasing compounds. *Antimicrob Agents Chemother* **51**: 4303-4307.

Oelgeschlager E & Rother M (2008) Carbon monoxide-dependent energy metabolism in anaerobic bacteria and archaea. *Arch Microbiol* **190**: 257-269.

Ojeda JF, Martinson D, Menscher E & Roop RM, 2nd (2012) The bhuQ gene encodes a heme oxygenase that contributes to the ability of Brucella abortus 2308 to use heme as an iron source and is regulated by Irr. *J Bacteriol*.

Otterbein LE (2009) The evolution of carbon monoxide into medicine. *Respir Care* **54**: 925-932.

Otterbein LE, Bach FH, Alam J, Soares M, Tao Lu H, Wysk M, Davis RJ, Flavell RA & Choi AM (2000) Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat Med* **6**: 422-428.

Otterbein LE, May A & Chin BY (2005) Carbon monoxide increases macrophage bacterial clearance through Toll-like receptor (TLR)4 expression. *Cell Mol Biol (Noisy-le-grand)* **51**: 433-440.

Pena AC, Penacho N, Mancio-Silva L, Neres R, Seixas JD, Fernandes AC, Romao CC, Mota MM, Bernardes GJ & Pamplona A (2012) A novel carbon monoxide-releasing molecule fully protects mice from severe malaria. *Antimicrob Agents Chemother* **56**: 1281-1290.

Piantadosi CA (2002) Biological chemistry of carbon monoxide. *Antioxid Redox Signal* **4**: 259-270.

Prockop LD & Chichkova RI (2007) Carbon monoxide intoxication: an updated review. *J Neurol Sci* **262**: 122-130.

Puri S & O'Brian MR (2006) The hmuQ and hmuD genes from Bradyrhizobium japonicum encode heme-degrading enzymes. *J Bacteriol* **188**: 6476-6482.

Ragsdale SW (2004) Life with carbon monoxide. *Crit Rev Biochem Mol Biol* **39**: 165-195.

Ratliff M, Zhu W, Deshmukh R, Wilks A & Stojiljkovic I (2001) Homologues of neisserial heme oxygenase in gram-negative bacteria: degradation of heme by the product of the pigA gene of Pseudomonas aeruginosa. *J Bacteriol* **183**: 6394-6403.

Raub JA, Mathieu-Nolf M, Hampson NB & Thom SR (2000) Carbon monoxide poisoning--a public health perspective. *Toxicology* **145**: 1-14.

Reynolds MF, Parks RB, Burstyn JN, Shelver D, Thorsteinsson MV, Kerby RL, Roberts GP, Vogel KM & Spiro TG (2000) Electronic absorption, EPR, and resonance raman spectroscopy of CooA, a CO-sensing transcription activator from R. rubrum, reveals a five-coordinate NO-heme. *Biochemistry* **39**: 388-396.

Ridley KA, Rock JD, Li Y & Ketley JM (2006) Heme utilization in Campylobacter jejuni. *J Bacteriol* **188**: 7862-7875.

Rodgers PA, Vreman HJ, Dennery PA & Stevenson DK (1994) Sources of carbon monoxide (CO) in biological systems and applications of CO detection technologies. *Semin Perinatol* **18**: 2-10.

Romão CC, Blattler WA, Seixas JD & Bernardes GJ (2012) Developing drug molecules for therapy with carbon monoxide. *Chem Soc Rev* **41**: 3571-3583.

Ryan MJ, Jernigan NL, Drummond HA, McLemore GR, Jr., Rimoldi JM, Poreddy SR, Gadepalli RS & Stec DE (2006) Renal vascular responses to CORM-A1 in the mouse. *Pharmacol Res* **54**: 24-29.

Ryter SW, Alam J & Choi AM (2006) Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. *Physiol Rev* **86**: 583-650.

Ryter SW & Otterbein LE (2004) Carbon monoxide in biology and medicine. *Bioessays* **26**: 270-280.

Ryter SW, Otterbein LE, Morse D & Choi AM (2002) Heme oxygenase/carbon monoxide signaling pathways: regulation and functional significance. *Mol Cell Biochem* **234-235**: 249-263.

Sandouka A, Fuller BJ, Mann BE, Green CJ, Foresti R & Motterlini R (2006) Treatment with CO-RMs during cold storage improves renal function at reperfusion. *Kidney Int* **69**: 239-247.

Santangelo R, Mancuso C, Marchetti S, Di Stasio E, Pani G & Fadda G (2012) Bilirubin: An Endogenous Molecule with Antiviral Activity in vitro. *Front Pharmacol* **3**: 36.

Sasakura Y, Hirata S, Sugiyama S, Suzuki S, Taguchi S, Watanabe M, Matsui T, Sagami I & Shimizu T (2002) Characterization of a direct oxygen sensor heme protein from Escherichia coli. Effects of the heme redox states and mutations at the heme-binding site on catalysis and structure. *J Biol Chem* **277**: 23821-23827.

Sawle P, Foresti R, Mann BE, Johnson TR, Green CJ & Motterlini R (2005) Carbon monoxide-releasing molecules (CO-RMs) attenuate the inflammatory response elicited by lipopolysaccharide in RAW264.7 murine macrophages. *Br J Pharmacol* **145**: 800-810.

Schlesinger G & Miller SL (1983) Prebiotic synthesis in atmospheres containing CH4, CO, and CO2. I. Amino acids. *J Mol Evol* **19**: 376-382.

Schmitt MP (1997) Utilization of host iron sources by Corynebacterium diphtheriae: identification of a gene whose product is homologous to eukaryotic heme oxygenases and is required for acquisition of iron from heme and hemoglobin. *J Bacteriol* **179**: 838-845.

Sjostrand T (1949) Endogenous formation of carbon monoxide in man. *Nature* **164**: 580.

Skaar EP, Gaspar AH & Schneewind O (2004) IsdG and IsdI, heme-degrading enzymes in the cytoplasm of Staphylococcus aureus. *J Biol Chem* **279**: 436-443.

Skaar EP, Gaspar AH & Schneewind O (2006) Bacillus anthracis IsdG, a heme-degrading monooxygenase. *J Bacteriol* **188**: 1071-1080.

Smith H, Mann BE, Motterlini R & Poole RK (2011) The carbon monoxide-releasing molecule, CORM-3 (RU(CO)(3) CL(glycinate)), targets respiration and oxidases in Campylobacter jejuni, generating hydrogen peroxide. *IUBMB Life* **63**: 363-371.

Soares MP & Bach FH (2009) Heme oxygenase-1: from biology to therapeutic potential. *Trends Mol Med* **15**: 50-58.

Srisook K, Han SS, Choi HS, Li MH, Ueda H, Kim C & Cha YN (2006) CO from enhanced HO activity or from CORM-2 inhibits both O2- and NO production and downregulates HO-1 expression in LPS-stimulated macrophages. *Biochem Pharmacol* **71**: 307-318.

Stanford SJ, Walters MJ, Hislop AA, Haworth SG, Evans TW, Mann BE, Motterlini R & Mitchell JA (2003) Heme oxygenase is expressed in human pulmonary artery smooth muscle where carbon monoxide has an anti-proliferative role. *Eur J Pharmacol* **473**: 135-141.

Suits MD, Pal GP, Nakatsu K, Matte A, Cygler M & Jia Z (2005) Identification of an Escherichia coli O157:H7 heme oxygenase with tandem functional repeats. *Proc Natl Acad Sci U S A* **102**: 16955-16960.

Sun B, Sun Z, Jin Q & Chen X (2008a) CO-releasing molecules (CORM-2)-liberated CO attenuates leukocytes infiltration in the renal tissue of thermally injured mice. *Int J Biol Sci* **4**: 176-183.

Sun B, Zou X, Chen Y, Zhang P & Shi G (2008b) Preconditioning of carbon monoxide releasing molecule-derived CO attenuates LPS-induced activation of HUVEC. *Int J Biol Sci* **4**: 270-278.

Sun BW, Chen ZY, Chen X & Liu C (2007) Attenuation of leukocytes sequestration by carbon monoxide-releasing molecules: liberated carbon monoxide in the liver of thermally injured mice. *J Burn Care Res* **28**: 173-181.

Taille C, El-Benna J, Lanone S, Boczkowski J & Motterlini R (2005) Mitochondrial respiratory chain and NAD(P)H oxidase are targets for the antiproliferative effect of carbon monoxide in human airway smooth muscle. *J Biol Chem* **280**: 25350-25360.

Tavares AF, Nobre LS & Saraiva LM (2012) A role for reactive oxygen species in the antibacterial properties of carbon monoxide-releasing molecules. *FEMS Microbiol Lett*.

Tenhunen R, Marver HS & Schmid R (1970) The enzymatic catabolism of hemoglobin: stimulation of microsomal heme oxygenase by hemin. *J Lab Clin Med* **75**: 410-421.

Urquhart P, Rosignoli G, Cooper D, Motterlini R & Perretti M (2007) Carbon monoxide-releasing molecules modulate leukocyte-endothelial interactions under flow. *J Pharmacol Exp Ther* **321**: 656-662.

Varadi J, Lekli I, Juhasz B, Bacskay I, Szabo G, Gesztelyi R, Szendrei L, Varga E, Bak I, Foresti R, Motterlini R & Tosaki A (2007) Beneficial effects of carbon monoxide-releasing molecules on post-ischemic myocardial recovery. *Life Sci* **80**: 1619-1626.

Vera T, Henegar JR, Drummond HA, Rimoldi JM & Stec DE (2005) Protective effect of carbon monoxide-releasing compounds in ischemia-induced acute renal failure. *J Am Soc Nephrol* **16**: 950-958.

Von Berg R (1999) Toxicology update. Carbon Monoxide. *J. Appl. Toxicol.* **19**: 379-386

Wagener FA, Volk HD, Willis D, Abraham NG, Soares MP, Adema GJ & Figdor CG (2003) Different faces of the heme-heme oxygenase system in inflammation. *Pharmacol Rev* **55**: 551-571.

Wandersman C & Stojiljkovic I (2000) Bacterial heme sources: the role of heme, hemoprotein receptors and hemophores. *Curr Opin Microbiol* **3**: 215-220.

Weaver LK (2009) Clinical practice. Carbon monoxide poisoning. *N Engl J Med* **360**: 1217-1225.

Wegele R, Tasler R, Zeng Y, Rivera M & Frankenberg-Dinkel N (2004) The heme oxygenase(s)-phytochrome system of Pseudomonas aeruginosa. *J Biol Chem* **279**: 45791-45802.

Weigel PH & Englund PT (1975) Inhibition of DNA replication in Escherichia coli by cyanide and carbon monoxide. *J Biol Chem* **250**: 8536-8542.

Wiesel P, Patel AP, DiFonzo N, Marria PB, Sim CU, Pellacani A, Maemura K, LeBlanc BW, Marino K, Doerschuk CM, Yet SF, Lee ME & Perrella MA (2000) Endotoxin-induced mortality is related to increased oxidative stress and end-organ dysfunction, not refractory hypotension, in heme oxygenase-1-deficient mice. *Circulation* **102**: 3015-3022.

Wilks A (2002) Heme oxygenase: evolution, structure, and mechanism. *Antioxid Redox Signal* **4**: 603-614.

Wu L & Wang R (2005) Carbon monoxide: endogenous production, physiological functions, and pharmacological applications. *Pharmacol Rev* **57**: 585-630.

Wyckoff EE, Lopreato GF, Tipton KA & Payne SM (2005) Shigella dysenteriae ShuS promotes utilization of heme as an iron source and protects against heme toxicity. *J Bacteriol* **187**: 5658-5664.

Wyckoff EE, Schmitt M, Wilks A & Payne SM (2004) HutZ is required for efficient heme utilization in Vibrio cholerae. *J Bacteriol* **186**: 4142-4151.

Xi Q, Tcheranova D, Parfenova H, Horowitz B, Leffler CW & Jaggar JH (2004) Carbon monoxide activates KCa channels in newborn arteriole smooth muscle cells by increasing apparent Ca2+ sensitivity of alpha-subunits. *Am J Physiol Heart Circ Physiol* **286**: H610-618.

Yachie A, Niida Y, Wada T, Igarashi N, Kaneda H, Toma T, Ohta K, Kasahara Y & Koizumi S (1999) Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. *J Clin Invest* **103**: 129-135.

Yet SF, Layne MD, Liu X, Chen YH, Ith B, Sibinga NE & Perrella MA (2003) Absence of heme oxygenase-1 exacerbates atherosclerotic lesion formation and vascular remodeling. *Faseb J* **17**: 1759-1761.

Zhang X, Migita CT, Sato M, Sasahara M & Yoshida T (2005) Protein expressed by the ho2 gene of the cyanobacterium Synechocystis sp. PCC 6803 is a true heme oxygenase. Properties of the heme and enzyme complex. *Febs J* **272**: 1012-1022.

Zhu W, Hunt DJ, Richardson AR & Stojiljkovic I (2000a) Use of heme compounds as iron sources by pathogenic neisseriae requires the product of the hemO gene. *J Bacteriol* **182**: 439-447.

Zhu W, Wilks A & Stojiljkovic I (2000b) Degradation of heme in gram-negative bacteria: the product of the hemO gene of Neisseriae is a heme oxygenase. *J Bacteriol* **182**: 6783-6790.

Results

Chapter 3|

A novel nitroreductase of *Staphylococcus aureus* with S-nitrosoglutathione reductase activity

This chapter was published in:

Tavares AFN, Nobre LS, Melo AMP & Saraiva LM (2009) A novel nitroreductase of *Staphylococcus aureus* with S-nitrosoglutathione reductase activity. *Journal of Bacteriology* **191**: 3403-3406.

Ana Filipa Tavares performed all the experimental work, with the exception of the *ntrA* mutant construction and phylogenetical analysis. Ana Filipa Tavares also participated in the writing of the manuscript.

Summary| In this study we report that inactivation of the putative nitroreductase SA0UHSC_00833 (*ntrA*) increases the sensitivity of *Staphylococcus aureus* to S-nitrosoglutathione (GSNO) and augments its resistance to nitrofurans. *S. aureus* NtrA is a bifunctional enzyme that exhibits nitroreductase and GSNO reductase activity. A phylogenetic analysis suggests that NtrA is a member of a novel family of nitroreductases that seems to play a dual role *in vivo*, promoting nitrofurans activation and protecting the cell against transnitrosylation.

3.1 Introduction

Staphylococcus aureus is a Gram-positive pathogen responsible for a large number of human infections that range from mild to potential lethal systemic infections. The incidence of methicillin-resistant *S. aureus* (MRSA) infections, which is rapidly increasing particularly among HIV/AIDS-infected patients (Crum-Cianflone *et al.*, 2007), reveals that the antibiotic of choice for the treatment of *S. aureus* is becoming ineffective and shows the need for using alternative compounds. Strains of *Staphylococcus* are sensitive to nitrofuran derivatives such as nitrofurazone and nitrofurantoin, and are utilized in the treatment of burns, skin grafts and genitourinary infections (Guay, 2001). The action of nitrofurans is dependent on the presence of specific microbial enzymes, the nitroreductases, which catalyze the reduction of the drug, a step that is essential for its activation. The activation of nitroaromatic compounds by bacterial nitroreductases is also used as a cancer therapy, since the cytotoxic hydroxylamine derivative compounds are able to destroy tumors (Johansson *et al.*, 2003).

In microorganisms subjected to nitrosative stress, S-nitrosoglutathione (GSNO) is formed by reaction of NO with the intracellular glutathione. The GSNO formed reacts with thiol-containing proteins promoting thiol nitrosation which modifies the function of proteins that are essential to many cellular processes. To control the level of S-nitrosylated proteins, organisms use S-nitrosoglutathione reductases (GSNOR), namely the widespread glutathione-dependent formaldehyde dehydrogenase (FALDH) also known as class III alcohol dehydrogenase (Liu *et al.*, 2001). However, GSNO is also a NADPH-dependent oxidizing substrate of other enzymes such as the thioredoxin system, glutathione peroxidase, γ-glutamyl transpeptidase, and xantine oxidase, indicating that GSNO reductase activity is frequently associated with

other enzymatic activities (Hou *et al.*, 1996; Nikitovic & Holmgren, 1996; Hogg *et al.*, 1997; Trujillo *et al.*, 1998).

Our microarray studies revealed that the staphylococcal gene SA0UHSC_00833 of *S. aureus* NCTC 8325 encoding a putative nitroreductase is induced by GSNO. Hence, we have analysed the *in vivo* role of this protein in the metabolism of GSNO and nitrofurans, and performed the biochemical characterization of the recombinant protein SA0UHSC_00833 (named NtrA).

3.2 Results and discussion

S. aureus NtrA is involved in GSNO metabolism

In a DNA microarray study of *S. aureus* NCTC 8325 we detected that GSNO significantly up-regulates the SA0UHSC_00833 gene (herein named *ntrA*) which encodes a putative nitroreductase (our unpublished data). To perform quantitative real-time RT-PCR analysis, cells of *S. aureus* NCTC 8325 were grown in LB to an OD_{600nm} = 0.4, and treated for 10 min with GSNO (150 µM). The bacterial strains, plasmids and oligonucleotides used in this study are listed in suplemmentary data in Tables S3.1 and S3.2 (see section 3.5). Real-time PCR experiments were performed with LightCycler^{R} FastStart DNA Master SYBR Green I Kit (Roche Applied Science), using 400 ng of cDNA prepare from total RNA and 0.5 µM of oligonucleotides SaNrNhe and SANrEco (Table S3.2) for the SA0UHSC_00833 (*ntrA*) gene, 16S_Fwd and 16S_Rev for *S. aureus* 16S rRNA. The results confirm that GSNO causes a strong transcriptional induction of the *ntrA* gene (13.4 ± 3.8-fold). The expression of a homolog gene in *S. aureus* COL, SACOL0874, was also reported to be induced by nitrosative stress conditions (Richardson *et al.*, 2006). Since these results suggest the involvement of the *ntrA* gene in nitrosative metabolism, a strain defective in the gene was constructed and analysed. For the construction of mutant, the *S.*

aureus NCTC 8325 SA0UHSC_00833 (*ntrA*) gene was PCR amplified using SA0833MutEco and SA0833MutBam oligonucleotides (Table S3.2), ligated to pSP64D-E (pSP64-SA0833), electroporated into *S. aureus* RN4220, and selected on Tryptone Soy Agar plates (TSA) containing erythromycin (10 µg/mL). The correct integration of pSP64-SA0833 into the chromosome of *S. aureus* was confirmed by PCR, and the generated mutant strain designated LMSA833. For the GSNO sensitivity tests, wild type and LMSA833 (Δ*ntrA*) were grown overnight in Tryptone Soy Broth (TSB), inoculated in fresh LB and exposed for 1 h to GSNO (100 µM). The cultures were treated for another 4 h with 1 mM of GSNO and serial dilutions were spotted on TSA. After overnight incubation, the plates revealed that Δ*ntrA* exhibited a higher degree of GSNO inhibition than the parental strain. Furthermore, expression of NtrA from the pMK4 plasmid restored the wild type phenotype (Figure 3.1). Nevertheless, we did not observe enhanced susceptibility of the *ntrA* mutant when using NO gas (data not shown).

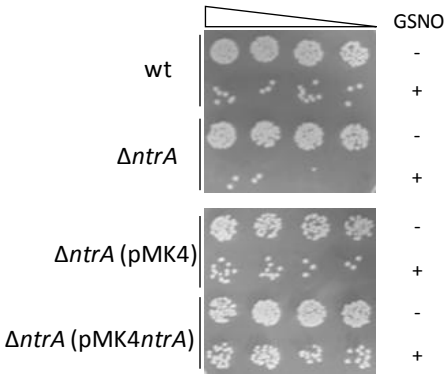


Figure 3.1| Sensitivity of *S. aureus ntrA* mutant (Δ*ntrA*) to GSNO. *S. aureus* wild type (wt), Δ*ntrA* and Δ*ntrA* harboring pMK4*ntrA* or the vector alone were grown in LB medium and treated with 1 mM of GSNO (+) or left untreated (-). Sensitivity tests were assayed after 4 h of GSNO treatment by plating on agar serial dilutions of the cultures.

S. aureus ntrA mutant is more resistant to nitrofurans

Since SA0UHSC_00833 is annotated as a putative nitroreductase, its role in the metabolism of nitrofurans was also examined. To this end, overnight cultures of *S. aureus* wild type (RN4220) and Δ*ntrA* (LMSA833)

grown in TSB were used to inoculate fresh LB medium, supplemented with nitrofurantoin (20 µg/mL) or nitrofurazone (25 µg/mL). In the absence of antibiotics, the wild type and mutant strains displayed similar growth kinetics. After the addition of nitrofurans the growth of both strains was immediately inhibited. While after 22 h the wild type showed almost limited recovery (recoveries of 17% and 9% for nitrofurantoin and nitrofurazone, respectively), the mutant strain had resumed growth at a rate similar to the untreated culture, exhibiting a recovery of 67% for cells treated with nitrofurantoin and of 71% for cells submitted to nitrofurazone (Figure 3.2).

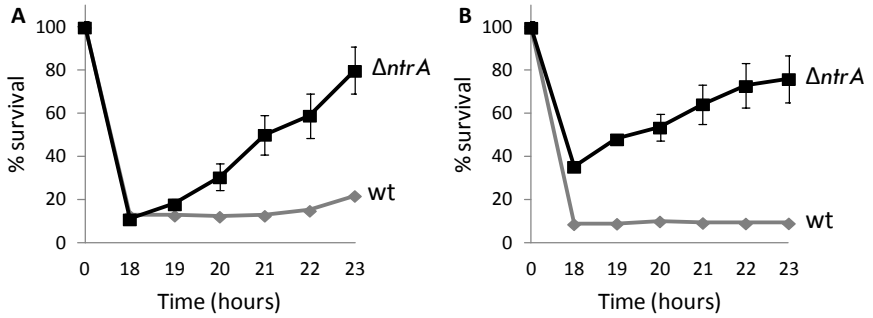


Figure 3.2| Resistance of *S. aureus ntrA* mutant (Δ*ntrA)* to nitrofurantoin and nitrofurazone. Wild type *S. aureus* RN4220 (◆) and Δ*ntrA* (■) were grown in LB medium and treated with 20 µg/mL nitrofurantoin (A) and 25 µg/mL nitrofurazone (B). The error bars represent the standard error of average values of at least two independent cultures.

Furthermore, cells of the *ntrA* mutant strain expressing NtrA, from a multi-copy plasmid bearing only the *ntrA* gene, regained the sensitivity to nitrofurans with a phenotype similar to the one observed for the wild type strain (data not shown). In conclusion, the presence of an active *ntrA* gene product contributes to the sensitivity of *S. aureus* to nitrofurans.

Mutation of *ntrA* decreases the nitroreductase activity of *S. aureus*

The activity of cell-free extracts of *S. aureus* wild type and *ntrA* mutant strain were analyzed by following the reduction of nitrofurantoin and nitrofurazone using NADH or NADPH as electron donors. To this end, cells from TSB overnight cultures of wild type and Δ*ntrA* (LMSA833) were harvested, resuspended in cold 20 mM Tris-HCl buffer pH 7.6 (buffer A) and incubated with lysostaphin (50 µg/mL) (37 ºC, 15 min). Following addition of DNAse (50 µg/mL), cells were centrifuged for 30 min (13000 *g,* 4ºC). The enzymatic assays were recorded in a Shimadzu UV-1700 spectrophotometer (25ºC) following the decrease of the nitrofuran absorbance (nitrofurazone (ϵ_{400nm}=12960 $M^{-1}cm^{-1}$) and nitrofurantoin (ϵ_{420nm}=12000 $M^{-1}cm^{-1}$)). The mixture contained buffer A, nitrofuran, NADPH or NADH (100 µM) and the reaction was initiated by addition of the cell extract. The NADPH-dependent activity was found to be higher than NADH-dependent activity and the higher activity values were measured when using nitrofurantoin. Inactivation of the *S. aureus ntrA* led to a significant decrease of the nitrofurantoin and nitrofurazone reductase activity values (Table 3.1), which correlates with the increase in the nitrofuran resistance observed in the *ntrA* mutant strain.

Table 3.1| Nitroreductase activity (nmol.min^{-1}.mg^{-1} protein) assayed in cell extracts of *S. aureus* wild type (wt) and *ntrA* mutant strain (Δ*ntrA*).

	Nitrofurantoin		Nitrofurazone	
S. aureus	NAPDH	NADH	NADPH	NADH
wt	9.5 ± 0.7	4.7 ± 0.5	4.8 ± 0.4	2.8 ± 0.4
Δ*ntrA*	5.6 ± 0.4	3.1 ± 0.2	2.7 ± 0.2	1.9 ± 0.3

S. aureus NtrA protein reduces GSNO and nitrofurans

To further characterize the function of the *S. aureus* NtrA, the protein was produced and characterized. Hence, *S. aureus* NCTC 8325 SA0UHSC_00833 (*ntrA*) gene was amplified using two oligonucleotides, SA0833NheIEx and SA0833EcoRIEx (Table S3.2), and cloned into pET28a(+) yielding pET28SA0833, that was transformed in *E. coli* BL21-Gold(DE3). Cells were grown, in LB with 30 µg/mL kanamycin, to an OD_{600nm} of 0.3 and induced for 7 h with isopropyl-ß-D-thio galactopyranoside (IPTG, 500 µM). Since the NtrA protein produced contain a His-Tag, the soluble extract was loaded onto a HisTrap Hp Column (GeHealthcare). Elution with ~200 mM imidazole yielded a pure fraction of NtrA**,** as judged by SDS-PAGE, which had an apparent molecular mass of ~21 kDa. The GSNO reductase activity of *S. aureus* NtrA was measured in buffer A plus *S. aureus* NtrA protein (0.6 µM), NADPH or NADH (250 µM) and GSNO (50-400 µM). The reaction was initiated by addition of GSNO and monitored following the oxidation of NAD(P)H (ϵ_{320nm}=6220 $M^{-1}cm^{-1}$). Activity values were corrected taking into account the reaction of GSNO with NAD(P)H. The kinetic data were fitted to the Michaelis-Menten equation using SigmaPlot. All values presented are the average of at least two assays. *S. aureus* NtrA enzyme showed to be specific for GSNO since no activity was seen for NO, S-nitrosocysteine and S-nitrosohomocysteine. Kinetic analysis of the *S. aureus* NtrA gave a K_M value (pH=7.6) for GSNO of ~ 181 µM (Table 3.2), a value which is lower than the one measured for the *E. coli* FALDH (K_M ~ 700

Table 3.2| GSNO reductase and nitroreductase activity of *S. aureus* NtrA.

	K_M (µM)	V_{max} (µmol.min^{-1}.mg^{-1})	k_{cat} (min^{-1})
GSNO	180.6 ± 43.4	1.5 ± 0.2	1.2
Nitrofurantoin	18.8 ± 4.8	13.7 ± 1.5	22.8
Nitrofurazone	25.7 ± 5.8	15.3 ± 1.6	25.5

μM, pH=7 (Liu *et al.*, 2001), thus indicating higher affinity of NtrA for this substrate. The nitroreductase activity of the purified *S. aureus* NtrA was also determined using nitrofurantoin and nitrofurazone as substrates (5-50 μM) and as electron donors NADPH or NADH (250 μM), which were used to initiate the reaction. Since the *S. aureus* NtrA (0.6 μM) was previously incubated with FMN/FAD (5 μM), all activities were corrected taking into account the chemical reduction of the nitrofuran compound generated by the same amount of flavin utilized in the protein mixture. We observed that while *S. aureus* NtrA incubated with FMN exhibited nitroreductase activity, no significant activity was measured when FAD was incorporated into NtrA. As observed for the GSNO reductase activity of *S. aureus* NtrA, the nitroreductase activities using NADH or NADPH as source of reducing equivalents were of the same order of magnitude (data not shown). The NADPH-dependent nitrofuran activity of the *S. aureus* NtrA (Table 3.2) is within the range of values usually observed. In particular, the only nitroreductase so far studied from *S. aureus*, the SA0UHSC_00366 protein (named NfrA), exhibits nitrofurazone and nitrofurantoin specific activities of 20 and 15 $\mu mol.min^{-1}.mg^{-1}$ protein, respectively, and the *E. coli* NsfA nitroreductase has a specific activity of ~80 $\mu mol.min^{-1}.mg^{-1}$ protein (Streker *et al.*, 2005).

The bifunctional *S. aureus* NtrA belongs to a novel family of nitroreductases

A search in the genome of *S. aureus* NCTC 8325 revealed that in addition to the SA0UHSC_00833 (NtrA), three other genes encoding putative nitroreductases are present, namely SA0UHSC_00366 (NfrA), SA0UHSC_02829, and SA0UHSC_02258. The four *S. aureus* nitroreductases gene products share a low percentage of sequence identity among them,

which ranges between 4% and 11%. Since the *E. coli* nitroreductases are well characterized (Whiteway *et al.*, 1998), we performed an amino acid sequence alignment with these enzymes which revealed that SA0UHSC_00366 (NfrA) shares the highest percentage of identity with *E. coli* b0851 (NfsA), SA0UHSC_02829 with *E. coli* b0578 (NfsB), and SA0UHSC_00833 (NtrA) and SA0UHSC_02258 present less than 10% identity with the two *E. coli* nitroreductases. A search, in the completed microbial genome databases, for homologs of *S. aureus* nitroreductases yielded several gene products with a percentage of sequence identity and similarity that varies between 17% and 42%, and 10% and 36%, respectively. A dendrogram was constructed based on the alignment of selected amino acid sequences of proteins found to be similar to SA0UHSC_00366 (NfrA), SA0UHSC_00833 (NtrA), SA0UHSC_02258, SA0UHSC_02829, *E. coli* NfsA and *E. coli* NfsB (identity within the selected sequences ranges between 4 and 90%) (Figure 3.3). The dendrogram suggests three main groups. The first contains *E. coli* NfsB, SA0UHSC_02829 and SA0UHSC_02258, being SA0UHSC_02258 the more distantly related member of this group, and the second branch includes *E. coli* NfsA and SA0UHSC_00366 (NfrA). The NtrA protein (SA0UHSC_00833) is located in a third independent group that does not contain any of the *E. coli* or *S. aureus* nitroreductases, thus suggesting that *S. aureus* NtrA is a member of a novel family of nitroreductases. Interestingly, the genome region comparison of the *ntrA* loci among the nine completely sequenced *S. aureus* genomes showed that in all cases the *ntrA* gene is flanked upstream by a gene encoding a protein similar to a 3-dehydroquinate dehydratase and downstream by a thioredoxin encoding gene, which is transcribed from the opposite strand.

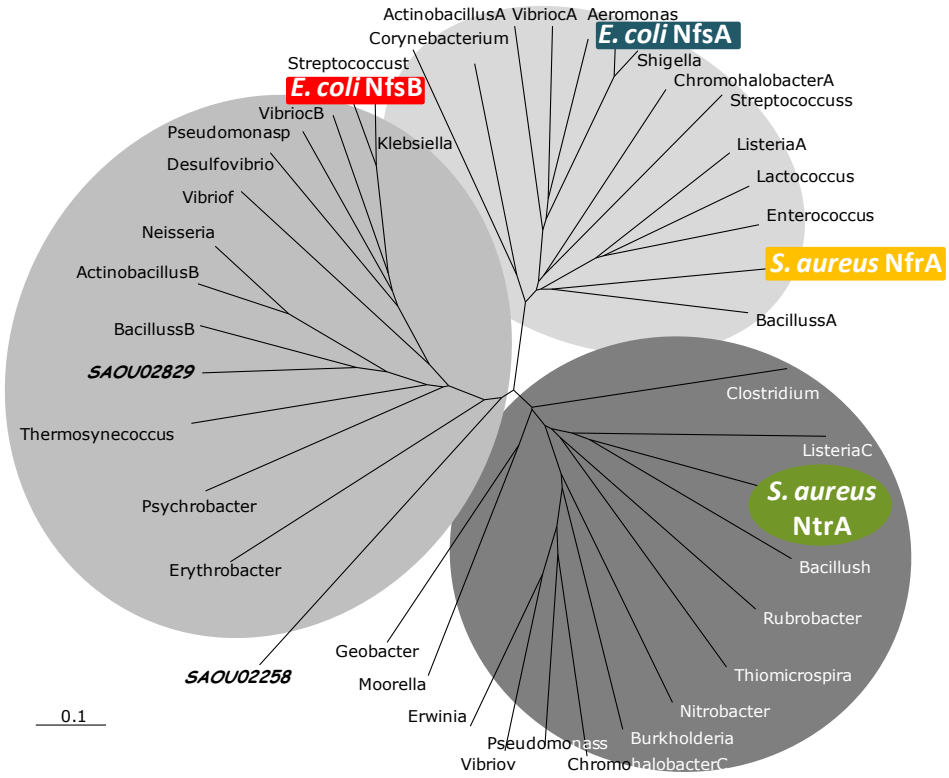


Figure 3.3| Dendrogram of representative nitroreductases families. Organism and protein sequence gi number corresponding to each abbreviation: Actinobacillus A (152979535) and B (152979206) – *Actinobacillus succinogenes*, Aeromonas – *Aeromonas salmonicida* (145298746); Bacillush – *Bacillus halodurans* (15612866), Bacilluss A (16080862) and B (16077850) – *Bacillus subtilis*; Burkholderia – *Burkholderia thailandensis* (83716954), Chromohalobacter A (92112337) and C (92113480) – *Chromohalobacter salexigens*; Corynebacterium – *Corynebacterium jeikeium* (68536654); Clostridium – *Clostridium difficile* (126700824); Desulfovibrio – *Desulfovibrio desulfuricans* G20 (78355133); *E. coli* NfsA – *E. coli* K-12 b0851 (16128819); *E. coli* NfsB – *E. coli* K-12 b0578 (16128561); Enterococcus – *Enterococcus faecalis* (29375756); Erythrobacter – *Erythrobacter litoralis* (85374565); Erwinia – *Erwinia carotovora* (50121266); Geobacter – *Geobacter uraniumreducens* (148263412); Klebsiella – *Klebsiella pneumoniae* (152969124); Lactococcus – *Lactococcus lactis* (125623424); Listeria A (16799400) and C (16799563) – *Listeria innocua*; Moorella – *Moorella thermoacetica* (83590668); Neisseria –

Neisseria gonorrhoeae (59800832); Nitrobacter – *Nitrobacter winogradskyi* (75676226); Pseudomonasp – *Pseudomonas putida* (148548471); Pseudomonass – *Pseudomonas stutzeri* (146282796); Psychrobacter – *Psychrobacter sp.* (148653797); Rubrobacter – *Rubrobacter xylanophilus* (108804130); SA0U02258 – *S. aureus* SA0UHSC_02258 (88195929); SA0U00366 – *S. aureus* SA0UHSC_00366 (88194164); SA0U002829 - *S. aureus* SA0UHSC_002829 (88196463); SA NtrA – *S. aureus* SA0UHSC_00833 (88194591); Shigella – *Shigella flexneri* (24112221); Streptococcuss – *Streptococcus sanguinis* (125718603); Streptococcust – *Streptococcus thermophilus* (55821160); Thermosynecoccus – *Thermosynecoccus elongatus* (22299744); Thiomicrospira – *Thiomicrospira crunogena* (78484399) ; Vibrioc A (15640734) and B (15601395) – *Vibrio cholera*; Vibriof – *Vibrio fischeri* (59713296); Vibriov – *Vibrio vulnificus* (27366397).

In conclusion, we have shown that *S. aureus* NtrA is a bifunctional enzyme able to catalyze the reduction of GSNO and nitrofurans. Although the existence of a single protein that combines two activities seems to be a common feature among the so far known GSNO reductases, the *S. aureus* NtrA is the first staphylococci nitroreductase shown to metabolize toxic nitrosothiols.

Even though the presence of NtrA contributes to the activation of nitrofurans, in the absence of these antibiotics NtrA acts as a defense system allowing the decomposition of GSNO formed endogenously, which occurs when *S. aureus* is exposed to nitrosative stress, and avoids the harmful effects caused by transnitrosylation reactions.

3.3 Acknowledgments

Work was funded by FCT project POCI/SAU-IMI/56088/2004 and by FCT studentships SFRH/BD/22425/2005 (LSN) and SFRH/BD/38457/2007 (AFNT).

3.4 References

Crum-Cianflone NF, Burgi AA & Hale BR (2007) Increasing rates of community-acquired methicillin-resistant Staphylococcus aureus infections among HIV-infected persons. *Int J STD AIDS* **18**: 521-526.

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

Guay DR (2001) An update on the role of nitrofurans in the management of urinary tract infections. *Drugs* **61**: 353-364.

Hogg N, Singh RJ, Konorev E, Joseph J & Kalyanaraman B (1997) S-Nitrosoglutathione as a substrate for gamma-glutamyl transpeptidase. *Biochem J* **323 (Pt 2)**: 477-481.

Hou Y, Guo Z, Li J & Wang PG (1996) Seleno compounds and glutathione peroxidase catalyzed decomposition of S-nitrosothiols. *Biochem Biophys Res Commun* **228**: 88-93.

Johansson E, Parkinson GN, Denny WA & Neidle S (2003) Studies on the nitroreductase prodrug-activating system. Crystal structures of complexes with the inhibitor dicoumarol and dinitrobenzamide prodrugs and of the enzyme active form. *J Med Chem* **46**: 4009-4020.

Liu L, Hausladen A, Zeng M, Que L, Heitman J & Stamler JS (2001) A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans. *Nature* **410**: 490-494.

Nikitovic D & Holmgren A (1996) S-nitrosoglutathione is cleaved by the thioredoxin system with liberation of glutathione and redox regulating nitric oxide. *J Biol Chem* **271**: 19180-19185.

Pinho MG, de Lencastre H & Tomasz A (2000) Cloning, characterization, and inactivation of the gene pbpC, encoding penicillin-binding protein 3 of Staphylococcus aureus. *J Bacteriol* **182**: 1074-1079.

Richardson AR, Dunman PM & Fang FC (2006) The nitrosative stress response of Staphylococcus aureus is required for resistance to innate immunity. *Mol Microbiol* **61**: 927-939.

Streker K, Freiberg C, Labischinski H, Hacker J & Ohlsen K (2005) Staphylococcus aureus NfrA (SA0367) is a flavin mononucleotide-dependent NADPH oxidase involved in oxidative stress response. *J Bacteriol* **187**: 2249-2256.

Sullivan MA, Yasbin RE & Young FE (1984) New shuttle vectors for Bacillus subtilis and Escherichia coli which allow rapid detection of inserted fragments. *Gene* **29**: 21-26.

Trujillo M, Alvarez MN, Peluffo G, Freeman BA & Radi R (1998) Xanthine oxidase-mediated decomposition of S-nitrosothiols. *J Biol Chem* **273**: 7828-7834.

Whiteway J, Koziarz P, Veall J, Sandhu N, Kumar P, Hoecher B & Lambert IB (1998) Oxygen-insensitive nitroreductases: analysis of the roles of nfsA and nfsB in development of resistance to 5-nitrofuran derivatives in Escherichia coli. *J Bacteriol* **180**: 5529-5539.

3.5 Supplementary data

Table S3.1| Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Source or Reference
E. coli		
XL2-Blue	*recA1 endA1* gyrA96 *thi-1 hsdR17* supE44 *relA1 lac* [F´*proAB lacI*^{q}*ZΔM15 Tn10* (Tet^{r}) Amy Cam^{r}]	Stratagene
BL21-Gold (DE3)	F^{-} *ompT hsdS*(r_B^{-} m_B^{-}) *dcm*$^{+}$ Tet^{r} *gal* λ(DE3) *endA* Hte	Stratagene
S. aureus		
NCTC 8325	Wild type (with 11-bp deletion in *rbsU*)	Laboratory stock
RN4220	NCTC8325-4-r (restriction negative derivate of NCTC8325) transformable by electroporation	Laboratory stock
LMSA833	RN4220 *ntrA*::Erm^{R}	This study
Plasmids		
pSP64D-E	Cloning vector carrying an erythromycin resistance cassette	(de Lencastre *et al.*, 1994; Pinho *et al.*, 2000)
pSP64-SA0833	DNA fragment (851 bp) containing the coding region of SA0UHSC_00833 gene cloned into pSP64D-E	This study
pET28a(+)	Protein expression vector carrying a $(His)_6$-tag at the N-terminal and a kanamycin resistance cassette	Novagen
pET28SA0833	*S. aureus* SA0UHSC_00833 gene cloned into pET28a(+)	This study
pMK4	Shuttle vector containing a chloramphenicol resistance cassette	BGSC (Sullivan *et al.*, 1984)
pMK4*ntrA*	SA0UHSC_00833 gene cloned into pMK4	This study

Table S3.2| Oligonucleotides used in this study.

Oligonucleotide	Sequence (5´-3´)
SaNrNhe	GTGCTGTCTTATGCTAGCGAAGTA
SANrEco	GATGTCCATGAATTCATGACTCAGC
16S_Fwd	TGGAGAGTTTGATCCTGGCTCAG
16S_Rev	TACCGCGGCTGCTGGCAC
SA0833MutEco	ATGTCACAGTTGATGAATTCGCAGA
SA0833MutBam	CTCATATCTCCGGATCCGTCTTT
SA0833NheIEx	CGATTTTAGATGGCTAGCCAACAAG
SA0833EcoRIEx	GATGTCCATGAATTCATGACTCAGC

Chapter 4|

Reactive oxygen species mediate bactericidal killing elicited by Carbon Monoxide-Releasing Molecules

This chapter was published in:

Tavares AFN, Teixeira M, Romão CC, Seixas JD, Nobre LS & Saraiva LM (2011) Reactive oxygen species mediate bactericidal killing elicited by Carbon Monoxide-Releasing molecules. *The Journal of Biological Chemistry* **286**: 26708-26717.

Ana Filipa Tavares performed all the experimental work and also contributed to the manuscript writing. EPR studies were done in collaboration with M Teixeira.

Summary| CO-Releasing Molecules (CO-RMs) were previously shown by us to be more potent bactericides than CO gas. This suggests a mechanism of action for CO-RM, which either potentiates the activity of CO or uses another CO-RM specific effect. We have also reported that CORM-2 induces the expression of genes related to oxidative stress. In the present study we intend to establish whether the generation of reactive oxygen species by CO-RMs may indeed result in the inhibition of bacterial cellular function. We now report that two CO-RMs (CORM-2 and ALF062) stimulate the production of ROS in *Escherichia coli*, an effect that is abolished by addition of antioxidants. Furthermore, deletion of genes encoding *E. coli* systems involved in reactive oxygen species scavenging, namely catalases and superoxide dismutases, potentiates the lethality of CORM-2 due to an increase of intracellular ROS content. CORM-2 also induces the expression of the *E. coli* DNA repair/SOS system *recA*, and its inactivation enhances toxicity of CORM-2. Moreover, fluorescence microscopy images reveal that CORM-2 causes DNA lesions to bacterial cells. We also demonstrate that cells treated with CORM-2 contain higher levels of free iron arising from destruction of iron-sulfur proteins. Importantly, we show that CO-RMs generate hydroxyl radicals in a cell-free solution, a process that is abolished by scavenging CO. Altogether, we provide a novel insight into the molecular basis of CO-RMs action by showing that their bactericidal properties are linked to cell damage inflicted by the oxidative stress that they are able to generate.

4.1 Introduction

Carbon monoxide (CO) is generated in the human body by the degradation of haem mediated by haem oxygenase (HO) enzymes. The endogenous CO production has benefits in the neural, cardiovascular and renal systems, having a cytoprotective effect in several pathologic conditions such as atherosclerotic vascular disease and inflammation (Li *et al.*, 2007). Nevertheless, high concentrations of CO are toxic resulting primarily from the binding of CO to haemoglobin and to cytochrome *c* oxidase, the terminal electron acceptor of the respiratory chain (Haldane, 1927; Wu & Wang, 2005). In mice brains it was observed that CO toxicity, associated with the binding of CO to the terminal oxidase, increases the generation of mitochondrial reactive oxygen species (ROS), namely hydrogen peroxide, augments the plasma's level of oxidized proteins and lipid peroxidation, and decreases the ratio reduced/oxidized glutathione. As expected, these effects are counteracted by the presence of superoxide dismutase and iron chelators (Zhang & Piantadosi, 1992). One of the three isoforms of mammalian haem oxygenase enzymes, HO-1, is inducible by several stress agents, including haem, heat shock, pro-inflammatory cytokines and hypoxia. Since these agents share the ability to directly or indirectly generate ROS, the elevated HO-1 expression is considered as a cellular indicator of oxidative stress (Ryter *et al.*, 2002; Wu & Wang, 2005). On the other hand, HO-1-derived CO has been reported to have remarkable benefits on microbial sepsis, as HO-1 deficient mice display enhanced susceptibility to polymicrobial infections and the administration of exogenous CO rescues the HO-1-deficient mice from sepsis-induced lethality (Chung *et al.*, 2008). Exogenous administration of CO, via CO-releasing molecules (CO-RMs), also showed to be able to kill bacteria (Nobre *et al.*, 2007), and the physiological consequences of the action of CO-RMs on

bacteria addressed by global transcriptional analysis revealed that CO-RMs induce a significant alteration on the bacterial transcriptome. In particular, induction of genes encoding two main redox-sensing regulators that control the bacterial oxidative response, SoxS and OxyR, occurs in cells exposed to CORM-2, and *E. coli* strains lacking *oxyR* and *soxS* genes exhibit enhanced susceptibility to CORM-2, altogether suggesting that CO potentiates the generation of reactive oxygen species (Nobre *et al.*, 2009).

The generation of intracellular ROS causes deterioration of DNA, lipids, and proteins and several targets have been linked to loss of cell integrity and/or viability (Cabiscol *et al.*, 2000). DNA is especially prone to iron-catalysed oxidation, as iron binds directly to the phosphodiester backbone where $^{\bullet}$OH radicals are subsequently generated (Imlay & Linn, 1988; Avery, 2011). In particular, guanine residues on DNA are targets of oxidation as well as thiol groups and iron-sulfur clusters of proteins (Cabiscol *et al.*, 2000; Brzoska *et al.*, 2006). Besides loss of function, denaturation of iron-sulfur clusters contributes itself to toxicity since the iron that is released upon oxidation of the clusters provokes additional oxidative damage via the Fenton reaction (Keyer & Imlay, 1996; Avery, 2011).

Nevertheless, bacteria have several mechanisms to avoid this outcome. The typical responses to ROS include DNA repair systems (*e.g.* RecA), the action of scavenging molecules (*e.g.* glutathione) and detoxification systems, like superoxide dismutase and catalase (Cabiscol *et al.*, 2000). Moreover, oxidative conditions have been shown to trigger the Isc and the Suf systems, which are responsible for the *de novo* assembly of clusters in nascent polypeptides (Johnson *et al.*, 2005; Ayala-Castro *et al.*, 2008).

In the present work we have exposed bacterial cultures to CO-releasing molecules and determined the effect of these compounds on cell viability upon addition of antioxidants, measured the level of ROS, analysed the deletion of ROS scavenging systems, observed the degree of DNA damage, evaluated the status of iron and iron-sulfur enzymes, and tested the generation of oxygen radicals by the CO-releasing molecules. Altogether, the data revealed that the bactericidal action of CO-releasing molecules relies on the generation of deleterious intracellular oxidative stress conditions.

4.2 Material and methods

Bacterial strains, growth conditions and viability assays

The bacterial strains used in this work are described in Table 4.1. All *Escherichia coli* cells, grown aerobically in minimal medium salts (MS) (Nobre *et al.*, 2007) at 37 ºC and 150 rpm, were cultured until reaching an optical density at 600 nm (OD_{600nm}) of 0.3 at which point the CO-releasing molecules were added. Tricarbonyldichlororuthenium (II) dimer (CORM-2) from Sigma-Aldrich (Sintra, Portugal) and tetraethylammonium molybdenum pentacarbonylbromide (ALF062) from Alfama (Oeiras, Portugal) (Nobre *et al.*, 2007) were used as CO donors; these compounds were freshly prepared as 10 mM stock solutions by dissolution in dimethyl sulfoxide, and methanol, respectively. $Ru(II)Cl_2(DMSO)_4$ from Strem Chemicals (Bischhein, France) was used as the inactive form of CORM-2 (iCORM-2) and the inactivated form of ALF062, named iALF062, was prepared by overnight vigorous mixing of the compound with methanol.

Table 4.1| *E. coli* strains used in this study.

***E. coli* strains**	**Description**	**Source or Reference**
K12 MG1655	Wild type strain	Laboratory stock
DSH7	Parental strain	(Yonezawa & Nishioka, 1999)
Δ*katEG*	DSH19,DSH7 *katE1,katG17*::Tn10	(Yonezawa & Nishioka, 1999)
Δ*sodAB*	DSH56, DSH7 ϕ(*sodA´-´lacZ*)49 Cm^r, ϕ(*sodB´-´*kan)/Δ2Km^r)	(Yonezawa & Nishioka, 1999)
BW25113	Parental strain	(Baba *et al.*, 2006)
Δ*iscS*	BW25113 *iscS*::Kan^r	(Baba *et al.*, 2006)
Δ*tonB*	BW25113 *tonB*::Kan^r	(Baba *et al.*, 2006)
Δ*iscR*	BW25113 *iscR*::Kan^r	(Baba *et al.*, 2006)
Δ*sufS*	BW25113 *sufS*:Kan^r	(Baba *et al.*, 2006)
Δ*recA*	BW25113 *recA*::Kan^r	(Baba *et al.*, 2006)

When required, together with CO-RMs, reduced haemoglobin (20 μM; Sigma-Aldrich), reduced glutathione (5 mM; Calbiochem, Lisbon, Portugal), glutamate (50 mM; Sigma-Aldrich), cysteine (600 μM; Sigma-Aldrich) and methionine (3 mM; Sigma-Aldrich) were added to the growth culture. To determine the cell viability of *E. coli* strains, the number of colony forming unit(s) (CFU) was evaluated in the presence of the compounds and their inactive forms. The percentage of survival was determined as the number of colonies obtained after CO-RMs treatment divided by the number of colonies formed in the cultures treated with the inactive form. Wild type *E. coli* cells were treated with 250 μM CORM-2 and 50 μM ALF062, while for the analysis of the *E. coli* mutant strains and their parent strains CORM-2 was used at a concentration of 150 μM. The experiments were performed in duplicate with at least two independent cultures. The results are presented as averaged values with error bars representing standard errors.

Endogenous ROS production

Cells of *E. coli* were treated for 1 h, in the presence of 250 µM CORM-2, 50 µM ALF062 or their respective inactive forms. *E. coli* mutant strains were treated with 150 µM of CORM-2 and iCORM-2 for 1 h and 4 h. Cells were harvested, washed twice with phosphate buffer (PBS) and resuspended in the same buffer. The probe 2´, 7'-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich) was then added to cell suspensions, at a final concentration of 10 µM, and fluorescence intensity (FI) was acquired during 2 h using a Varian Eclipse 96-well spectrofluorimeter (wavelength of excitation at 485 nm and of emission at 538 nm). To determine the ROS variation the FI of cultures treated with control compounds were subtracted from those of cells treated with CO-RMs. The FI was normalized in relation to the OD_{600nm} of each culture. These experiments were done in duplicate with at least two different biological samples.

Quantitative real-time RT-PCR

For real-time reverse transcription (RT)-PCR experiments, 0.5 µg of *E. coli* total RNA, from two independent cultures, grown with 250 µM CORM-2 and control compound for 1 h, was used to synthesize cDNA with the First Strand cDNA Synthesis Kit for RT-PCR from Roche Applied Science (Lisbon, Portugal). Real-time PCR experiments were performed in a LightCycler Instrument using LightCycler^{R} FastStart DNA Master SYBR Green I Kit according to the manufacturer's instructions (Roche Applied Science). The amplification reactions were carried out using the previously synthesized cDNA as the initial template, and each reaction mixture contained 0.5 µM specific oligonucleotides (recA_up 5´-CCTTGCGGCACGTATGATGA-3´ and recA_low 5´-GGCGCAGCGATTTTGTTCTT-3´), 2 mM $MgCl_2$, and hot-start PCR mix (Roche

Applied Science). The expression ratio of the target gene was determined relatively to a reference gene, the *E. coli* 16S rRNA, whose transcription remains invariant under the tested conditions. The samples were assayed in triplicate.

DNA damage assays

For DNA damage assays, *E. coli* cultures were exposed for 1 h to 8 mM H_2O_2 (as control), 250 µM CORM-2 and iCORM-2 and treated and analysed according to the manufacturer's instructions of hOGG1 Flare™ assay kit (Trevigen, UK). Briefly, cells were centrifuged, washed and resuspended in PBS to obtain a bacterial concentration of 10^8 CFU/mL. The cells were mixed with low agarose, immersed in a lysis solution for 1 h and then submitted to electrophoresis for 1 h at 12 V in TAE buffer. The SYBR® GREEN I dye was used for DNA visualization and the images acquired with a fluorescence microscope (Leica).

Reaction of CORM-2 with free thiol groups

The reduction of Ellman's reagent (DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); Sigma-Aldrich) by free thiol groups releases 5-thio-2-nitrobenzoic acid (TNB), which can be monitored spectrophotometrically at 412 nm (Ellman, 1959). Increasing amounts of CORM-2, iCORM-2 and iodocetamide were mixed with 100 µM glutathione (GSH) in 100 mM phosphate buffer (pH 7). After 1 h at room temperature, 500 µM DTNB was added and the absorbance at 412 nm was measured. Iodocetamide is an irreversible thiol-modifying compound and was used as a positive control. Samples and standard curves

were done in duplicate. Control reactions performed in the absence of GSH showed no absorbance.

EPR studies:

Free iron detection

The intracellular free iron content was determined by EPR in whole cells of *E. coli* grown for 1 h with 250 µM CORM-2 and iCORM-2, collected by centrifugation, resuspended in 9 mL of fresh MS and treated with 1 mL 0.2 M deferoxamine mesylate (Sigma-Aldrich) for 10 min at 37 ºC. Cells were then washed once with ice-cold 20 mM Tris-HCl (pH 7.4) and resuspended in 0.4 mL of the same buffer supplemented with 10% (v/v) glycerol. Aliquots of 0.3 mL were transferred to EPR tubes and immediately frozen in liquid nitrogen. The EPR spectra were obtained on a Bruker EMX spectrometer equipped with an Oxford Instruments continuous flow helium cryostat and recorded at 9.7 GHz microwave frequency, 20 mW microwave power, modulation of amplitude of 2 mT at 95 K. The iron content of each sample was determined by measuring the amplitude of the EPR signal and normalizing it for the total protein concentration, which was determined by the bicinchoninic acid method (BCA) (Smith *et al.*, 1985). The results represent the average of five independent cultures.

EPR spin-trap analysis

The 5-tert-butoxycarbonyl 5-methyl-1-pyrroline N-oxide (BMPO) from Applied Bioanalytical Labs (Bradenton, USA) was used as a spin-trap for the detection of reactive oxygen species. This spin trap allows one to distinguish between the superoxide anion and the hydroxyl (Zhao *et al.*, 2001). Experiments were conducted with 5 mM BMPO, 2 mM of CORM-2 and iCORM-2, and when required 1 mM haemoglobin. EPR spectra were obtained for

samples of ALF062 (199 μM in H_2O plus 0.7% methanol) mixed with BMPO (25 mM), which were previously bubbled with oxygen or degassed with nitrogen. Samples were loaded into a quartz flat cell and EPR spectra were recorded, at room temperature, on the Bruker EMX spectrometer and recorded at 9.8 GHz microwave frequency, 2 mW microwave power and modulation of amplitude of 0.1 mT.

Enzymatic assays

E. coli cultures treated for 1 h with 250 μM CORM-2 and iCORM-2 were washed and resuspended in the respective assay buffer before being disrupted in a French press. The aconitase activity was measured, under anaerobic conditions, using the indirect method described by Gardner *et al*. (Gardner, 2002), that follows the production of NADPH at 340 nm (ε_{340nm}= 6220 $M^{-1}cm^{-1}$). To this end, samples were added to the reaction mixture that contained 50 mM Tris-HCl (pH 7.6), 0.6 mM $MnCl_2$, 0.2 mM $NADP^+$ and 1 U isocitrate dehydrogenase (Sigma-Aldrich). The reaction was initiated with 30 mM sodium citrate. One unit of aconitase activity was defined as 1 μmol NADPH formed per min at 25ºC. The activity of glutamate synthase (GOGAT) was determined by the formation of $NADP^+$, following the decrease in absorbance at 340 nm (ε_{340nm}= 6220 $M^{-1}cm^{-1}$) (Meister, 1985). The activity was measured in reaction mixtures containing the lysate samples in 100 mM Tris-HCl (pH 7.8) buffer plus 10 mM glutamine and 0.2 mM NADPH, and initiated by addition of 5 mM α-ketoglutarate. One unit of GOGAT activity was defined as 1 nmol NADPH consumed per min at 25ºC. The glutamate dehydrogenase (GDH) activity was determined as the above described GOGAT activity, using 5 mM ammonium chloride instead of glutamine. Activities were assayed in a

Shimadzu UV-1700 and normalized to the total cellular protein lysate content determined by BCA assays (Smith *et al.*, 1985).

4.3 Results

Bactericidal CO-RMs induce ROS

Previous studies have shown that CO-RMs alter the transcription of several genes involved in oxidative stress response, suggesting that CO-RMs elicit the production of reactive oxygen species (Nobre *et al.*, 2009). To infer if biological antioxidants attenuate the bactericidal effect of CO-RMs, we have evaluated the viability of *E. coli* cells upon treatment with two CO donors, namely CORM-2 and ALF062 (Figure 4.1), in the presence of exogenous reduced glutathione (GSH) and cysteine. The data showed that GSH was able to restore the viability of the microbial cells by approximately 95%. Furthermore, supplementation of the culture medium with cysteine allowed a cell growth recovery of approximately 80% (Figure 4.2A and B). These results revealed that the presence of antioxidants causes reduction of the CO-RMs efficiency. Since *E. coli* cells submitted to oxidative stress develop a methionine auxotrophy (Masip *et al.*, 2006), and it was previously detected that CORM-2 interferes with the *E. coli* methionine metabolism (Nobre *et al.*, 2009), we have now analysed the growth behavior of cells exposed to CORM-2 in the presence of methionine. We observed that addition of this amino acid abolishes the action of the bactericide as viability of bacteria treated with CORM-2 increased significantly in the methionine supplemented culture (Figure 4.2A).

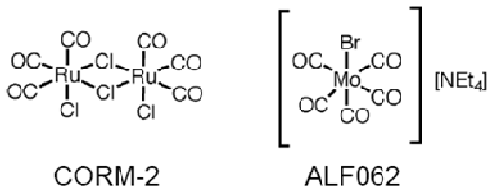


Figure 4.1| Chemical structure of CO-RMs used in this study.

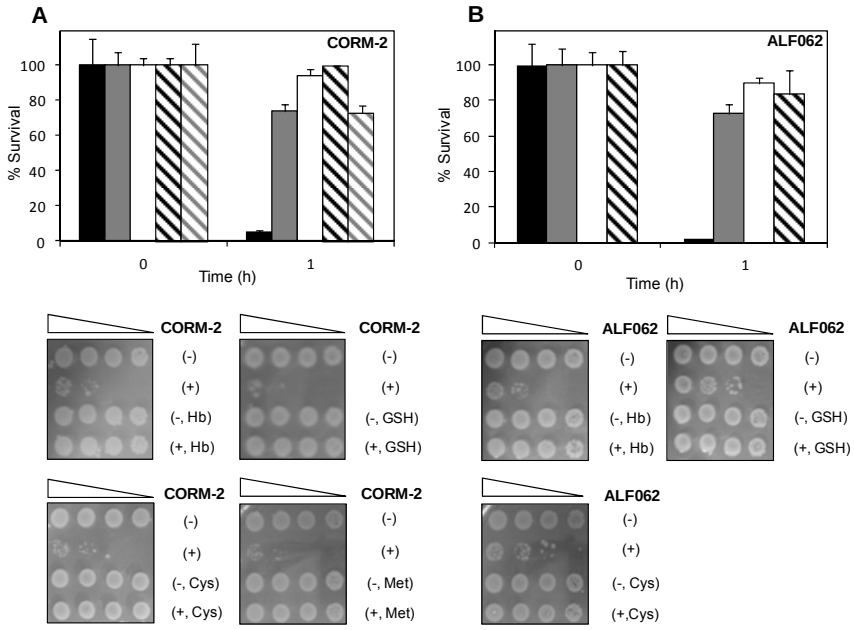


Figure 4.2| Lethality of CO-releasing molecules is abolished by antioxidants. *E. coli* cells were grown under aerobic conditions and exposed to 250 µM CORM-2 (A) and 50 µM ALF062 (B) for 1 h, in a medium containing the CO-releaser (black bars), and supplemented with haemoglobin (Hb, grey bars), glutathione (GSH, white bars), cysteine (Cys, black striped bars) and methionine (Met, grey striped bars). Survival percentage was calculated in relation to cultures treated with the respective inactive compound. The results represent the average of, at least, two independent cultures performed in duplicate, and error bars represent the standard error of the mean values obtained. Sensitivity tests performed in cultures treated for 1 h with CO-RMs (+) and inactive forms (-) are also shown.

Next, we have examined the level of endogenous reactive oxygen species formed in cells treated with CORM-2 and ALF062. The data revealed a significant increase of ROS content in cells exposed to either compound, (Figure 4.3A and B). Importantly, the level of ROS formed was dependent on the CORM-2 concentration utilized (Figure 4.3C). Furthermore, a strong decrease of ROS production was observed upon addition of glutathione

(Figure 4.3A and B). Quite importantly, the generation of ROS by CO-RMs was shown to be strictly dependent on CO since cells treated with inactive compounds, namely iCORM-2 and iALF062, that are CO depleted molecules, exhibited ROS levels similar to that of untreated cells (FI/OD_{600nm} ~ 30 at 120 min). Furthermore, when the medium was supplemented with the CO scavenger molecule haemoglobin, the ROS formation decreased more than 80% for both CORM-2 and ALF062 (Figure 4.3A and B).

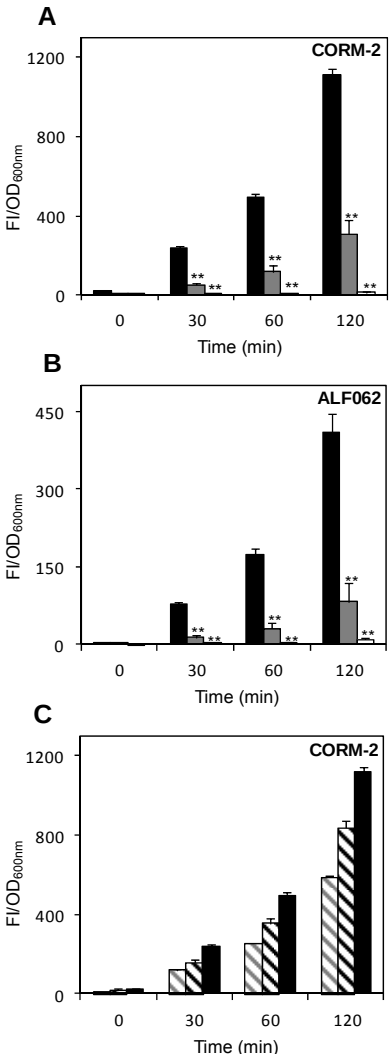


Figure 4.3| CO-RMs increase intracellular ROS content. ROS were quantified in *E. coli* cells treated, for 1 h, with 250 µM CORM-2 (A) and 50 µM ALF062 (B) with the CO-releasers (black bars) and in the presence of haemoglobin (grey bars) or glutathione (white bars). In (C) cells were treated with increased concentrations of CORM-2, 150 µM (grey striped bars), 200 µM (black striped bars) and 250 µM (black bars). The fluorescence intensities (FI) are represented as the subtraction of cultures treated with the inactive compound from cultures exposed to CO-releasing molecules and normalized in relation to the OD_{600nm} of the respective culture. Each data bars represents the average of three independent measurements with the corresponding standard errors (**P <0.01).

Since the activity of glutamate dehydrogenase was recently reported to decrease under oxidative stress (Mailloux *et al.*, 2009), we tested this activity that was found to be indeed lowered by 26% in cells treated with CORM-2 (Figure 4.4A). Moreover, supplementation of *E. coli* culture with glutamate, which is the substrate of GDH, abolishes the effect of CORM-2 (Figure 4.4B).

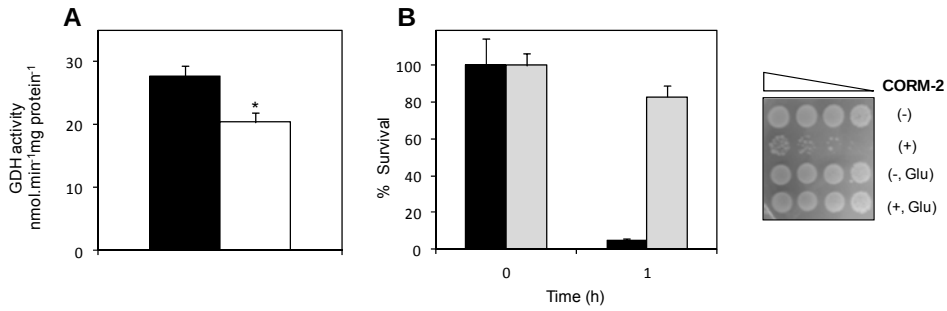


Figure 4.4| Glutamate dehydrogenase activity is lower in cells treated with CORM-2. (A) Activity of glutamate dehydrogenase (GDH) in cell extracts of *E. coli* treated for 1 h with iCORM-2 (black bars) and CORM-2 (white bars). The error bars represent the standard error of the mean values obtained (*P <0.05). (B) Effect of 250 µM CORM-2 on *E. coli* viability of aerobically grown cells exposed to the CO releaser in the absence (black bars) and in the presence of glutamate (Glu, grey bars). The percentage of survival was calculated in relation to cultures treated with the respective inactive compound, and error bars represent the standard error of the mean values obtained. Sensitivity tests performed in cultures treated, for 1 h, with CO-RM-2 (+) and iCORM-2 (-) are also presented.

Altogether, these results showed that cells exposed to CORM-2 are under oxidative stress. As both CORM-2 and ALF062 caused to *E. coli* cells similar oxidative stress conditions the subsequent experiments were conducted only with CORM-2.

Deletion of superoxide dismutase and catalase genes increase CORM-2 lethality

The observation that CO-RMs increase ROS production led us to hypothesize that strains deleted in the major antioxidant systems should be more prone to killing by CO-RMs. Therefore, we have analysed the phenotype of *E. coli* strains deleted in catalases *(*Δ*katEG*) and superoxide dismutases *(*Δ*sodAB*), in the presence of CORM-2.

The data regarding viability of Δ*katEG* and Δ*sodAB* mutants showed that these strains have increased susceptibility to CORM-2 when compared to the wild type, and that the effect is higher for the catalases deficient strain (Figure 4.5A). In agreement, CORM-2 treated cells of Δ*katEG* have higher amounts of ROS (Figure 4.5B and C). However, the ROS content of the Δ*sodAB* mutant strain did not vary, a result that may be due to the fact that superoxide anions do not directly oxidize the DCFH fluorescent probe (LeBel *et al.*, 1992). Therefore, it was concluded that the addition of CORM-2 to bacterial cells leads to an intracellular oxidative stress status that is exacerbated in the absence of oxidative defense systems.

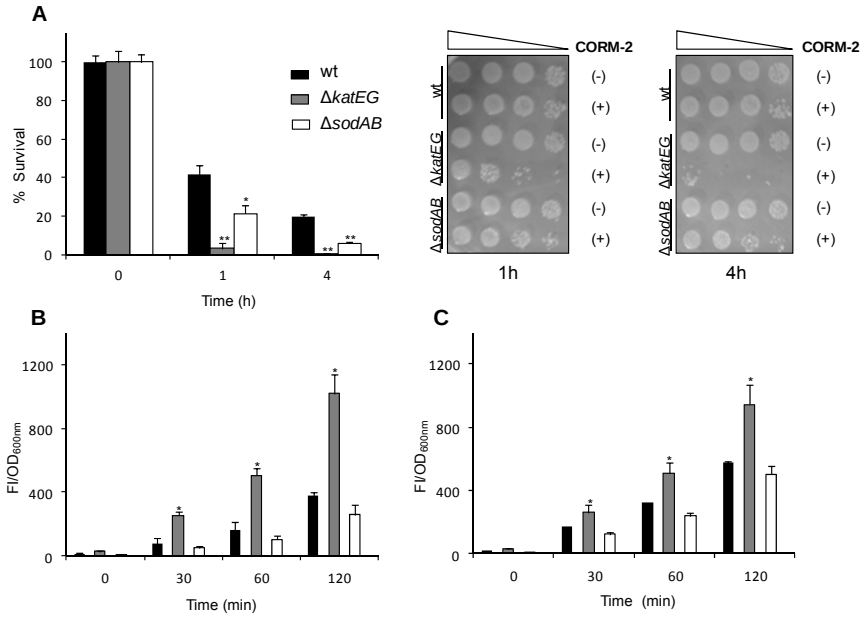


Figure 4.5| Bactericidal effect of CORM-2 is enhanced upon deletion of ROS scavenger systems. (A) Cell viability of the parental *E. coli* strain (black bars) and mutant strains, Δ*katEG* (grey bars) and Δ*sodAB* (white bars) were determined upon treatment with 150 µM CORM-2 and iCORM-2 for 1 h and 4 h. For the same times, sensitivity tests were performed in cultures treated with CORM-2 (+) and iCORM-2 (-). ROS intracellular content of parental (black bars), Δ*katEG* (grey bars) and Δ*sodAB* (white bars) strains determined in cells exposed to 1 h (B) and 4 h (C) to 150 µM CORM-2 and iCORM-2. The fluorescence intensities (FI) are represented as a subtraction of the cultures treated with iCORM-2 from cultures treated with CORM-2 and normalized in relation to OD_{600nm} of each culture. The error bars represent the standard error of the average values obtained from at least two independent biological samples performed in duplicate (*P <0.05,**P <0.01).

CORM-2 induces DNA damage

DNA is one of the main targets of ROS and since we found that CO-RMs increase the ROS production in *E. coli* cells, we examined if the effect of CORM-2 was potentiated in a *recA* mutant strain. RecA participates in the double-strand break repair, catalyzing the homologous recombination and function as a regulatory protein to induce the SOS response to DNA damage (Courcelle & Hanawalt, 2003). We observed that CORM-2 increases the expression of *recA* by ~2.5 fold and that the deletion of *recA* yielded an *E. coli* strain that exhibited increased susceptibility to CORM-2-treated cells (Figure 4.6A). Hence, we have analysed if CORM-2-derived ROS caused DNA lesions. To this end, DNA damage was assayed in cells treated with CORM-2, iCORM-2 and H_2O_2 (which was used as a control) using the glycosylase OGG1 enzyme that detects 8-oxoguanine, a base modification that usually occurs upon DNA damage. The images acquired by fluorescence microscopy of cells treated with CORM-2 and hydrogen peroxide revealed the presence of long comet tails characteristic of damaged DNA. In contrast, cells exposed to the inactive compound, iCORM-2, exhibited images where the supercoiled form of undamaged DNA was visualized (Figure 4.6B).

Thus, the data revealed that CORM-2 is a DNA-damaging drug, which is due to the ROS species that are being generated.

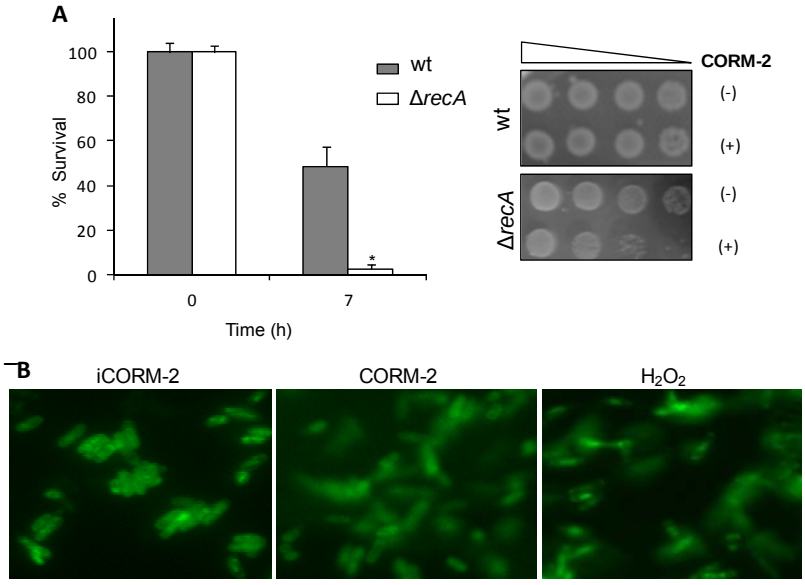


Figure 4.6| CORM-2 causes damages to bacterial DNA. (A) Cell viability of *E. coli recA* mutant (white bars) and its parental strain (grey bars) grown under aerobic conditions and exposed to 150 µM CORM-2 and iCORM-2 was determined after 7 h. Error bars represent the standard error of the mean values obtained (*P <0.05). Sensitivity tests were also performed in cultures treated with CORM-2 (+) and iCORM-2 (-). (B) Fluorescent microscopy of *E. coli* cells challenged, for 1 h, with 250 µM CORM-2, iCORM-2 and H_2O_2.

CORM-2 interferes with iron homeostasis

The formation of ROS, particularly the generation of hydroxyl radicals, is usually associated with the presence of free iron that acts as a catalyst in the Fenton reaction. Hence, the intracellular levels of free iron upon exposure to CORM-2 were evaluated by means of whole cell EPR. The cells treated with CORM-2 have a 4-fold higher content of free iron than cells treated with the compound devoid of CO (Figure 4.7).

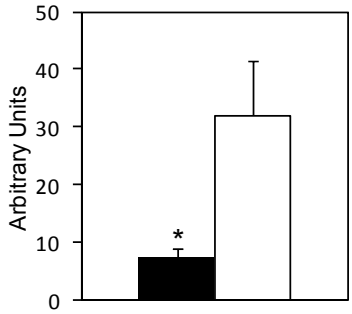


Figure 4.7| CORM-2 increases the level of intracellular free iron. Determination of the iron content of E. coli cells challenged with 250 µM CORM-2 (white bars) and iCORM-2 (black bars) by EPR in whole cells. Arbitrary units correspond to the amplitude of the EPR signal normalized for total protein concentration. The results are representative of five independent biological samples (*P <0.05).

The higher level of free iron detected in cells treated with CORM-2 may come from extracellular sources due to alterations in iron import or have an intracellular origin arising from destruction of iron containing proteins. To test the first hypothesis, we have examined the viability of cells lacking *tonB*, which is one of the major iron import systems of *E. coli* (Moeck & Coulton, 1998). However, no effect was observed on the viability of CORM-2-treated *E. coli tonB* mutant (Figure 4.8A), and no differences were detected in ROS production in the same mutant when compared with its parental strain (Figure 4.8B and C). These results indicate that exogenous iron does not play a significant role in oxidative damage-mediated cell killing. We have next evaluated if upon exposure of cells to CORM-2 denaturation of iron-sulfur clusters were supplying iron intracellularly for ROS generation. To this end, we have analysed the impact of CORM-2 on the phenotype of *E. coli* strains deleted in genes involved in Fe-S assembly, namely the cysteine desulfurase *iscS*, a gene product that acts as the sulfur donor for biogenesis of Fe-S clusters, *iscR*, the regulator of the *iscRSUA* operon, and s*ufS*, an *iscS* paralogue putatively required for Fe-S assembly under stress conditions (Schwartz *et al.*, 2000). Upon exposure to CORM-2 the behavior of the strains lacking *iscR* and s*ufS* remained essentially unaltered when compared to their respective

parental strains (Figure 4.8A). The viability of the CORM-2-treated Δ*iscS* strain was significantly higher and exhibited a lower content of ROS (Figure 4.8A-C). These results are in agreement with a decrease content of Fe-S centres originated from the absence of *iscS*, which translates into a diminished CORM-2 toxicity.

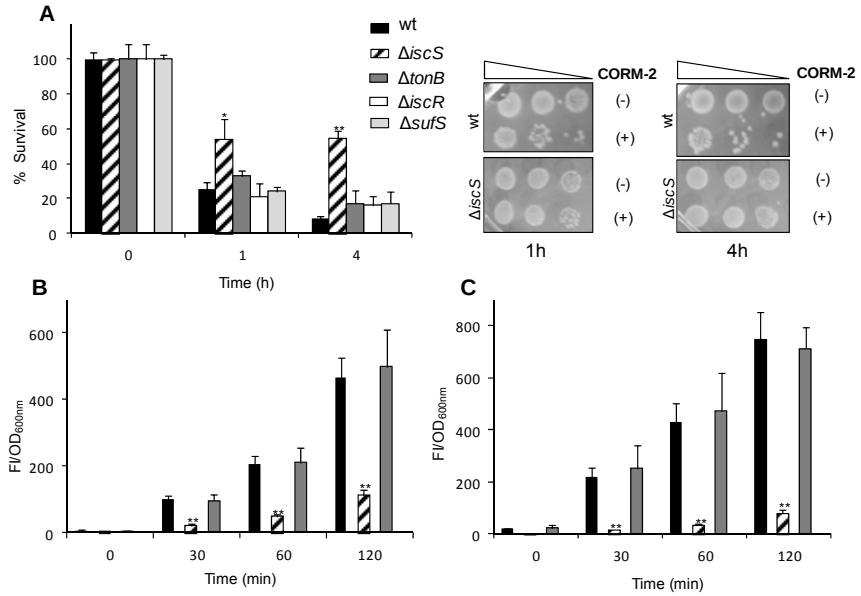


Figure 4.8| CORM-2 efficiency depends on iron-sulfur cell content. (A) Viability of *E. coli* parental strain (black bars), Δ*iscS* (striped bars), Δ*tonB* (dark grey bars), Δ*iscR* (white bars) and Δ*sufS* (light grey bars) determined upon exposure to 150 μM CORM-2 and iCORM-2, for 1 h and 4 h. Sensitivity tests were also performed in Δ*iscS* cells treated with CORM-2 (+) and iCORM-2 (-), for 1h and 4 h. ROS intracellular content evaluated in the parental strain (black), Δ*iscS* (striped), Δ*tonB* (dark grey) upon exposure for 1 h (B) and 4 h (C) to 150 μM CORM-2 and iCORM-2. The fluorescence intensities (FI) represent the subtraction of the cultures treated with iCORM-2 from cultures treated with CORM-2 normalized in relation to OD_{600nm} of the respective culture. The error bars are the standard error of the average values obtained from, at least, two independent biological samples performed in duplicate (*P <0.05, **P <0.01).

The higher level of intracellular iron in cells exposed to CORM-2 suggests that Fe-S containing enzymes are being degraded. This is corroborated by the lower activity of two enzymes measured in cells treated with the CO-releaser, namely aconitase and glutamate synthase (GOGAT). We observed that exposure of cell extracts to this bactericide resulted in a significant decrease on the activity of the two enzymes (~75% and 82% for aconitase and GOGAT, respectively) while no alterations occurred when using iCORM-2 (Figure 4.9). Overall, these results revealed that the ROS-associated action of CORM-2 relies on the intracellular iron released from Fe-S clusters, with concomitant decrease in the enzymatic activity of key metabolic enzymes of *E. coli*, and that iron-sulfur centres constitute a source of iron that potentiates the Fenton reaction.

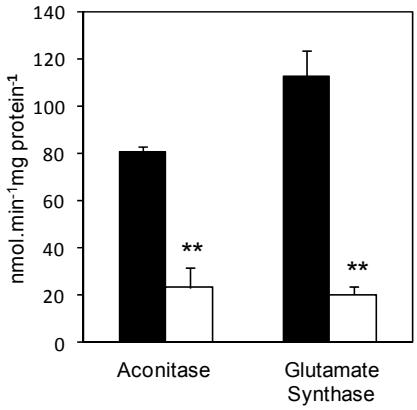


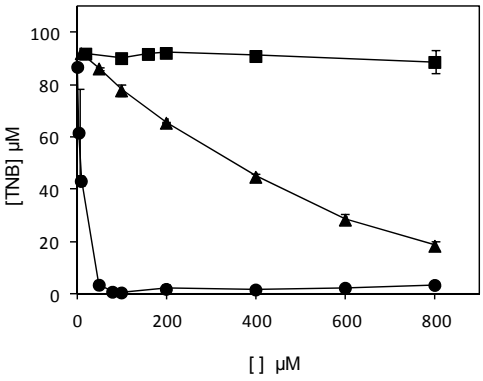
Figure 4.9| CORM-2 lowers the activity of iron-sulfur containing proteins. Aconitase and glutamate synthase activities determined in cells extracts of *E. coli* challenged for 1 h with CORM-2 (white bars) and iCORM-2 (black bars). The results are the average of, at least, three independent biological samples (**P <0.01).

In vitro reaction of CORM-2 with free thiol groups

Since we observed that cysteine supplementation abolished the action of CORM-2 (Figure 4.2A), we sought to investigate whether CORM-2 could react *in vitro* with thiol groups. To this end, the Ellman's reagent, DTNB, which is reduced by free thiol groups to TNB, was added to a buffer solution containing CORM-2 in the presence of GSH. While CORM-2 inhibited the

reduction of Ellman's reagent similar concentrations of iCORM-2 did not impair DTNB reduction (Figure 4.10). This result shows that thiol groups are no longer able to react with DNTB suggesting that CORM-2 generates species that promote oxidation of thiols.

Figure 4.10| CORM-2 impairs reduction of thiols by the Ellman's reagent. Production of TNB, upon incubation, for 1 h, of increased amounts of iodoacetamide (▲), CORM-2 (●) and iCORM-2 (■) with glutathione were determined in the presence of the Ellman´s reagent. Error bars represent the standard error of average values.



CORM-2 and ALF062 generate *per se* hydroxyl radicals

We next asked the question whether CO-RMs were able to generate radicals *in vitro, i.e*. in the absence of cells. To this end, the EPR spectra of samples containing CORM-2 and the BMPO spin trap were done. The results showed that the mixture of CORM-2 with BMPO gives rise to a EPR signal attributable to the formation of a BMPO-OH adduct (Figure 4.11A), while no signal was detected upon mixture of iCORM-2 with the same spin trap (Figure 4.11B). Notably, the signal arising from the BMPO-OH adduct was completely quenched upon incubation of CORM-2 with haemoglobin (Figure 4.11C). Hence, these results indicate that *in vitro* CORM-2 produces radicals of the hydroxyl type and that CO is associated with its generation.

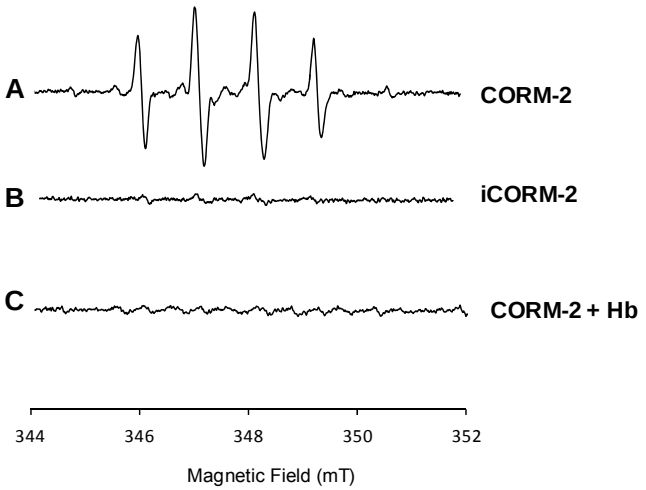


Figure 4.11| CORM-2 generates ROS *in vitro*. EPR spectra of samples containing 2 mM CORM-2 (A), iCORM-2 (B) and CORM-2 plus haemoglobin (Hb, C) acquired in the presence of the spin trap, BMPO.

To further analyse this issue we did similar experiments with ALF062. The signal of the BMPO-OH adduct was observed upon bubbling O_2 (Figure S4.1 in Supplementary Data), while in a degassed solution no EPR spectrum was seen. In this case it is clear that the OH radicals arise from the reaction of the electron-rich metal in the $[Mo(CO)_5Br][NEt_4]$ complex with molecular oxygen in the protic solvent H_2O.

However, the formation of OH radicals from CORM-2 solution and its dependence on CO is not so obvious. We propose that these OH radicals result from the reduction of O_2 by low valent Ru species, which intermediate the water-gas shift reaction initiated with the attack of water on one of the CO ligands of the $Ru^{II}(CO)_3$ moieties of CORM-2 (Johnson *et al.*, 2007; Seixas, 2010; Santos-Silva *et al.*, 2011). Addition of haemoglobin removes CO from $Ru^{II}(CO)_3$ forming oxygen stable $Ru^{II}(CO)_2$ complexes which are no longer attacked by water under these conditions. Thus, the formation of radicals is inhibited by haemoglobin through scavenging of CO.

4.4 Discussion

Recently, CO-releasing molecules (CO-RMs) based on transition metal carbonyls, were shown to have bactericidal properties against several pathogens including *E. coli, S. aureus* and *Pseudomonas aeruginosa* (Nobre *et al.*, 2007; Desmard *et al.*, 2009). These effects have been attributed to CO release since the compounds lacking CO ligands are no longer bacterial inhibitors. Moreover, DNA microarrays indicate that CO is effectively delivered to intracellular targets causing a plethora of transcriptional modifications as judged by the analysis of the data acquired in cells of *E. coli* exposed to CO-RMs (Davidge *et al.*, 2009; Nobre *et al.*, 2009). The present work adds relevant information as it shows that CO-RMs stimulate the *in vivo* production of ROS and that the main cellular antioxidant glutathione confers protection against the killing effect of CO-RMs.

Importantly, ROS generation from the pioneering CORM-2 increases with the concentration of the CO-RM and is abolished once the CO groups are absent or scavenged by haemoglobin, providing a clear indication that CO is required for the formation of reactive oxygen species. Our data also show that *E. coli* strains mutated in genes that encode ROS detoxifiers, namely in superoxide dismutases (*sodAB)* and catalases (*katEG)* exhibited a sharp decreased survival indicating that impairment of ROS detoxification potentiates the effect of CORM-2.

Furthermore, CORM-2 was found to activate *recA* transcription and we observed that the bactericidal effect of CORM-2 is accentuated by knocking out *recA*, most probably due to the inability of triggering the SOS response. Accordingly, we observed by a fluorimetric assay that cells treated with CORM-2 have a higher content of damaged DNA when compared to cells

exposed to the inactive form of the compound. The presence of degraded DNA may be explained by the ROS generated by the bactericide as these species are known to cause DNA lesions (Imlay & Linn, 1988).

The results also revealed that cells treated with CORM-2 have higher levels of free iron, which does not arise from exogenous sources but from the disruption of iron-sulfur containing proteins. One of such damaged proteins was shown to be aconitase, a member of the dehydratase family of proteins characterized by a solvent-exposed oxygen-labile $[4Fe\text{-}4S]^{2+}$ cluster (Gardner & Fridovich, 1991), that upon exposure to CORM-2 exhibited a significantly lower *in vivo* activity. Moreover, the *iscS* mutant showed higher resistance to CORM-2, possibly due to the lower abundance of Fe-S clusters in this mutant and to the activation of the *suf* operon through positive regulation of the apo-form of IscR (Schwartz *et al.*, 2000; Jang & Imlay, 2010). Hence, the toxicity of CORM-2 is related to the content of denaturated Fe-S clusters.

Carbon monoxide is a classical inhibitor of the aerobic respiration and consequently it is a source of ROS, as demonstrated for mitochondria and embryonic kidney cells (D'Amico *et al.*, 2006). However, our results show that CORM-2 generates hydroxyl radicals *per se*, and this ability seems to be not restricted to this CO-releaser compound since not only ALF062 but also other transition metal carbonyls, like $Na[Mo(CO)_3(histidinate)]$ and $Na_3[Mo(CO)_3(citrate)]$, are reported to promote hydroxyl radical formation (Seixas, 2010). More importantly, we proved that the radical formation from CORM-2 is intimately associated with the presence of CO ligands as no radical species were observed when the inactive compound devoid of CO, iCORM-2, was used and upon addition of the haemoglobin, a CO chelator.

Furthermore, *in vitro* experiments demonstrate that in the presence of CORM-2 thiol groups become unable to react with Ellman's reagent, thus

suggesting that CORM-2 oxidizes free thiol groups of amino acids, most probably due to its ability to produce ROS *in vitro*. These results are in agreement with the fact that thiol donors, like cysteine, are able to cancel the effects of CORM-2 on bacterial growth, which was observed by us in the present work and by other authors when using CORM-3 against *P. aeruginosa* (Desmard *et al.*, 2009).

The work described herein shows that bacterial cells treated with CORM-2 have higher amounts of ROS. The fact that other authors did not observe any increase in H_2O_2 production in *P. aeruginosa* cells exposed to CORM-3 may be due to the experimental conditions utilized. Nevertheless, the addition of the antioxidant N-acetyl cysteine (NAC) abolished the CORM-3-mediated inhibition of bacterial growth (Desmard *et al.*, 2009). Moreover, they observed that *P. aeruginosa* cells treated with CORM-3 caused the impairment of the respiratory chain, which usually leads to generation of ROS (Desmard *et al.*, 2009).

Interestingly, several reports have demonstrated that many antibiotics mediate cell death through hydroxyl radical formation in bacteria and that there exists a relationship between antibiotic susceptibility and production of ROS (Becerra & Albesa, 2002; Kohanski *et al.*, 2007; Dwyer *et al.*, 2009). Hence, like other antibiotics, the lethal effect of CO-releasing compounds seems to rely on the production of ROS.

In conclusion, we have demonstrated that CO-RMs stimulate, in a non-cellular medium, the formation of hydroxyl radicals in a CO-dependent mode. The radicals that are generated account for the bactericidal properties of CORM-2 by damaging DNA and iron-sulfur proteins; the consequent increase of intracellular free iron promotes ROS via the Fenton reaction and for survival

bacteria need to bring into play cellular mechanisms of oxidant protection. Altogether, the herein presented results provide a molecular basis of the CO-RMs antibacterial action.

4.5 Acknologments

The authors are grateful to Prof. Nishioka for providing the *E. coli* DSH7, DSH19 and DSH56 strains. Work was funded by FCT project PTDC/BIA-PRO/098224/2008 and by FCT studentships SFRH/BD/38457/2007 (AFNT) and SFRH/BPD/69325/2010 (LSN).

4.6 References

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

Ayala-Castro C, Saini A & Outten FW (2008) Fe-S cluster assembly pathways in bacteria. *Microbiol Mol Biol Rev* **72**: 110-125.

Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL & Mori H (2006) Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* **2**: 2006 0008.

Becerra MC & Albesa I (2002) Oxidative stress induced by ciprofloxacin in Staphylococcus aureus. *Biochem Biophys Res Commun* **297**: 1003-1007.

Brzoska K, Meczynska S & Kruszewski M (2006) Iron-sulfur cluster proteins: electron transfer and beyond. *Acta Biochim Pol* **53**: 685-691.

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

Chung SW, Liu X, Macias AA, Baron RM & Perrella MA (2008) Heme oxygenase-1-derived carbon monoxide enhances the host defense response to microbial sepsis in mice. *J Clin Invest* **118**: 239-247.

Courcelle J & Hanawalt PC (2003) RecA-dependent recovery of arrested DNA replication forks. *Annu Rev Genet* **37**: 611-646.

D'Amico G, Lam F, Hagen T & Moncada S (2006) Inhibition of cellular respiration by endogenously produced carbon monoxide. *J Cell Sci* **119**: 2291-2298.

Davidge KS, Sanguinetti G, Yee CH, Cox AG, McLeod CW, Monk CE, Mann BE, Motterlini R & Poole RK (2009) Carbon monoxide-releasing antibacterial molecules target respiration and global transcriptional regulators. *J Biol Chem* **284**: 4516-4524.

Desmard M, Davidge KS, Bouvet O, Morin D, Roux D, Foresti R, Ricard JD, Denamur E, Poole RK, Montravers P, Motterlini R & Boczkowski J (2009) A carbon monoxide-releasing molecule (CORM-3) exerts bactericidal activity against Pseudomonas aeruginosa and improves survival in an animal model of bacteraemia. *Faseb J* **23**: 1023-1031.

Dwyer DJ, Kohanski MA & Collins JJ (2009) Role of reactive oxygen species in antibiotic action and resistance. *Curr Opin Microbiol* **12**: 482-489.

Ellman GL (1959) Tissue sulfhydryl groups. *Arch Biochem Biophys* **82**: 70-77.

Gardner PR & Fridovich I (1991) Superoxide sensitivity of the Escherichia coli aconitase. *J Biol Chem* **266**: 19328-19333.

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

Haldane JB (1927) Carbon Monoxide as a Tissue Poison. *Biochem J* **21**: 1068-1075.

Imlay JA & Linn S (1988) DNA damage and oxygen radical toxicity. *Science* **240**: 1302-1309.

Jang S & Imlay JA (2010) Hydrogen peroxide inactivates the Escherichia coli Isc iron-sulphur assembly system, and OxyR induces the Suf system to compensate. *Mol Microbiol* **78**: 1448-1467.

Johnson DC, Dean DR, Smith AD & Johnson MK (2005) Structure, function, and formation of biological iron-sulfur clusters. *Annu Rev Biochem* **74**: 247-281.

Johnson TR, Mann BE, Teasdale IP, Adams H, Foresti R, Green CJ & Motterlini R (2007) Metal carbonyls as pharmaceuticals? [Ru(CO)3Cl(glycinate)], a CO-releasing molecule with an extensive aqueous solution chemistry. *Dalton Trans*: 1500-1508.

Keyer K & Imlay JA (1996) Superoxide accelerates DNA damage by elevating free-iron levels. *Proc Natl Acad Sci U S A* **93**: 13635-13640.

Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA & Collins JJ (2007) A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* **130**: 797-810.

LeBel CP, Ischiropoulos H & Bondy SC (1992) Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. *Chem Res Toxicol* **5**: 227-231.

Li C, Hossieny P, Wu BJ, Qawasmeh A, Beck K & Stocker R (2007) Pharmacologic induction of heme oxygenase-1. *Antioxid Redox Signal* **9**: 2227-2239.

Mailloux RJ, Singh R, Brewer G, Auger C, Lemire J & Appanna VD (2009) Alpha-ketoglutarate dehydrogenase and glutamate dehydrogenase work in tandem to modulate the antioxidant alpha-ketoglutarate during oxidative stress in Pseudomonas fluorescens. *J Bacteriol* **191**: 3804-3810.

Masip L, Veeravalli K & Georgiou G (2006) The many faces of glutathione in bacteria. *Antioxid Redox Signal* **8**: 753-762.

Meister A (1985) Glutamate synthase from Escherichia coli, Klebsiella aerogenes, and Saccharomyces cerevisiae. *Methods Enzymol* **113**: 327-337.

Moeck GS & Coulton JW (1998) TonB-dependent iron acquisition: mechanisms of siderophore-mediated active transport. *Mol Microbiol* **28**: 675-681.

Nobre LS, Seixas JD, Romao CC & Saraiva LM (2007) Antimicrobial action of carbon monoxide-releasing compounds. *Antimicrob Agents Chemother* **51**: 4303-4307.

Nobre LS, Al-Shahrour F, Dopazo J & Saraiva LM (2009) Exploring the antimicrobial action of a carbon monoxide-releasing compound through whole-genome transcription profiling of Escherichia coli. *Microbiology* **155**: 813-824.

Ryter SW, Otterbein LE, Morse D & Choi AM (2002) Heme oxygenase/carbon monoxide signaling pathways: regulation and functional significance. *Mol Cell Biochem* **234-235**: 249-263.

Santos-Silva T, Mukhopadhyay A, Seixas JD, Bernardes GJ, Romao CC & Romao MJ (2011) CORM-3 reactivity toward proteins: the crystal structure of a Ru(II) dicarbonyl-lysozyme complex. *J Am Chem Soc* **133**: 1192-1195.

Schwartz CJ, Djaman O, Imlay JA & Kiley PJ (2000) The cysteine desulfurase, IscS, has a major role in in vivo Fe-S cluster formation in Escherichia coli. *Proc Natl Acad Sci U S A* **97**: 9009-9014.

Seixas JDdS (2010) Development of CO-Releasing Molecules for the treatment of inflammatory diseases. Vol. PhD thesis: Instituto de Tecnologia Quimíca e Biológica da Universidade Nova de Lisboa.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ & Klenk DC (1985) Measurement of protein using bicinchoninic acid. *Anal Biochem* **150**: 76-85.

Wu L & Wang R (2005) Carbon monoxide: endogenous production, physiological functions, and pharmacological applications. *Pharmacol Rev* **57**: 585-630.

Yonezawa Y & Nishioka H (1999) Sensitivities and gene-expressions of Escherichia coli mutants deficient in DNA repair and reactive oxygen species scavenging capacity exposed to natural sunlight. *J Toxicol Environ Health A* **57**: 237-245.

Zhang J & Piantadosi CA (1992) Mitochondrial oxidative stress after carbon monoxide hypoxia in the rat brain. *J Clin Invest* **90**: 1193-1199.

Zhao H, Joseph J, Zhang H, Karoui H & Kalyanaraman B (2001) Synthesis and biochemical applications of a solid cyclic nitrone spin trap: a relatively superior trap for detecting superoxide anions and glutathiyl radicals. *Free Radic Biol Med* **31**: 599-606.

4.7 Supplementary data

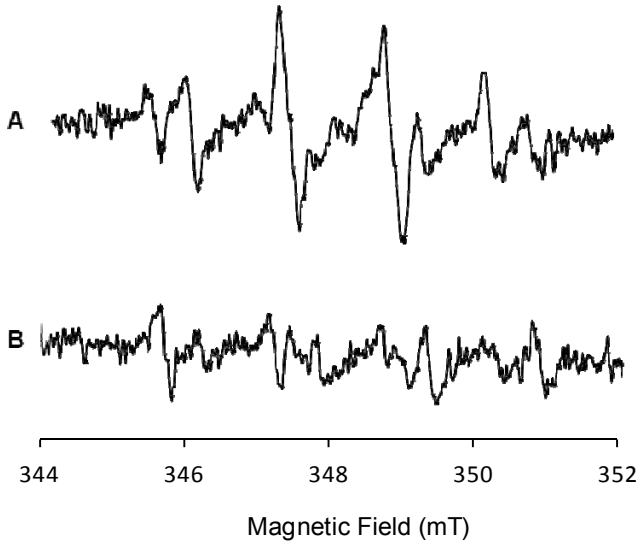


Figure S4.1| ALF062 generates ROS *in vitro*. EPR spectra of ALF062 in an oxygenated (A) and degassed solution (B).

Chapter 5|

Fighting

Helicobacter pylori with

Carbon Monoxide-Releasing Molecules

This chapter is part of a manuscript in preparation:

Tavares AFN, Parente M, Justino MC**,** Oleastro M, Nobre LS & Saraiva LM (2013) *Fighting Helicobacter pylori* with Carbon Monoxide-Releasing Molecules.

Ana Filipa Tavares performed all the experimental work, with the exception of the oxygen consumption assays.

Summary| *Helicobacter pylori* is a pathogen that establishes long life infections responsible for chronic gastric ulcer diseases and a proved risk factor for gastric carcinoma, which is a leading cause of cancer-related death in the world. In spite of the use of a multi-antibiotic therapeutics, the increasing number of resistant *H. pylori* strains makes urgent the development of alternative treatments. We report that *H. pylori* is killed by carbon-monoxide releasing molecules, such as CORM-2 and CORM-3, with minimal inhibitory concentration (MIC) of 200 and 420 mg/L, respectively. In particular, CORM-2 is shown to inhibit the urease activity and bacterial respiration of *H. pylori*. Analysis of several clinical isolates of *H. pylori* that are resistant to metronidazole showed to be susceptible to CORM-2 treatment. Moreover, the MIC values for metronidazole of *H. pylori* clinical strains significantly decrease upon exposure to sub-lethal doses of CORM-2. In key cells of the innate immune system, such as macrophages, CORM-2 alone and together with metronidazole was found to strongly reduce the survival of *H. pylori*. Thus, CO-RMs represent a novel class of antimicrobial therapeutic agents with potential to be clinically useful in the treatment of *H. pylori* infections.

5.1 Introduction

Helicobacter pylori is a pathogen that colonizes the gastric mucosa of humans and is ubiquitous in over half the world's population. Once acquired, *H. pylori* establishes lifelong infections that are the major cause of gastric and duodenal ulcer diseases, and malignant gastric cancer (Graham, 2000; Kusters *et al.*, 2006; McColl, 2010; Sachs & Scott, 2012). *H. pylori* uses several factors that enable colonization (Clyne *et al.*, 2007; Fischer *et al.*, 2009; Molnar *et al.*, 2010). In particular, the activity of the nickel-dependent urease is essential for the survival and pathogenesis of the bacterium as this enzyme hydrolyses urea to ammonia, which neutralizes the stomach acidity (Stingl & De Reuse, 2005; Zanotti & Cendron, 2010). The more widely used antibiotics for treatment of *H. pylori* are metronidazole, clarithromycin, amoxicillin and tetracycline (Rimbara *et al.*, 2011). Other antibiotics such as ciprofloxacin, levofloxacin and furazolidone are also utilized, in particular, as alternative or second line treatment (Dore *et al.*, 2012; Fakheri *et al.*, 2012; Gisbert *et al.*, 2013). However, when given as monotherapy none of these drugs are effective enough to eradicate *H. pylori*. Hence, infections with *H. pylori* are usually treated with a combination of drugs, which consists of two or three antibiotics together with an acid-suppressive drug (a proton pump inhibitor, *e.g.* omeoprazole) (Rimbara *et al.*, 2011). However, the efficacy of these multiple antibiotic therapies is decreasing mainly due to the crescendo occurrence of antibiotic-resistant *H. pylori* strains. Especially, metronidazole resistant strains are a major cause of *H. pylori* treatment failure (Megraud, 2004; Graham *et al.*, 2007).

The endogenous production of carbon monoxide (CO), via the mammalian haem oxygenase (HO), exerts benefits in the neural, cardiovascular and renal systems (Li *et al.*, 2007). Moreover, it has remarkable

impact on microbial sepsis as HO-1 deficient mice display enhanced susceptibility to polymicrobial infections, and the administration of exogenous CO rescues the HO-1-deficient mice from sepsis-induced lethality (Chung *et al.*, 2008). The CO-releasing molecules (CO-RMs) are metal carbonyls that have been used to administrate CO exogenously (Mann, 2010; Motterlini & Otterbein, 2010), and have potential medical applications. More recently, CO-RMs were demonstrated to kill pathogens such as *Escherichia coli*, *Staphylococcus aureus* (Nobre *et al.*, 2007), and *Pseudomonas aeruginosa* (Desmard *et al.*, 2009). The antimicrobial action of CO-RMs results from the binding of CO to haemoproteins, including those of the respiratory chain, together with the generation of intracellular oxidative stress, which deteriorates DNA, lipids, and proteins (Tavares *et al.*, 2011). In this work, the bactericidal effect of CO-RMs was analysed for several *H. pylori* strains, and the mechanisms that underpin the antimicrobial properties were addressed. The effect of CO-RM on *H. pylori* during infection of animal cell lines was also studied.

5.2 Material and methods

Reagents

Two different sources of CO were used, namely the tricarbonylchloro(glycinate)ruthenium (II), CORM-3 (Alfama), and the tricarbonyldichlororuthenium (II) dimer, CORM-2 (Sigma). These compounds were freshly prepared as a 10 or 20 mM stock solution by dissolution in pure distilled water and dimethyl sulfoxide (DMSO), respectively. Dichlorotetrakis(dimethyl sulfoxide) ruthenium (II) (Strem chemicals) dissolved in DMSO, was used as the inactive form of Ru-based CORM-2 (iCORM-2). To take into consideration that CORM-2 is a dimer, the iCORM-2 concentration

used in each assay was always twice the concentration of CORM-2. Metronidazole (MTZ), amoxicillin (AMX), and clarithromycin (CH) (all from Sigma) were dissolved in pure distilled water.

Bacterial strains, growth conditions and viability assays

Helicobacter pylori 26695 was used as the reference strain and the clinical strains were isolated from human gastric biopsies, the latter belonging to the Instituto Nacional de Saúde Doctor Ricardo Jorge collection, Portugal. All *H. pylori* strains were routinely cultured, at 37ºC, under a microaerobic atmosphere (6% O_2, 7% CO_2, 3.5% H_2 and 83.5% N_2) created by means of an Anoxomat system (MART Microbiology). Growth was performed in horse blood-agar (HBA, Oxoid) plates and in brain heart infusion (BHI, Oxoid) liquid medium, both supplemented with an antibiotics/fungicide cocktail. The cocktail consisted in a mixture of vancomycin (12.5 mg/L), polymyxin B (0.3 mg/L), trimethoprim (6.3 mg/L) and amphotericin B (5.0 mg/L). The liquid medium was further supplemented with 10% (v/v) decomplemented fetal calf serum (FCS, Gibco-Invitrogen) or with 0.2% β-cyclodextrin (βCD, Sigma).

To analyse the susceptibility of *H. pylori* to CO-RMs, cells cultured on HBA plates, for 24 h were used to inoculate 10 mL of BHI-FCS liquid media contained in 25 cm^3 cell culture flasks (Nunc). After a 16 h incubation period, these cultures were used as starter cultures to inoculate *H. pylori* in 10 mL BHI-βCD at an optical density at 600 nm (OD_{600nm}) ~0.05. Immediately after inoculation, CORM-3, CORM-2, iCORM-2 and/or MTZ were added to cultures and growth was monitored by recording the OD_{600nm} for 20 h. At selected times, the number of viable cells was evaluated by measuring the colony-forming unit per milliliter (CFU/mL) formed upon plating serial dilutions on HBA plates, which were incubated three days, at 37ºC, under a microaerobic

atmosphere. When indicated, the *H. pylori* growth was performed as described above in the presence of 5 mM reduced glutathione (Sigma) and 5 mM cysteine (Fluka). The *H. pylori* morphology was examined after staining the bacteria with the Gram-staining kit (Fluka) and using a Leica DMRB microscope with 100 x objective.

Determination of the minimal inhibitory concentration and minimal bactericidal concentration

The minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) were determined for CORM-3, CORM-2 and antibiotics accordingly with (Albertson *et al.*, 1998). To this end, starting cultures of *H. pylori* 26695 and clinical isolates, prepared as described above, were used to inoculate fresh BHI-βCD medium to an OD_{600nm} of ~0.05, and aliquots of 1.2 mL were distributed in 24 well-plates (Sarstedt). For each antibiotic the following range of concentrations were used with increasing doubling concentrations: MTZ 0.5-256 mg/L, AMX 0.001-0.250 mg/L and CH 0.001-0.250 mg/L. CORM-3 and CORM-2 were used from 50-600 mg/L with 50 mg/L intervals. After microaerobic incubation for 72 h, at 37ºC and 90 rpm, MICs were determined by reading the optical density at 600 nm. For the MBCs determination, 10 µL of each culture was then applied on HBA plates and incubated for another 72 h.

Oxygen consumption assays

H. pylori cultures grown for 15 h in BHI-βCD were harvested by centrifugation (5 min, 12000 × g, 4 °C), washed and resuspended in 10 mM potassium phosphate buffer (pH 7.0). Bacterial suspensions were incubated, for 5 min, with CORM-2 (25 mg/L), iCORM-2 (50 mg/L) or left untreated. The

O_2 consumption of *H. pylori* was stimulated by addition of sodium pyruvate (5 mM), and monitored in a Rank Broths oxygen electrode (Hansatech).

Urease activity assay

The urease activity was measured in *H. pylori* 26695 cultures, grown as described previously, for 15 h, in BHI-βCD in the absence and in the presence of 200 mg/L CORM-2 or iCORM-2, which, before analysed, were diluted in the same medium, to achieve a final concentration of 1 x 10^8 CFU/mL (OD_{600nm} ~0.1). The effect of CORM-2 was also analysed *in vitro*, using increasing concentrations of CORM-2. In this case, a *H. pylori* suspension was prepared in PBS at 2x10^8 CFU/mL (OD_{600nm} ~0.2), using cells grown for 24 h on HBA plates, and treated with CORM-2 (0, 2.5, 5, 12.5, 25, 50, 100 and 200 mg/L) for 15 min.

Urease activity was determined by mixing 50 µL of each cellular suspension with 500 µL of a buffered solution (pH 6.9) containing 0.7 mM KH_2PO_4 (Roth), 0.7 mM Na_2HPO_4 (Roth), 300 mM urea (Sigma) and 0.1 mM phenol red (Sigma) for 30 min and assessed spectrophotometrically at 560 nm (Gu *et al.*, 2004).

Assay of *Helicobacter pylori* viability in macrophages

Murine macrophage cell line RAW264.7 (ATCC Tib71) was seeded with 5 x 10^5 cells per well, in 24-well plates (Sarstedt) containing Dulbecco's modified Eagle's medium with 4.5 g/L glucose and 110 mg/mL sodium pyruvate (DMEM glutamax™, Gibco-Invitrogen) supplemented with 10% FCS, 70 U/mL penicillin and 70 µg/mL streptomycin (Gibco-Invitrogen), and grown for 3 h at 37º C in humidified 5% CO_2 atmosphere. At this point, macrophages were activated with 0.3 µg/mL gamma interferon (IFN-γ, Sigma), for 12 h.

Cultures of *H. pylori* 26695, grown as described above for the viability assays, in the presence of CORM-2, iCORM-2 and/or MTZ for 15 h, were washed three times with PBS (pH 7.4) and resuspended in infection medium (DMEM glutamax™ supplemented with 10% FCS, without addition of antibiotics), at an initial bacterial content of ~$5x10^8$ CFU/mL). These bacterial suspensions (100 µL) were used to infect macrophages contained in the same medium, at a multiplicity of infection (MOI) of ~100, for 3 and 6 h, at 37°C and 5% CO_2. The bacterial content was determined by plating serial dilutions onto HBA plates, which were incubated for 3 days at 37ºC under microaerobic conditions. After counting the number of viable bacteria, the values were normalized to the initial value of CFU/mL obtain (before cultures were used to infect macrophages), and the percentage survival was determined by dividing the number of colonies obtained for treated cultures by those counted for untreated cultures, assuming that the CFU/mL of untreated cultures correspond to 100 % survival.

Statistical analyses

Statistical analyses were performed with GraphPad Prism 5 (GraphPad Software), using, as indicated in each case, either One-way or Two-way ANOVA followed by a Bonferroni multiple comparison test. The Mann Whitney *t* test was used for the analysis of the MIC and MBC data, with the significance threshold at $P < 0.05$ (95 % confidence level). Data are presented as mean ± standard error (SE), with exception for MICs and MBCs that are presented as medians.

5.3 Results and discussion

Helicobacter pylori viability is inhibited by CO-RMs

To examine how CO-RMs affect the growth of *H. pylori,* CORM-2 and CORM-3 were added to cultures of *H. pylori* 26695 growing in BHI-βCD and incubated in a microaerobic atmosphere containing 6% O_2, 7% CO_2, 3.5% H_2 and 83.5% N_2. Both CO-RMs inhibited bacterial viability during a 20 h period, in a concentration-dependent manner (Figure 5.1). The effect mediated by CORM-2 was stronger than that exerted by CORM-3. Exposure of *H. pylori* to 200 mg/L CORM-2, for 15 h, induced a 4-log loss of viability (Figure 5.1A), while for the same period of time 300 mg/L CORM-3 lowered the cell viability by 2-logs (Figure 5.1B). Interestingly, for the same period of time, the decrease of the *H. pylori* bacterial count exerted by 300 mg/L CORM-2 was comparable to that caused by 1.5 mg/L

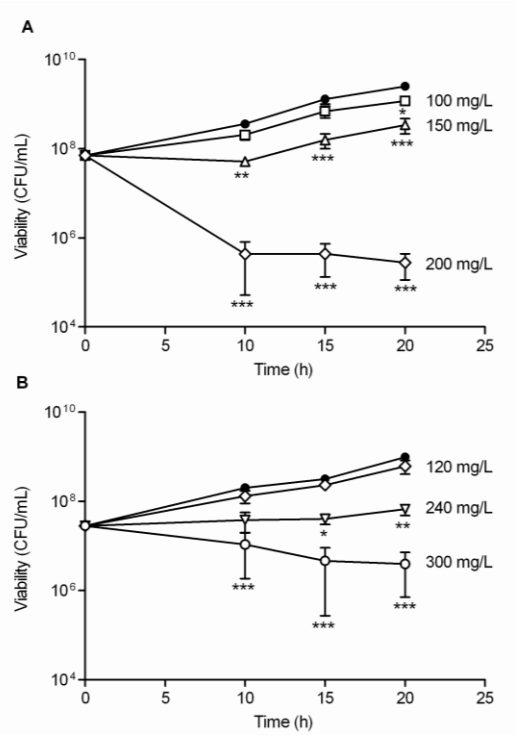


Figure 5.1| CO-RMs affect *H. pylori* 26695 viability. Cell viability of *H. pylori* 26695 left untreated (filled circle) and treated with 100, 150 and 200 mg/L CORM-2 (open square, triangle and diamond, respectively) (A), and after exposure to 120, 240 and 300 mg/L CORM-3 (open diamond, inverted triangle and circle, respectively) (B). The number of viable cells were determined for four biological samples and are expressed as means ± SE. *p<0.05, **p<0.01 and ***p<0.001 (Two-way ANOVA and Bonferroni test).

metronidazole (Figure 5.1A and S5.1).

Next, we determined for *H. pylori* 26695 the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) for the two CO-RMs, having obtaining the following values: for CORM-2 a MIC of 200 mg/L and MBC of 250 mg/L, and for CORM-3 a MIC of 420 mg/L and MBC of 510 mg/L. Furthermore, the ratio MBC/MIC was lower than 4 revealing the bactericidal character of the two drugs (Pankey & Sabath, 2004).

Since some antibiotics were report to induce in *H. pylori* the formation of coccoid forms (She *et al.*, 2001), we tested whether CO-RMs could promote similar modifications. However, even after 20 h exposure to 200 mg/L CORM-2, the shape of the treated cells remained unaltered and the presence of coccoid forms was not observed (data not shown).

CO-RMs used in this study are ruthenium-containing compounds that release CO when exposed to biological systems. To infer if the transition metal contributed to the antimicrobial effect induced by CO-RMs, the growth of *H. pylori* was analysed in the presence of dichlorotetrakis(dimethyl sulfoxide) ruthenium (II), the iCORM-2. However, iCORM-2 elicited no growth impairment up to 400 mg/L (Figure S5.2). Although ruthenium is not toxic by itself, we have previously demonstrated that the presence of the transition metal is needed to elicit the antimicrobial action, which relies on effects mediated by a network that involves CO liberation and ROS formation (Tavares *et al.*, 2011).

These data revealed that CO-RMs are effective antimicrobials against *H. pylori*, as previously reported for other pathogens as *E. coli*, *S. aureus* and *P. aeruginosa* (Nobre *et al.*, 2007; Desmard *et al.*, 2009). Since *H. pylori* was more effectively killed by CORM-2, the following experiments were performed with this compound.

CORM-2 hinders *H. pylori* respiration

Given the high affinity of CO to haem-containing proteins, we next examined whether the lower viability of *H. pylori* in the presence of CO-RMs was mediated by inhibition of the cellular respiration. *H. pylori* cells, cultured as described above, were grown to the mid-exponential phase (~15 h), harvested, resuspended in buffer, and the O_2 consumption rates determined by amperometric measurements with Clark-type O_2 electrode. Cells left untreated and stimulated with pyruvate had a considerable oxygen reductase specific activity (~1.2 nmol O_2/min/CFU). Incubation of the cells with 25 mg/L CORM-2 caused more than 50 % decrease in oxygen consumption when compared with untreated and iCORM-2-treated cells (Figure 5.2A). Similar findings have been reported for other bacteria as *Campylobacter jejuni* and *E. coli* (Davidge *et al.*, 2009; Smith *et al.*, 2011).

These results showed that CORM-2 inhibits cellular respiration, which is expected to induce the formation of reactive oxygen species (ROS). Furthermore, due to its chemical nature CO-RMs self-generated ROS that impact on bacterial survival (Tavares *et al.*, 2011). In agreement, we observed that supplementation of *H. pylori* growth medium with antioxidant agents such as glutathione and cysteine prevented bacterial killing (Figure 5.2B).

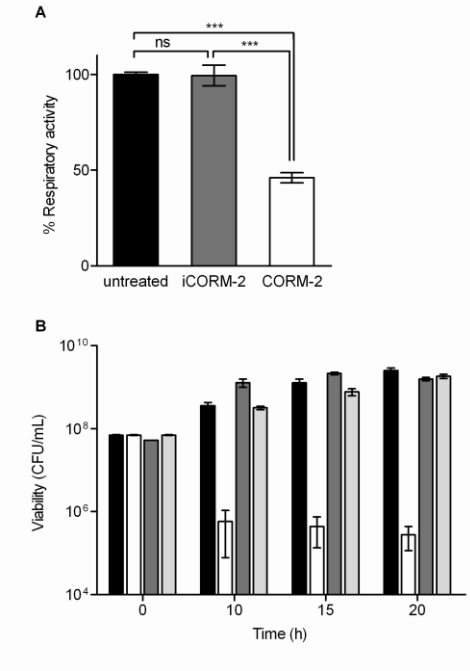


Figure 5.2| CORM-2 inhibits the *H. pylori* 26695 respiration. (A) Cellular suspensions of *H. pylori* were left untreated (black) and treated for 5 min with 25 mg/L CORM-2 (white) and the inactive form, 50 mg/L iCORM-2 (grey). Oxygen consumption was measured, after the addition of piruvate (5 mM) in a Clark-type O_2 electrode for two biological samples of *H. pylori* assayed in triplicate. Values are expressed as means ± SE.***p<0.001, ns - non significant (One-way ANOVA and Bonferroni test). (B) *H. pylori* viability in BHI-βCD medium (black) and after exposure to 200 mg/L CORM-2 alone (white) and in the presence of 5 mM glutathione (dark grey) or 5 mM cysteine (light grey). Values represent average of three biological samples with the respective SE.

CORM-2 inhibits the urease activity of *H. pylori*

In spite of the antimicrobial effect of CO-RMs on bacteria, the cellular targets remain largely unknown. *H. pylori* expresses significant amounts of a nickel-containing urease, which is a key enzyme in the metabolism of this pathogen during gastric colonization (Stingl & De Reuse, 2005). Since CO binds transition metals, we next determined whether CORM-2-derived CO altered the *H. pylori* urease activity. To this end, the urease activity of *H. pylori* grown in BHI-βCD and left untreated or treated for 15 h with iCORM-2 and with CORM-2 was compared. The results showed that while no differences were observed upon exposure to iCORM-2, the urease activity suffered a decrease of ~65% in cells treated with 200 mg/L CORM-2 (Figure 5.3A).

To determine the half-maximal inhibitory concentration (IC_{50}) of CORM-2 for the urease activity, cells of *H. pylori* grown for 24 h on HBA plates were used to prepare cell suspensions that were incubated for 15 min with increasing concentrations of CORM-2. Using CORM-2 concentrations up to 200 mg/L, a value of IC_{50} of 6 ± 1 mg/L was obtained (Figure 5.3B).

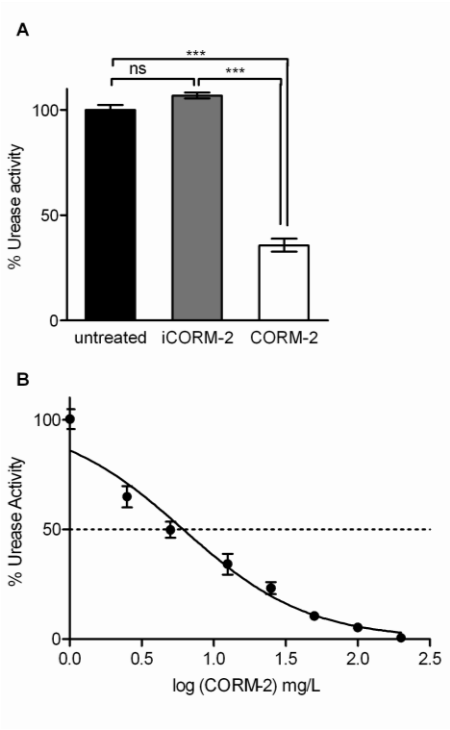


Figure 5.3| Inhibitory effect of CORM-2 on urease activity of *H. pylori* 26695. (A) Urease activity was measured in *H. pylori* cells left untreated (black), treated for 15 h with 400 mg/L iCORM-2 (grey) and 200 mg/L CORM-2 (white). The results represent the average of three biological samples performed in duplicate, and error bars represent the SE. ***p<0.001, ns - non significant (One-way ANOVA and Bonferroni test). (B) Urease activity of *H. pylori* cell suspensions treated, for 15 min, with several concentrations of CORM-2 (see Materials and methods). The results are the average of five biological samples and error bars represent SE.

These data revelead that the antimicrobial effect of CORM-2 on *H. pylori* is also associated with inhibition of urease activity, most probably due to the binding of CO to nickel for which CO has high affinity (Ermler *et al.*, 1998; Drennan *et al.*, 2001). Due to the crucial role of urease in the persistence of *H. pylori* in the gastric niche (Stingl & De Reuse, 2005), an urease inhibitor such as CORM-2 gains relevance as a novel way to control infections. Moreover, and

contrary with other potent urease inhibitors such as acetohydroxamic acid and fluorofamide (Phillips *et al.*, 1993; Pope *et al.*, 1998), CORM-2 is stable under the acidic conditions encountered by *H. pylori*, representing a clear advantage for *H. pylori* treatment {Johnson, 2007 #47; Funaioli, 1999 #48.

CORM-2 is able to kill metronidazole-resistant *H. pylori* strains

H. pylori is a pathogen with a high degree of adaptation to the human stomach, which is reflected in high diversity of phenotype and antibiotic-resistance of the clinical isolates. Hence, we next investigated the toxicity of CO-RMs towards *H. pylori* clinical strains isolated from human gastric biopsies with different degree of MTZ resistance (Table 5.1). The behavior of the clinical isolates when treated with CORM-2 differed significantly, exhibiting MIC_{CORM-2} between 100 and 200 mg/L (Table 5.1). Moreover, CORM-2 acted as a bactericidal since the MBC/MIC ratios determined for all isolates were lower than four (Table S5.1). More importantly, while the MTZ-susceptible strains had the highest MICs to CORM-2 (MIC_{CORM-2} = 200 mg/L) (Table 5.1), the growth of MTZ-resistant clinical isolates (MIC_{MTZ} > 8 mg/L) was inhibited by lower concentrations of CORM-2 ($MIC_{CORM-2} \leq 150$ mg/L). The only exception occurred for the MTZ-resistant strain 4597, which exhibited the same MIC for CORM-2 than the MTZ-susceptible strains (Table 5.1).

Table 5.1| MICs of CORM-2 and metronidazole to the reference strain 26695 and clinical isolates of *H. pylori.*

	MIC (mg/L)	
Strain	**CORM-2**	**MTZ***
26695	200	8
5599	200	2
5611	150	64
5846	100	16
4597	200	32
4574	150	32
5587	100	32

*S: Sensitive (MIC< 8 mg/L)

R: Resistant (MIC> 8 mg/L)

Combination of CORM-2 and antibiotics attenuates *in vitro H. pylori* viability

Since *H. pylori* infections are usually eradicate by means of triple or even quadruple therapies in which at least two drugs are antibiotics, we sought to analyse the effect of CORM-2 as an adjuvant of the currently used antibiotics. For this purpose, CORM-2 was conjugated with each one of the three most commonly antibiotics used to treat *H. pylori*, namely MTZ, amoxicillin (AMX) and clarithromycin (CH), and the MIC and MBC values were evaluated.

To this end, *H. pylori* 26695 was exposed to sub-lethal doses of CORM-2 and, for each antibiotic, the MICs and MBC values were determined (Table S5.2). The addition of MTZ (1.5 mg/L) for 15 h resulted in a decrease of less than 2-log while a non significant alteration occurred with CORM-2 (100 mg/L) alone (Figure 5.4). Simultaneous exposure to the two drugs resulted in a four-log drop in recovered viable bacteria after 20 h of treatment (Figure 5.4). The effect of 100 mg/L CORM-2 in MTZ translated into a reduction of 50% of the MIC_{MTZ} (Figure 5.5 and Table S5.2). Likewise, conjugation of CORM-2 with AMX and CH led to a significant decrease of the MIC and MBC (Figure 5.5 and Table S5.2) that, like for MTZ, was more pronounced for higher concentrations of CORM-2 further confirming the influence of the drug on the measure values (Figure 5.5). The effect of combining MTZ with CORM-2 was also tested for all *H. pylori* clinical isolates. At least, a twofold decrease of the MIC_{MTZ} was observed, with the highest MTZ resistant strains exhibiting the more significant drop of the MIC and MBC values (Figure 5.6). For example, the clinical isolate *H. pylori* 5611 (MIC_{MTZ} = 64 mg/L) become susceptible (MIC_{MTZ} = 8 mg/L) when the MTZ was administrated together with 100 mg/L CORM-2 (Tables S5.3 and S5.4).

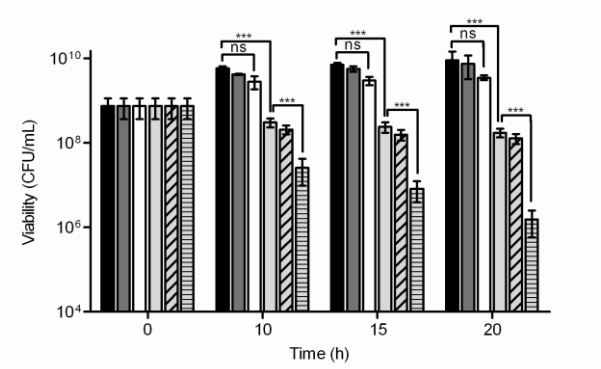


Figure 5.4| Effect of combined metronidazole and CORM-2 treatment on *H. pylori* viability. Cell viability of *H. pylori* 26695 left untreated (black) and exposed to iCORM-2 (dark grey), 100 mg/L CORM-2 (white), 1.5 mg/L metronidazole (light grey), 1.5 mg/L metronidazole and 200 mg/L iCORM-2 (light grey, diagonal strips) and 1.5 mg/L metronidazole and 100 mg/L CORM-2 (light grey, horizontal strips). The number of viable cells were determined for four independent cultures and are expressed as means ± SE. ***p<0.001 (Two-way ANOVA and Bonferroni test).

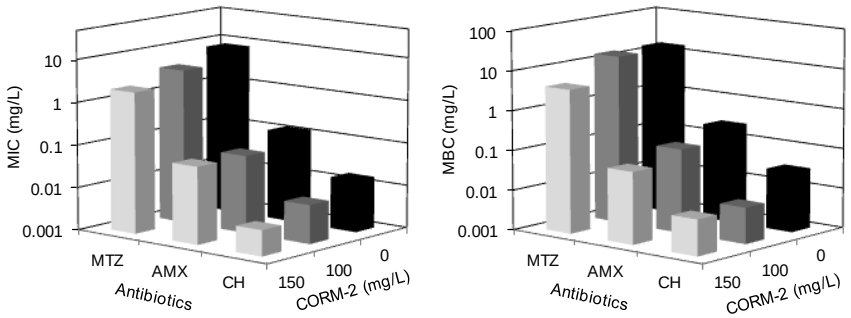


Figure 5.5| CORM-2 decreases the MIC and MBC values of metronidazole, amoxicillin and clarithromycin for *H. pylori* 26695. (A) MICs and (B) MBCs of *H. pylori* 26995 for metronidazole, amoxicillin and clarithromycin determined in the absence (black) and in the presence of 100 mg/L (dark grey) and 150 mg/L CORM-2 (light grey). Results representing the median of five biological samples are significantly different in all cases (p<0.05 in Mann Whitney *t* test).

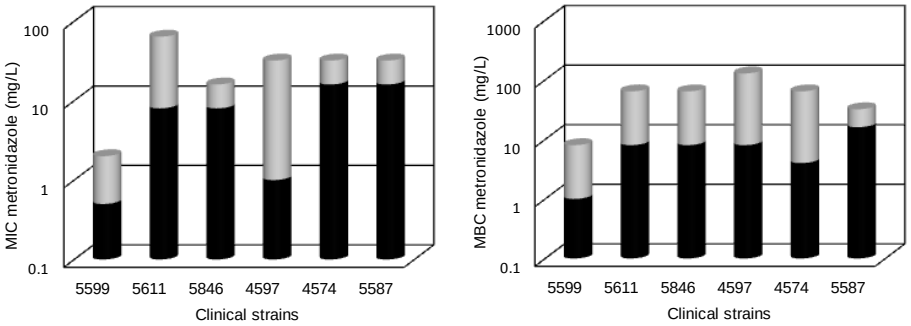


Figure 5.6| CORM-2 lowers the MIC and MBC of metronidazole for *H. pylori* clinical isolates. (A) MIC of metronidazole was determined for each *H. pylori* clinical isolate in the absence (grey) and in the presence of 50 mg/L (strains 5846 and 5587), 100 mg/L (strains 5611 and 4574) and 150 mg/L CORM-2 (strains 5599 and 4597) (black). (B) MBC of metronidazole obtained for *H pylori* clinical isolates, in the absence (grey) and in the presence of 100 mg/L (strains 5846 and 5587) and 150 mg/L (strains 5611, 4574, 4597 and 5599) CORM-2 (black). In all cases, values representing the median of five biological samples were significantly different ($p<0.05$ in Mann Whitney *t* test).

CO-RM treated *H. pylori* compromises *in vivo* bacterial survival

Upon pathogen invasion the mammalian immune system triggers an arsenal of weapons aiming to eliminate the harmful microbe. Professional phagocytes, such as macrophages, play a key role due to the damage inflicted to invaders. Since *H. pylori* is known to trigger the innate immune system it is important to evaluate the effect of CORM-2 upon bacterial infection of macrophages. Hence, the viability of CORM-2-treated *H. pylori* was analysed in the murine macrophage cell line RAW264.7. To this end, *H. pylori* 26695 cells remained unexposed, and exposed to the inactive form iCORM-2, and to CORM-2 were incubated with activated macrophages and their viability evaluated by colony formation after 3 and 6 h post-infection. For similar viable bacterial loads, the iCORM-2-treated *H. pylori* was as resistant to macrophages as untreated bacterial cells. On the contrary, the survival of the CORM-2-

treated *H. pylori* was approximately 98% lower in comparison to cells exposed to the inactive form iCORM-2 (Figure 5.7).

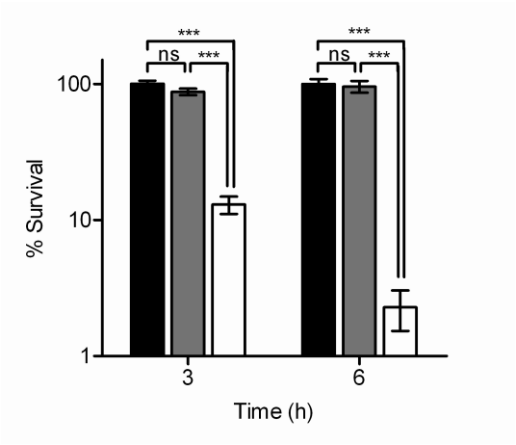


Figure 5.7| CORM-2-treated *H. pylori* 26695 is more susceptible to macrophage killing. Macrophages RAW264.7, pre-activated with IFN-γ (0.3 μg/mL) for 12 h, were infected (MOI ~100) with *H. pylori* untreated (black), treated with 400 mg/L iCORM-2 (grey) or 200 mg/L CORM-2 (white). Bacterial survival was determined after 3 and 6 h of infection for five biological samples analysed in triplicate. Error bars represent the SE. ***p<0.001, ns - non significant (Two-away ANOVA and Bonferroni test).

Combined CORM-2-MTZ strongly reduces *in vivo* survival of *H. pylori*

Given that the combination of CORM-2 with MTZ reduced the *in vitro* viability of *H. pylori*, we sought to determine the survival of the double-treated *H. pylori* when in contact with activated macrophages. Sub-lethal concentrations of CORM-2 conjugated with MTZ led to a ~98% decrease in bacterial count, while no significant alteration was seen when infecting with cells treated with CORM-2 or iCORM-2 alone, and a ~30% survival was observed for cells exposed to MTZ alone or MTZ plus the inactive form of

CORM-2 (Figure 5.8). This allows concluding that the co-administration of MTZ-CORM-2 renders *H. pylori* more susceptible to *in vivo* macrophage killing.

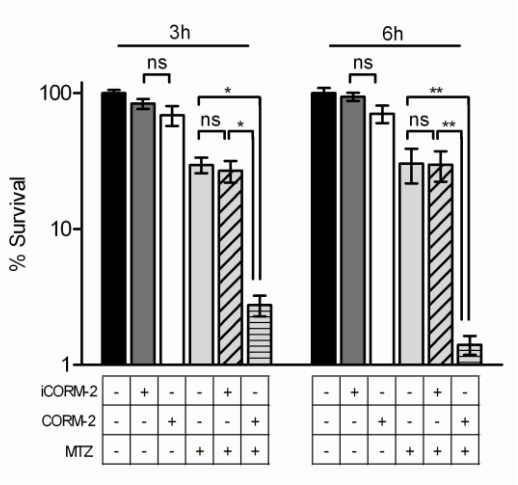


Figure 5.8| *H. pylori* 26695 treated with metronidazole plus CORM-2 are higly susceptible to macrophage killing. Macrophages RAW264.7, pre-activated with IFN-γ (0.3 µg/mL) for 12 h, were infected at a MOI of ~100 with *H. pylori* untreated (black), treated with 200 mg/L iCORM-2 (dark grey), 100 mg/L CORM-2 (white), 1.5 mg/L metronidazole (light grey), metronidazole with iCORM-2 (grey, diagonal strips) and metronidazole with 100 mg/L CORM-2 (grey, horizontal strips). Bacterial survival was determined after 3 and 6 h of infection. The results are the average of five biological samples performed in triplicate, and error bars represent the SE. *p<0.05, **p<0.01, ns - non significant (Two-way ANOVA and Bonferroni test).

In conclusion, this work provides evidences that CO-RMs constitute a promising therapeutic strategy for the treatment of *H. pylori*, as they are efficient antimicrobials both alone and as co-adjuvants with the antibiotics currently used to treat *H. pylori*. This is important data since chronic colonization with *H. pylori* antibiotic-resistant strains is difficult to eradicate and combined therapies are among the more effective means to combat resistant strains.

5.4 Acknowledgments

The work was funded by Project Grants PEst-OE/EQB/LA0004/2011, PTDC/BIA-PRO/098224/2008 and PTDC/SAU-MII/098086/2008 from Fundação para a Ciência e Tecnologia (FCT), and by FCT fellowships SFRH/BD/38457/2007 (AFNT), SFRH/BD/81192/2011 (MP), and SRFH/BPD/69325/2010 (LN).

5.5 References

Albertson N, Wenngren I & Sjostrom JE (1998) Growth and survival of Helicobacter pylori in defined medium and susceptibility to Brij 78. *J Clin Microbiol* **36**: 1232-1235.

Chung SW, Liu X, Macias AA, Baron RM & Perrella MA (2008) Heme oxygenase-1-derived carbon monoxide enhances the host defense response to microbial sepsis in mice. *J Clin Invest* **118**: 239-247.

Clyne M, Dolan B & Reeves EP (2007) Bacterial factors that mediate colonization of the stomach and virulence of Helicobacter pylori. *FEMS Microbiol Lett* **268**: 135-143.

Davidge KS, Sanguinetti G, Yee CH, Cox AG, McLeod CW, Monk CE, Mann BE, Motterlini R & Poole RK (2009) Carbon monoxide-releasing antibacterial molecules target respiration and global transcriptional regulators. *J Biol Chem* **284**: 4516-4524.

Desmard M, Davidge KS, Bouvet O, Morin D, Roux D, Foresti R, Ricard JD, Denamur E, Poole RK, Montravers P, Motterlini R & Boczkowski J (2009) A carbon monoxide-releasing molecule (CORM-3) exerts bactericidal activity against Pseudomonas aeruginosa and improves survival in an animal model of bacteraemia. *Faseb J* **23**: 1023-1031.

Dore MP, Tadeu V, Are B, Mura I, Fanciulli G, Massarelli G & Piana A (2012) Efficacy of a "Rescue" Ciprofloxacin-Based Regimen for Eradication of Helicobacter pylori Infection after Treatment Failures. *Gastroenterol Res Pract* **2012**: 484591.

Drennan CL, Heo J, Sintchak MD, Schreiter E & Ludden PW (2001) Life on carbon monoxide: X-ray structure of Rhodospirillum rubrum Ni-Fe-S carbon monoxide dehydrogenase. *Proc Natl Acad Sci U S A* **98**: 11973-11978.

Ermler U, Grabarse W, Shima S, Goubeaud M & Thauer RK (1998) Active sites of transition-metal enzymes with a focus on nickel. *Curr Opin Struct Biol* **8**: 749-758.

Fakheri H, Bari Z & Sardarian H (2012) A modified bismuth-containing quadruple therapy including a short course of furazolidone for Helicobacter pylori eradication after sequential therapy failure. *Helicobacter* **17**: 264-268.

Fischer W, Prassl S & Haas R (2009) Virulence mechanisms and persistence strategies of the human gastric pathogen Helicobacter pylori. *Curr Top Microbiol Immunol* **337**: 129-171.

Gisbert JP, Perez-Aisa A, Bermejo F, Castro-Fernandez M, Almela P, Barrio J, Cosme A, Modolell I, Bory F, Fernandez-Bermejo M, Rodrigo L, Ortuno J, Sanchez-Pobre P, Khorrami S, Franco A, Tomas A, Guerra I, Lamas E, Ponce J & Calvet X (2013) Second-line Therapy With Levofloxacin After Failure of Treatment to Eradicate Helicobacter pylori Infection: Time Trends in a Spanish Multicenter Study of 1000 Patients. *J Clin Gastroenterol* **47**: 130-135.

Graham DY (2000) Helicobacter pylori infection is the primary cause of gastric cancer. *J Gastroenterol* **35 Suppl 12**: 90-97.

Graham DY, Lu H & Yamaoka Y (2007) A report card to grade Helicobacter pylori therapy. *Helicobacter* **12**: 275-278.

Gu Q, Xia HH, Wang WH, Wang JD, Wong WM, Chan AO, Yuen MF, Lam SK, Cheung HK, Liu XG & Wong BC (2004) Effect of cyclo-oxygenase inhibitors on Helicobacter pylori susceptibility to metronidazole and clarithromycin. *Aliment Pharmacol Ther* **20**: 675-681.

Kusters JG, van Vliet AH & Kuipers EJ (2006) Pathogenesis of Helicobacter pylori infection. *Clin Microbiol Rev* **19**: 449-490.

Li C, Hossieny P, Wu BJ, Qawasmeh A, Beck K & Stocker R (2007) Pharmacologic induction of heme oxygenase-1. *Antioxid Redox Signal* **9**: 2227-2239.

Mann BE (2010) *Carbon Monoxide: An Essential Signalling Molecule*: Springer.

McColl KE (2010) Clinical practice. Helicobacter pylori infection. *N Engl J Med* **362**: 1597-1604.

Megraud F (2004) H pylori antibiotic resistance: prevalence, importance, and advances in testing. *Gut* **53**: 1374-1384.

Molnar B, Galamb O, Sipos F, Leiszter K & Tulassay Z (2010) Molecular pathogenesis of Helicobacter pylori infection: the role of bacterial virulence factors. *Dig Dis* **28**: 604-608.

Motterlini R & Otterbein LE (2010) The therapeutic potential of carbon monoxide. *Nat Rev Drug Discov* **9**: 728-743.

Nobre LS, Seixas JD, Romao CC & Saraiva LM (2007) Antimicrobial action of carbon monoxide-releasing compounds. *Antimicrob Agents Chemother* **51**: 4303-4307.

Pankey GA & Sabath LD (2004) Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of Gram-positive bacterial infections. *Clin Infect Dis* **38**: 864-870.

Phillips K, Munster DJ, Allardyce RA & Bagshaw PF (1993) Antibacterial action of the urease inhibitor acetohydroxamic acid on Helicobacter pylori. *J Clin Pathol* **46**: 372-373.

Pope AJ, Toseland CD, Rushant B, Richardson S, McVey M & Hills J (1998) Effect of potent urease inhibitor, fluorofamide, on Helicobacter sp. in vivo and in vitro. *Dig Dis Sci* **43**: 109-119.

Rimbara E, Fischbach LA & Graham DY (2011) Optimal therapy for Helicobacter pylori infections. *Nat Rev Gastroenterol Hepatol* **8**: 79-88.

Sachs G & Scott DR (2012) Helicobacter pylori: Eradication or Preservation. *F1000 Med Rep* **4**: 7.

She FF, Su DH, Lin JY & Zhou LY (2001) Virulence and potential pathogenicity of coccoid Helicobacter pylori induced by antibiotics. *World J Gastroenterol* **7**: 254-258.

Smith H, Mann BE, Motterlini R & Poole RK (2011) The carbon monoxide-releasing molecule, CORM-3 (RU(CO)(3) CL(glycinate)), targets respiration and oxidases in Campylobacter jejuni, generating hydrogen peroxide. *IUBMB Life* **63**: 363-371.

Stingl K & De Reuse H (2005) Staying alive overdosed: how does Helicobacter pylori control urease activity? *Int J Med Microbiol* **295**: 307-315.

Tavares AF, Teixeira M, Romao CC, Seixas JD, Nobre LS & Saraiva LM (2011) Reactive oxygen species mediate bactericidal killing elicited by carbon monoxide-releasing molecules. *J Biol Chem* **286**: 26708-26717.

Zanotti G & Cendron L (2010) Functional and structural aspects of Helicobacter pylori acidic stress response factors. *IUBMB Life* **62**: 715-723.

5.6 Supplementary data

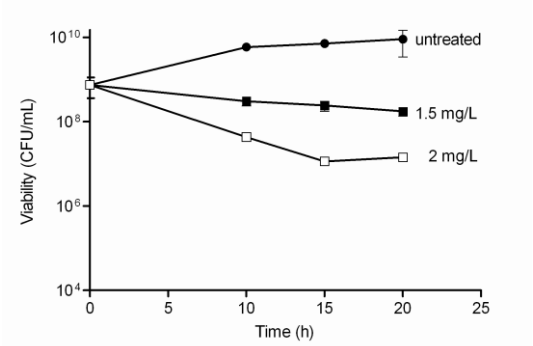


Figure S5.1|Effect of MTZ on *H. pylori* viability. *H. pylori* 26695, grown as described in Material and Methods, was treated with 1.5 and 2 mg/L metronidazole (black and white squares, respectively) or left untreated (black circle). Cell viability was analysed at the indicated times by determining CFU/mL. Values represent average values of two biological samples with the respective SE.

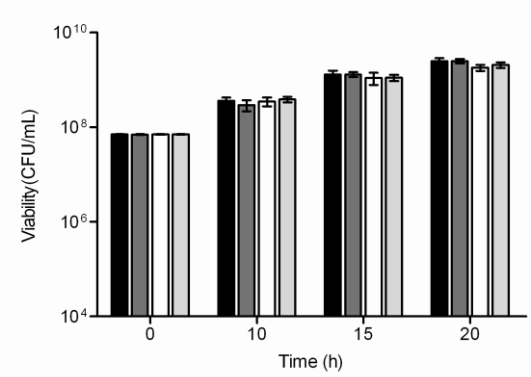


Figure S5.2| iCORM-2 has no effect on *H. pylori* viability. *H. pylori* 26695 left untreated (black) and after exposure to 200, 300 and 400 mg/L iCORM-2 (dark grey, white and light grey bars, respectively). Cell viability was determined as described in Material and Methods. Values represent the average values of three biological samples with SE.

Table S5.1| MIC and MBC of CORM-2 and metronidazole (MTZ) to the reference strain 26695 and clinical isolates of *H. pylori*.

	CORM-2 (mg/L)			MTZ* (mg/L)		
Strain	**MIC**	**MBC**	**MBC/MIC**	**MIC**	**MBC**	**MBC/MIC**
26695	200	250	1.3	8	16	2
5599	200	200	1.0	2	8	4
5611	150	200	1.3	64	64	1
5846	100	150	1.5	16	64	4
4597	200	200	1.0	32	128	4
4574	150	200	1.3	32	64	2
5587	100	150	1.5	32	32	1

*S: Sensitive (MIC<8 mg/L)

R: Resistant (MIC>8 mg/L)

Table S5.2| MIC and MBC of metronidazole (MTZ), clarithromycin (CH) and amoxicillin (AMX) for *H. pylori* 26695 determined in the presence of sub-lethal doses of CORM-2.

CORM-2 (mg/L)	MIC (mg/L)			MBC (mg/L)			MBC/MIC		
	MTZ	**CH**	**AMX**	**MTZ**	**CH**	**AMX**	**MTZ**	**CH**	**AMX**
0	8	0.016	0.125	16	0.032	0.25	2	2	2
100	4	0.008	0.064	16	0.008	0.125	4	1	2
150	2	0.004	0.064	4	0.008	0.064	2	2	1

Table S5.3| MIC of metronidazole with sub-lethal doses of CORM-2 for *H. pylori* clinical isolates.

CORM-2	MIC (mg/L)					
(mg/L)	5599	5611	5846	4597	4574	5587
0	2	64	16	32	32	32
50	2	16	8	32	32	16
100	1	8	-	4	16	-
150	0.5	-	-	1	-	-

Table S5.4| MBC of metronidazole with sub-lethal doses of CORM-2 for *H. pylori* clinical isolates.

CORM-2	MBC (mg/L)					
(mg/L)	5599	5611	5846	4597	4574	5587
0	8	64	64	128	64	32
50	8	64	16	64	64	32
100	2	16	8	16	32	16
150	1	8	-	8	4	-

Discussion

Chapter 6|

General discussion

Part of this Chapter is based on:

Tavares AFN, Nobre LS & Saraiva LM (2012) A role for reactive oxygen species in the antibacterial properties of Carbon Monoxide-Releasing Molecules. *FEMS Microbiology Letters* **336**:1-10.

Antibiotics revolutionized the treatment of infectious diseases having saved countless lives since their introduction in clinical practice in the middle of the last century. However, due to its widespread use many antibiotics are not as effective as they used to be, because bacteria have developed mechanisms to avoid the deleterious effects of these drugs (*e.g.* mutations in existing genes and acquisition of resistance genes from other organisms). In order to overcome this problem, it is crucial to have a deeper understanding of the mechanisms of antibiotic action and to develop new approaches to fight antibiotic-resistant bacteria.

6.1 The role of a novel *S. aureus* nitroreductase

In an era of great concern about resistance of *S. aureus* to several anti-infective agents, it is interesting to note that, even after several decades of clinical use, nitrofurans are still active against strains resistant to other drugs such as MRSA (Shah & Wade, 1989; Flournoy & Robinson, 1990; Johnson *et al.*, 1993; Johnson *et al.*, 1999; Guay, 2001; Barisic *et al.*, 2003; Ionescu *et al.*, 2010). Nitrofurans action is dependent on the reduction of the nitro group by bacterial nitroreductases (see section 1.2). Indeed**,** early studies have demonstrated an inverse correlation between nitrofurantoin MICs of urinary tract pathogens and their nitroreductase activity levels (McOsker *et al.*, 1989). Later, Whiteway and co-workers established a direct link between mutations in *nfsA* and *nfsB* nitroreductases of *E. coli*, and the development of bacterial resistance to nitrofurans (Whiteway *et al.*, 1998). Importantly, *S. aureus* clinical strains are usually susceptibility to both nitrofurantoin and nitrofurazone, which suggests the presence of enzymes capable of activating these nitroaromatic compounds to yield toxic species (Guay, 2001; Barisic *et al.*, 2003; Ionescu *et al.*, 2010). In fact, a search in the genome of *S. aureus*

NCTC 8325 revealed the presence of four putative nitroreductases, namely SAOUHSC_00833, SAOUHSC_02829, SAOUHSC_02258 and SAOUHSC_00366. To date, the *S. aureus* nitroreductase SAOUHSC_00366, named NfrA, was the only one studied, which showed the ability to catalyze the NADPH-dependent reduction of nitrofurantoin and nitrofurazone (Table 6.1) (Streker *et al.*, 2005). In this thesis, the role of the putative nitroreductase SAOUHSC_00833 of *S. aureus* in nitrofurans metabolism was addressed (Chapter 3). *S. aureus* SA0UHSC_00833, which was herein named NtrA, was demonstrated to reduce nitrofurans with values that are within the range of activities usually observed for bacterial nitroreductases (Table 6.1).

Table 6.1| Bacterial nitroreductase activities.

		Activity ($\mu mol.min^{-1}.mg^{-1}$ of protein)		
Bacteria	**NTR**	**Nitrofurantoin**	**Nitrofurazone**	**References**
E. coli	**NfsA**	82	73	(Zenno *et al.*, 1996a)
	NfsB	21*	13*	(Zenno *et al.*, 1996b)
S. aureus	**NfrA**	15	20	(Streker *et al.*, 2005)
	NtrA	14	15	(Tavares *et al.*, 2009)
H. pylori	**RdxA**	0.0010	0.0019	(Olekhnovich *et al.*, 2009)
	FrxA	2.0	0.5	(Sisson *et al.*, 2002)
B. subtillis	**NfrA1**	ND	97	(Zenno *et al.*, 1998)

NADPH was used as electron donor

* NADH was used as electron donor

ND – not determined

Since NtrA was capable of reducing nitrofurans under aerobic conditions, it could be inferred that this enzyme belongs to the family of oxygen-insensitive nitroreductases (see section 1.2.1). A previous phylogenetic analysis of the putative bacterial nitroreductases genes divided oxygen-

insensitive nitroreductases into two main groups that are represented by NfsA and NfsB of *E. coli* (Roldan *et al.*, 2008). Interestingly, the amino acid sequence of NtrA has no significant homology with either NfsA or NfsB family of nitroredutases, and our phylogenetic analysis revealed that *S. aureus* NtrA belongs to a novel family of bacterial nitroreductases (Figure 3.3). This family includes several other putative nitroreductases such as those from *Listeria inocua, Rubrobacter xylanophilus* and *Nitrobacter winogradskyi* (Table 6.2 and Figure 3.3).

Similar to nitroreductases of the group represent by NfsB of *E. coli*, the *S. aureus* NtrA was able to use both NADH and NADPH as a source of reducing equivalents, unlike those from NfsA group that only could use NADPH (see section 1.2.1). Interestingly, FMN cofactor was reported to be the most effective electron acceptor in *S. aureus* NtrA, as previously acknowledged to nitroreductases from both NfsA and NfsB groups (Zenno *et al.*, 1996a; Watanabe *et al.*, 1998; Streker *et al.*, 2005).

In this work, *S. aureus* NtrA was shown to contribute to the *in vivo* antimicrobial activity of nitrofurans. Indeed, the *ntrA* mutation in *S. aureus* resulted in an increased nitrofuran resistance which is consistent with the involvement of NtrA in the activation of these antibiotics (Figure 3.2). In agreement with the increased resistance of *S. aureus ntrA* mutant to nitrofurans, lower nitroreductase activity was measured in cell extracts of this mutant strain (Table 3.1). Moreover, mutation of *nfrA* (SAOUHSC_00366) of *S. aureus* also led to a higher resistance to nitrofurans (our unpublished results). Hence, it is here recognized a relation between nitroreductases and the bacterial susceptibility to nitrofurans in *S. aureus*.

Table 6.2| Percentage of identity (I) and similarity (S) of bacterial nitroreductases.

	%	*Ec* NfsA	*Bs* NfrA1	*Sa* NfrA	*Ec* NfsB	*Sa* SAOUHSC 02829	*Hp* FrxA	*Hp* RdxA	*Sa* SAOUHSC 02258	*Sa* NtrA	*Li* lin0488
Ec	I		39	34	10	10	9	11	8	9	12
NfsA	S		61	67	21	26	25	29	19	21	21
Bs	I			40	11	8	5	8	7	10	10
NfrA1	S			64	25	27	25	26	21	21	25
Sa	I				10	10	10	8	6	7	7
NfrA	S				24	29	28	26	22	22	21
Ec	I					20	18	19	7	7	11
NfsB	S					35	39	39	25	22	26
Sa **SAOUHSC 02829**	I						10	12	10	11	11
	S						26	25	27	22	26
Hp	I							21	10	9	11
FrxA	S							45	26	27	26
Hp	I								12	10	11
RdxA	S								25	22	24
Sa **SAOUHSC 02258**	I									12	9
	S									25	28
Sa	I										25
NtrA	S										42

Ec- Escherichia coli; *Sa – Staphylococcus aureus*; *Bs – Bacillus subtillis*; *Hp – Helicobacter pylori; Li – Listeria inocua.*

Further studies, as growth phenotype of strains mutated in the other two *S. aureus* nitroreductases, namely SAOUHSC_02829 and SAOUHSC_02258, as well as its biochemical characterization need to be performed to evaluate their contribution to *S. aureus* nitrofuran activation. Furthermore, it would be interestingly to analyse whether there is a relation between the degree of nitrofuran resistance in *S. aureus* clinical strains and mutations in the genes codifying for nitroreductases.

Nitroreductases co-administration could be used as a therapeutic strategy to increase bacterial susceptibility to nitrofurans, which could be relevant in case of multiple antibiotic resistances. Interestingly, co-administration of *E. coli* nitroreductases for direct anticancer therapy has already been suggested, as they are also able to promote activation of prodrugs in tumor cells (Johansson *et al.*, 2003; Searle *et al.*, 2004; Vass *et al.*, 2009).

Besides the role of *S. aureus* NtrA in nitrofurans activation, this work also showed the involvement of NtrA in the metabolism of GSNO, an important biological NO donor. GSNO induced the transcription of *ntrA* gene, as also previously seen in a DNA microarray study using S-nitroso N-acetyl DL-penicillamine (SNAP) as NO donor (Richardson *et al.*, 2006). In addition, we observed that H_2O_2 induced the transcription of *ntrA* gene (our unpublished results), consistent with earlier reports of the involvement of nitroreductases in oxidative stress response and cellular redox balancing (see section 1.2.1), such as, *E. coli* NfsA, *Rhodobacter capsulatus* NprA, *B. subtilis* NfrA and *S. aureus* NfrA (Liochev *et al.*, 1999; Mostertz *et al.*, 2004; Perez-Reinado *et al.*, 2005; Streker *et al.*, 2005).

In this work, *S. aureus* NtrA was demonstrated to be able to detoxify GSNO (Table 3.2). In *S. aureus*, only flavohemoglobin Hmp was until now reported to counteract nitrosative stress (Goncalves *et al.*, 2006). Interestingly, our group has recently shown that Hmp is also involved in antibiotics action, by enhancing the antimicrobial activity of several imidazoles such as miconazole (Nobre *et al.*, 2010).

The fact that a single protein combines two activities seems to be a common factor among GSNO reductases. Indeed, GSNO reductase activity is always found in proteins that exhibit another enzymatic activity, namely, in

glutathione-dependent formaldehyde dehydrogenase (FALDH), glutathione peroxidase, xanthine oxidase and thioredoxin system (Hou *et al.*, 1996; Nikitovic & Holmgren, 1996; Trujillo *et al.*, 1998; Liu *et al.*, 2001; Laver *et al.*, 2012). However, *S. aureus* NtrA is the first case of a nitroreductase displaying GSNO reductase activity. The K_M obtained in this work for *S. aureus* NtrA is in the same range of values usually observed for other GSNO reductases, despite the great variety of values reported (Table 6.3). *S. aureus* NtrA detoxifies GSNO with higher affinity than the *E. coli* FALDH, with a K_M approximately 4-fold lower than that reported for the *E. coli* FALDH (Table 6.3) (Liu *et al.*, 2001). The K_M of *S. aureus* NtrA for GSNO is similar to that of the *Saccharomyces cerevisiae* FALDH, whereas the human and rat FALDHs have higher affinities (Table 6.3) (Jensen *et al.*, 1998; Fernandez *et al.*, 2003). Concerning thioredoxins, the K_M values for GSNO as a substrate for *M. tuberculosis*, human and *Plasmodium falciparum* were reported to be lower than that for *S. aureus* NtrA (Table 6.3) (Attarian *et al.*, 2009).

Table 6.3|K_M for GSNO reductases.

Bacteria	GSNO reductase	K_M (μM)	References
S. aureus	NtrA	181	(Tavares *et al.*, 2009)
E. coli	FALDH	700	(Liu *et al.*, 2001)
Saccharomyces cerevisiae	FALDH	150	(Fernandez *et al.*, 2003)
Human	FALDH	27	(Fernandez *et al.*, 2003)
	Thioreroxin	60	(Attarian *et al.*, 2009)
Rat	FALDH	28	(Jensen *et al.*, 1998)
M. tuberculosis	Thioreroxin	4	(Attarian *et al.*, 2009)
Plasmodium falciparum	Thioreroxin	31	(Attarian *et al.*, 2009)

The mechanism by which nitroreductase NtrA detoxifies this S-nitrosothiol (GS-N=O), which resembles the nitroso intermediate (R-N=O), may be analogous to the nitrofuran reduction via the hydroxylamine intermediate (see section 1.2 - Figure 1.5). Additionally, the *S. aureus* NtrA seems to be specific to GSNO, since no activity was observed for NO, S-nitrosocysteine and S-nitrosohomocysteine. Similar results were obtained for the *E. coli* FALDH (Liu *et al.*, 2001).

Our data also demonstrated that NtrA affords *in vivo* protection as a mutant strain exhibited increased susceptibility to GSNO killing (Figure 3.1). Similar results were reported for the FALDH mutant strains of *Saccharomyces cerevisiae*, *Streptococcus pneumoniae* and *Haemophilus influenza* (Liu *et al.*, 2001; Kidd *et al.*, 2007; Stroeher *et al.*, 2007). *H. pylori* mutants in thioredoxin systems also had increased susceptibility to GSNO (Comtois *et al.*, 2003).

In summary, this work showed that the *S. aureus* NtrA is a bifunctional enzyme involved in nitrofurans activation and protection against nitrotrosative stress.

6.2 Dissecting the bactericidal action mechanism of Carbon Monoxide-Releasing Molecules

Previous work have shown that CO-RMs may constitute a therapeutic strategy to control bacterial infections due to its effectiveness in eliminating pathogens as *E. coli*, *S. aureus* and *P. aeruginosa* (Nobre *et al.*, 2007; Desmard *et al.*, 2009). In this thesis, the bactericidal mechanism underlying the action of CO-RMs was analysed (Chapter 4). So far, the antimicrobial effect of CO-RMs has been attributed mainly to the inhibition of the aerobic respiratory chain through the binding of CO to terminal oxidases (Davidge *et al.*, 2009; Desmard *et al.*, 2009). Indeed, CO-RMs were shown to decrease the

respiratory rates in *E. coli*, *P. aeruginosa* and *C. jejuni* (Davidge *et al.*, 2009; Desmard *et al.*, 2009; Smith *et al.*, 2011).

In mammals, the binding of CO to terminal oxidases with consequent inhibition of mitochondrial respiration and increased ROS production has been reported (Gutterman, 2005; Boczkowski *et al.*, 2006; Zuckerbraun *et al.*, 2007; Lo Iacono *et al.*, 2011). The ROS produced are proposed to act as a second messenger for CO, mediating activation of signaling pathways (Boczkowski *et al.*, 2006; Zuckerbraun *et al.*, 2007). The activation of the signal transduction pathways, such as the mitogen-activated protein kinases (MAPK) mediate the anti-inflammatory, cytoprotective and anti-proliferative effects that CO exerted (Soares & Bach, 2009). In particular, administration of CO-RMs to cultured smooth muscle from human airways inhibits muscle proliferation by stimulating mitochondrial production of ROS (Taille *et al.*, 2005). Interestingly, addition of N-acetylcysteine partially impairs the effects of CORM-2, suggesting a role for ROS in the anti-proliferative effect of CO in human airway smooth muscle (Taille *et al.*, 2005). The anti-inflammatory effects of CO have also been reported to be dependent on ROS generated via inhibition of the cytochrome *c* oxidase since the effects are abolished when the ROS generation is impaired (Zuckerbraun *et al.*, 2007; Bilban *et al.*, 2008). Recently, it was established that CO confers protection in acute lung injury by promoting activation of autophagy also via mitochondrial ROS formation (Lee *et al.*, 2011).

The data presented in this thesis reveal that oxidative stress mediates the bactericidal effect elicited by CO-RMs. Indeed, *E. coli* cells exposed to CO-RMs, namely CORM-2 and ALF062, contained higher level of intracellular ROS, which was abolished by adding the antioxidant glutathione and the CO scavenger, hemoglobin (Figure 4.3). Importantly, control compounds devoided

of CO were unable to generate ROS. In agreement with these results, Smith and co-workers detected H_2O_2 production in *C. jejuni* after exposure to CORM-3 (Smith *et al.*, 2011). More recently, increased ROS formation upon CORM-2 treatment was also reported for *P. aeruguinosa* (Murray *et al.*, 2012). In that study, although the treatment with the antioxidant cysteine only partly inhibited ROS formation, N-acetylcysteine was able to completely prevent the ROS production (Murray *et al.*, 2012). Contrary to these findings, Desmard and co-workers reported that in *P. aeruginosa,* CO-RMs, such as CORM-2, CORM-3 and CORM-A1, did not induce increase of ROS content (Desmard *et al.*, 2011). However, the authors observed that N-acetylcysteine, cysteine and glutathione, well-known by their antioxidant properties, canceled the bactericidal effect of CO-RMs, which suggests that ROS are contributing to their antimicrobial action. The fact that these authors did not detect ROS production is most probably due to the conditions used in the assay. In those experiments, the fluorescence probe was added during the growth of bacteria that last for 60 min, and only then the cells were washed and incubated with CO-RM. As this probe crosses the membranes and is oxidized by several agents, the 60 min of incubation within cells is expected to significantly decrease the amount of probe molecules left available to be oxidized by the ROS produced by the CO-RM, therefore strongly limiting the detection of these species (Desmard *et al.*, 2011).

Other evidences linking the action of CO-RMs to the generation of ROS have been presented in this thesis. In particular, when ROS production was inhibited by antioxidants as glutathione and cysteine, the lethality of the CO-RMs was abolished (Figure 4.2). Moreover, strains deleted in ROS detoxifying systems, namely catalase and superoxide dismutase had increased susceptibility to CORM-2, highlighting the contribution of ROS to drug killing

(Figure 4.5). Additionally, CORM-2 was shown to induce DNA damage and to interfere with iron homeostasis (Figure 4.6, 4.7, 4.8 and 4.9). The intracellular levels of free iron increased by four times upon CORM-2 treatment, and Fe-S centres were shown to be one of the iron sources (Figure 4.7 and 4.9). Moreover, among the bacterial systems that remediate hydroxyl radical damage, *recA*, which is involved in triggering the DNA damage response, was shown to be required to counteract CO-RMs action (Figure 4.6). Finally, CORM-2 promoted oxidation of free thiols groups (Figure 4.10). Interestingly, reuterin, an antimicrobial compound that induces oxidative stress, was also described to oxidize thiol groups of proteins and small molecules, losing its antimicrobial effect upon supplementation of *E. coli* cultures with cysteine (Schaefer *et al.*, 2010). Besides the critical role of cysteine in thiol-redox systems, this amino acid is also essential in the structure of Fe-S clusters, which are a well known target of oxidative stress (Figure 4.9). Altogether, these results allowed concluding that the mechanisms that underlay the killing effect of CO-RMs on bacteria include the production of ROS.

Since CO-RMs mediate bacterial cell death through ROS formation, it is predictable that the gene expression profile of *E. coli* in the presence of CO-RMs under aerobic conditions share similarities with the transcriptional response to hydrogen peroxide (Table 6.4) (Zheng *et al.*, 2001; Davidge *et al.*, 2009; Nobre *et al.*, 2009; Wang *et al.*, 2009). In particular, genes involved in folding and stability of proteins, one of ROS main targets, such as the heat shock proteins *hptX, ibpA* and *ibpB,* and chaperones like *dnaK, dnaJ* and *hslO* were induced by oxidative stress and CO-RMs. The *yqhD* gene encoding an alcohol dehydrogenase proposed to protect cells against lipid oxidation, and *yeeD,* gene coding for a redox protein that regulates the formation of disulfide bonds, were also induced by CORM-2 and H_2O_2 (Zheng *et al.*, 2001; Perez *et*

al., 2008; Nobre *et al.*, 2009; Wang *et al.*, 2009). Furthermore, CORM-2 induced the expression of genes related with the two major transcriptional regulators of oxidative stress, OxyR and SoxRS (Nobre *et al.*, 2009). In particular, CORM-2 increased the expression of the *soxS* gene and of members of the SoxRS regulon, such as the *marAB* operon, that encodes a multiple antibiotic resistance protein, and *micF* coding for a major outer membrane porin (Nobre *et al.*, 2009). Moreover, the *E. coli soxS* mutant was shown to be more susceptibility to CORM-2 than the parental strain (Nobre *et al.*, 2009). Although CORM-2 induced the transcription of *oxyR*, no gene of the regulon was found altered under the microarray experimental conditions utilized (Nobre *et al.*, 2009). Nevertheless, *oxyR* mutant exhibited enhanced susceptibility to CORM-2 (Nobre *et al.*, 2009). Previous studies in *E. coli* demonstrated that OxyR-regulated genes such as *dps*, *katG, grxA*, *ahpCF* and *trxC* are induced in cells exposed to sub-lethal concentrations of H_2O_2 (Zheng *et al.*, 2001; Wang *et al.*, 2009). Our unpublished results also revealed that when cells were treated with a sub-lethal 150 μM dose of CORM-2, up-regulation of *katG* and *ahpC* were observed. Moreover, a significant induction of these genes (*katG* and *ahpC*) was also seen after treating *E. coli* cells with another CO-RM, namely ALF062 (our unpublished results).

E. coli genes linked with the metabolism of cysteine, which plays a critical role in protection of bacteria against oxidative stress, were also altered upon CO-RMs treatment. Indeed, the transcription of *sbp*, involved in sulfate uptake, which is crucial to cysteine biosynthesis, was induced in both CO-RMs microarray experiments and was one of the most induced genes in the presence of H_2O_2 (Table 6.4) (Zheng *et al.*, 2001; Davidge *et al.*, 2009; Nobre *et al.*, 2009). Moreover, CORM-2 up-regulates a significant number of genes involved in sulphur metabolism, such as *tauABC*, *ssuAD* and *cysWA,* being the

latter also up-regulated in the presence of H_2O_2 (Nobre *et al.*, 2009; Wang *et al.*, 2009). Furthermore, CO-RMs seem to interfere with the metabolism of methionine, as judged by alterations observed in the expression of methionine biosynthesis related genes. In particular, *metF*, which is involved in methionine biosynthesis, was highly induced by the two CO-releasing molecules (Davidge *et al.*, 2009; Nobre *et al.*, 2009). CORM-2 also induced several other methionine–related genes, namely *metNI*, *metBL* and *metR*, the latter regulating the transcription of genes involved in methionine biosynthesis (Nobre *et al.*, 2009). Consistent with these data, the deletion of *metR*, *metI* and *metN* enhances the susceptibility of *E. coli* cells to CORM-2 (Nobre *et al.*, 2009). Although no methionine related gene had their transcription altered under oxidative stress conditions, it has been demonstrated that the oxidative stress is associated with methionine auxotrophy (Hondorp & Matthews, 2004). Interestingly, our results revealed that methionine supplementation abolishes the CO-RMs bactericidal activity (Figure 4.2). Overall, these results indicate that CO-RMs interfere with cysteine and methionine biosynthetic pathways.

The *E. coli spy*, encoding a periplasmic protein that is induced by cell envelope stress, is also up-regulated in the presence of CO-releasing molecules and H_2O_2, suggesting that the integrity of cell envelope is affected under both stress conditions (Davidge *et al.*, 2009; Nobre *et al.*, 2009; Wang *et al.*, 2009). Another periplasmatic protein involved in cell envelope stress, *cpxP* was induced in the presence of CO-RMs (Davidge *et al.*, 2009; Nobre *et al.*, 2009). Interestingly, *cpxP* was the most up-regulated gene during n-butanol stress, which also generates an increase in ROS formation (Rutherford *et al.*, 2010). Altogether, these previous findings reinforce the proposal that oxidative stress is beyond CO-RMs action in bacteria.

Table 6.4| *E. coli* genes induced by CO-RMs and associated with oxidative stress.

CO-RMs	Gene name	Protein	Fold change
CORM-2	*spy*	Envelope stress induced periplasmic protein	30
	sbp	Sulfate transporter subunit	11
	oxyR	Hydrogen peroxide-inducible genes activator	4
	soxS	DNA binding transcriptional dual regulator	15
	micF	Regulatory antisense sRNA affecting *ompF* expression	4
	marA	Multiple antibiotic resistance protein	10
	marB	Multiple antibiotic resistance protein	7
	ibpA	16 kDa heat shock protein A	19
	ibpB	16 kDa heat shock protein B	79
	dnaK	Chaperone Hsp70, co-chaperone with DnaJ	3
	dnaJ	Chaperone protein Hsp40, co-chaperone with DnaK	4
	hslO	Hsp33-like chaperonin	5
	cysW	Sulfate/thiosulfate transporter subunit W	3
	cysA	Sulfate/thiosulfate transporter subunit A	5
	yqhD	Alcohol dehydrogenase, NAD(P)-dependent	4
	yeeD	Predicted redox protein	3
	metB	Cystathionine gamma-synthase, PLP-dependent	5
	metL	Bi-functional aspartate kinase II/homoserine dehydrogenase II	3
	metN	D-methionine transport ATP binding protein	10
	metI	D-methionine transport system permease	7
	metF	5,10-Methylenetetrahydrofolate reductase	18
	metR	DNA-binding transcriptional activator, homocysteine-binding	21
CORM-3	*spy*	Envelope stress induced periplasmic protein	3
	sbp	Sulfate transporter subunit	3
	metF	5,10-Methylenetetrahydrofolate reductase	11
	htpX	Heat shock protein	3

Adapted from (Tavares *et al.*, 2012).

250 µM CORM-2 (Nobre *et al.*, 2009) and 30 µM CORM-3 (Davidge *et al.*, 2009).

In addition to the increase of ROS production in *E. coli* cells upon CO-RMs treatment, it is herein shown that CO-RMs are able to produce hydroxyl radicals *per se* in a CO-dependent mode, as the presence of haemoglobin prevented their formation (Figure 4.11). These findings might explain the better efficiency of CO-RMs as bactericides than that of CO gas (Nobre *et al.*, 2007; Desmard *et al.*, 2009). The generation of hydroxyl radicals from CORM-2 is proposed to result from the reduction of oxygen by the reduced ruthenium species, which mediate the water–gas shift reaction that is initiated with the attack of water on one of the CO ligands of the $Ru^{II}(CO)_3$ moieties of CORM-2. Moreover, the ROS formation is not exclusively linked to the presence of ruthenium in the CO-RM, as ALF062, a Mo-containing CO-RM, also induces the formation of hydroxyl radicals. In this case, it is plausible that hydroxyl radicals originate from the reaction of the electron-rich metal in the $[Mo(CO)_5Br]$-[NEt4] complex with oxygen.

Interestingly, antibiotics from the ß-lactams, aminoglycosides and quinolones classes have been reported to mediate bacterial cell death through the stimulation of the deleterious hydroxyl radical formation (Becerra & Albesa, 2002; Dwyer *et al.*, 2007; Kohanski *et al.*, 2007; Dwyer *et al.*, 2009). Indeed, the efficiency of these drugs was found to be dependent on ROS production since addition of antioxidants as thiourea decreased their lethality (Kohanski *et al.*, 2007). The free intracellular ferrous iron released from Fe-S clusters was also established as a source for Fenton-mediated hydroxyl radical formation promoted by these antibiotics (Dwyer *et al.*, 2007; Kohanski *et al.*, 2007).

Overall, the results compiled in this thesis undoubtedly revealed that the formation of ROS by CO-RMs contribute to their killing properties and need to be considered when using this class of compounds. The outline of the

CO-RMs effect on bacteria disclosed so far is represented in Figure 6.1. Independently of their pharmacological applications, CO-RMs no longer should be seen as simple CO delivery systems. To which point in animal cells the cytoprotective and potent anti-inflammatory properties of CO-RMs are linked to ROS formation is an open question that requires investigation to fully understand the mode of action of this novel class of compounds that exhibit a wide range of therapeutic properties.

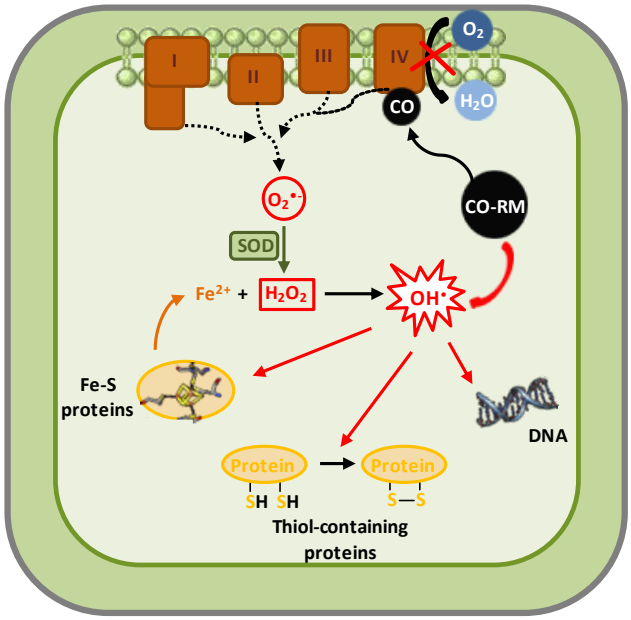


Figure 6.1| Schematic representation of CO-RMs effect on bacteria. CO-RMs release CO that binds to terminal oxidases inhibiting respiration and enhancing the production of superoxide. Superoxide dismutase (SOD) converts superoxide to hydrogen peroxide, which via the Fenton reaction generates hydroxyl radicals ($OH^{\cdot}$). CO-RMs also produce hydroxyl radicals *per se*. ROS damage DNA, oxidize thiol groups and degrade iron-sulfur (Fe-S) containing proteins releasing free iron that promotes the Fenton reaction. Adapted from (Tavares *et al.*, 2012).

6.3 Carbon Monoxide-Releasing Molecules as antimicrobial agents against *H. pylori*

In this thesis, the effect of exogenous CO via CO-RMs was analysed on the pathogen *H. pylori* (Chapter 5). The results herein presented demonstrated that CO-RMs are able to eliminate *H. pylori* in a dose-dependent manner, while a CO-depleted compound has no antimicrobial properties (Figure 5.1 and S5.2). Importantly, CORM-2 is shown to be a potent antibacterial molecule against clinical isolated strains of *H. pylori*, some of which displayed resistance to metronidazole (Table 5.1). Interestingly, no morphological alteration was found upon CORM-2 treatment, as reported for other antimicrobial agents, such as aspirin and NO (Wang *et al.*, 2003). Our group has recently reported that although NO was able to efficiently eliminate *H. pylori,* no coccoid forms were observed (Justino *et al.*, 2012). As previously reported for *E. coli*, *P. aeruginosa* and *C. jejuni,* CORM-2 were shown to decrease the respiratory rates in *H. pylori* (Figure 5.2) (Davidge *et al.*, 2009; Desmard *et al.*, 2009; Smith *et al.*, 2011).

The bactericidal effect of CORM-2 on *H. pylori* cultures occurs at higher concentrations (200 mg/L ± 400 μM) than those required to kill *E. coli* or *S. aureus* (250 μM). These results are most probably due to experimental conditions regarding growth medium and the growth of bacteria, since *H. pylori* is a fastidious bacteria that requires a complex growth media. Nonetheless, further assays presented in this work revealed that *H. pylori* cells previously treated with CORM-2 had increased sensitivity to macrophage killing (Figure 5.7). Importantly, the bactericidal effect of CORM-2 on *H. pylori* occurs at concentrations which are usually not cytotoxic to eukaryotic cells, as the treatment of macrophages or smooth muscle cells with CO-RMs at concentrations up to 500 μM is reported to promote a non significant

cytotoxicity (Motterlini *et al.*, 2002; Motterlini *et al.*, 2005; Desmard *et al.*, 2009). Nevertheless, a recent study shows that only 20 μM CORM-2 or the respective inactive compound compromise the cell viability of human and canine kidney cells (Winburn *et al.*, 2012). Further studies need to be conducted in order to clarify the toxicological profile of CO-RMs.

Besides iron, CO is known to bind other transition metals in biological systems such as nickel, as well documented for nickel containing CO-dehydrogenase (Ermler *et al.*, 1998; Drennan *et al.*, 2001). In accordance, the *H. pylori* urease, a nickel containing enzyme was found to have its activity inhibited upon CORM-2 treatment (Figure 5.3). Due to the urease crucial role in survival and pathogenesis of *H. pylori* (see section 1.1.3), an urease inhibitor constitutes a potential strategy to control infections caused by this bacterium. In fact, many urease inhibitors have been investigated in the past decades, like phosphorodiamidates, hydroxamic acid derivatives and imidazoles, but part of them are prevented from *in vivo* utilization due to their toxicity, instability and harmful side effects (Amtul *et al.*, 2002; Amtul *et al.*, 2007).

One of the mechanisms of CO-RMs bactericidal action relies on the generation of ROS, as herein proved. Accordingly, antioxidants such as glutathione and cysteine showed to suppress the antimicrobial effect of CORM-2 on *H. pylori* (Figure 5.2). Recently, CORM-3 was reported to be unable to inhibit the growth of the microaerophilic pathogen *C. jejuni.* However, it is possible that the presence in the growth media of high concentrations of methionine and cysteine (~0.7 mM and ~3 mM, respectively) prevented the bactericidal effect of CORM-3 (Smith *et al.*, 2011)

Importantly, we found that when CORM-2 is combined with the most commonly antibiotics used against *H. pylori* namely metronidazole, clarithromycin and amoxicillin, bacteria cell death is enhanced and antibiotic

resistance could be relieved (Figure 5.5 and Table S5.2). Moreover, *H. pylori* cells, previously treated with sub-lethal doses of metronidazole and CORM-2, were more susceptible to macrophage killing (Figure 5.8).

Some examples of higher efficiency of drugs when combined with CO-RMs have been recently reported. The ruthenium-based carbonyl ALF492 is proposed to be an effective adjuvant for treatment of cerebral malaria when used in combination with the antimalarial drug artesunate (Pena *et al.*, 2012). More recently, CORM-2 has been reported to act as an adjuvant when combined with tobramycin, a drug commonly used to treat *P. aeruginosa* lung infections (Murray *et al.*, 2012). Additionally, aspirin which has been reported to inhibit the growth of *H. pylori*, enhances bacteria susceptibility to metronidazole (Wang *et al.*, 2003).

Altogether, the work developed during this thesis allowed concluding that CO-RMs represent a novel therapeutic strategy against bacterial pathogens by mediating cell death through ROS formation. In particular, CO-RMs seem to eliminate *H. pylori*, as it has already been recognized to *E. coli*, *S. aureus* and *P. aeruginosa*. Nevertheless, the mechanisms underlying CO-RMs action need to be further explored, particularly under anaerobic conditions. Furthermore, it would be interesting to elucidate CO targets besides the well-known haem proteins, and the nickel containing protein – urease, herein described. Moreover, for the clinical application in humans, novel CO-RMs need to be prepared in order to minimize doses and maximize not only efficacy but also safety.

6.4 References

Amtul Z, Rahman AU, Siddiqui RA & Choudhary MI (2002) Chemistry and mechanism of urease inhibition. *Curr Med Chem* **9**: 1323-1348.

Amtul Z, Follmer C, Mahboob S, Atta Ur R, Mazhar M, Khan KM, Siddiqui RA, Muhammad S, Kazmi SA & Choudhary MI (2007) Germa-gamma-lactones as novel inhibitors of bacterial urease activity. *Biochem Biophys Res Commun* **356**: 457-463.

Attarian R, Bennie C, Bach H & Av-Gay Y (2009) Glutathione disulfide and S-nitrosoglutathione detoxification by Mycobacterium tuberculosis thioredoxin system. *FEBS Lett* **583**: 3215-3220.

Barisic Z, Babic-Erceg A, Borzic E, Zoranic V, Kaliterna V & Carev M (2003) Urinary tract infections in South Croatia: aetiology and antimicrobial resistance. *Int J Antimicrob Agents* **22 Suppl 2**: 61-64.

Becerra MC & Albesa I (2002) Oxidative stress induced by ciprofloxacin in Staphylococcus aureus. *Biochem Biophys Res Commun* **297**: 1003-1007.

Bilban M, Haschemi A, Wegiel B, Chin BY, Wagner O & Otterbein LE (2008) Heme oxygenase and carbon monoxide initiate homeostatic signaling. *J Mol Med (Berl)* **86**: 267-279.

Boczkowski J, Poderoso JJ & Motterlini R (2006) CO-metal interaction: Vital signaling from a lethal gas. *Trends Biochem Sci* **31**: 614-621.

Comtois SL, Gidley MD & Kelly DJ (2003) Role of the thioredoxin system and the thiol-peroxidases Tpx and Bcp in mediating resistance to oxidative and nitrosative stress in Helicobacter pylori. *Microbiology* **149**: 121-129.

Davidge KS, Sanguinetti G, Yee CH, Cox AG, McLeod CW, Monk CE, Mann BE, Motterlini R & Poole RK (2009) Carbon monoxide-releasing antibacterial molecules target respiration and global transcriptional regulators. *J Biol Chem* **284**: 4516-4524.

Desmard M, Davidge KS, Bouvet O, Morin D, Roux D, Foresti R, Ricard JD, Denamur E, Poole RK, Montravers P, Motterlini R & Boczkowski J (2009) A carbon monoxide-releasing molecule (CORM-3) exerts bactericidal activity against Pseudomonas aeruginosa and improves survival in an animal model of bacteraemia. *Faseb J* **23**: 1023-1031.

Desmard M, Foresti R, Morin D, Dagouassat M, Berdeaux A, Denamur E, Crook SH, Mann BE, Scapens D, Montravers P, Boczkowski J & Motterlini R (2011) Differential antibacterial activity against Pseudomonas aeruginosa by carbon monoxide-releasing molecules. *Antioxid Redox Signal* **16**: 153-163.

Drennan CL, Heo J, Sintchak MD, Schreiter E & Ludden PW (2001) Life on carbon monoxide: X-ray structure of Rhodospirillum rubrum Ni-Fe-S carbon monoxide dehydrogenase. *Proc Natl Acad Sci U S A* **98**: 11973-11978.

Dwyer DJ, Kohanski MA, Hayete B & Collins JJ (2007) Gyrase inhibitors induce an oxidative damage cellular death pathway in Escherichia coli. *Mol Syst Biol* **3**: 91.

Dwyer DJ, Kohanski MA & Collins JJ (2009) Role of reactive oxygen species in antibiotic action and resistance. *Curr Opin Microbiol* **12**: 482-489.

Ermler U, Grabarse W, Shima S, Goubeaud M & Thauer RK (1998) Active sites of transition-metal enzymes with a focus on nickel. *Curr Opin Struct Biol* **8**: 749-758.

Fernandez MR, Biosca JA & Pares X (2003) S-nitrosoglutathione reductase activity of human and yeast glutathione-dependent formaldehyde dehydrogenase and its nuclear and cytoplasmic localisation. *Cell Mol Life Sci* **60**: 1013-1018.

Flournoy DJ & Robinson MC (1990) In vitro antimicrobial susceptibilities of 349 methicillin-resistant Staphylococcus aureus isolates from veterans. *Methods Find Exp Clin Pharmacol* **12**: 541-544.

Goncalves VL, Nobre LS, Vicente JB, Teixeira M & Saraiva LM (2006) Flavohemoglobin requires microaerophilic conditions for nitrosative protection of Staphylococcus aureus. *FEBS Lett* **580**: 1817-1821.

Guay DR (2001) An update on the role of nitrofurans in the management of urinary tract infections. *Drugs* **61**: 353-364.

Gutterman DD (2005) Mitochondria and reactive oxygen species: an evolution in function. *Circ Res* **97**: 302-304.

Hondorp ER & Matthews RG (2004) Oxidative stress inactivates cobalamin-independent methionine synthase (MetE) in Escherichia coli. *PLoS Biol* **2**: e336.

Hou Y, Guo Z, Li J & Wang PG (1996) Seleno compounds and glutathione peroxidase catalyzed decomposition of S-nitrosothiols. *Biochem Biophys Res Commun* **228**: 88-93.

Ionescu R, Mediavilla JR, Chen L, Grigorescu DO, Idomir M, Kreiswirth BN & Roberts RB (2010) Molecular characterization and antibiotic susceptibility of Staphylococcus aureus from a multidisciplinary hospital in Romania. *Microb Drug Resist* **16**: 263-272.

Jensen DE, Belka GK & Du Bois GC (1998) S-Nitrosoglutathione is a substrate for rat alcohol dehydrogenase class III isoenzyme. *Biochem J* **331 (Pt 2)**: 659-668.

Johansson E, Parkinson GN, Denny WA & Neidle S (2003) Studies on the nitroreductase prodrug-activating system. Crystal structures of complexes with the inhibitor dicoumarol and dinitrobenzamide prodrugs and of the enzyme active form. *J Med Chem* **46**: 4009-4020.

Johnson JR, Berggren T & Conway AJ (1993) Activity of a nitrofurazone matrix urinary catheter against catheter-associated uropathogens. *Antimicrob Agents Chemother* **37**: 2033-2036.

Johnson JR, Delavari P & Azar M (1999) Activities of a nitrofurazone-containing urinary catheter and a silver hydrogel catheter against multidrug-resistant bacteria characteristic of catheter-associated urinary tract infection. *Antimicrob Agents Chemother* **43**: 2990-2995.

Kidd SP, Jiang D, Jennings MP & McEwan AG (2007) Glutathione-dependent alcohol dehydrogenase AdhC is required for defense against nitrosative stress in Haemophilus influenzae. *Infect Immun* **75**: 4506-4513.

Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA & Collins JJ (2007) A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* **130**: 797-810.

Laver JR, McLean S, Bowman LA, Harrison LJ, Read RC & Poole RK (2012) Nitrosothiols in bacterial pathogens and pathogenesis. *Antioxid Redox Signal*.

Lee SJ, Ryter SW, Xu JF, Nakahira K, Kim HP, Choi AM & Kim YS (2011) Carbon monoxide activates autophagy via mitochondrial reactive oxygen species formation. *Am J Respir Cell Mol Biol* **45**: 867-873.

Liochev SI, Hausladen A & Fridovich I (1999) Nitroreductase A is regulated as a member of the soxRS regulon of Escherichia coli. *Proc Natl Acad Sci U S A* **96**: 3537-3539.

Liu L, Hausladen A, Zeng M, Que L, Heitman J & Stamler JS (2001) A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans. *Nature* **410**: 490-494.

Lo Iacono L, Boczkowski J, Zini R, Salouage I, Berdeaux A, Motterlini R & Morin D (2011) A carbon monoxide-releasing molecule (CORM-3) uncouples mitochondrial respiration and modulates the production of reactive oxygen species. *Free Radic Biol Med* **50**: 1556-1564.

McOsker CC, Pollack JR & Andersen JA (1989) Inhibition of bacterial protein synthesis by nitrofurantoin macrocrystals: an explanation for the continued efficacy of nitrofurantoin. *Royal Society of Medicine International Congress and Symposium Series* **154**: 33-44.

Mostertz J, Scharf C, Hecker M & Homuth G (2004) Transcriptome and proteome analysis of Bacillus subtilis gene expression in response to superoxide and peroxide stress. *Microbiology* **150**: 497-512.

Motterlini R, Clark JE, Foresti R, Sarathchandra P, Mann BE & Green CJ (2002) Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities. *Circ Res* **90**: E17-24.

Motterlini R, Mann BE & Foresti R (2005) Therapeutic applications of carbon monoxide-releasing molecules. *Expert Opin Investig Drugs* **14**: 1305-1318.

Murray TS, Okegbe C, Gao Y, Kazmierczak BI, Motterlini R, Dietrich LE & Bruscia EM (2012) The carbon monoxide releasing molecule CORM-2 attenuates Pseudomonas aeruginosa biofilm formation. *PLoS One* **7**: e35499.

Nikitovic D & Holmgren A (1996) S-nitrosoglutathione is cleaved by the thioredoxin system with liberation of glutathione and redox regulating nitric oxide. *J Biol Chem* **271**: 19180-19185.

Nobre LS, Seixas JD, Romao CC & Saraiva LM (2007) Antimicrobial action of carbon monoxide-releasing compounds. *Antimicrob Agents Chemother* **51**: 4303-4307.

Nobre LS, Al-Shahrour F, Dopazo J & Saraiva LM (2009) Exploring the antimicrobial action of a carbon monoxide-releasing compound through whole-genome transcription profiling of Escherichia coli. *Microbiology* **155**: 813-824.

Nobre LS, Todorovic S, Tavares AF, Oldfield E, Hildebrandt P, Teixeira M & Saraiva LM (2010) Binding of azole antibiotics to Staphylococcus aureus flavohemoglobin increases intracellular oxidative stress. *J Bacteriol* **192**: 1527-1533.

Olekhnovich IN, Goodwin A & Hoffman PS (2009) Characterization of the NAD(P)H oxidase and metronidazole reductase activities of the RdxA nitroreductase of Helicobacter pylori. *Febs J* **276**: 3354-3364.

Pena AC, Penacho N, Mancio-Silva L, Neres R, Seixas JD, Fernandes AC, Romao CC, Mota MM, Bernardes GJ & Pamplona A (2012) A novel carbon monoxide-releasing molecule fully protects mice from severe malaria. *Antimicrob Agents Chemother* **56**: 1281-1290.

Perez-Reinado E, Blasco R, Castillo F, Moreno-Vivian C & Roldan MD (2005) Regulation and characterization of two nitroreductase genes, nprA and nprB, of Rhodobacter capsulatus. *Appl Environ Microbiol* **71**: 7643-7649.

Perez JM, Arenas FA, Pradenas GA, Sandoval JM & Vasquez CC (2008) Escherichia coli YqhD exhibits aldehyde reductase activity and protects from the harmful effect of lipid peroxidation-derived aldehydes. *J Biol Chem* **283**: 7346-7353.

Richardson AR, Dunman PM & Fang FC (2006) The nitrosative stress response of Staphylococcus aureus is required for resistance to innate immunity. *Mol Microbiol* **61**: 927-939.

Roldan MD, Perez-Reinado E, Castillo F & Moreno-Vivian C (2008) Reduction of polynitroaromatic compounds: the bacterial nitroreductases. *FEMS Microbiol Rev* **32**: 474-500.

Rutherford BJ, Dahl RH, Price RE, Szmidt HL, Benke PI, Mukhopadhyay A & Keasling JD (2010) Functional genomic study of exogenous n-butanol stress in Escherichia coli. *Appl Environ Microbiol* **76**: 1935-1945.

Schaefer L, Auchtung TA, Hermans KE, Whitehead D, Borhan B & Britton RA (2010) The antimicrobial compound reuterin (3-hydroxypropionaldehyde) induces oxidative stress via interaction with thiol groups. *Microbiology* **156**: 1589-1599.

Searle PF, Chen MJ, Hu L, Race PR, Lovering AL, Grove JI, Guise C, Jaberipour M, James ND, Mautner V, Young LS, Kerr DJ, Mountain A, White SA & Hyde EI (2004) Nitroreductase: a prodrug-activating enzyme for cancer gene therapy. *Clin Exp Pharmacol Physiol* **31**: 811-816.

Shah RR & Wade G (1989) Reappraisal of the risk/benefit of nitrofurantoin: review of toxicity and efficacy. *Adverse Drug React Acute Poisoning Rev* **8**: 183-201.

Sisson G, Goodwin A, Raudonikiene A, Hughes NJ, Mukhopadhyay AK, Berg DE & Hoffman PS (2002) Enzymes associated with reductive activation and action of nitazoxanide, nitrofurans, and metronidazole in Helicobacter pylori. *Antimicrob Agents Chemother* **46**: 2116-2123.

Smith H, Mann BE, Motterlini R & Poole RK (2011) The carbon monoxide-releasing molecule, CORM-3 (RU(CO)(3) CL(glycinate)), targets respiration and oxidases in Campylobacter jejuni, generating hydrogen peroxide. *IUBMB Life* **63**: 363-371.

Soares MP & Bach FH (2009) Heme oxygenase-1: from biology to therapeutic potential. *Trends Mol Med* **15**: 50-58.

Streker K, Freiberg C, Labischinski H, Hacker J & Ohlsen K (2005) Staphylococcus aureus NfrA (SA0367) is a flavin mononucleotide-dependent NADPH oxidase involved in oxidative stress response. *J Bacteriol* **187**: 2249-2256.

Stroeher UH, Kidd SP, Stafford SL, Jennings MP, Paton JC & McEwan AG (2007) A pneumococcal MerR-like regulator and S-nitrosoglutathione reductase are required for systemic virulence. *J Infect Dis* **196**: 1820-1826.

Taille C, El-Benna J, Lanone S, Boczkowski J & Motterlini R (2005) Mitochondrial respiratory chain and NAD(P)H oxidase are targets for the antiproliferative effect of carbon monoxide in human airway smooth muscle. *J Biol Chem* **280**: 25350-25360.

Tavares AF, Nobre LS, Melo AM & Saraiva LM (2009) A novel nitroreductase of Staphylococcus aureus with S-nitrosoglutathione reductase activity. *J Bacteriol* **191**: 3403-3406.

Tavares AF, Nobre LS & Saraiva LM (2012) A role for reactive oxygen species in the antibacterial properties of Carbon Monoxide-Releasing Molecules. *FEMS Microbiol Lett*.

Trujillo M, Alvarez MN, Peluffo G, Freeman BA & Radi R (1998) Xanthine oxidase-mediated decomposition of S-nitrosothiols. *J Biol Chem* **273**: 7828-7834.

Vass SO, Jarrom D, Wilson WR, Hyde EI & Searle PF (2009) E. coli NfsA: an alternative nitroreductase for prodrug activation gene therapy in combination with CB1954. *Br J Cancer* **100**: 1903-1911.

Wang S, Deng K, Zaremba S, Deng X, Lin C, Wang Q, Tortorello ML & Zhang W (2009) Transcriptomic response of Escherichia coli O157:H7 to oxidative stress. *Appl Environ Microbiol* **75**: 6110-6123.

Wang WH, Wong WM, Dailidiene D, Berg DE, Gu Q, Lai KC, Lam SK & Wong BC (2003) Aspirin inhibits the growth of Helicobacter pylori and enhances its susceptibility to antimicrobial agents. *Gut* **52**: 490-495.

Watanabe M, Nishino T, Takio K, Sofuni T & Nohmi T (1998) Purification and characterization of wild-type and mutant "classical" nitroreductases of Salmonella typhimurium. L33R mutation greatly diminishes binding of FMN to the nitroreductase of S. typhimurium. *J Biol Chem* **273**: 23922-23928.

Whiteway J, Koziarz P, Veall J, Sandhu N, Kumar P, Hoecher B & Lambert IB (1998) Oxygen-insensitive nitroreductases: analysis of the roles of nfsA and nfsB in development of resistance to 5-nitrofuran derivatives in Escherichia coli. *J Bacteriol* **180**: 5529-5539.

Winburn IC, Gunatunga K, McKernan RD, Walker RJ, Sammut IA & Harrison JC (2012) Cell damage following carbon monoxide releasing molecule exposure: implications for therapeutic applications. *Basic Clin Pharmacol Toxicol* **111**: 31-41.

Zenno S, Koike H, Kumar AN, Jayaraman R, Tanokura M & Saigo K (1996a) Biochemical characterization of NfsA, the Escherichia coli major nitroreductase exhibiting a high

amino acid sequence homology to Frp, a Vibrio harveyi flavin oxidoreductase. *J Bacteriol* **178**: 4508-4514.

Zenno S, Koike H, Tanokura M & Saigo K (1996b) Gene cloning, purification, and characterization of NfsB, a minor oxygen-insensitive nitroreductase from Escherichia coli, similar in biochemical properties to FRase I, the major flavin reductase in Vibrio fischeri. *J Biochem* **120**: 736-744.

Zenno S, Kobori T, Tanokura M & Saigo K (1998) Purification and characterization of NfrA1, a Bacillus subtilis nitro/flavin reductase capable of interacting with the bacterial luciferase. *Biosci Biotechnol Biochem* **62**: 1978-1987.

Zheng M, Wang X, Templeton LJ, Smulski DR, LaRossa RA & Storz G (2001) DNA microarray-mediated transcriptional profiling of the Escherichia coli response to hydrogen peroxide. *J Bacteriol* **183**: 4562-4570.

Zuckerbraun BS, Chin BY, Bilban M, d'Avila JC, Rao J, Billiar TR & Otterbein LE (2007) Carbon monoxide signals via inhibition of cytochrome c oxidase and generation of mitochondrial reactive oxygen species. *Faseb J* **21**: 1099-1106.

ITQB-UNL | Av. da República, 2780-157 Oeiras, Portugal
Tel (+351) 214 469 100 | Fax (+351) 214 411 277

www.itqb.unl.pt